Synthesis and Optimization of Group B Streptococcus Capsular Polysaccharide Fragments Using the Glyconeer

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry, Pharmacy
of Freie Universität Berlin

by

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December 2019

This work was performed between June 2016 and June 2019 in GlycoUniverse GmbH & Co KGaA and in the Department of Biomolecular Systems of Max Planck Institute of Colloids and Interfaces under the guidance of Prof. Dr. Peter H. Seeberger.

1st Reviewer: Prof. Dr. Peter H. Seeberger

2nd Reviewer: Prof. Dr. Christoph Tzschucke

Date of oral defense: 17.03.2020

Acknowledgments

I would like to acknowledge Professor Dr. Peter H. Seeberger for giving me the opportunity to work in his group, for providing an environment at the forefront of innovation and technology, and for making this PhD thesis possible.

I acknowledge Dr. Mario Salwiczek for scientific guidance and for his support throughout the period of my PhD.

I am grateful to Mr. Sascha Meyer for his support in the early stages of my PhD.

I sincerely thank Prof. Dr. Christoph Tzschucke for agreeing to review this thesis.

I am deeply thankful to Tanistha Gupta for the close collaboration and assistance in designing and executing the majority of the experiments conducted for this thesis and for her support throughout the whole time of my PhD, since the very first day of work in Arnimallee 22 untill the submission of this thesis. The continuous sharing of thoughts, ideas, tasks, responsibilities, joys, and sorrows, was a major help in overcoming the challenges of these past three and a half years.

I am grateful to Dr. Roberto Adamo for taking interest in my project, for the productive collaboration, for giving constructive feedback and support. I am also grateful to my fellow PhD student Linda Delbino for fruitful discussions and collaborations.

I am thankful to Prof. Sabine Flitsch for hosting me at the Manchester Institute of Biotechnology in Manchester and guiding my studies in enzymatic sialylation. I am also thankful to Dr. Peter Both and to my fellow PhD student Andrea Marchesi for their valuable participation in this collaboration.

I am very grateful to Dr. Maria Bräutigam for her prompt availability to assist me in writing the Zusammenfassung as well as for scientific discussions and sharing of ideas during my PhD.

I am thankful to all the colleagues at the Department of Biomolecular Systems of MPIKG for their professionalism, assistance and support.

I would like to thank all my fellow PhD students from GLYCOVAX network for sharing their experiences and engaging in creating a truly collaborative and friendly group. I am also grateful to all the PIs involved in the GLYCOVAX consortium as well as ALTA, the logistics partner, providing the means for this network to flourish.

I acknowledge the funding received from the European Union's Framework Programme for Research and Innovation Horizon 2020 (2014-2020) under the Marie Sklodowska-Curie Grant Agreement No. 675671.

Last but certainly not the least, I am deeply thankful to my friends and my family who supported me from the very first moment and encouraged me to pursue my dreams with the most unconditional support. From them I learn the most essential and extraordinary theory any scientist can devise.

Publications

Scientific Publications

J. Louçano, P. Both, A. Marchesi, L. del Bino, R. Adamo, S. Flitsch, M. Salwiczek, P. Seeberger. Automated glycan assembly of *Streptococcus pneumoniae* type 14 capsular polysaccharide fragments. (submitted to RSC Advances)

Scientific Conferences and Symposia

Synthesis of Group B *Streptococcus* type III capsular polysaccharide related glycans. (**Oral**) "A. Corbella" International Summer School on Organic Synthesis, Gargnano, Italy, June 2019.

Optimization of Automated Glycosylation Cycles in the Glyconeer Automated Synthesizer. (**Poster**) International Carbohydrate Symposium, Lisbon, July 2018.

Towards Automated Synthesis of Type IV Group B Streptococcus Polysaccharide. (**Poster**) International GlycoBioTec Symposium, Berlin, February 2017.

Abstract

Group B *Streptococcus* (GBS) is a major neonatal pathogen and a leading cause of infant mortality worldwide. Management of GBS infection relies on the prophylactic and therapeutic use of antibiotics. This approach has limited efficiency and increases antibiotic resistance. Carbohydrate conjugate vaccines are consensually regarded as the best possible alternative and several formulations have been and are now in clinical trials.

In general, carbohydrate vaccine development suffers from shortfalls associated with the availability oligosaccharide libraries. Chemically pure glycans enable the prosecution of structure-immunogenicity studies that elucidate minimal epitopes and the intermolecular interactions responsible for recognition and immunity. One of the most promising methodologys to produce libraries of glycans is Automated Glycan Assembly (AGA). In this work, glycans from GBS are produced by AGA using the commercial synthesizer Glyconeer. The entire process is optimized since the production of building blocks to the automated oligosaccharide synthesis process.

Ready-to-use building blocks (BBs) are required in bulk to do automated experiments continuously. The synthesis of BBs can be achieved with a variety of different methods and the choice is usually based on the chemist preference and experience. This work optimizes and streamlines the strategies for the synthesis of building blocks for AGA bearing a thiolate aglycon as a leaving group. The synthetic schemes are completed and upscaled with minimal column purifications.

This thesis goes on to describing the optimization of elongation cycles that introduce each BB in the growing oligosaccharide chain during AGA. The first target is GBS type IV capsular polysaccharide. In the synthesis of GBS type IV, the major bottleneck was the assemble of the Glc-α-1,4-Gal cis linkage. A trisaccharide containing this linkage was successfully synthesized. However, the low yields obtained rendered the elongation of the chain unsuccessful.

The other target structures were related with GBS type III. With only three different BBs, four different GBS type III fragments were assembled. A sialic acid moiety present in the native polysaccharide was introduced enzymatically, proving that the combination of enzymatic sialylation with AGA is an expeditious approach to the preparation of complex glycans for vaccine development.

Overall, the work in this thesis is a contribution to the standardization of the chemical work involved in AGA and a steppingstone for the fast and cheap production of GBS related glycans.

Zusammenfassung

Die zur Lancefield-Gruppe B gehörenden Streptokokken (GBS) sind ein neonatales Pathogen und eine Hauptursache für die weltweite Kindersterblichkeit. Die Behandlung von GBS-Infektionen beruht auf prophylaktischem und therapeutischem Einsatz von Antibiotika. Die Effizienz dieses Ansatzes ist begrenzt und führt zu einem Anstieg von Resistenzen. Polysaccharid-Konjugat-Impfstoffe werden als beste Alternative zum Einsatz von Antibiotika gesehen und mehrere GBS-Impfstoff-Formulierungen werden derzeit in klinischen Studien evaluiert.

Ein generelles Problem bei der Entwicklung von Polysaccharid-Impfstoffen ist die begrenzte Zugänglichkeit zu Oligosaccharid-Bibliotheken. Chemisch reine Glykane erlauben die Durchführung von Struktur-Immunogenitäts-Studien, die das minimale Epitop und die intermolekularen Interaktionen, die für Erkennung und Immunität verantwortlich sind, aufklären. Eine der vielversprechendsten Methoden, um Glykan-Bibliotheken aufzubauen, ist die automatisierte Festphasensynthese von Glykanen (Automated Glycan Assembly, AGA). In dieser Arbeit werden GBS-Glykane durch den Einsatz von AGA hergestellt und der gesamte Prozess, von der Synthese der Monosaccharid-Bausteine bis zum automatisierten Prozess der Oligosaccharid-Synthese, optimiert.

Gebrauchsfertige Monosacharid-Bausteine werden in großem Maßstab für die kontinuierliche Ausführung automatisierter Experimente benötigt. Für die Synthese der Bausteine steht eine Vielzahl an Methoden zur Verfügung und die Entscheidung, eine Methode zu nutzen, basiert in vielen Fällen auf der Präferenz und Erfahrung des einzelnen Chemikers. In dieser Arbeit werden Synthesestrategien für Bausteine, die im AGA eingesetzt werden und eine Thiol-Abgangsgruppe haben, optimiert und rationalisiert. Die Syntheseschemata werden vervollständigt und in großem Maßstab mit wenigen säulenchromatographischen Aufreinigungsschritten durchgeführt.

Darüber hinaus wird in dieser Arbeit die Optimierung der Elongations-Zyklen, die die einzelnen Bausteine während des AGA in die wachsende Oligosaccharidkette einführen, beschrieben. Das erste Zielmolekül ist das GBS Typ IV kapsuläre Polysaccharid. Die Herausforderung bei der Synthese dieses Polysaccharids ist die

Glc-α-1,4-Gal-cis-Verknüpfung. Ein Trisaccharid, das diese Verknüpfung enthält, wurde erfolgreich synthetisiert. Jedoch zeigen die geringen Ausbeuten, dass die Elongation der Zuckerkette nicht erfolgreich war.

Die weiteren Zielmoleküle in dieser Arbeit gehören zu der GBS Typ III. Mit nur drei unterschiedlichen Bausteinen, wurden drei GBS Typ III-Fragmente dargestellt. Ein Sialinsäure-Rest, der im natürlichen Polysaccharid vorkommt, wurde enzymatisch eingeführt. Dies zeigt, dass die Kombination aus enzymatischer Sialylierung mit AGA einen schnellen Zugang zu komplexen Glykanen für die Impfstoffentwicklung darstellt.

Zusammenfassend ist die vorgelegte Arbeit ein Beitrag zur Standardisierung der chemischen Schritte, die Teil des AGA sind und ein Sprungbrett für die schnelle und preisgünstige Produktion von Glykanen, die mit der GBS verwandt sind.

Contents

List of figures

List of schemes

List of tables

Abbreviations

Ac	Acetyl group
ACN	Acetonitrile
AGA	Automated Glycan Assembly
Ar	Aromatic
BB	Building Block
cat.	Catalyst
CIP	Contact ion pair
CMPI	2-Chloro-1-methylpyridinium idodide
CPS	Capsular Polysaccharide
d	Doublet (relative to NMR peaks)
DABCO	1,4-Diazabicyclo[2.2.2]octane
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
dd	Doublet of doublets (relative to NMR peaks)
DMAP	4-(Dimethylamino)pyridine
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
dt	Doublet of triplets (relative to NMR peaks)
EOD	Early-onset disease (relative to Group B Streptococcus infection)
equiv	Equivalents
Fmoc	Fluorenylmethyloxycarbonyl
GBS	Group B Streptococcus
GBSIII	Group B *Streptococcus* type III
h	Hours
HMBC	Heteronuclear Multiple-Bond Correlation spectroscopy
HSQC	Heteronuclear Single Quantum Correlation spectroscopy
IAP	Intrapartum antibiotic prophylaxis
Lev	Levaloyl
LOD	Late-onset disease (relative to Group B Streptococcus infection)
m	Multiplet (relative to NMR peaks)
Mbp	5-tert-butyl-2-methylphenyl
min	Minutes
NIS	*N*-Iodosuccinimide
NMR	Nuclear Magnetic Resonance spectroscopy
ppm	Parts per million
Py	Pyridin
q	Quartet (relative to NMR peaks)
rf	Retention factor
rt	Room temperature
s	Singlet (relative to NMR peaks)
Sp14	Streptococcus pneumoniae type 14
SSIP	Solvent-separated ion pair
t	Triplet (relative to NMR peaks)
TBAI	Tetrabutylammonium iodide
TBS	t-Butyl-dimethylsilyl
TCA	Trichloroacetyl
TFA	Trifluoroacetic acid
TLC	Thin Layer Chromatography
TMSOTf	Trimethylsilyltrifluoromethanesulfonate
δ	Chemical shift

a journey of a thousand miles begins with a single step

(Chinese proverb)

1. Introduction

1.1. Group B Streptococcus

1.1.1. Epidemiology and Management

Streptococcus agalactiae or Group B Streptococcus (GBS) is a Gram-positive bacterium that is often part of the normal human gastrointestinal and vaginal flora. Since the 1970s, GBS has emerged as a major neonatal pathogen and today it is responsible for at least 409 000 maternal/fetal/infant infection cases (**Figure 1.1**) and 147 000 stillbirths and infant deaths globally.[1] GBS invasive disease in infants is classified in early-onset disease (EOD), when it develops on days 0-6 after birth, and late-onset disease (LOD), when it develops between the 7th and the 89th day of life. The most common manifestations of EOD are pneumonia and sepsis. Newborns with EOD frequently develop respiratory failure and septic shock. LOD is more likely associated with meningitis, which can lead to chronic neurological sequelae or even death. GBS is also responsible for infections in pregnant women and is increasingly associated with infections in elderly and immunocompromised populations.[2]

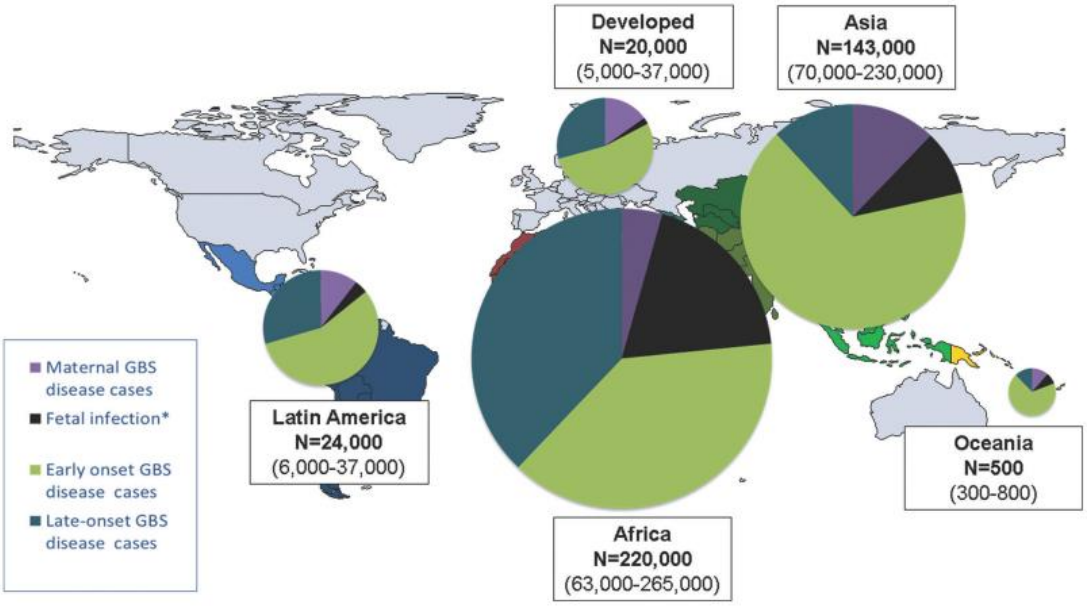


Figure 1.1 Estimated number GBS disease cases in pregnant/postpartum women, fetuses and infants in 2015.[1]

Maternal colonization is the main transmission pathway in EOD, with vertical transmission occurring at or just before birth. The most widely used strategy for prevention of GBS disease is the use of intrapartum antibiotic prophylaxis (IAP) based on GBS colonization status. Pregnant women are subjected to a microbiological screening with a rectovaginal swab at 35-37 weeks´ gestation. If they test positive for GBS or if they present relevant clinical risk factors (such as previous infant with GBS disease, preterm labour, maternal pyrexia) they are given benzylpenicillin or ampicillin intrapartum. The implementation of IAP significantly decreased the number of EOD events. However, no impact was observed in cases of LOD, and GBS persists as the leading cause of mortality and morbidity in neonates. Furthermore, the microbiological screening policies and the coverage of IAP is substantially heterogeneous worldwide. In the UK, IAP is given based on risk factors and no universal screening is recommended. On the other hand, many low- and middle-income countries have no IAP policy or report a low coverage for its implementation. The major obstacles are in countries that lack healthcare professionals and infrastructure or where most births occur at home.

An important drawback of the IAP is the increase in antibiotic resistance. In case of GBS, only a small number of isolates resistant to β-lactam antibiotics (penicillin and analogues) have been reported. However, there is an increasing resistance to erythromycin and clindamycin, which are the alternative options for penicillin-allergic women. In this context, a vaccine against GBS may provide an ideal solution, with a higher chance for global coverage and a possibility of higher efficacy against EOD and LOD.[3]

1.1.2. Capsular Polysaccharides

Capsular polysaccharides are the most promising vaccine targets in GBS and therefore it is important to understand their structure and role. The capsule constitutes a powerful virulence factor in GBS. It helps evading the immune system of the host by preventing the binding of complement proteins, decreasing the phagocytic activity of neutrophils, and promoting intracellular survival inside

dendritic cells.[2] Chemically, the capsule is made of a capsular polysaccharide (CPS) that mimics the host epitopes. GBS has been classified in serotypes according to the structure and immunogenicity of the CPS. To date, ten different serotypes are known: Ia, Ib, and II–IX. All serotypes are associated with colonization in pregnant women with serotype distribution subjected to variation according to the geographical region. The global estimated prevalence of maternal colonization is 18%, with lower prevalence in Eastern and Southern Asia (around 11%).[4] The global incidence of invasive GBS disease in infants is 0.49 per 1000 live births and is higher in Africa and lower in Asia. Serotype III alone accounts for over half of the cases of invasive disease by GBS (62%), followed by serotypes Ia, Ib, V and II. Together, these 5 serotypes account for 97% of the disease-causing isolates. Serotype Ia and V are dominant invasive isolates in non-pregnant cases. The prevalence of serotype IV is rising in invasive cases both in neonates and adults.[2,5]

1.1.3. Structure of GBS Capsular Polysaccharides

The chemical structures of GBS CPSs is described for all the serotypes and represented in **Figure 1.2**.[6,7] Except for type VIII, which bears a rhamnose unit, all the repeating units are built with four monosaccharides (*i.e.* Glc*p*, Gal*p*, Glc*p*NAc, and Neu*p*NAc) and the glycosidic linkage pattern is unique to each serotype. The repeating units accommodate one or two side chains and the Neu*p*NAc-α-2,3-Gal motif is always found at the terminus of one of the side chains.

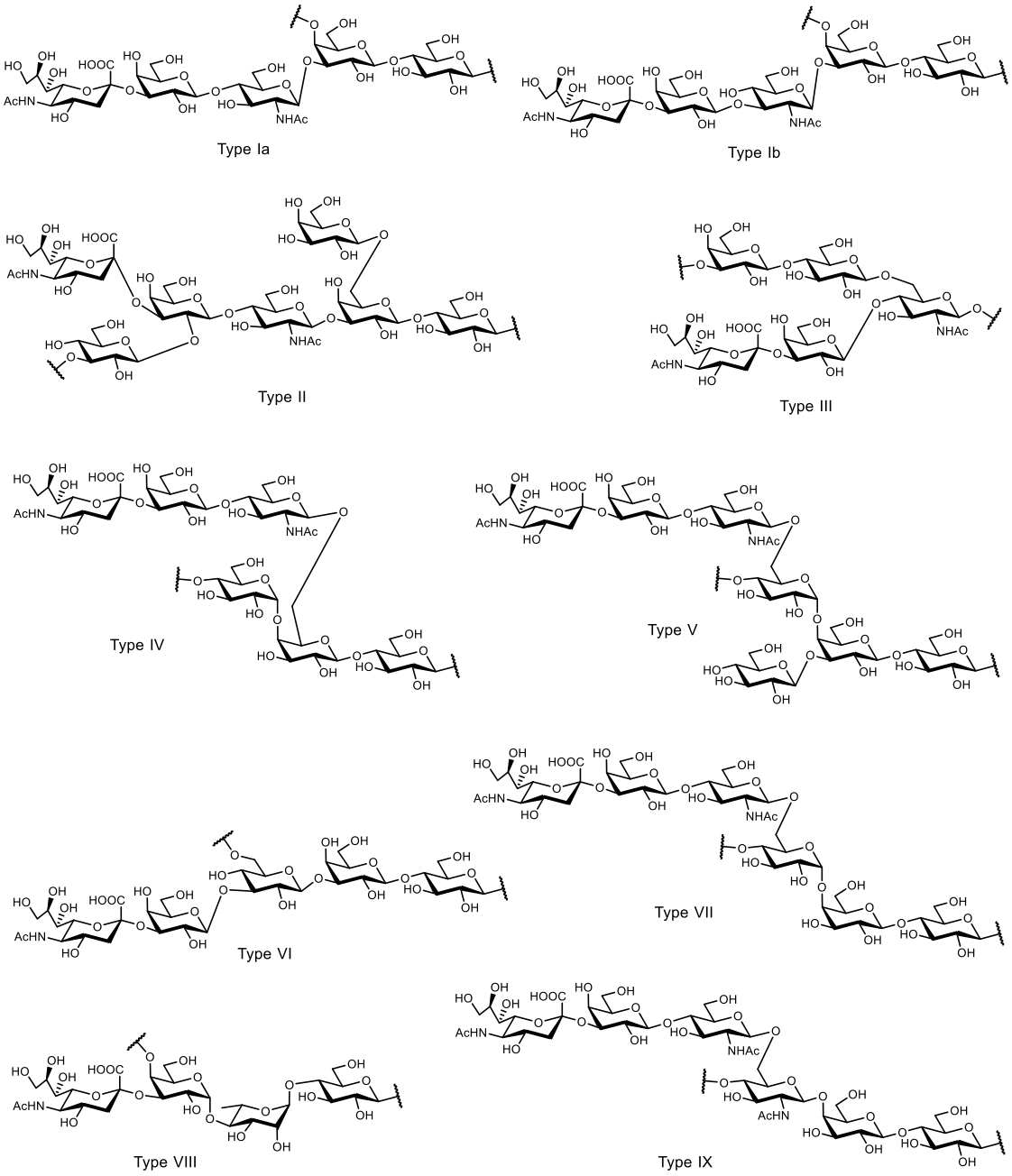


Figure 1.2 Repeating unit of the CPS of all the GBS serotypes.

1.1.4. Progress on GBS Vaccine Development

The research in CPS based GBS vaccines has been going on for decades. In 1976, Baker and Kasper showed that low levels of maternal antibodies against type III CPS were strongly correlated with neonatal susceptibility to GBS EOD or LOD.[8] This finding provided the proof of concept that maternal vaccination could be a

suitable effective strategy to prevent GBS infection in newborns, supporting the rationale for the development of a vaccine against GBS using CPS as antigen. After this, several vaccines have been in clinical trials. In the first generation, unmodified type-specific polysaccharides were used. Purified native type Ia, II or III polysaccharides were injected in healthy adult volunteers, including pregnant women. Soon it was observed that unconjugated carbohydrate vaccines generate a heterogeneous response among the patients and are not capable of inducing B-cell memory response, particularly in infants. The immunogenicity of polysaccharides as human vaccines is enhanced by conjugation to protein carriers, and a second generation of vaccines conjugated to tetanus toxoid (TT) was tested. The third generation came with the pursuit of a broadly effective vaccine against the most common disease-causing strains: a multivalent conjugate formulation.[9]

More recently, a trivalent formulation (Ia, Ib, III) was tested in phase I and II clinical trials and showed favourable safety and immunogenicity in non-pregnant and pregnant women.[10] A hexavalent formulation (Ia, Ib, II, III and V) that covers 97% of the invasive disease cases is currently undergoing phase I/II clinical trials in pregnant women (NCT03765073).

The development of an anti-GBS vaccine suitable for immunization during pregnancy has been listed as a priority by the World Health Organization (WHO). An effective vaccine would induce the production of functionally active antibodies that could cross the placenta and provide protection to the infant. The most promising vaccine candidates able to confer protection and induce functionally active antibodies are represented by capsular polysaccharide (CPS) and surface proteins.

1.1.5. Structure-Immunogenicity Studies

Carbohydrate-based vaccines are typically obtained as heterogeneous and complex mixtures of poly- or oligosaccharides covalently linked to the immunogenic carrier protein through non-specific methods. Consequently, knowledge about correlation between structure and immunological properties is scarce. The most prevalent serotype within invasive GBS isolates is type III. Synthetic work has been done

to prepare libraries of fragments from the GBS type III polysaccharide (GBS PSIII) a substantial amount of studies about GBS PSIII minimum epitope has been conducted.

With access to chemically pure GBS PSIII fragments, the protective epitope was elucidated by means of SPR, competitive ELISA, and STD-NMR experiments, and complemented with MD simulations. GBS PSIII forms a helical structure where the negatively charged sialic acid residue of the α-NeuNAc-(2→3)-β-D-Galp branch points outwards the trisaccharide backbone structure →4-β-D-Glcp-(1→6)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→. A sialic acid-dependent functional epitope was found to be fully contained within four consecutive monosaccharide units deriving from the PSIII backbone and one branched disaccharide present in this sequence. The length dependent affinity to mAbs, once attributed to the existence of a conformational epitope, is now attributed to a multivalence effect.[11]

These findings have critical implications in understanding the protective immunity against GBS type III and in the design of conjugate vaccines. Similar studies for other serotypes are scarce or non-existent. The structural complexity of carbohydrates, with α and β linkages, and with an array of possible branching sites, results in highly specific spatial motifs that are serologically distinct, even when few monosaccharides are at stake. For instance, each GBS serotype induces a type specific response, even when the structural similarity is high (e.g. types IV and VII).[12] The existence of helical conformational epitopes, which implies the need for longer oligosaccharides, has been considered a challenge for epitope-mapping studies.[13] The elucidation of functional epitopes requires elaborate synthetic efforts to procure chemically pure oligosaccharides. This has been a major bottleneck for the advancement of knowledge in this field.

1.2. Production of Carbohydrates in the Context of Vaccine Development

Carbohydrates play a pivotal role in recognition processes associated with adhesion, cell-cell interactions, infection and immunity[14] and have been exploited for protective vaccination for decades. Currently, there are several vaccines in the

market based on carbohydrate antigens such as *Haemophilus influenza* type B (Hib), *Streptococcus pneumoniae*, and *Neisseria meningitidis*.

Conventional carbohydrate vaccine development and manufacture relies on the cultivation of microorganisms and subsequent extraction of antigens through biochemical and microbiological methods. Cell cultures constitute an important source of carbohydrate antigens since they allow for large scale production and access to complex structures. However, antigen isolation and depolymerization is complex, costly, and results in a heterogeneous mixture of glycans. Moreover, the presence of contaminants may result in undesired effects on the immune response and introduces safety concerns.[15]

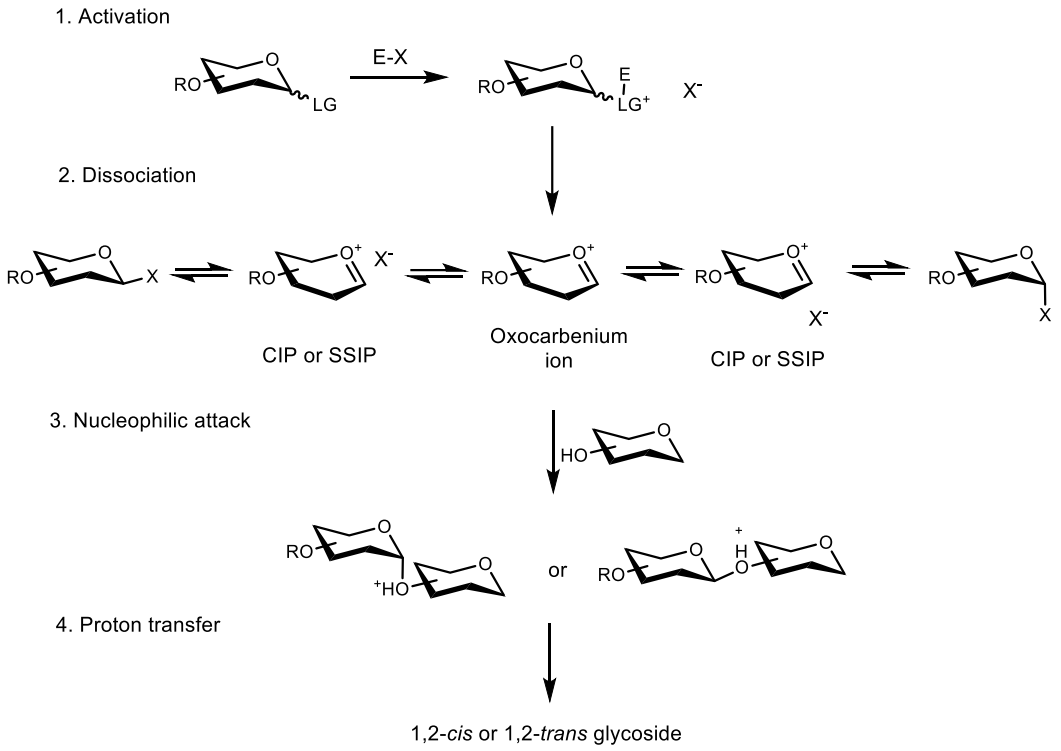
Chemical synthesis provides access to chemically pure oligosaccharides that can be used to do epitope mapping and structure-immunogenicity relationship studies such as with GBS type III.[11] The importance of synthetic carbohydrates was further highlighted with the approval of a chemically synthesized oligosaccharide Hib vaccine in Cuba in 2004.[16]

1.2.1. Chemical Synthesis of Carbohydrates

The key step in carbohydrate synthesis is the glycosylation reaction where a glycosyl donor (electrophile) reacts with a glycosyl acceptor (nucleophile). The formation of the glycosidic bond generates a stereogenic centre. Therefore, a tight control of regio- and stereoselectively is required.

Mechanism of glycosylation

The formation of the glycosidic bond happens upon displacement of a suitable leaving group. The reaction develops in four steps as depicted in **Scheme 1.1**. [17,18]



Scheme 1.1 Simplified outline of glycosylation mechanism. E-X is the activator. CIP: contact ion pair. SSIP: solvent separated ion pair.

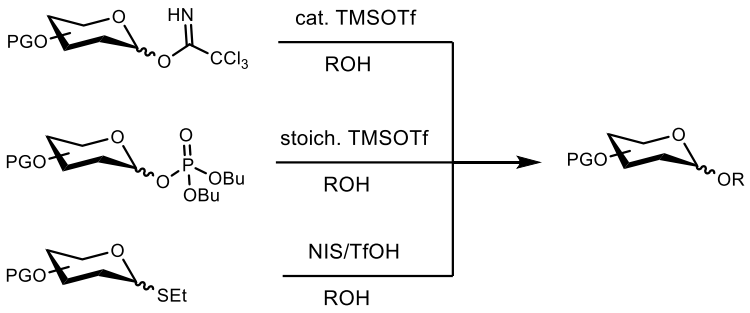
In the first step, the donor is activated as a result of the interaction of the LG with the activator. Under particular conditions, the acceptor attacks the donor at this step in a S_N2 fashion, leading to a stereospecific reaction with inversion of configuration. Most of the time, the reaction proceeds with the second step where the activated leaving group dissociates. This is a typically irreversible transformation and the rate-limiting step. It leads to the generation of a glycosyl cation that resonates into the oxocarbenium ion form. In turn, the oxocarbenium ion can interact with the counter ion to form contact ion pairs (CIP) or solvent separated ion pairs (SSIP) where either the alpha or beta are free to form a covalent bond giving rise to the alpha or beta anomers. In the third step, the nucleophilic attack determines the stereochemical outcome. The oxocarbenium ion, with its sp^2-hybridized anomeric carbon, allows the attack from both faces. The ion-pairs will favour one of the faces over the other and the covalent species will undergo a S_N2-type transformation with inversion of configuration. On the other hand, the acceptor electronic, steric and conformational properties translate into an inherent reactivity and stereochemical preference. As a balance between all these factors, the outcome

is often a mixture of anomers. In the fourth step, a proton transfer renders the glycosidic bond formation irreversible.[17,19]

In order to gain control on the glycosylation product, both donor and acceptor are protected with selected protecting groups (PGs) and a leaving group that have a direct effect on the overall reactivity of the building block as well as on the stereo- and regioselectivity.

The choice of the leaving group

The classic Fischer glycosylation where a free sugar is activated by hydrochloric acid or the Koenigs-Knorr glycosylation where a glycoside halide is activated with silver carbonate have been supplanted by more efficient methods. Nowadays, a number of leaving groups has been developed and currently, imidates, phosphates, and thiolates are amongst the most popular ones.



Scheme 1.2 Activation of imidate, phosphate and thiolate glycosyl donors.

Glycosyl trichloroacetimidates can be readily prepared through the addition of the anomeric hydroxyl to trichloroacetonitrile in the presence of an inorganic (such as NaH, K_2CO_3 or $CsCO_3$) or organic base (such as DBU). Imidates require a strong Lewis acid for activation such as TMSOTf, $BF_3{\cdot}OEt_2$, Tf_2O or AgOTf.[20] Trichloroacetimidates are more reactive than trifluoracetimidates.[20] This difference in reactivity has been exploited to perform one-pot oligosaccharide synthesis where a trichloroacetimidate donor was activated with $Yt(OTf)_3$ in the presence of a trifluoroacetimidate acceptor. The resulting disaccharide was activated by adding $Bi(OTf)_3$ and a new monosaccharide donor to generate a trisaccharide. The same sequential transformation was achieved with $Bi(OTf)_3$ as activator in both steps

where the temperature served as a switch to the selective activation (lower temperature activation of trichloroacetimidate and higher temperature activation of trifluoroacetimidate) .[21]

Glycosyl phosphates are highly reactive donors that can be prepared by the epoxidation of a glycal with DMDO followed by opening of the epoxide with a dialkylphosphate.[22] Alternatively, phosphates can be prepared from thioglycosides by activation in the presence of dialkylphosphate.[23] Phosphate donors give high yielding couplings upon activation with stoichiometric amounts of TMSOTf. When such a strong acidic media poses a problem, phosphate donors can be activated under mild neutral conditions in concentrated solutions of lithium perchlorate in organic solvents, or with a silylated acceptor and a catalytic amount of TfOH.[24]

Thioglycosides are versatile glycosyl donors. The anomeric thiolate is stable towards a variety of conditions used for protecting-group manipulations and is installed at the early stages of the building block synthetic scheme. Thiolates are easily prepared from the peracetylated sugar by reaction with the appropriate thiol in the presence of BF_3·OEt. On the other hand, thioglycosides are easily activated with thiophilic species under mild conditions. The soft sulfur atom provides easy and selective reactivity with soft electrophiles. Activator systems for thioglycosides include NIS/TfOH, NBS/TfOH, and AgOTf.[20] Thioglycoside reactivity can be tuned by manipulating the aglycon group. An ethyl thioglycoside was selectively activated in the presence of a toluyl thioglycoside acceptor, that was subsequently activated in a second glycosylation reaction.[25] The sulphur substituent is also important to avoid aglycon transfer when a thioglycoside is used as an acceptor. Bulky substituents such as 2,6-demethylphenyl are able to prevent aglycon transfer.[26]

Controlling the stereochemical outcome

In a glycosylation reaction, both donor and acceptor possess inherent preferences for the formation of either the α or β anomer. Mannose has an inherent preference to form α-glycosides whereas glucose and galactose have an inherent preference to form β-glycosides.[27] One of the big challenges of carbohydrate synthesis is to articulate environmental variables in order to create conditions that

enhance or override inherent preferences and lead to stereoselective glycosidic bond formation.

The formation of 1,2-trans glycosidic linkages is made easy by installing a participating group at O2. By neighbouring group participation, an acyl group at O2 stabilizes the oxocarbenium ion generating an acyloxonium ion that resolves upon attack of the nucleophile from the available trans-face. Donors with acyl protecting groups at O2 generally show high 1,2-trans stereoselectivity.[17] The stereoselective outcome is also affected by remote substituents, particularly those with strong electron-withdrawing effect, steric hindrance or remote participation. Although remote participation can be weaker than for O2 substituents[17], it has been successfully used previously. For instance, α-glucosides are successfully installed when the glucoside building block bears an acetyl group at O6. The α/β ratio rises from 8:1 to 11:1 when the O3 position is also acetylated. In the case of galactose building blocks, O4-acetylation leads to α-galactosides.[28]

The solvent can play an important role in defining the stereochemical outcome of a glycosylation. Ethers such as diethyl ether, dioxane and tetrahydrofurane stabilize the oxocarbenium ion by generating an equatorial O-linked intermediate. The β face becomes sterically blocked and the α glycoside is preferentially formed. On the other hand, nitrile solvents such as acetonitrile form an axial glycosyl nitrilium cation and thus promote β-glycoside formation.

Several other factors including temperature, amount, and type of activator used can influence the stereochemical outcome of glycosylations. Kinetically controlled reactions at low temperatures favour the formation of β linkages, particularly for glucose and galactose. However, opposite results have also been reported. [17,27]

Synthesis of oligosaccharides

Oligosaccharide chains can be prepared following a linear or a convergent approach. In the linear stepwise approach, each monosaccharide is added sequentially. After glycosylation, additional synthetic steps may be required to

transform the resulting disaccharide into the donor or acceptor for the next step. The cycle repeats for as long as the target oligosaccharide chain. This approach is very straightforward but can lead to a drastic drop in yields as the sequence gets longer. In a convergent strategy, smaller oligosaccharides are pre-synthesized and linked together by a glycosylation reaction. The strategy provides time savings due to the use of oligomeric building blocks and can be particularly useful for the synthesis of sequences with two or more repeating units.

To expedite oligosaccharide synthesis, efforts have been made to develop strategies that reduce or eliminate the manipulations in between glycosylation steps. These strategies explore the relative reactivity of the building blocks based on their leaving groups and protecting groups as well as the inherent reactivity of the hydroxyl groups of the acceptors.

Chemoselective activation can be achieved using the armed-disarmed concept. Armed donors are equipped with electron donating groups (e.g. benzyl ether) and can be selectively activated in the presence of disarmed donors, which display electron withdrawing groups (e.g. acyl groups). The disarmed building block can be activated in a subsequent glycosylation reaction without the need for additional transformations. Another strategy is the use of different leaving groups that can be activated in a selective fashion. Thiocyanate glycosyl donor, for example, is activated over an S-thiazolinyl acceptor that can be activated in a subsequent glycosylation. Fluoro and S-phenyl orthogonal leaving groups can be used in sequential glycosylations by alternating the use of the thioglycoside donors, activated with NIS/AgOTf, with the use of fluoride donors, activated with $Cp_2Hf_2Cl_2$/AgOTf.[17]

One-pot glycosylations are interesting approaches to oligosaccharide synthesis. Knowledge of the relative reactivities of a library of glycosylating agents is the basis for the sequential one-pot combination of building blocks that can serve as glycosylating agents as well as nucleophiles. Despite conceptually attractive, the strategy requires many different building blocks. Moreover, each building block requires adjustments in reaction temperature and time which further complicates glycan syntheses.[29]

An important strategy to avoid lengthy chromatographic purifications in between glycosylation steps is the supported synthesis. A solid support allows for the use of excess building blocks and reagents that are easily washed away by filtration. At the same time, it reduces the oligosaccharide synthesis to a simplified iterative process setting the scene for an automated platform.

1.3. Automated Glycan Assembly

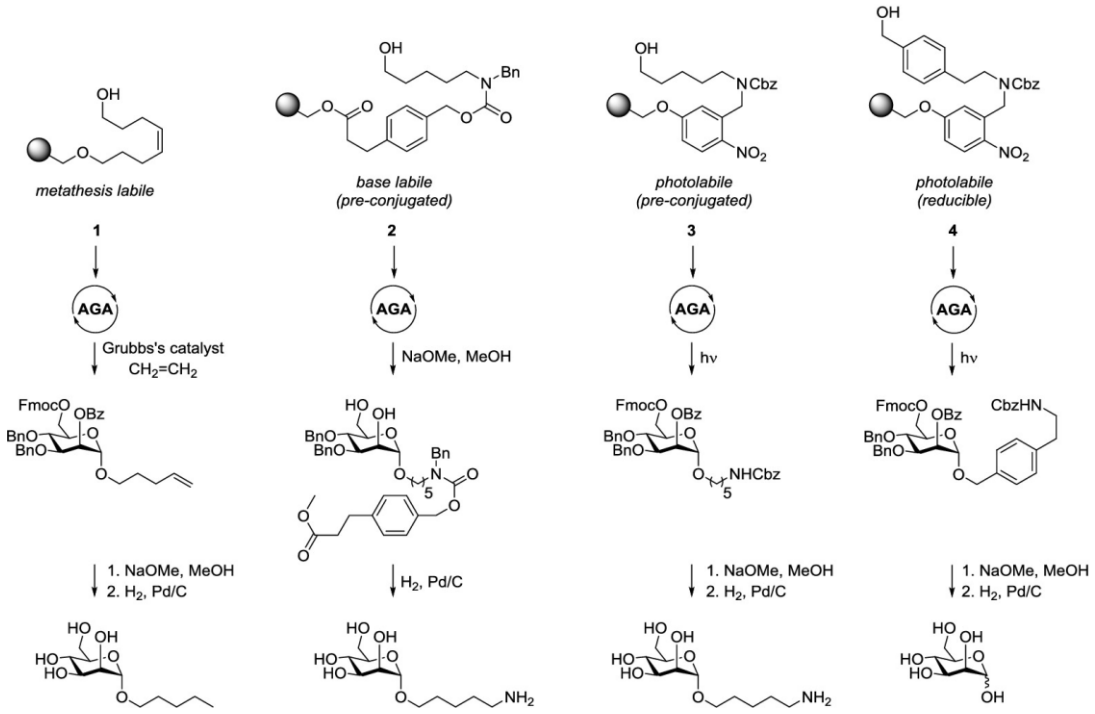
Automated Glycan Assembly (AGA) is based on a solid support equipped with a linker that is used to attach successively one building block after another with all synthetic manipulations being executed by automated instrumentation. Upon completion of AGA, the resulting oligosaccharide is cleaved from the solid support and purified by chromatography. The solid support can be equipped with either a nucleophile (acceptor-bound approach) or a glycosylation agent (donor-bound approach). The former approach proved to be more convenient as glycosyl donors are more prone to undergo side reactions and can be used in excess to ensure higher yields[30].

Solid phase and linker

With stability towards a wide range of chemical conditions and with established efficiency in the peptide field, the Merrifield resin is widely used for solid-phase carbohydrate synthesis. The resin is functionalized with a linker that provides the first point of attachment for the growing oligosaccharide chain. At the same time, the linker bears a functionality that allows for cleavage of the glycan from the resin at the end of chain elongation. This functionality is key as it must be orthogonal to all the chemistry used during the oligosaccharide synthesis.

Some of the most popular linkers are depicted in **Scheme 1.3**. The metathesis labile linker has been used in the synthesis of a wide range of structures, but it cannot be used with thioglycoside donors as they are activated in an electrophilic environment (NIS/TfOH) that reacts with the double bond. The pre-conjugated

base-labile and photolabile linkers generate oligosaccharides with a 5-aminopentanyl chain that can be used as a handle for the preparation of microarrays or conjugation with proteins. The photolabile "reducible" linker provides glycans with free reducing ends. The big advantage of photolabile linkers is the exceptional orthogonality and the mild of cleaving conditions.[30,31]



Scheme 1.3 Linkers for automated glycan assembly. Adapted from ref. 32.[32]

Building blocks

Thioglycosides, glycosyl phosphates and glycosyl imidates have been successfully used in AGA. Thioglycosides are activated with NIS/TfOH while phosphates and imidates are activated with TMSOTf in stoichiometric or catalytic amounts respectively. Thioglycosides are particularly interesting from the commercial point of view as they are stable for long periods of time and offer the possibility of large-scale production and storage. At the same time, thioglycosides react at higher temperatures than phosphates and imidates which reflects in time savings during cooling steps of the automated synthesis.[30,33]

To ensure stereoselectivity, the building blocks are equipped with participating protecting groups, generally benzoyl, trichloroacetamide or acetyl groups. The temporary protecting group of choice is 9-fluorenylmethyloxycarbonyl (Fmoc) as it is quantitatively cleaved with Et_3N or piperidine in only five minutes. Other temporary protecting groups are used for branching building blocks such as levulinoyl esters (Lev), which are cleaved with hydrazine, and naphtyl ether (Nap), cleaved with DDQ[30,33].

1.4. The Glyconeer: The First Commercial Automated Glycan Synthesizer

1.4.1. Description of the Hardware

The Glyconeer (**Figure 1.3**) is a versatile automated platform designed to address the challenges of oligosaccharide solid-phase synthesis. The Glyconeer delivers solvents and reagents into a reaction vessel, incubates the reaction vessel contents at a controlled temperature, mixes the contents of the reaction vessel through gas bubbling, and drains the resulting solutions into the waste.

Solvents and reagents are delivered in a fast and accurate manner through a pressure-driven system. Argon pushes solvents and reagents through valves and tubes that are inert to volatile and corrosive reagents. The pressure of argon is controlled by the software and determines the flow rate of the corresponding solvent or reagent. There are eight positions available for solvents. The reagents are divided in three blocks: the basic block, consisting of 6 positions for basic reagents; the acid block, consisting of six positions for acidic reagents; the aqueous block, consisting of four positions for reagents containing water. The acidic block contains a thermoelectric cooling device (Peltier) where two reagent bottles can be stored at 0 °C (intended for activator solutions).

Building blocks are loaded into specific vials that are placed in a carousel with 64 vial positions. The carousel positions the appropriate vial below a two-way

needle that can deliver solvents to dissolve solid building blocks and uptakes the building block solution and delivers it to the reaction vessel.

Figure 1.3 The automated solid phase oligosaccharide synthesizer Glyconeer.

The central piece of the Glyconeer is the reaction vessel (RV), located in the centre of the instrument. The RV is a 15 mL triple jacketed cylindrical vessel fitted with a frit at the bottom. The inner compartment closes with a conical cap that has two openings, each connected to a PTFE line: an exhaust line that leads to the exterior and an "in line" through which solvents, reagents and building blocks are delivered into the RV. The temperature of the RV is controlled through a coolant that flows through the middle compartment. The coolant is pumped by an external cryostat with a temperature range from -40 °C to +80 °C. The external compartment of the RV is a vacuum compartment to increase the insulation.

Below the RV, an on-line UV detector measures the UV transmittance of the solution discharged from the RV after cleavage of the Fmoc protecting group. The decrease in transmittance caused by the generated dibenzofulvene provides an indirect measure of the coupling efficiency.

1.4.2. Pre-Programmed Modules

The synthesis of oligosaccharides using the Glyconeer is based on the repetition of an elongation cycle that incorporates one building block into the growing oligosaccharide chain. During the elongation cycle, the building block is activated and glycosylates the hydroxyl group of the acceptor, and the temporary protecting group is removed to prepare the introduction of another building block during the following elongation cycle. These repetitive tasks are done according to a synthesis plan that is scheduled in the Glycosoft (the software that operates the Glyconeer) by combining pre-programmed modules in the appropriate order. Each module completes a specific task. When scheduling a synthesis, the software enables the user to modify a certain number of variables in each module. The pre-programmed modules are described in **Table 1.1**.

Table 1.1 Description of the pre-programmed modules used in the Glyconeer

Module	**Description**
1. Resin swelling	DCM is delivered to the reaction vessel and the resin is incubated for 30 min at 25 °C.
2. Acid Wash	The resin is washed with a solution of TMSOTf in DCM at -20 °C.
3. Glycosylation with thioglycosides	Building block and the activator solution (NIS/TfOH) are delivered to the reaction vessel at temperature T1 and incubated for a period of time t1. The temperature is then raised to T2 and an incubation time of t2 min is followed.
4. Glycosylation with phosphates	Building block and the activator solution (TMSOTf in DCM) are delivered to the reaction vessel at temperature T1 and incubated for a period of time t1. The temperature is then raised to T2 and an incubation time of t2 min is followed.
5. Capping	Resin is washed with a 10% solution of pyridine in DMF and then capped by acetylation with a solution of Ac_2O and MsOH in DCM at 25 °C.
6. Fmoc Deprotection	Fmoc is cleaved with piperidine 20% (v/v) in DMF.
7. Lev Deprotection	Lev is deprotected with 0.15 M hydrazine acetate in Py/AcOH/H_2O

In the “resin swelling” module, DCM is delivered to the RV and the resin is allowed to swell. The incubation time is standardized (30 min) but can be changed by the user. The “acid wash” module washes the resin and reaction vessel with a

solution of TMSOTf to quench any base left over from previous steps that would interfere with acid-mediated activation[34]. In the "glycosylation" module, DCM is delivered to the RV while the temperature is set to the appropriate value. When the temperature is reached, DCM is drained, and the BB solution and activator are sequentially added. The temperature is gradually increased to drive the glycosylation to completion. The "capping" module washes the resin with a solution of pyridine in DMF and proceeds with acetylation of the unreacted hydroxyl groups with acetic anhydride and methanesulfonic acid[35]. The "Fmoc deprotection" module delivers a solution of piperidine in DMF that cleaves the Fmoc group. The "Lev deprotection" promotes cleavage of Lev with hydrazine acetate in a pyridine/acetic acid buffer.

1.4.3. Validation of the Glyconeer

The Glyconeer was validated with the synthesis of motifs of the *Mycobacterium tuberculosis* cell surface and α-Gal epitopes expressed in cancer cells.[34] The three oligosaccharides (**Figure 1.4**) constitute various degrees of complexity and each requires several different building blocks. The linear hexasaccharide **1.15** contains multiple 1,2-trans linkages, hexasaccharide **1.14** is a branched structure with 1,2-trans linkages, and trisaccharide **1.16** contains both 1,2-trans and 1,2-cis linkages. The three oligosaccharides were assembled in the Glyconeer with the BBs depicted in **Figure 1.4**. After the synthesis, they were cleaved from the solid support in a photoreactor and purified by normal phase HPLC to eliminate deletion sequences and unwanted stereoisomers. The protected hexasaccharide **1.12** was obtained in 38% yield. The protected branched hexasaccharide **1.11** was obtained in 24% yield. The protected trisaccharide **1.13** was obtained in 25% yield (yields based on resin loading). The protecting groups were removed by methanolysis with NaOMe followed by hydrogenation with Pd/C. Purification by reverse-phase HPLC afforded the fully deprotected conjugation-ready glycans.

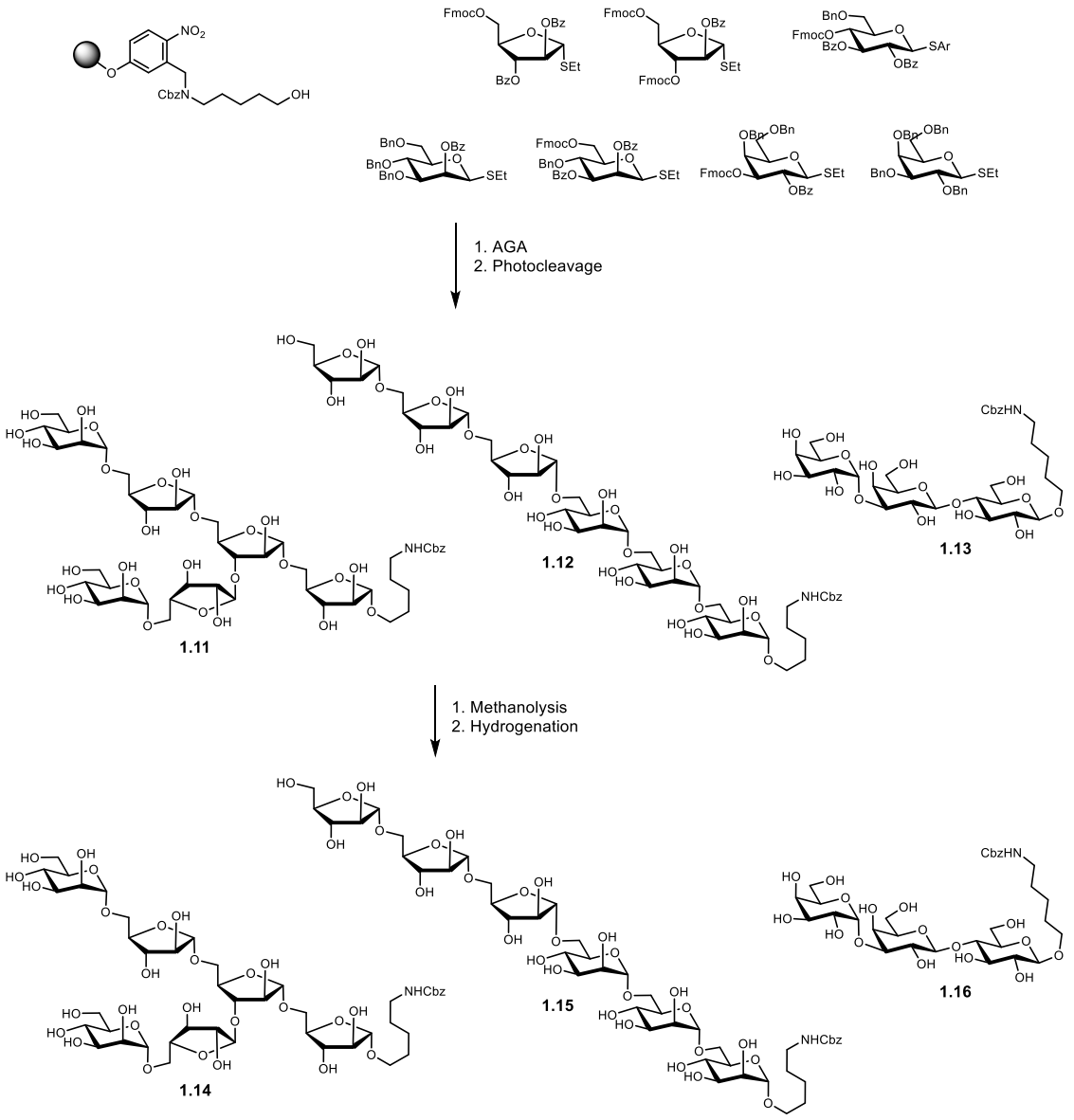


Figure 1.4 Glycans synthesized with the Glyconeer.[34]

1.5. Aim of the Thesis

Chemically defined oligosaccharides are pivotal for the elucidation of functional epitopes and for the development of safer and more efficient carbohydrate vaccines. The overall goal of this work is to develop and standardize methods for automated glycan assembly in order to used them as tools to advance the field of vaccine development, particularly in the case of Group B Streptococcus.

In the first part of the thesis, the focus is on standardizing the production of differentially protected building blocks. The methods are critically revisited, alternative strategies are compared, procedures are tested for reproducibility and scalability.

In the second and third chapters of this thesis, the emphasis is on the automated glycan assembly. The target is the synthesis of oligosaccharides from type IV and type III GBS capsular polysaccharide using the Glyconeer. Special attention is given to develop standardized conditions for the formation of each glycosidic linkage. In the case of GBS type III, different frameshifts of the repeating unit are synthesized. The obtained glycans can be used in structural and immunological studies.

2. Synthesis of Building Blocks

2.1. Introduction

One of the biggest advantages of automation is time saving. The Glyconeer constitutes a platform for rapid production of oligosaccharides. In a few hours, disaccharides, trisaccharides, or tetrasaccharides can be synthesized. Compared to solution-phase methods, AGA makes use of a large excess of donor (typically 6.5 to 10 equivalents). Starting materials, that is, building blocks and functionalized resin, are required in bulk to do automated experiments in a continuous way.

"Approved" building blocks are differentially protected building blocks that can be used in AGA in a versatile and reliable fashion. Also, they should be accessible cheaply and fast: this translates into reproducible and high yielding synthetic schemes. Thioglycosides as commonly used glycosyl donors. Fluorenylmethyloxycarbonyl (Fmoc) and levulinoyl (Lev) groups are convenient temporary protecting groups. Acyl groups such as benzoyl, acetyl, or trichloroacetyl (TCA) are used as permanent participating groups and benzyl ethers are used as permanent non-participating groups.[30,33] Protecting group manipulations in carbohydrate chemistry have been extensively studied and there are published procedures for the transformations needed to prepare the "approved" building blocks. However, the lack of mechanistic novelty led to a declining interest in optimizing those procedures.

This chapter deals with pre-automation work necessary for the synthesis of GBS type IV CPS. Aiming at generating knowledge for routine production and developing standard operating procedures in an industrial setting, the main goals are the optimization and upscaling of procedures. Special emphasis is given to developing crystallization conditions to avoid chromatographic purifications and facilitate large scale production, to reduce the amount of reagents in order to reduce cost in large scale production, and to assess reproducibility to allow for competitive price calculations.

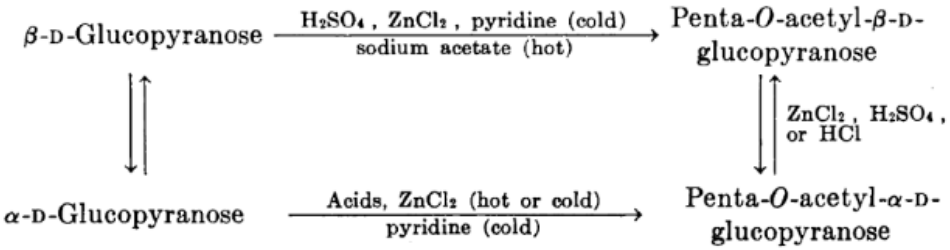
In the first part of this chapter, the focus is on the first two transformations of the free monosaccharides, peracetylation and thioglycosylation. In the second part, a retrosynthetic analysis of GBS type IV CPS is discussed as well as the synthesis of

the corresponding building blocks. In the last part, the upscaling of the resin functionalization is presented.

2.2. Peracetylation and Thioglycosylation of Monosaccharides

Thioglycosides are the most common used glycosyl donors in AGA. They are bench stable, which means they can be mass produced for commercial purposes, reinforcing the need for standard large-scale production procedures. Because thioglycosides are compatible with most of the conditions used in protecting group manipulations, they are installed in the earlier steps of the synthetic schemes. Thioglycosides are easily prepared by reaction of the peracetylated sugar with an appropriate thiol in the presence of a suitable acid. Therefore, the first reactions to produce an "approved" building block from a free sugar are peracetylation and thioglycosylation.

Acetyl esterification is a very common reaction and usually very straightforward. However, the acetylation of reducing sugars has a higher complexity due to the existence of anomers and regioisomers (namely pyranosides and furanosides). The most widely used methods for peracetylation are: i) Ac_2O and NaOAc at high temperature; ii) Ac_2O and pyridine; c) Ac_2O and acid catalyst. Each method gives a different ratio of α/β anomers (**Scheme 2.1**).



Scheme 2.1 Peracetylation of D-glucopyranose leads to α or β pentaacetate. [36]

Sodium acetate catalyses the anomerization of the free sugar but not in the acetylated stage. As the β anomer is more reactive, it is acetylated first and, in these conditions, the β peracetate is obtained. In acid medium, anomerization of the

acetates takes place and leads to the thermodynamic product which, in case of glucose and galactose, is the α peracetate. The pyridine method does not seem to promote isomerization as it is reported to afford the same α/β ratio as the parent free sugar.[36,37]

Acid catalysis appears as an attractive method for the peracetylation since it avoids the use of stoichiometric amounts of pyridine (whose toxicity is well known) and requires no heating, compared to the sodium acetate method. The use of metal triflates[38] and particularly $In(OTf)_3$[39] leads to high yielding peracetylations with very simple procedures. $In(OTf)_3$ has low toxicity, is non-corrosive and water tolerant. Therefore, it was anticipated that it could be used in large scale peracetylations. Here a scale up study of this method, together with a comparison with sodium acetate and pyridine methods is presented.

2.2.1. Peracetylation of Monosaccharides

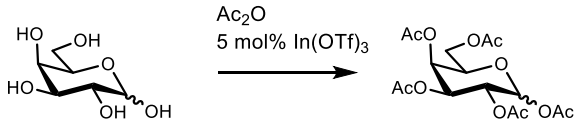
Indium Triflate Method

Peracetylation of galactose was performed in neat Ac_2O with 5% $In(OTf)_3$ following the reported procedure[39] (**Table 2.1**). The work up was difficult, as the large excess of acetic anhydride required a large volume of sodium carbonate solution for neutralization. To avoid this problem, the amount of Ac_2O was reduced to eight equivalents. Because the anhydride was being used both as reagent and solvent, the decrease of Ac_2O was compensated with the use of an organic solvent. DCM, toluene and ACN were tested. In the three cases, the reaction was complete by TLC. However, after isolation of the product, the reaction with toluene gave a lower yield. The reaction with ACN was faster so it was chosen to proceed with optimization.

Keeping ACN a solvent, the amount of $In(OTf)_3$ catalyst was reduced to 2.5% and then 1.3% (**Table 2.2** entries 1 and 4). The experiments showed complete conversion and high isolated yields. The amount of Ac2O was further reduced to seven equivalents with similar results. The procedure was then scaled up to a 50 g

batch leading to the isolation of the galactose pentaacetate in quantitative yield (**Table 2.2**, entry 4).

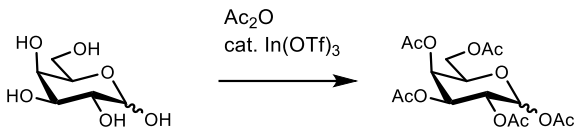
Table 2.1 Solvent screening in D-galactose peracetylation with $In(OTf)_3$.



Entry	Ac_2O	Solvent	Reaction time	Yield	α/β Ratio
1	30 equiv	neat	1.5 h	88%	7:1
2	8 equiv	DCM	ON	98%	10:1
3	8 equiv	Toluene	5.5 h	77%	10:1
4	8 equiv	ACN	3 h	93%	10:1

Just seven equivalents of Ac_2O and just 1.3 mol% of $In(OTf)_3$ are enough to drive the reaction to completion in a short amount of time (1.5 to 2h). As the reaction proceeds, the reaction mixture evolves from a suspension to a solution and this observation can be safely used as an indicator of the endpoint. It is speculated that the first acetylation is rate limiting. The first acetylation should increase the solubility of the sugar and, once in solution, the subsequent acetylation steps should be fast. This is consistent with the fact that only the starting material and the final product can be observed by TLC, regardless of the progress stage of the reaction. The reaction can be easily scaled up although the extraction procedure becomes more inconvenient as the scale increases.

Table 2.2 Optimization of D-galactose peracetylation with $In(OTf)_3$.



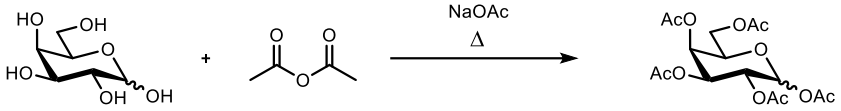
Entry	Ac_2O	$In(OTf)_3$	Reaction time	Yield	α/β Ratio
1	10 equiv	2.5%	2 h	79%[a]	10:1
2	10 equiv	1.3%	2.5 h	quant.	10:1
3	7 equiv	1.3%	1.5 h[b]	96%	8:1
Scale up to 50 g					
4	7 equiv	1.3%	2 h	quant.	8:1

[a]Complete conversion by TLC, lower yield attributed to losses during work up. [b]Cooling bath removed 30 min earlier than before.

Sodium acetate method

D-Galactose was peracetylated with acetic anhydride and sodium acetate following a procedure described for glucose[40]. The free sugar was added to a refluxing mixture of acetic anhydride and NaOAc. Upon precipitation with ice, β-D-galactopyranose pentaacetate was isolated (**Table 2.3**, entry 1). The highest yield was obtained when the reaction mixture was quenched with sodium bicarbonate and extracted with DCM (entry 2). Crystallization of the crude so obtained led to 60% isolated yield. The lower yield compared with the $In(OTf)_3$ method is attributed to the formation of isomeric penta-acetetates of D-galactose, namely D-galactofuranose, which also occurs with the pyridine method[36].

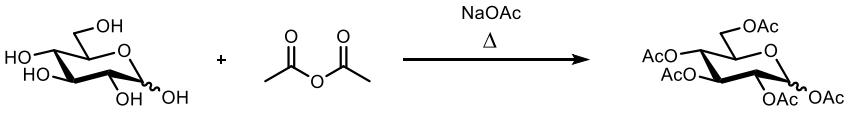
Table 2.3 Reaction conditions for peracetylation of D-galactose with NaOAc.



Entry	Scale	Isolation	Yield	α/β Ratio
1	2 g	Precipitation with ice and filtration	38%	Pure β
2	2 g	Extraction with organic solvent	60%	Pure β

The peracetylation of glucose with the sodium acetate method led to the isolation of pure β-D-glucopyranose pentaacetate in 71% after crystallization (**Table 2.4**, entry 1). The filtrate obtained after isolating the crystals was identified as a mixture of α and β D-glucopyranose pentaacetates and accounted for 25% yield. When this reaction was scaled up to 50 g, the pure β D-glucopyranose pentaacetate was isolated in 76% yield (entry 2).

Table 2.4 Reaction conditions for peracetylation of D-glucose with NaOAc.



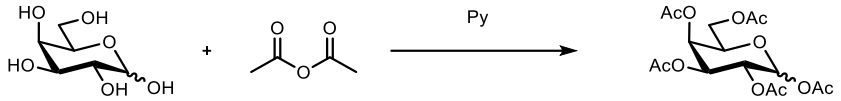
Entry	Scale	Yield	α/β Ratio
1	2 g	71%	Pure β
2	50 g	76%	Pure β

Pyridine method

Pyridine is a volatile liquid that is toxic by inhalation, causes irritation to the skin and the eyes, and causes infertility in male humans. Pyridine is highly flammable and forms explosive mixtures with air. Thus, handling large volumes of pyridine creates a series of risks. Still, the pyridine method is one of the most widely used for acetylation of sugars.

When the pyridine method was used with galactose, an α/β ratio favouring the α anomer was observed (**Table 2.5**). The yields were very high both in small large scale.

Table 2.5 Reaction conditions for peracetylation of D-glucose with the pyridine method.



Entry	Scale	Reaction time	Yield	α/β Ratio
1	2 g	ON	88%	2.5:1
2	10 g	ON	97%	2.8:1

2.2.2. Thioglycosylation

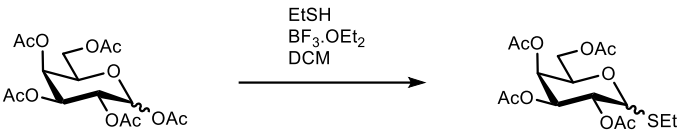
Several thioglycosides have been used as donors, with ethyl, toluyl and 5-tert-butyl-2-methylphenyl (Mpb) being the most common thioglycoside aglycons. The substituent on the sulphur can provide orthogonality within different thioglycosides. For instance, an ethyl thioglycoside can be selectively activated in the presence of a toluyl thioglycoside acceptor, that is subsequently activated in a second glycosylation reaction.[25] The sulphur substituent is also known to affect aglycon transfer. Bulky substituents such as Mpb prevent aglycon transference when the thioglycoside is used as an acceptor increasing the yield of the intended glycosylation.[26] In order to establish standard "approved" building blocks for AGA, the question of which aglycon leads to a more appropriate leaving group was posed. To answer that question, ethyl, toluyl and Mpb thioglycosides of galactose and glucose were synthesized.

The synthesis of the thioglycosides was accomplished by combining the peracetates of D-galactose and D-glucose with the appropriate thiols in the presence of boron trifluoride etherate.

Galactose

In the first attempt to generate ethyl thiogalactoside, α-D-galactose pentaacetate was used as a starting material. After incubating for 18 h, the reaction was not complete and a 42% conversion was observed by NMR (**Table 2.6**, entry 1). When the β-galactose pentaacetate was used, the reaction was over in 3.5 h and ethyl thiogalactoside was isolated in 95% yield as a 1:1 α/β mixture (entry 2). In an effort to find a stereoselective procedure, the reaction was repeated at 0 °C. However, no change in the α/β ratio was observed (entry 3).

Table 2.6 Thioglycosylation of D-galactose pentaacetate with EtSH.



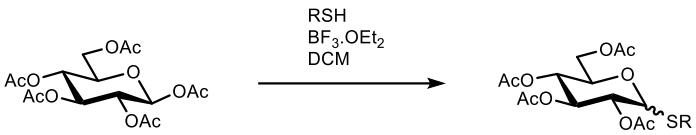
Entry	Pentaacetate α/β ratio	Temperature	Time	Yield	Product α/β ratio
1	25:1	rt	18 h	42%[a]	4:3[a]
2	Beta	rt	3.5 h	95%	1:1
3	Beta	0 °C	3.5 h	95%	1:1

[a]Estimated by NMR analysis of the crude reaction mixture.

Glucose

The lack of stereoselectivity was also observed with glucose (**Table 2.7**, entry 1). The resulting α and β thioglucosides could not be purified by crystallization. Also, they showed a similar rf value which hindered purification by column chromatography. When toluyl thiol (entry 2) or Mpb thiol (entry 3) were used, the reaction was selective and the β-thioglucoside could be purified by crystallization. The toluyl glucoside gave a higher isolated yield.

Table 2.7 Thioglycosylation of D-glucose pentaacetate with EtSH.



Entry	R	Purification and yield
1	Et	Column: α 22%, β 12%, α/β mixed fractions 37%
2	Tol	Crystallization: β 73%
3	Mpb	Crystallization: β 53%

2.2.3. Discussion

Peracetylation and thioglycosylation are the first transformations in the route to the production of thioglycosides. Hence, universal, robust and high-yielding procedures that can be scaled up to multi gram production are very important to reduce the cost of building blocks.

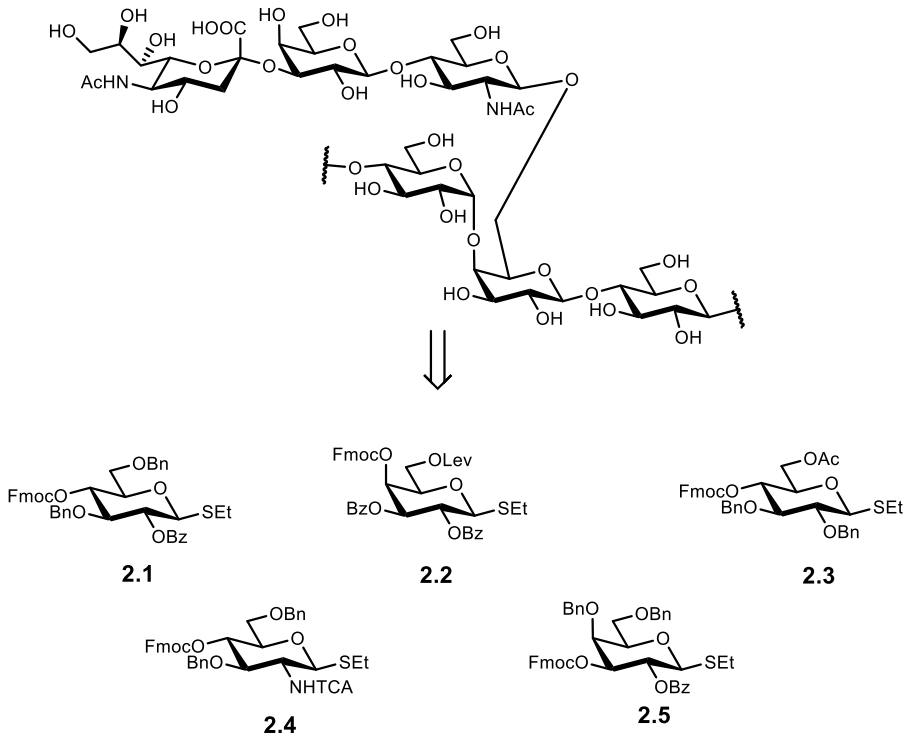
Peracetylations with $In(OTf)_3$ led to very high yields with very simple procedures (that is, no heating or special equipment were required). The method is stereoselective for the α peracetate and when it was reacted with the thiol it showed very low reactivity. The higher stability of the alpha anomer prevents the conversion into the thioglycoside under the tested conditions. This finding implicates that α promoting peracetylation methods (i.e. acid methods) are not appropriate when thioglycosides are to be generated. The pyridine method is easy to set up and gives good yields. Furthermore, the method is reliable and scalable. The resulting ratios for galactose favour the α anomer but it can vary according to the α/β ratio of the parent sugar. The pyridine method requires manipulation of large volumes of pyridine, which is more problematic for large scale reactions. The NaOAc method requires heating and yields can vary. For example, galactose forms isomeric acetates such as D-galactofuranose which decrease the yield of the target galactopyranose pentaacetate. On the other hand, the NaOAc method is selective for the β peracetate, which reacts easily in the thioglycosylation step. Hence, the NaOAc method is recommended as the method of choice for the preparation of thioglycosides.

The generation of ethyl glycosides of galactose and glucose led to mixtures of α/β anomers without any selectivity. Furthermore, the products were not crystalline and had to be purified by column chromatography. On the other hand, the reaction with toluyl and Mpb thiols gave stereo selective formation of the corresponding thiogalactosides (unpublished data) and thioglucosides. Moreover, the thioglycosides crystalized and were easily isolated by filtration with toluyl derivatives giving higher isolated yields. From a building block synthesis perspective, the use of toluyl thioglycosides proved to be the best option.

2.3. Synthesis of Building Blocks to Assemble GBS type IV Capsular Polysaccharide

2.3.1. Retrosynthetic Analysis

A retrosynthetic analysis of the repeating unit of GBS type IV CPS led to the design of the building blocks **2.1**-**2.5** as depicted in **Scheme 2.2**.

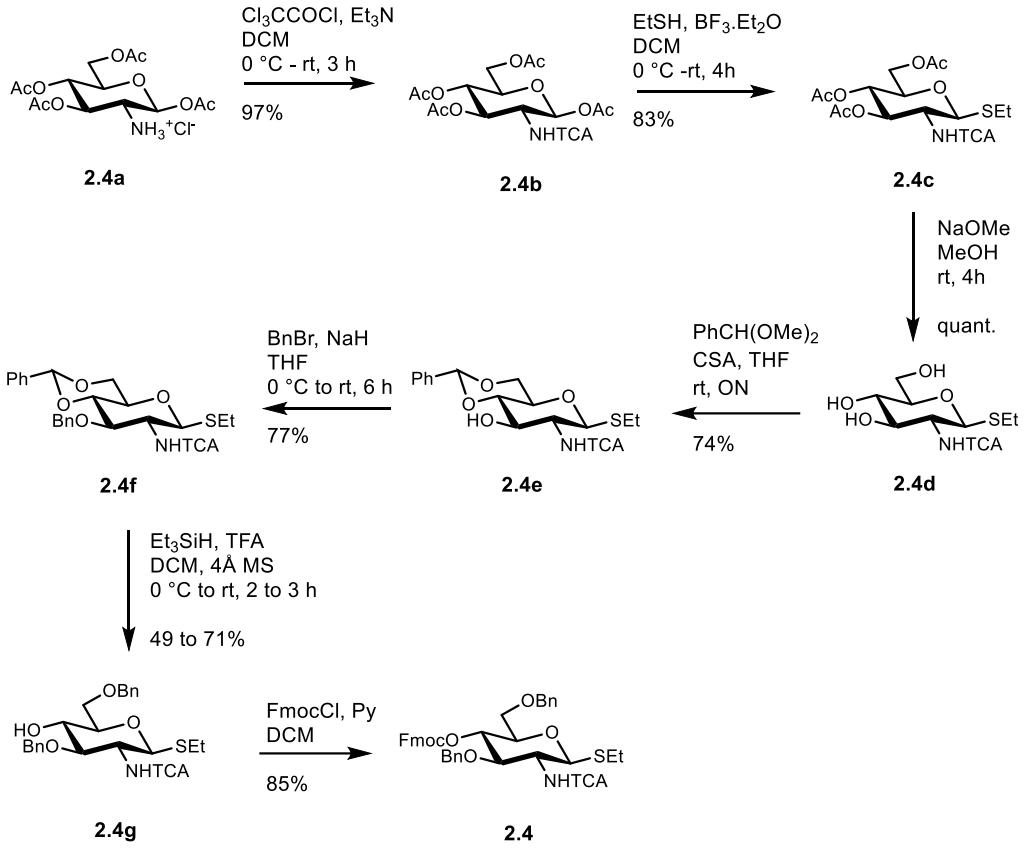


Scheme 2.2 Retrosynthetic analysis of the repeating unit of GBS type IV CPS.

Fluorenylmethyloxycarbonyl (Fmoc) was used as the temporary protecting group of choice. To assure stereoselectivity in the glycosidic bond formation, benzoyl (Bz) groups were used in O2 for β linkages in glucose and galactose. For glucosamine, the trichloroacetyl (TCA) group served as participating group. To generate the α-glucoside, an acetyl group was used in O6 as it is known to enhance the alpha stereoselectivity via remote participation.[28]

2.3.2. Synthesis of the Glucosamine Building Block

Building block **2.4** was prepared from peracetylated glucosamine hydrochloride derivative **2.4a** in seven steps (**Scheme 2.3**). The synthetic route was scouted in a 10 g batch and scaled up to 40 g batch.



Scheme 2.3 Synthetic route to glucosamine building block **2.4**.

The introduction of the TCA group was performed with trichloroacetyl chloride and triethylamine in DCM. The literature reports 82% isolated yield after

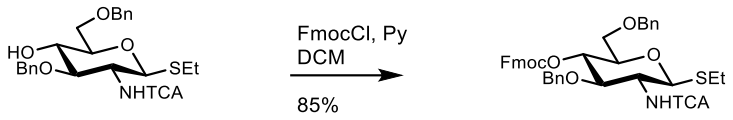
crystallization.[41] However, here the crude product after work up showed high purity by NMR. Avoiding crystallization led to an increase of isolated yield from 82% (reported) to 90% (in the 10 g batch) or 97% (in the 40 g batch). Compound **2.4b** so obtained was successfully transformed into the corresponding ethyl thioglycoside **2.4c**. The product was selectively generated as the β anomer and isolated in 81% yield (10 g batch) and 84% yield (40 g batch) after crystallization. This is in agreement with reported 85% yield.[42]

Thioglycoside **2.4c** was deacetylated using Zemplén conditions and isolated in quantitative yields without the need for any purification procedure. It was then transformed into the benzylidene intermediate **2.4e** by reacting with benzaldehyde dimethyl acetal in the presence of camphorsulfonic acid (CSA). This reaction is typically performed in DMF using a sulfonic acid catalyst.[43] Here, THF was used as a solvent to avoid the use of DMF. TLC analysis of the reaction mixture showed trace amounts of the starting material **2.4d** even after 18 h of reaction. Crystallization of the crude material, obtained after aqueous work-up, led to the benzylidene compound **2.4e** in 72% (10 g batch) or 74% (40 g batch) yield. The difference between these values and literature values (85% after column chromatography[43]) support the choice of crystallization as a purification method.

The introduction of benzyl group was done with BnBr and NaH in THF. In this particular case, the amount of NaH required for the reaction had to be superstoichiometric as the hydride base is strong enough to deprotonate both the alcohol and the amide proton. Regarding the solvent, THF and DMF lead to different reaction paths. In THF, the alcohol reacts with benzyl bromide and the O-benzyl derivative is formed. The use of DMF favours N-alkylation.[44] The benzylated compound **2.4f** was isolated by crystallization in 75% (10 g batch) or 77% yield (40 g batch). Reductive cleavage of the benzylidene ring to release the 4-OH group was achieved with Et_3Si and TFA. Crystallization from EtOH gave the unreacted **2.4f** and the intended product **2.4g** was purified by column chromatography resulting in a yield of 58% (10 g batch) or 71% (40 g batch). In the last transformation of the synthetic route, **2.4g** was reacted with FmocCl in the presence of pyridine to give final building block **2.4**. The isolation of the final compound **2.4** by crystallization proved cumbersome. In the first trial (**Table 2.8**, entry 1), crystallization with

EtOAc/hexane 1:4 failed, as well as EtOAc/hexane 1:1. Crystallization with ethanol was successful in generating crystals but the recovery was very low and was contaminated with a side product originating from the excess of FmocCl reagent. In a second reaction (**Table 2.8**, entry 2), the amount of FmocCl was reduced to 1.1 equivalents and the reaction was complete by TLC. Crystallization from EtOAc/hexane 1:2 failed to afford the product. Surprisingly, in the large scale reaction (**Table 2.8**, entry 3), the product crystalized in a pure state from EtOAc/hexane 1:1, after 13 days at 4 °C and was isolated in 70% yield. To conclude, crystallization of **2.4** showed to be unreliable and difficult to implement as a routine purification strategy.

Table 2.8 Scanned conditions for the introduction of Fmoc group on 4-OH group of glucosamine building block **2.4**.



2.4vii **2.4**

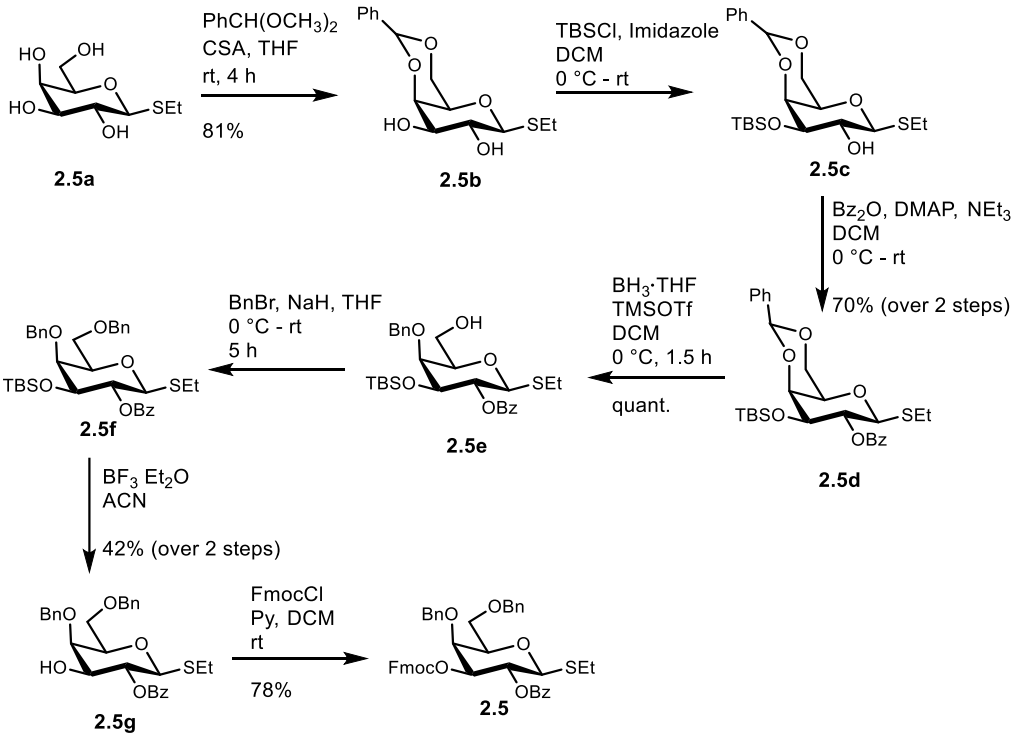
Entry	Scale	Conditions[a]	Crystallization
1	470 mg	FmocCl (2 eq)	EA/Hex 1:4 → failed EA/Hex 1:1 → failed EtOH → product and Fmoc reagent co-crystalized, low recovery
2	470 mg	FmocCl (1.1 eq)	EA/Hex 1:2 → failed
3	5.8 g	FmocCl (1.1 eq)	EA/Hex 1:1 → crystallized on the 13th day, 70% isolated yield

2.3.3. Synthesis of the Galactose Building Blocks

The synthesis of galactose building block **2.5** was based on a reported procedure for the preparation of the toluyl analogue.[45] Here, the synthetic route is tested and optimized starting from the ethyl thioglycoside **2.5a** (**Scheme 2.4**).

The introduction of the benzylidene acetal was performed with benzaldehyde dimethyl acetal and camphorsulfonic acid (CSA) in THF, as previously optimized at GlycoUniverse.[46] The reaction was performed on 40 g scale and the benzylidene **2.5b** was isolated by crystallization in 81% yield.

The following steps were performed in a small batch (5 g) and then scaled up to a large 20 g batch. The introduction of the t-butyl-dimethylsilyl (TBS) group was performed with the corresponding silyl chloride. The conversion was quantitative by TLC after 17 h aqueous work up led to the target intermediate **2.5c** together with substoichiometric amounts of the TBSCl or, most likely, the corresponding hydrolyzed derivative. To decrease the excess of silyl reagent and get a cleaner sample of **2.5c**, the reaction was repeated with only one equivalent of TBSCl. After 24h, the conversion was not complete.

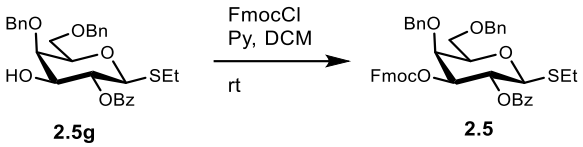


Scheme 2.4 Synthetic route to galactose building block **2.5**.

The crude sample of **2.5c** (as obtained in the first trial) was used as such in the benzoylation and TLC indicated quantitative conversion. The benzoyl derivative **2.5d** crystallized from EtOH in 66% (5 g batch) or 70% (20 g batch) over two steps. The benzylidene ring was subsequently opened by reductive cleavage with borane tetrahydrofuran and TMSOTf to give **2.5e** in 97% (5 g batch) or quantitative yield (20 g batch). In the next step, the 6-OH group was benzylated. The conversion into **2.5f** was complete and the crude product was treated with boron trifluoride etherate to cleave the TBS group and expose the 3-OH group. The resulting compound **2.5g** was purified by crystallization and was obtained in 42% yield (over

2 steps). The introduction of the Fmoc protecting group was subject for optimization (**Table 2.9**). While the reference procedure employed 2.5 equivalents of FmocCl for the transformation[23], it was hypothesized that the reaction could work with less reagent. When galactoside **2.5g** was reacted with 1.1 equivalents of FmocCl, the reaction did not go to completion after 24 h of incubation time. When 1.5 equivalents were used, TLC showed complete conversion after 2h. The low isolated yield (48%) in the small batch (entry 3) can be explained by the excess of solvent used in the crystallization. In the large-scale batch (entry 4), the amount of solvent was decreased in proportion to the scale and the isolated yield for the final BB **2.5** was 78%.

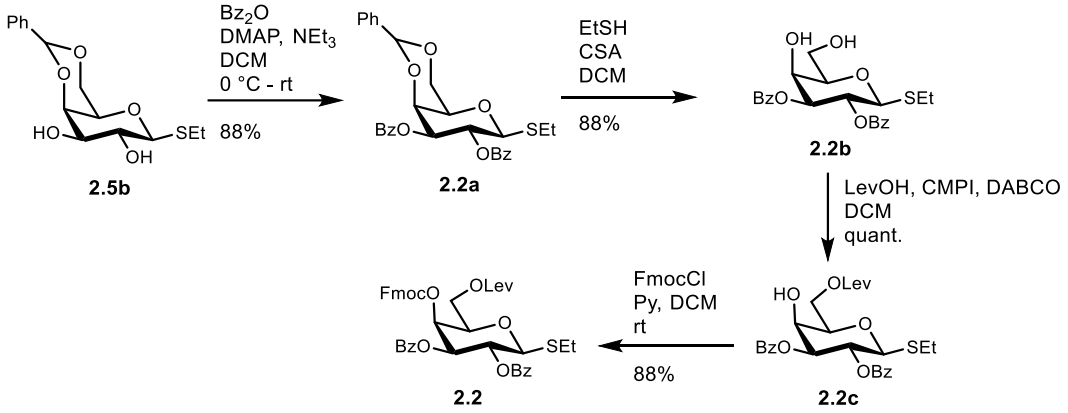
Table 2.9 Optimization of Fmoc introduction reaction.



2.5g **2.5**

Entry	Scale	FmocCl (equiv)	Time	Results	Isolated Yield
1	180 mg	1.1	Overnight	Not complete (TLC)	-
2	180 mg	1.5	2 h	Complete (TLC)	-
3	740 mg	1.5	5 h	Complete (TLC)	48%
4	8.3 g	1.5	5 h	Complete (TLC)	78%

The synthesis of the branching building block **4.2** followed the synthetic route depicted in **Scheme 2.5**.

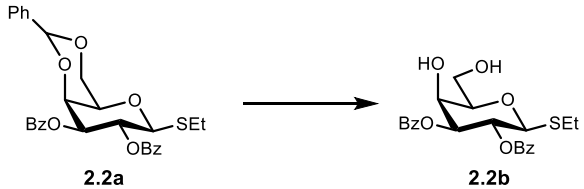


2.5b **2.2a** **2.2b** **2.2c** **2.2**

Scheme 2.5 Synthetic route to galactose building block **2.2**.

The benzylidene intermediate **2.5b** was di-benzoylated with benzoic anhydride. The resulting derivative **2.2a** crystalized from EtOAc/hexane 1:3 in 88% yield. The next transformation was the removal of the benzylidene acetal. In the first attempt, the reaction was done in DCM with H_2O and TFA (**Table 2.10**, entry 1). When the reaction was followed by TLC, no starting material could be seen. However, after aqueous workup, the TLC showed the presence of the starting material. Purification by column gave the target diol **2.2b** in only 36% yield.

Table 2.10 Optimization of the benzylidene removal to prepare derivative **2.2b**.



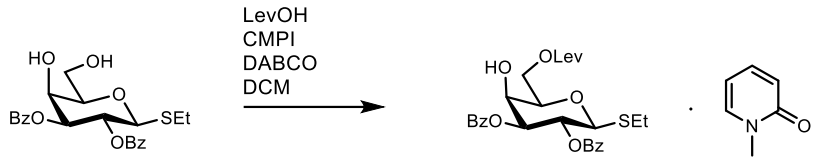
2.2a **2.2b**

Entry	Conditions	Work up	Yield
1	DCM/Water TFA	$NaHCO_3$ (stable emulsion)	36%
2	EtSH/CSA DCM	NaOH 10% (stable emulsion)	Decomposition
3		No work up	40% (filtered from reaction mixture) 48% (crystallization of the filtrate) Total: 88%
4	EtSH/CSA DCM	No work up	84% filtered from reaction mix

A different method was tried and benzylidene intermediate **2.2a** was combined with EtSH and CSA in DCM (**Table 2.10**, entry 2). The reaction was stirred overnight, and a white precipitate formed. An extraction with 10% NaOH was done to neutralize and remove the excess of thiol. During the extraction process, a stable emulsion was formed. Brine was added but only upon resting for two to three hours the emulsion broke. After work up, TLC showed the product had evolved into several different structures. The strong basic medium had likely led to benzoyl migrations to the O-4 and O-6 as well as hydrolysis of one or both the benzoyl ester. The same reaction was repeated, and the white precipitate formed was directly filtered and washed with DCM, without prior work up (**Table 2.10**, entry 3). The solid was identified as the pure product (40%). The filtrate was evaporated and crystalized

with DCM/hexane leading to the isolation of a second portion of the product (47%). To try and isolate the majority of the product in the first filtration, the reaction was done with less solvent (**Table 2.10**, entry 4). Upon completion of the reaction, the solid was directly filtered from the reaction mixture and washed with cold hexane to give 84% pure product. The same conditions were applied to a large batch (48 g) and the isolated yield was 75%.

Table 2.11 Optimization of the introduction of Lev protecting group to generate **2.2c**.



2.2b **2.2c**

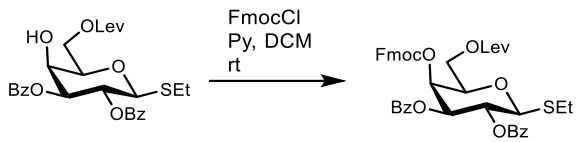
Entry	Scale	Conditions	Yield
1	1 g	LevOH (1.2 equiv) CMPI (2.5 equiv) DABCO (4.0 equiv) DCM (anh. 0.11 M)	quant.
2	1 g	LevOH (1.1 equiv) CMPI (1.2 equiv) DABCO (2.4 equiv) DCM (anh. 0.31 M)	quant.
3	28 g	LevOH (1.1 equiv) CMPI (1.2 equiv) DABCO (2.5 equiv) DCM (anh. 0.31 M)	17% (CMPI was likely inactivated by water contamination from DABCO.)

The levulinoyl ester at O6 was introduced using 2-chloro-1-methylpyridinium iodide (CMPI). A recent procedure[47] was reproduced in the first trial (**Table 2.11**, entry 1) which led to a complete conversion. After aqueous work up, the galactoside derivative **2.2c** was isolated in a 1:1 mixture with the by-product N-methyl-2-pyridinone. The procedure followed uses a large excess of the CMPI and DABCO. However, according to the original paper by Teruaki Mukaiyama[48] only 1.2 equivalents of the CMPI and 2.4 equivalents of base are required. To economize reagents, a new experiment using those conditions was done (**Table 2.11**, entry 2). The concentration of the reaction was also increased to facilitate scale up. The reaction was complete and the levulinoyl-galactoside **2.2c** was isolated in a

quantitative yield. When the reaction was scaled up, a water contamination in DABCO inactivated the CMPI and the yield was low (17%, after column).

The last transformation was the introduction of the Fmoc protecting group. In the case of the glucosamine building block **2.4**, it was possible to use as little as 1.1 equivalents of FmocCl for this transformation. For the galactoside **2.2c**, 1.1 equiv of FmocCl did not lead to complete conversion after 24h (**Table 2.12**, entry 1). Increasing the reagent to 1.5 equiv led to a complete conversion (**Table 2.12**, entry 2). In both cases, crystallization with EtOAc/Hexane or EtOAc/EtOH led to a mixture of the **2.2** and impurities from the FmocCl reagent. The reaction was successfully scaled up and the product was isolated by column in 88% yield.

Table 2.12 Introduction of Fmoc protecting in building block **2.2**.



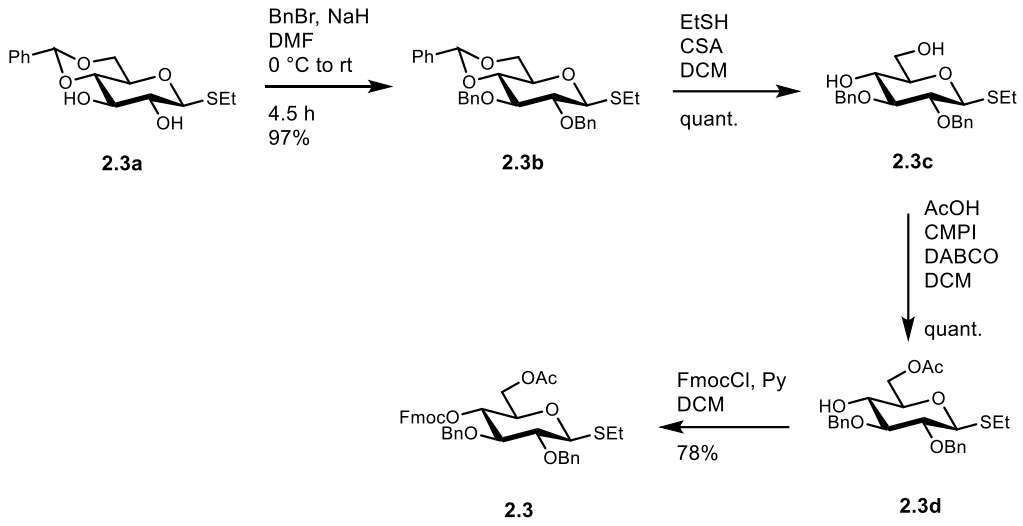
2.2c **2.2**

Entry	Scale	FmocCl	Time	Results
1	1 g	1.5 equiv	18 h	Complete conversion
2	1 g	1.1 equiv	24 h	Complete conversion

2.3.4. Synthesis of the Glucose Building Blocks

To generate the α glucoside linkage, thioglucoside **2.3** was designed and synthesized from the commercial benzylidene intermediate **2.3a** according to the route in **Scheme 2.6**.

The first transformation was the di-O-benzylation, a reaction typically performed in DMF. Inspired by the results with glucosamine building block, where benzylation was successful in THF, a series of experiments were conducted to ascertain whether DMF could be replaced (**Table 2.13**).

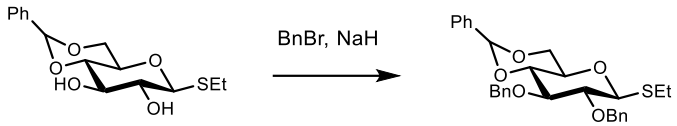


Scheme 2.6 Synthetic route to glucose building block **2.3**.

When the diol **2.3a** was combined with NaH and BnBr in THF, the reaction was not complete after 24 h and the starting material was recovered in 50% by crystallization (**Table 2.13**, entry 1). To try and facilitate the reaction, catalytic tetrabutylammonium iodide was used to generate the more reactive benzyl iodide (entry 2). Although the strategy works for other sugar derivatives,[49] it was not successful here. In fact, TLC analysis of the reaction mixture after 96 h showed the presence of the unreacted starting material and the formation of unwanted side products. Only when the reaction was performed in DMF, the conversion was complete. After work up, DMF was removed under reduced pressure and the dibenzyl intermediate **2.3b** crystallized from EtOH in 88% yield (entry 3).

In the scale up experiments (entries 4, 5, and 6), the conversion was complete (by TLC) and good reproducibility was observed. However, evaporating DMF became cumbersome and an isolation strategy was developed. After work up with DCM and aqueous NH_4Cl, the organic layer was evaporated under reduced pressure until all DCM was removed. Afterwards, a 6:1 mixture of EtOH:H_2O was dropwise added to the resulting DMF solution, under vigorous stirring. Under these conditions, the derivative **2.3b** precipitated and was isolated by filtration. A final wash with cold hexane to remove the mineral oil from NaH gave the product in yields from 87 to 97%, in different batch sizes (10 to 40 g).

Table 2.13 Benzylation of glucoside derivative **2.3a**.

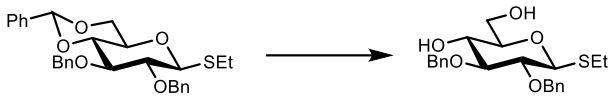


2.3a **2.3b**

Entry	Scale	NaH	BnBr	Solvent	Conditions	Results
1	1 g	5 equiv	3 equiv	THF	24 h	50% Starting material recovered
2	1 g			THF	$INBu_4$ 1 mol% 96 h	Incomplete conversion and side products formation
3	1 g			DMF	18 h	**2.3ii** 88%
4	10 g	3 equiv	2.2 equiv	DMF	2.5 h	**2.3ii** 97%
5	20 g				2.5 h	**2.3ii** 87%
6	40 g				4.5 h	**2.3ii** 96%

The following step was the removal of the benzylidene group. In the first attempt, hydrolysis in DCM spiked with H_2O and TFA was performed, based on a reported procedure.[28] This strategy resulted in incomplete conversion and 46% of the starting material was isolated, along with 29% of the expected diol **2.3c** (**Table 2.14**, entry 1). The procedure was repeated with double the reported reaction time (4.5 h) but again the reaction was far from complete and the product was isolated in 51% yield (entry 2). When the reaction was performed at a higher temperature, TLC analysis was consistent with the previous experiments (entry 3). Another strategy using p-TSA in an homogeneus medium (MeOH/DCM) was attempted (entry 4) and led to the diol **2.3c** in an unsatisfying yield of 50%. A third method, with EtSH, gave the best results. The diol **2.3c** was isolated as a colourless oil in quantitative yield after column purification (entry 5). The reaction was scaled up to 28 g to afford the product in a reproducible quantitative yield (entry 6).

Table 2.14 Removal of benzylidene group to generate **2.3c**.

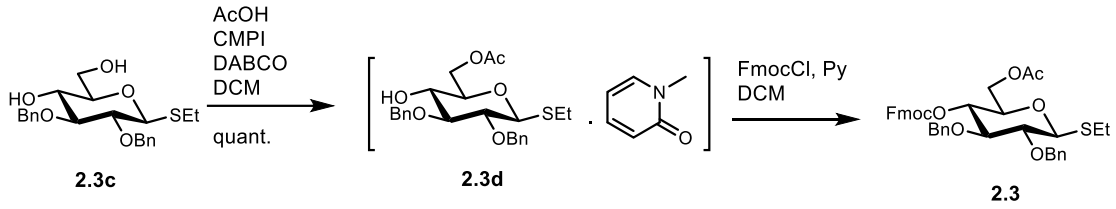


2.3b **2.3c**

Entry	Scale	Reagents	Conditions	Results
1	2 g	DCM/Water/TFA 0.11M, 34eq, 32eq	0 °C, 2 h	**2.3c** 29%[a] Reported yield: 82%[28]
2	1 g	DCM/Water/TFA 0.11M, 34eq, 32eq	0 °C, 4.5 h	**2.3c** 51% [b]
3	1 g	DCM/Water/TFA 0.11M, 34eq, 32eq	rt, 6 h	Incomplete reaction by TLC. Not quantified.
4	1 g	p-TSA, MeOH/DCM 1 eq, 4:3 0.15 M	35 °C 24 h	**2.3c** 50% [b] Reported yield: 83%[50]
5	2 g	EtSH, CSA, DCM (0.3M)	rt, 18 h	**2.3c** quant.
6	28 g	EtSH, CSA, DCM (0.3M)	rt, 18 h	**2.3c** quant.

[a] Estimated by NMR of the crystallized mixture of product and starting material. [b] Isolated yields by column chromatography.

Introduction of the remote-participating group at O6 and the Fmoc at O4 were performed as described in the literature for an identical building block.[28] The acetyl group was introduced using 2-chloro-1-methylpyridinium idodide (CMPI) to activate the acetic acid, at -15 °C. Under these conditions, the reaction is selective for the more reactive primary alcohol. Upon aqueous work up, the 6-O-acetyl derivative **2.3d** was isolated in a mixture with the by-product N-methyl-2-pyridone. The proportion of the mixture was estimated by NMR and based on that, the yield was estimated to be quantitative (**Scheme 2.7**).



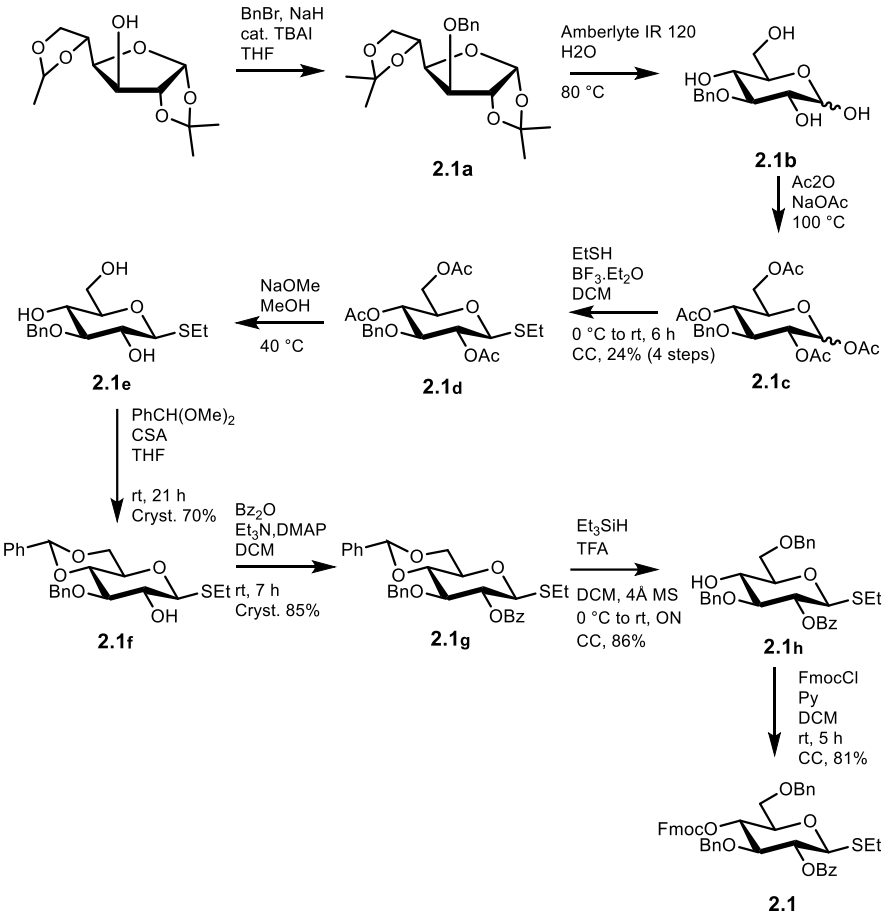
2.3c **2.3d** **2.3**

Scheme 2.7 Introduction of 6-O-acetyl and 4-O-Fmoc groups in glucose building block.

Fmoc group was introduced by combining the crude compound **2.3d** with FmocCl and pyridine. In the scouting batch, TLC showed complete conversion and a clean reaction. The product was purified by crystallization from

DCM/Isopropanol/hexane and obtained as white crystals in 60% yield. In the larger batch, the product was purified by flash chromatography which increased the isolated yield to 78%.

Building block **2.1** was synthesized from glucose diacetonide to facilitate the selective benzylation at O3 (**Scheme 2.8**).



Scheme 2.8 Synthetic route to glucose building block **2.1**.

Thioglycoside **2.1d** was obtained by benzylation, acidic hydrolysis, peracetylation, and thioglycosylation. The benzylation was performed with BnBr and NaH in THF with cat. NBu_4I. The conversion was complete the reaction was clean by TLC. Acid treatment with Amberlyte IR 120 cleaved the acetals and led to the more stable pyranose ring. Peracetylation with Ac_2O and NaOAc gave the tetraacetate **2.1c** as a 1:4 α/β mixture. The introduction of the ethylthio leaving group led to mixture of the unreacted α tetraacetate **2.1c**, and the α and β

thioglycosides. The β anomer **2.1d** was isolated by column chromatography in 24% over 4 steps.

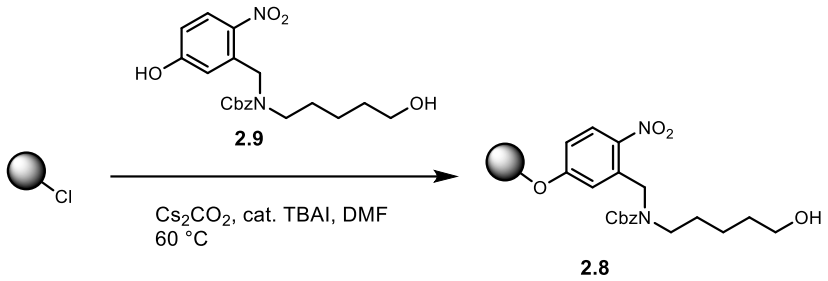
Thioglycoside **2.1d** was subjected to methanolysis under Zemplén conditions. After two hours of reaction, TLC showed absence of the starting material and a single long spot for a new product. After work up, NMR showed a mixture of the expected compound **2.5e** and a monoacetyl intermediate. The crude material was again subjected to the Zemplén conditions. After 6h at 40 °C, TLC showed a single round spot. After neutralization, the obtained material was identified by NMR as the pure triol **2.5e**.

Introduction of the benzylidene protecting group was achieved using benzaldehyde dimethyl acetal and camphorsulfonic acid (CSA) in THF, according to the procedure used previously for galactose and glucosamine. Compound **2.1f** was purified by crystallization and isolated in 70% yield. Position O2 was benzoylated with benzoic anhydride, triethylamine and catalysis of DMAP in DCM. The resulting ester **2.1g** crystalized and was obtained in 85% yield. The benzylidene acetal was cleaved with Et_3Si and TFA and compound **2.1h** was isolated by column chromatography in 86% yield. Introduction of Fmoc group with FmocCl and pyridine led to the final building block **2.1** which was purified by column chromatography and isolated in 81% yield.

2.4. Functionalization of the solid phase

The most frequently used solid support in AGA is a cross-linked polystyrene resin (Merrifield resin) functionalized with a photocleavable linker (compound **2.8**). The linker has a 5-aminopentanol chain that provides the first hydroxyl acceptor in the AGA process. The nitrogen is connected to a photolabile *o*-nitrobenzyl moiety that is, in turn, directly connected to the polystyrene polymer through an ether bond. The nitrogen is protected with a Cbz group. Here, the reproducibility and scalability of the functionalization of Merrifield resin with **2.9** is addressed.

Resin **2.8** was prepared as reported[43]. Compound **2.9** was activated with Cs_2CO_3 in DMF. Tetrabutylammonium iodide replaces the chlorine and is subsequently displaced by the phenolate of **2.9** (**Scheme 2.9**). A sample of the resulting resin **2.8** was used for loading quantification following an established procedure[51,52].



Scheme 2.9 Functionalization of the Merrifield resin with a photocleavable linker.

The reaction was first done in a 5 g batch and the resulting loading was 0.40 mmol/g. The reaction was then scaled up to a 50 g batch. Extra care was taken in the washing and drying steps to avoid contaminations that could interfere with the loading determination. The loading for the 50 g batch was also 0.40 mmol/g.

The procedure for functionalization of the Merrifield resin with the photocleavable linker showed a high reproducibility and scalability, with maximum functionalization yield.

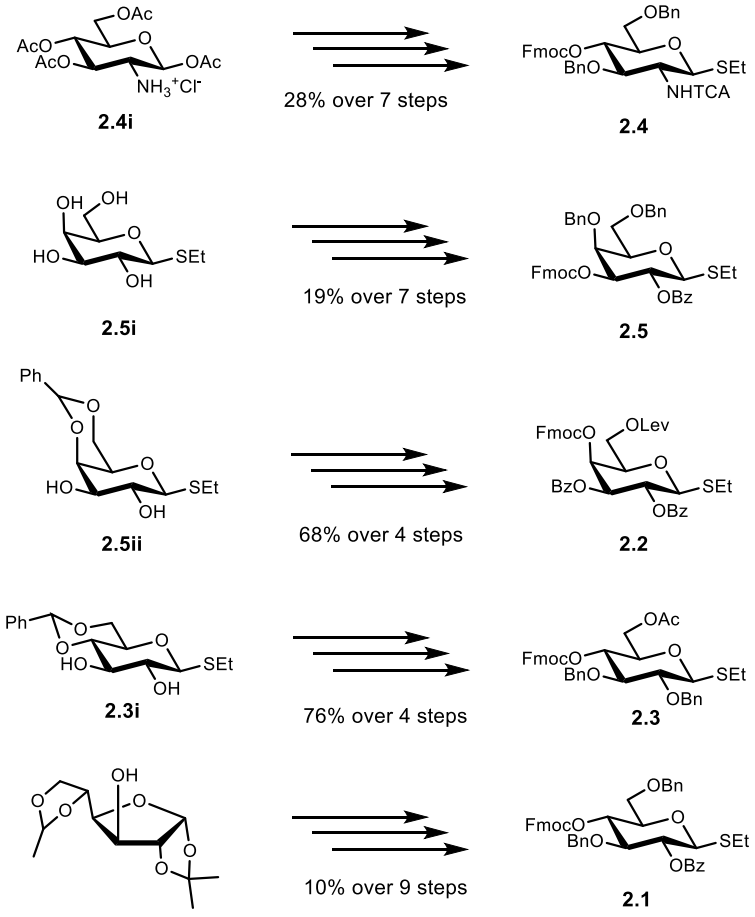
2.5. Conclusion

The number of procedures for carbohydrate protecting group manipulations in the literature is very vast and can be overwhelming. With the work here described, procedures from the literature are optimized and combined in the best way possible to establish a streamlined and universal strategy for the synthesis of approved building blocks for AGA.

The very first transformations of free monosaccharides are peracetylation and thioglycosylation. The sodium acetate method proved to be the best choice for the peracetylation as it is high yielding and highly β selective, which guaranties success

in the introduction of the thioglycoside. Toluyl group was established as the aglycon of choice for thioglycosides due to the selective formation of the β anomer in glucose and galactose, and high yields in crystallization. Exception is made for glucosamine, for which the thioglycosylation with ethyl thiol led to stereoselective formation of the β-thioglycoside.

In general, the efforts to implement crystallization led to synthetic schemes with slightly lower yields but minimal chromatographic purifications. Standard procedures were optimized and successfully applied for similar transformations in different building blocks. For example, the benzoylation procedure was used for galactose, glucose, and glucosamine derivatives. The procedure proved to be robust enough to be used in single and di-benzoylations and the resulting esters were easily isolated by crystallization. In benzylation reactions, the case where DMF cannot be replaced by THF was identified (2,3-di-O-benzylation of glucose). In other cases, THF was successfully used.



Scheme 2.10 Overview of the building block synthesis.

Building block **2.4** was obtained in 28% yield over seven steps with only one chromatographic purification. Building block **2.5** was obtained in 19% over seven steps from **2.5a** without any column purification. The benzylidene intermediate **2.5b**, which was optimized and upscaled, was used to prepare building block **2.2** in 68% over four steps with only one column purification in the last step. The glucose building block **2.3** was obtained from intermediate **2.3a** in 76% over four steps and required three column purifications. Finally, glucose building block **2.1** was obtained from the glucose diacetonide in 10% over nine steps with three column purifications.

Merrifield resin functionalized with a photocleavable linker was prepared on large scale with 100% yield and is now cheaply available at GlycoUniverse.

With bulk amounts of building blocks and solid support in hands full focus can be turned to experimentation in the Glyconeer.

2.6. Experimental Section

General Materials and Methods

All reagents and solvents were acquired from commercial sources, unless stated otherwise. Anhydrous solvents were dried with 10% (m/v) pre-activated 4 or 3 Å molecular sieves. Amberlite IR-120 (Across Organics) protonic exchange resin was rinsed thoroughly with methanol before use. TLC was performed on 0.25 mm Kieselgel 60 F 254 glass-supported plates (Macherey-Nagel), with detection via UV light (254 nm) and H_2SO_4 (10% in EtOH) staining. Flash chromatography purifications were performed with silica gel VWR 60-200 μm or Fluka 200-430 mesh (for difficult separations). NMR spectra were obtained using Ascend 400 (Bruker) and Agilent 400 MHz NMR Magnet (Agilent Technologies) spectrometers at 400 MHz (^{1}H) and 100 MHz (^{13}C) or a Varian 600 (Agilent) at 600 MHz (^{1}H) and 150 MHz (^{13}C), or a Ascend 700 (Bruker) at 700 MHz (^{1}H) and 176 MHz (^{13}C). $CDCl_3$ or D_2O were used as solvents and chemical shifts (δ) referenced to residual non-

deuterated solvent peak unless stated otherwise. NMR chemical shifts (δ) are reported in ppm and coupling constants (J) are reported in Hz. Assignments were supported by COSY and HSQC experiments and compared with literature data when available. ESI-HRMS were performed with a Xevo G2-XS Q-Tof (Waters).

Peracetylation

α/β Ratios were estimated by NMR.

General procedure for the $In(OTf)_3$ method

D-Galactose was taken in a 3-neck rb flask and flushed with Ar. Anhydrous ACN was added under Ar to give a 0.33 M mixture. The resulting white suspension stirred in an ice bath for 5 min. Ac_2O was added and the reaction mixture was further stirred for another 5 min. Indium triflate was added and after 30 min the ice bath was removed. When the reaction was complete, Na_2CO_3 20% (aq) was added and the mixture stired for c.a. 1h. The aqueous layer was isolated and extracted with DCM (2X). The combined organic layers were washed with Brine (2X), dried over $MgSO_4$, evaporated and dried in high vacuum. The resulting oil was identified as the pure product.

Sodium acetate method

Acetic anhydride (10 equiv) was refluxed with NaOAc (0.5 equiv) and D-galactose or D-glucose was portion wise added over 15 min. The mixture was refluxed for an appropriate amount of time (see **Table 2.3** and **Table 2.4**) and then cooled to rt. The reaction was quenched by addition of ice. The product precipitated from the mixture and was filtered and recrystalized from ethanol. Alternatively, the reaction mixture was diluted with EtOAc and washed with sat. aq. $NaHCO_3$ (3X), brine, dried over $MgSO_4$, concentrated on rotavap and further dried under high vacuum.

Pyridine method

D-Galactose was taken in a 3-neck rb flask and flushed with Ar. Pyridine (anhydrous, 10 equiv) was added and the resulting white suspension stirred in an ice bath (ca. 10 min). Acetic anhydride (10 equiv) was dropwise added over 5 min and after 30 min the cooling bath was removed. The reaction mixture was stirred at rt and followed by TLC. When the conversion was complete, the reaction mixture was concentrated on the rotavap and co-evaporated with toluene. The resulting material was combined with EtOAc and extracted with HCl 1 M (2X) then with sat. $NaHCO_3$ (2X) then with brine. The organic layer was dried over $MgSO_4$, evaporated on rotavap and dried under high vacuum overnight to afford the product as a colorless oil.

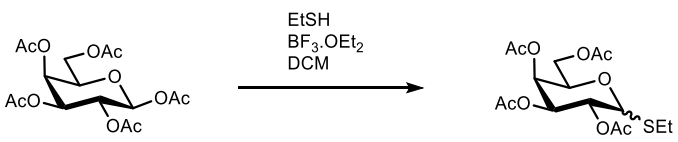
D-Galactopyranose pentaacetate analytical data in agreement with the literature[39]. D-Glucopyranose pentaacetate analytical data in agreement with the literature[39,40].

Thioglycosylation

General procedure for thioglycosylation

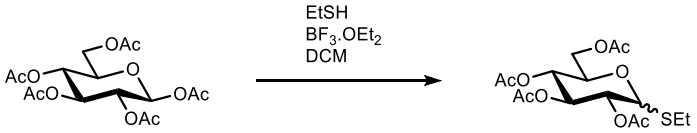
A 0.2 M solution of the parent peracetylated glycoside in DCM (anhydrous) was cooled in an ice bath for ca. 10 min. The appropriate thiol (1.5 equiv) was added and then boron trifluoride etherate was dropwise added over 5 min. After 15 min, the cooling bath was removed, and the reaction mixture was stirred at rt until completion by TLC or for a maximum of 24 h. The reaction mixture quenched with H_2O. The organic layer was washed with sat. aq. $NaHCO_3$ (2x) and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The thioglycoside was purified by crystallization (preferentially) or column chromatography.

Ethyl 2,3,4,6-tetra-O-acetyl-1-thio-D-galactopyranoside



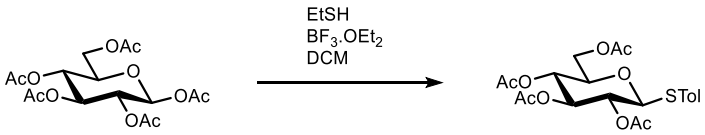
The product was purified by column chromatography (0 to 50% EtoAOc in Hexane). to give a 1:1 α/β mixture of the thioglycoside (95% yield). Analytical data in agreement with the literature[53].

Ethyl 2,3,4,6-tetra-O-acetyl-1-thio-D-glucopyranoside



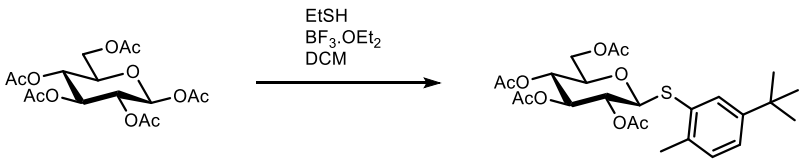
The product was purified by column chromatography (EtOAc/hexane) to give α 22%, β 12%, α/β mixed fractions 37%. Analytical data in agreement with the literature.

Toluyl 2,3,4,6-tetra-O-acetyl-1-thio- β-D-glucopyranoside



Purified by crystallization. The crude material was dissolved in hot EtOAc (7 mL) and hexane (25 mL) was then dropwise added. Resulting suspension was kept in the fridge overnight then filtered to afford the pure product (73%). Analytical data in agreement with the literature.

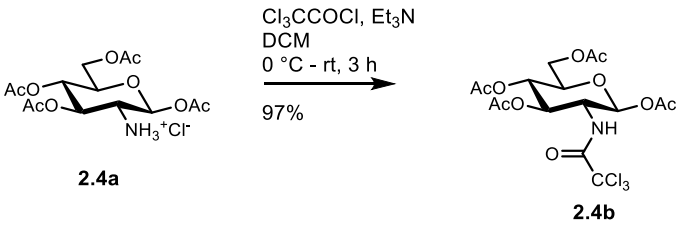
5-tert-Butyl-2-methylphenyl 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside



Purified by crystallization from hot EtOH. After 24 h in the fridge, the suspension was filtered to afford the pure product (53%). Analytical data in agreement with the literature.

Synthesis of BB 2.4

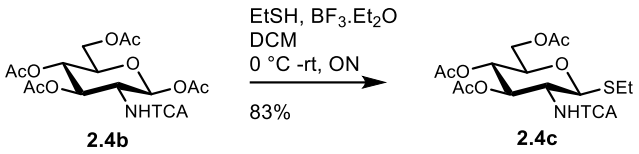
1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-trichloroacetamido-D-glucopyranose **2.4b**



1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-amino-D-glucopyranose hydrochloride **2.4a** (40.1 g, 104 mmol) was weighed in a 3-neck 1L rb flask then flushed with Ar. DCM (anhydrous, 380 mL) was added and the resulting suspension stirred in an ice/water bath for 10 min. Triethylamine (29.3 mL, 210 mmol) was added and after 5 min trichloroacetyl chloride (15.8 mL, 141 mmol) was dropwise added over 10 min. The reaction mixture was stirred for another 3 h. The reaction was quenched with water (350 mL). After a few minutes stirring, the organic layer was isolated and extracted with sat. $NaHCO_3$ (2X 350 mL), HCl 1N (2X 350 mL) and brine (350 mL). The organic layer was then dried over $MgSO_4$, concentrated on rotavap to a yellow oil and dried on high vacuum overnight. The resulting off white foam was identified as the pure product (50.1 g, 97%).

^{1}H NMR (400 MHz, Chloroform-d) δ 7.36 (d, *J* = 9.6 Hz, 1H, NH), 5.80 (d, *J* = 8.8 Hz, 1H, H1), 5.43 (dd, *J* = 10.9, 9.4 Hz, 1H, H-3), 5.14 (t, *J* = 9.7 Hz, 1H, H-4), 4.32 (ddd, *J* = 10.9, 9.6, 8.8 Hz, 2H, H-2), 4.27 (dd, *J* = 12.5, 5.0 Hz, 1H, H-6a), 4.15 (dd, *J* = 12.5, 2.2 Hz, 1H, H-6b), 3.90 (ddd, *J* = 10.1, 5.0, 2.2 Hz, 1H, H-5), 2.10 (s, 2H), 2.09 (s, 2H), 2.07 (s, 2H), 2.05 (s, 2H).

Ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-trichloroacetamido-1-thio-β-D-gluco-pyranose **2.4c**

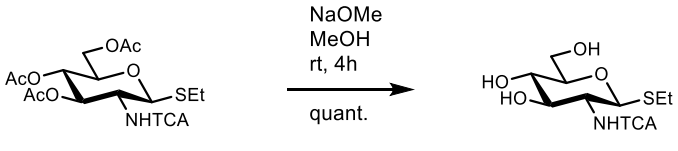


2.4b **2.4c**

1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranose **2.4b** (49.0 g, 99.5 mmol) was weighed in 3-neck 1 L rb flask then flushed with Ar. DCM (anhydrous, 22 mL) was added and the resulting pale yellow solution stirred in an ice bath for 15 min. Ethanethiol (7.9 mL, 110 mmol) was added and boron trifluoride etherate (9.9 mL, 80 mmol) was dropwise added over 5 min. After ca. 30 min, the cooling bath was removed, and the solution was stirred overnight. The reaction was quenched with H_2O (100 mL). The organic layer was isolated and extracted wit sat. $NaHCO_3$ (2X 100 mL) and with brine (100 mL). Organic layer was dried over $MgSO_4$, concentrated under reduced pressure, and dried in high vacuum overnight. The crude solid was dissolved in EtOAc (60 mL) at a 50 °C bath. Then, hexane (ca 300 mL) was added dropwise while stirring vigorously. The resulting suspension was kept in the fridge overnight then filtered to afford the pure product (40.6 g, 83%).

^{1}H NMR (400 MHz, Chloroform-d) δ 6.93 (d, *J* = 9.3 Hz, 1H, NH), 5.33 (t, *J* = 10.0 Hz, 1H), 5.12 (t, *J* = 9.7 Hz, 1H), 4.67 (d, *J* = 10.3 Hz, 1H, H-1), 4.25 (dd, *J* = 12.4, 5.1 Hz, 1H, H-6a), 4.15 (dd, *J* = 12.3, 2.4 Hz, 1H, H-6b), 4.11 (q, *J* = 10.2 Hz, 1H, H-2), 3.75 (ddd, *J* = 10.1, 5.1, 2.4 Hz, 1H, H-5), 2.81 – 2.63 (m, 2H, SCH_2), 2.08 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.27 (t, *J* = 7.4 Hz, 3H, CH_3).

Ethyl 2-deoxy-2-trichloroacetamido-1-thio-β-D-glucopyranose **2.4d**



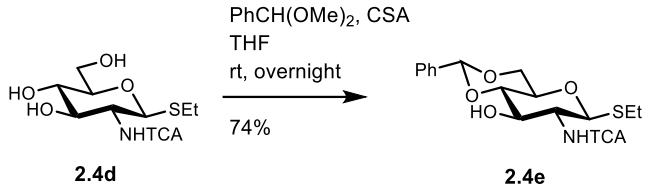
2.4c **2.4d**

Ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-trichloroacetamido-1-thio-β-D-glucopyranose **2.4c** (39.0 g, 78.8 mmol) was weighed in a 3-neck 500 mL rb flask and flushed with Ar. Anhydrous MeOH (190 mL) was added and then sodium methoxide

(57.5 mL, 0.5 M in MeOH). The resulting mixture was stirred at rt. After 1h, TLC (DCM/EA 9:1) showed complete conversion (product rf = 0.021). The reaction mixture was filtered through Amberlyte IR 120 (ca 25 g, previously washed with 2X 50 mL MeOH) until pH = 6. The resin was washed with MeOH (2X ca. 20 mL) and the washings were combined with the reaction mixture. Solvent was evaporated on rotavap and the resulting off-white solid dried in high vacuum. The solid was identified as the pure product **2.4d** (28.8 g, 99%).

^{1}H NMR (400 MHz, Deuterium Oxide) δ 4.77 (m, 1H, H-1), 3.92 (m, 1H), 3.81 (t, J = 10.2 Hz, 1H, H3), 3.77 – 3.68 (m, 2H, H6), 3.56 – 3.41 (m, 2H), 2.84 – 2.64 (m, 2H, SCH_2), 1.24 (t, J = 7.4 Hz, 3H, CH_3).

Ethyl 4,6-*O*-benzylidene-2-deoxy-2-trichloroacetamido-1-thio-β-D-gluco-pyranoside **2.4e**



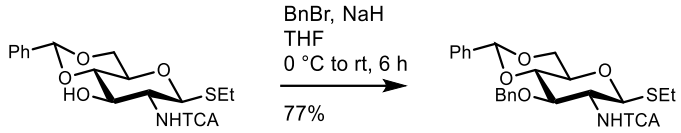
2.4d **2.4e**

Ethyl 2-deoxy-2-trichloroacetamido-1-thio-β-D-glucopyranose **2.4d** (26.6 g, 72.1 mmol) and camphorsulfonic acid (2.96 g, 12.7 mmol) were dissolved in THF (anhydrous, 140 mL). Benzaldehyde dimethyl acetal (21.5 mL, 143 mmol) was added and the resulting solution was stirred at rt overnight. After 18h, TLC analysis of the crude mixture showed traces of starting material. The reaction was quenched with sat. aq. $NaHCO_3$ (25 mL). The aqueous layer was isolated and extracted with EtOAc (25 mL). Combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, concentrated on rotavap and dried under high vacuum. The resulting white solid was crystallized from DCM/Hex to afford the pure ethyl 4,6-*O*-benzylidene-2-deoxy-2-trichloroacetamido-1-thio-β-D-glucopyranoside (4.03g, 74%).

^{1}H NMR (700 MHz, DMSO-d_6) δ 8.88 (d, *J* = 9.3 Hz, 1H, -NH), 7.46 (dd, *J* = 7.4, 2.2 Hz, 2H, -Ar), 7.41 – 7.35(m, 3H, -Ar), 5.62 (s, 1H, -PhCH), 5.53 (d, *J* = 6.3 Hz, 1H, -

OH), 4.78 (d, *J* = 10.4 Hz, 1H, H-1), 4.22 (dd, *J* = 10.1, 5.0 Hz, 1H, H-6a), 3.84 (td, *J* = 9.3, 6.3 Hz, 1H, H-3), 3.73 (q, *J* = 9.7 Hz, 2H, H-2, H-5), 3.51 (t, *J* = 9.3 Hz, 1H, H-4), 3.39 (td, *J* = 9.8, 5.1 Hz, 1H, H-6b), 2.70 – 2.57 (m, 2H, -SCH_2), 1.18 (t, *J* = 7.4 Hz, 3H, -CH_3).

Ethyl 3-O-benzyl-4,6-O-benzylidene-2-deoxy-2-trichloroacetamido-1-thio-β-D-glucopyranoside **2.4f**



2.4e **2.4f**

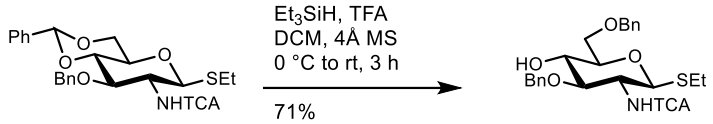
Ethyl 4,6-O-benzylidene-2-deoxy-2-trichloroacetamido-1-thio-β-D-glucopyranoside **2.4e** (20.1 g, 44.0 mmol) was weighed in a 3-neck rb flask then flushed with Ar. THF (anhydrous, 196 mL) was added to form a colorless solution that was stirred in an ice bath for 10 min. NaH 60% in mineral oil (4.57 g, 114 mmol) was added and after 10 min, benzyl bromide (7.8 mL, 65.7 mmol) was dropwise added over 10 min. After 1.5h, the cooling bath was removed. After the mixture was stirred for another 4 h, TLC showed complete conversion (Hex/EA 4:1, rf 0.61). Reaction mixture was diluted with DCM (100 mL) and quenched sat. aq NH_4Cl (110 mL). The aqueous phase was then isolated and extracted with DCM (100 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated on rotavap to a yellow solid. Crystallization from EA/Hex (740 mL/300 mL) afforded the pure product **2.4f** as a white crystalline solid (13.4 g, 56%). Filtrate was concentrated and dried and the resulting yellow solid crystallized from EA/Hex (165 mL/410 mL) to afford a second fraction of the product **2.4f** as a yellowish precipitate (5.16 g, 21%).

^{1}H NMR (400 MHz, Chloroform-d) δ 7.51 – 7.48 (m, 2H), 7.43 – 7.37 (m, 3H), 7.33 – 7.27 (m, 5H), 6.86 (d, J = 8.2 Hz, 1H, NH), 5.60 (s, 1H, PhCH), 5.01 (d, J = 10.4 Hz, 1H, H-1), 4.91 (d, J = 11.3 Hz, 1H, PhC*H*H), 4.72 (d, J = 11.3 Hz, 1H, PhC*H*H), 4.38 (dd, J = 10.5, 4.9 Hz, 1H, H-6a), 4.15 (t, J = 9.4 Hz, 1H, H-3), 3.84 – 3.75 (m, 2H, H-4,

H-6b), 3.71 (td, J = 10.0, 8.1 Hz, 1H, H-2), 3.58 (td, J = 9.7, 5.0 Hz, 1H, H-5), 2.83 – 2.63 (m, 2H, SCH_2), 1.27 (t, J = 7.4 Hz, 3H, CH_3).

^{13}C NMR (101 MHz, Chloroform-d) δ 161.81, 137.79, 137.26, 129.22, 128.62, 128.45, 128.43, 128.10, 126.12, 101.39, 83.53, 82.65, 77.48, 77.37, 77.16, 76.84, 74.93, 70.62, 68.71, 57.51, 24.82, 15.24.

Ethyl 3,6-di-O-benzyl-2-deoxy-2-trichloroacetamido-1-thio-β-D-glucopyranoside **2.4g**

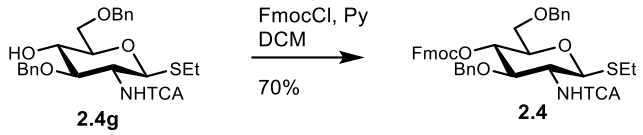


2.4f **2.4g**

Ethyl 3-O-benzyl-4,6-O-benzylidene-2-deoxy-2-trichloroacetamido-1-thio-β-D-glucopyranoside **2.4f** (11.9 g, 21.7 mmol) was combined with DCM (anhydrous, 170 mL) in the presence of 4 Å molecular sieves and the resulting suspension was stirred for 10 min and triethylsilane (14.0 mL, 87.7 mmol) was added. The reaction mixture was cooled in an ice bath and after 30 min, TFA (7.0 mL, 91 mmol) was dropwise added over 5 min. The cooling bath was removed after 15 min and the reaction mixture was stirred at rt. After 3h, TLC showed the product and trace amounts of the starting material. The reaction mixture was quenched by dropwise addition Et_3N (ca. 40 mL) till no more gas evolved. Then the reaction mixture was diluted with DCM (130 mL) and washed with H_2O (2X 300 mL), aq. sat. $NaHCO_3$ (2X 300 mL), and brine (300 mL). The organic layer was dried over $MgSO_4$, concentrated on rotavap and dried in high vacuum. Purification by column chromatography (Hex/EtOAc 6:1 to 2:1) gave the pure compound **2.4g** (8.47 g, 71%).

^{1}H NMR (700 MHz, DMSO-*d*$_6$) δ 9.06 (d, *J* = 9.0 Hz, 1H, -NHCO), 7.35 (d, *J* = 5.3 Hz, 3H, -Ar), 7.29 – 7.28 (m, 4H, -Ar), 7.25 – 7.22 (m, 1H, -Ar), 5.56 (d, *J* = 6.3 Hz, 1H, -OH), 4.80 (dd, *J* = 11.1, 1.9 Hz, 1H, -CH_2Bn), 4.68 (d, *J* = 9.9 Hz, 1H, H-1), 4.61 (dd, *J* = 11.1, 6.2 Hz, 1H), 4.54 (d, *J* = 1.3 Hz, 2H), 3.77 (dd, *J* = 11.1, 1.5 Hz, 1H), 3.58 (dd, *J* = 11.0, 5.7 Hz, 1H), 3.43 – 3.36 (m, 2H), 2.63 (ddq, *J* = 42.3, 12.8, 7.4 Hz, 2H), 1.22 – 1.18 (m, 3H).

Ethyl 3,6-di-O-benzyl-2-deoxy-2-trichloroacetamido-4-*O*-(9-fluorenyl-methoxy-carbonyl)-1-thio-β-D-glucopyranoside **2.4**



Compound **2.4g** (5.80 g, 10.6 mmol) was kept under high vacuum for ca. 1 h then DCM (anhydrous, 75 mL) was added and the resulting pale yellow solution kept under Ar. Pyridine (anhydrous, 2.6 mL, 32 mmol) was added and then FmocCl (3.01 g, 11.6 mmol) was added. In a few seconds the solution became bright yellow. After a few minutes the solution turned orange and then redish. The solution was stirred at rt and after 3 h TLC showed complete conversion. The reaction was diluted with DCM (75 mL), extracted with water (2X 150 mL) and brine (150 mL), dried over $MgSO_4$, and filtered. The solvent was removed on rotavap to afford a yellow oil that was co-evaporated with toluene (3X 15 mL) and then dried in high vacuum overnight. The resulting yellow vitreous solid was crystallized from EtOAc (11 mL) and Hexane (11 mL) at 70 °C. After 4 days in the fridge and after inducing crystallization with spatula, the crystalline product was filtered and washed with cold EtOAc/Hex 1.1 (ca. 60 mL). After drying in the desiccator under vaccum, the solid was identified as the pure product (5.67 g, 70%).

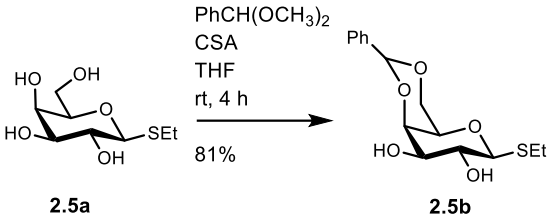
^{1}H NMR (500 MHz, Chloroform-d) δ 7.75 (ddt, J = 7.7, 3.0, 0.9 Hz, 2H), 7.57 (dd, J = 7.5, 1.0 Hz, 1H), 7.52 (dd, J = 7.5, 1.0 Hz, 1H), 7.39 (td, J = 7.5, 1.0 Hz, 2H), 7.36 – 7.17 (m, 12H), 6.93 (d, J = 7.9 Hz, 1H, NH), 5.04 (d, J = 10.3 Hz, 1H, H-1), 4.94 (dd, J = 9.9, 9.0 Hz, 1H, H-4), 4.64 (s, 2H, 3-OC*H*$_2$Ph), 4.53 (s, 2H, 6-OC*H*$_2$Ph), 4.33 (dd, J = 9.6, 6.4 Hz, 1H, Fmoc C*H*H), 4.29 (dd, J = 9.6, 6.4 Hz, 1H, Fmoc C*H*H), 4.24 (dd, J = 10.0, 9.0 Hz, 1H, H-3), 4.11 (t, J = 7.2 Hz, 1H, Fmoc CH), 3.77 (ddd, J = 9.9, 5.2, 3.8 Hz, 1H, H-5), 3.70 – 3.61 (m, 3H, H-2, H-6a, H-6b), 2.73 (qq, J = 12.7, 7.4 Hz, 2H, SCH_2), 1.29 (t, J = 7.4 Hz, 3H, CH_3).

^{13}C NMR (126 MHz, Chloroform-d) δ 161.80 (C=O amide), 154.39 (C=O carbonate), 143.38, 143.19, 141.42, 141.38, 137.96, 137.41, 128.55, 128.45, 128.05, 127.97, 127.77, 127.76, 127.32, 127.30, 125.23, 125.12, 120.22, 120.20, 92.41 (Cl_3C),

82.56 (C1), 78.88 (C3), 77.41 (C2), 76.26 (C4), 74.84 (3-OCH_2), 73.70 (6-OCH_2), 70.24 (Fmoc CH_2), 69.71 (C6), 57.74 (C5), 46.77 (Fmoc CH), 24.88 (SCH_2), 15.31 (CH_3).

Synthesis of BB 2.5

Ethyl 4,6-O-benzylidene-1-thio-β-D-galactopyranoside **2.5b**



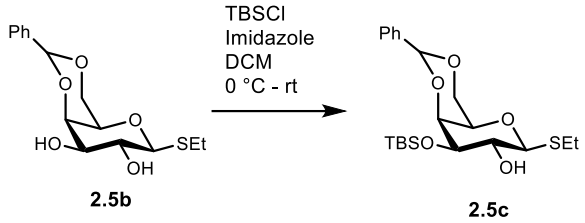
2.5a **2.5b**

Ethyl β-D-galactoside **2.5a** (40.2 g, 179 mmol) was taken in a 500 mL rb flask. CSA (7.26 g, 31.2 mmol) was added, then THF (anhydrous, 300 mL), then $PhCH(OCH_3)_2$ (55.0 mL, 366 mmol). The resulting mixture was stirred at rt for 4h. The reaction mixture poured into 1 L Erlenmeyer flask then EtOAc (200 mL) was added followed by sat. $NaHCO_3$ (200 mL). The organic layer was isolated and extracted with sat. $NaHCO_3$, (250 mL), water (2X250 mL) and brine (2X250 mL). Then dried over $MgSO_4$, concentrated on rotavap and the resulting white solid dried in high vacuum overnight. The crude material was dissolved in boiling DCM (ca. 340 mL). Hexane was subsequently dropwise added (650 mL) under constant stirring allowing the formation of a fine precipitate. The resulting suspension was kept in the fridge overnight. The white solid was then filtered and washed with cold hexane/DCM 4:1 (ca 300 mL). The solid was dried under vacuum overnight to afford the pure product (45.3 g, 81% yield).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.52 – 7.43 (m, 2H), 7.36 (dd, J = 4.8, 2.2 Hz, 3H), 5.51 (s, 1H, PhCH), 4.32 (d, J = 9.6 Hz, 1H, H-1), 4.31 (dd, J = 12.5, 1.5 Hz, 1H, H-6a), 4.20 (dd, J = 3.7, 1.2 Hz, 1H, H-4), 4.00 (dd, J = 12.6, 1.9 Hz, 1H, H-6b), 3.79 (t, J = 9.4 Hz, 1H, H-2), 3.65 (dd, J = 9.2, 3.7 Hz, 1H, H-4), 3.47 (q, J = 1.6 Hz, 1H, H-5), 2.89 – 2.66 (m, 4H, SCH_2, 2-OH, 3-OH), 1.33 (t, J = 7.5 Hz, 3H, CH_3).

^{13}C NMR (101 MHz, Chloroform-d) δ 137.72, 129.39, 128.39, 126.54, 101.55 (Ph*C*H), 85.38 (C1), 75.73, 73.94, 70.16, 69.75, 69.40, 23.54 (SCH_2), 15.36 (CH_3).

Ethyl 3-O-tert-butyldimethylsilyl-4,6-O-benzylidene-1-thio-β-D-galacto-pyranoside **2.5c**

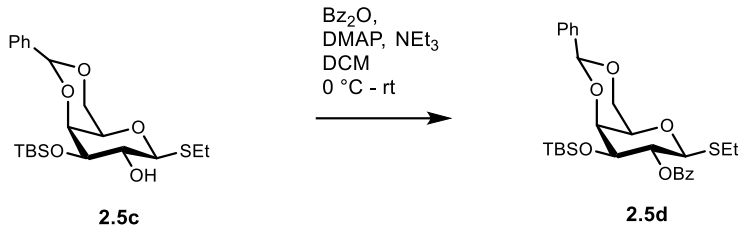


Ethyl benzylidene 1-thio-β-D-galactoside (20.0g, 64.0 mmol) was taken in a rbf and flushed with Ar. DCM (anhydrous, 230 mL) was added and the resulting suspension was stirred under an ice bath for ca. 10 min. TBSCl (14.5 g, 96.2 mmol) was added followed by imidazole (9.18 g, 135 mmol). After ca. 20 min, the cooling bath was removed, and the suspension was stirred overnight. After 17h, TLC showed complete conversion. The reaction mixture was quenched with sat. aq. NaHCO3 (100 mL). The aqueous layer was extracted with DCM (100 mL) and the combined organic layers were washed with brine (200 mL), dried over MgSO4, concentrated on rotavap and dried under high vacuum. The resulting colourless oil was identified as the product.

^{1}H NMR (700 MHz, Chloroform-*d*) δ 7.50 (ddd, J = 8.0, 1.6, 0.6 Hz, 2H), 7.38 – 7.31 (m, 3H), 5.50 (s, 1H, PhCH), 4.35 (d, J = 9.5 Hz, 1H, H-1), 4.34 (dd, J = 12.4, 1.5 Hz, 1H, H-6a), 4.07 (dd, J = 3.6, 0.9 Hz, 2H, H-4), 4.01 (dd, J = 12.4, 1.9 Hz, 1H, H-6a), 3.88 (t, J = 9.3 Hz, 1H, H-2), 3.72 (dd, J = 9.0, 3.6 Hz, 1H, H-3), 3.47 (q, J = 1.7 Hz, 1H, H-5), 2.82 (dq, J = 12.6, 7.4 Hz, 1H, SC*H*H), 2.73 (dq, J = 12.6, 7.5 Hz, 1H, , SCH*H*), 1.31 (t, J = 7.5 Hz, 3H, CH_3), 0.92 (s, 9H, t-BuSi), 0.13 (s, 3H, $SiCH_3$), 0.13 (s, 3H, $SiCH_3$).

^{13}C NMR (176 MHz, Chloroform-d) δ 138.10, 128.86, 128.18, 126.26, 126.19, 101.06 (Ph*C*H), 85.39 (C1), 76.94 (C4), 75.59 (C3), 70.34 (C5), 69.55 (C6), 68.95 (C2), 25.85 ($SiC(CH_3)_3$), 23.24 (SCH_2), 18.34 ($SiC(CH_3)_3$), 15.35 (SCH_2CH_3), -4.24 ($SiCH_3$), -4.53 ($SiCH_3$).

Ethyl 2-O-benzoyl-3-O-tert-butyldimethylsilyl-4,6-O-benzylidene-1-thio-β-D-galactopyranosie **2.5d**

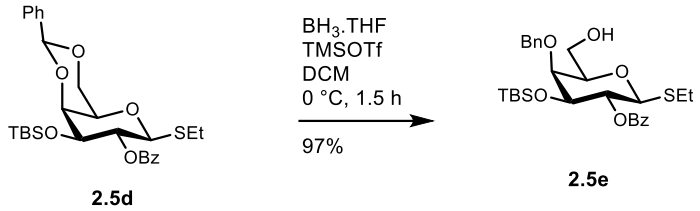


Crude starting material was dissolved in DCM (anhydrous, 160 mL) under Ar. The solution was cooled in an ice bath and benzoic anhydride (29.1 gm 129 mmol), DMAP (1.44 g, 12.8 mmol) and triethylamine (27 mL, 194 mmol) were successively added. After 1h, the cooling bath was removed, and the reaction was stirred at rt overnight. The reaction washed with sat. aq. $NaHCO_3$ (160 mL). The aqueous layer was extracted with DCM (160 mL) and the combined organic layers were washed with brine (300 mL). The organic layer was dried over $MgSO_4$, concentrated on rotavap and dried in high vacuum. Crystallization from ethanol gave **2.5d** (24.0 g, 70% over 2 steps).

^{1}H NMR (700 MHz, Chloroform-*d*) δ 8.04 (dd, J = 8.2, 1.1 Hz, 2H), 7.57 – 7.52 (m, 3H), 7.44 (td, J = 7.6, 1.7 Hz, 2H), 7.41 – 7.34 (m, 3H), 5.60 (t, J = 9.6 Hz, 1H, H-2), 5.53 (s, 1H, PhCH), 4.56 (d, J = 9.9 Hz, 1H, H-1), 4.39 (dd, J = 12.2, 1.6 Hz, 1H, H-6a), 4.14 (dd, J = 3.7, 1.1 Hz, 1H, H-4), 4.05 (dd, J = 12.2, 1.8 Hz, 1H, H-6b), 4.02 (d, J = 9.0, 2.6 Hz, 1H, H-3), 3.55 (q, J = 1.5 Hz, 1H, H-5), 2.91 (dq, J = 12.3, 7.5 Hz, 1H, SC*H*H), 2.75 (dq, J = 12.3, 7.5 Hz, 1H, SCH*H*), 1.26 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 0.75 (s, 9H), 0.05 (s, 3H), -0.12 (s, 3H).

^{13}C NMR (176 MHz, Chloroform-d) δ 165.39 (C=O), 138.02, 133.01, 130.45, 129.87, 128.97, 128.40, 128.27, 126.38, 101.21 (Ph*C*H), 82.90 (C1), 76.98 (C4), 73.64 (C3), 70.36 (C5), 70.20 (C2), 69.52 (C6), 25.56 ($SiC(CH_3)_3$), 22.86 (SCH_2), 18.04 ($Si(CH_3)_3$), 15.00 (SCH_2CH_3), -4.44 ($SiCH_3$), -4.59 ($SiCH_3$).

Ethyl 2-O-benzoyl-3-O-tert-butyldimethylsilyl-4-O-benzyl-1-thio-β-D-galactopyranoside **2.5e**



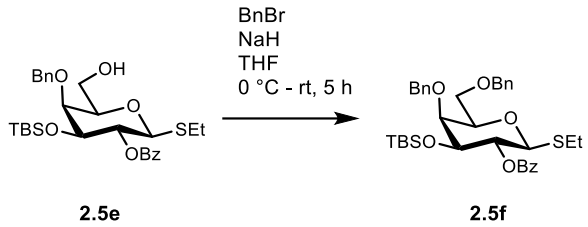
2.5d **2.5e**

Ethyl 3-O-tert-butyldimethylsilyl-4,6-O-benzylidene-1-thio-β-D-galacto-pyranoside (20.9 g, 39.4 mmol) was weighed in a 3-neck 250 mL rb flask and flushed with Ar. Then DCM (anhydrous, 97 mL) was added and the resulting colorless solution stirred in an ice bath for 15 min. Borane tetrahydrofuran (1 M in THF, 160 mL) was then dropwise added over 10 min followed by the addition of TMSOTf (3.6 mL, 20 mmol). After 1.5 h TLC showed complete conversion. The reaction was quenched by dropwise addition of a 1:10 mixture of NEt_3/MeOH until no further gas evolved. The resulting solution was concentrated under reduced pressure, redissolved in DCM (60 mL), washed with sat. aq. $NaHCO_3$ (60 mL), brine (60 mL), and dried over $MgSO_4$. The solution was then concentrated unde reduced pressure and dried under high vacuum to afford the product as a white foam (22.0 g, quant.).

^{1}H NMR (700 MHz, Chloroform-*d*) δ 8.05 (dd, J = 8.4, 1.3 Hz, 1H), 7.59 – 7.53 (m, 1H), 7.47 – 7.42 (m, 3H), 7.41 – 7.34 (m, 4H), 7.33 – 7.28 (m, 1H), 5.66 (br s, 1H, H-2), 5.10 (d, J = 11.7 Hz, 1H, 4-OC*H*HPh), 4.60 (d, J = 11.7 Hz, 1H, 4-OCH*H*Ph), 4.51 (d, J = 9.4 Hz, 1H, H-1), 3.98 (brd, J = 9.4 Hz, 1H, H-3), 3.85 (dd, J = 11.2, 6.7 Hz, 1H, H-6a), 3.78 (dd, J = 2.8, 1.1 Hz, 1H, H-4), 3.61 (ddd, J = 6.6, 5.2, 1.2 Hz, 1H, H-5), 3.57 (dd, J = 11.2, 5.3 Hz, 1H, H-6b), 2.76 (dq, J = 12.4, 7.4 Hz, 1H, SC*H*H), 2.69 (dq, J = 12.4, 7.5 Hz, 1H, SCH*H*), 1.21 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 0.79 (s, 9H), 0.12 (s, 3H), -0.07 (s, 3H).

^{13}C NMR (176 MHz, Chloroform-d) δ 165.31 (C=O), 138.45, 132.90, 130.23, 129.74, 128.36, 128.24, 127.93, 127.71, 83.70 (C1), 78.96 (C5), 76.80 (C4), 75.70 (C3), 74.78 (4-OCH_2Ph), 70.85 (C2), 62.16 (C6), 25.47 (SiC(CH_3)$_3$), 23.55 (SCH_2), 17.74(SiC(CH_3)$_3$), 14.77(SCH_2CH_3), -4.05 ($SiCH_3$), -5.10 ($SiCH_3$).

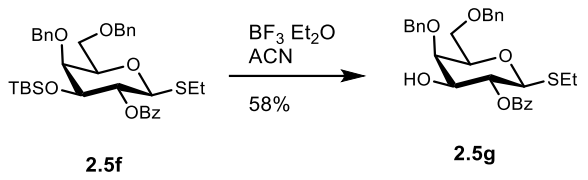
Ethyl 2-O-benzoyl-3-O-tert-butyldimethylsilyl-4,6-di-O-benzyl-1-thio-β-D-galactopyranoside **2.5f**



2.5e **2.5f**

Ethyl 2-O-benzoyl-3-O-tert-butyldimethylsilyl-4-O-benzyl-1-thio-β-D-galactopyranoside **2.5e** (21.0 g, 39.4 mmol) was taken in a 3-neck rb flask and flushed with Ar for 5 min. THF (anhydrous, 190 mL) was added resulting in a colorless solution that was stirred in an ice bath for 5 min. NaH (60% in mineral oil, 4.11 g, 103 mmol) was then added forming a grey suspension. After another 5 min BnBr (5.2 mL, 44 mmol) was dropwise added over 5 min. The cooling bath was removed after 30 min and 4 h later, TLC (Hex/EA 4:1) showed the product at rf = 0.89 and no starting material. The reaction mixture was diluted with DCM (100 mL) and quenched by dropwise addition of sat. aq. NH_4Cl (200 mL). The aqueous phase extracted with DCM (200 mL) and the combined organic layers were washed with brine (400 mL), dried over $MgSO_4$, evaporated and dried in high vacuum overnight to afford a yellowish oil. The crude product **2.5f** was used as such in the next step.

Ethyl 2-O-benzoyl-4,6-di-O-benzyl-1-thio-β-D-galactopyranoside **2.5g**



2.5f **2.5g**

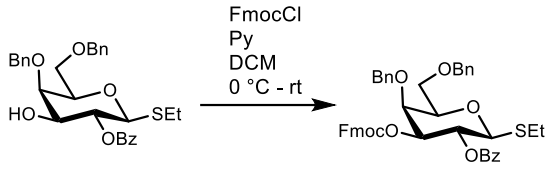
The crude material **2.5f** (39.4 mmol based on 100% yield) was taken in MeCN (anhydrous, 213 mL) and the resulting pale yellow solution was stirred in an ice bath for ca. 10 min. Boron trifluoride etherate (5.4 mL, 43.8 mmol) was added and the reaction mixture was quenched after 7 min by dropwise addition of sat. aq. $NaHCO_3$ (215 mL). The aqueous layer was isolated and extracted with DCM (215 mL). The combined organic layers were washed with brine (400 mL), dried over

$MgSO_4$, concentrated on rotavap and dried under high vacuum. The crude material was crystalized from EtOAc/Hex 1:2 to afford the pure **2.5g** (9.33 g, 47% over two steps).

^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 7.98 (dd, J = 8.2, 1.0 Hz, 2H), 7.67 (tt, J = 7.7, 7.3, 1.3 Hz, 1H), 7.58 – 7.50 (m, 2H), 7.41 – 7.17 (m, 10H), 5.55 (d, J = 5.4 Hz, 1H, 3-OH), 5.19 (t, J = 9.7 Hz, 1H, H-2), 4.93 (d, J = 11.4 Hz, 1H, PhC*H*H), 4.70 (d, J = 10.0 Hz, 1H, H-1), 4.52 (d, J = 11.5 Hz, 1H, PhC*H*H), 4.50 (d, J = 12.1 Hz, 1H, PhC*H*H), 4.44 (d, J = 12.0 Hz, 1H, PhC*H*H), 3.97 (ddd, J = 9.7, 5.5, 3.1 Hz, 1H, H-3), 3.89 (td, J = 6.1, 1.1 Hz, 1H, H-5), 3.83 (dd, J = 3.2, 1.1 Hz, 1H, H-4), 3.54 (d, J = 6.1 Hz, 2H, H-6), 2.68 – 2.52 (m, 2H, SCH_2), 1.14 (t, J = 7.4 Hz, 3H, CH_3).

^{13}C NMR (126 MHz, DMSO-*d*$_6$) δ 165.14, 138.98, 138.20, 133.23, 130.05, 129.35, 128.61, 128.24, 128.13, 127.63, 127.62, 127.49, 127.35, 82.77, 77.34, 76.70, 74.42, 72.61, 72.29, 71.67, 68.82, 40.02, 39.85, 39.69, 39.52, 39.35, 39.19, 39.02, 23.51, 15.10.

Ethyl 2-O-benzoyl-4,6-di-O-benzyl-1-thio-β-D-galactopyranoside **2.5**



2.5g **2.5**

Pyridine (4.0 mL, 49 mmol) and FmocCl (6.38 g, 24.7 mmol) were sequentially added to a solution of ethyl 2-O-benzoyl-4,6-di-O-benzyl-1-thio-β-D-galactopyranoside **2.5g** (8.37 g, 16.4 mmol) in DCM (anhydrous, 136 mmol) under inert atmosphere. The colorless solution turned yellow and after a few minutes turned red. After 4.5 h stirring at rt, TLC showed complete conversion. The reaction mixture was diluted with DCM (70 mL), extracted with water (3 X 200 mL) and brine (200 mL). Then dried over $MgSO_4$, filtered and the solvent was removed on rotavap. The resulting yellow oil was co-evaporated with toluene (3 X 20 mL) and dried in high vacuum (72 h). The resulting yellow solid was crystallized from EtOH (300 mL, 70 °C) to afford the product as an off-white solid (10.5 g, 87%).

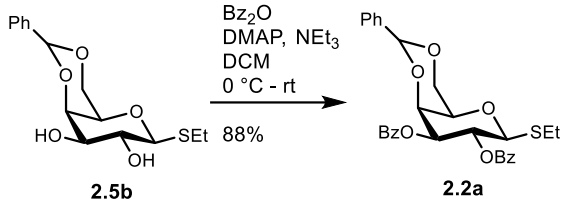
^{1}H NMR (400 MHz, Chloroform-d) δ 8.09 – 7.98 (m, 2H), 7.73 – 7.63 (m, 2H), 7.58 – 7.48 (m, 1H), 7.49 – 7.26 (m, 16H), 7.10 – 7.15 (m, 2H), 5.75 (t, J = 9.9 Hz, 1H, H-2), 5.07 (dd, J = 10.0, 3.0 Hz, 1H, H-3), 4.79 (d, J = 11.5 Hz, 1H, PhC*H*H), 4.60 (d, J = 9.9 Hz, 1H, H-1), 4.56 – 4.44 (m, 3H, $PhCH_2$, PhCH*H*), 4.30 (dd, J = 10.4, 7.1 Hz, 1H, H-6a), 4.22 (dd, J = 10.4, 7.8 Hz, 1H, H-6b), 4.14 (d, J = 2.9 Hz, 1H, H-4), 4.06 (t, J = 7.4 Hz, 1H, H-5), 3.82 (t, J = 6.6 Hz, 1H, Fmoc-CH), 3.67 (d, J = 6.6 Hz, 2H, Fmoc-CH_2), 2.74 (dtt, J = 19.8, 12.5, 7.5 Hz, 2H, SCH_2), 1.23 (t, J = 7.4 Hz, 3H, CH_3).

^{13}C NMR (101 MHz, Chloroform-d) δ 165.34, 154.63, 143.36, 142.92, 141.30, 141.19, 138.01, 137.81, 133.31, 130.04, 129.66, 128.56, 128.48, 128.40, 128.25, 127.99, 127.97, 127.90, 127.82, 127.20, 127.17, 125.27, 125.05, 120.05, 83.81, 79.11, 77.45, 77.33, 77.13, 76.81, 75.17, 74.07, 73.65, 70.19, 68.65, 68.14, 46.55, 23.97, 14.89.

HRMS (ESI) m/z calcd. for $[M+Na]^+$ 753.2498. Found 753.2498.

Synthesis of BB 2.2

Ethyl 2,3-di-O-benzoyl-4,6-O-benzylidene-1-thio-β-D-galactopyranoside **2.2a**



2.5b **2.2a**

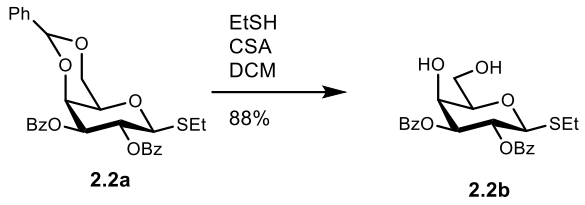
A solution of compound **2.5b** (34.2 g, 109 mmol) and Bz_2O (73.6 g, 3 equiv) in DCM (anhydrous, 340 mL) was stirred under Ar. Et_3N (60 mL, 4 equiv) and DMAP (740 mg, 3 mol% per OH group) were added and the reaction was stirred at rt. After 4 h, the mixture was washed with HCl 1 M (2 X 300 mL), sat. aq. $NaHCO_3$ (2 X 300 mL), and brine (300 mL). The organic layer was dried over MgSO4, concentrated under reduced pressure and dried under high vacuum. The resulting solid was

crystallized from EtOAc/Hex 1:2 to give pure compound **2.2a** as a white solid (50.4 g, 88%).

^{1}H NMR (700 MHz, Chloroform-d) δ 7.99 – 7.96 (m, 4H), 7.52 – 7.47 (m, 4H), 7.40 – 7.33 (m, 7H), 5.96 (t, J = 9.9 Hz, 1H, H-2), 5.54 (s, 1H), 5.40 (dd, J = 10.0, 3.5 Hz, 1H, H-3), 4.73 (d, J = 9.9 Hz, 1H, H-1), 4.63 (dd, J = 3.5, 1.1 Hz, 1H, H-4), 4.42 (dd, J = 12.4, 1.6 Hz, 1H, H-6a), 4.10 (dd, J = 12.4, 1.8 Hz, 1H, H-6b), 3.73 (q, J = 1.6 Hz, 1H, H-5), 2.95 (dq, J = 12.3, 7.5 Hz, 1H, SC*H*H), 2.81 (dq, J = 12.3, 7.5 Hz, 1H, SCH*H*), 1.31 (t, J = 7.5 Hz, 3H, $SCH_2C\mathit{H_3}$).

^{13}C NMR (176 MHz, Chloroform-d) δ 166.29 (C=O), 165.47 (C=O), 137.73, 133.49, 133.28, 130.05, 129.92, 129.70, 129.29, 129.17, 128.53, 128.47, 128.33, 126.43, 101.17 (4,6-O-*C*HPh), 83.07 (C1), 74.01, 73.99 (C4, C2), 70.08 (C5), 69.35 (C6), 67.33 (C3), 23.08 (CH_2), 15.02 (CH_3).

Ethyl 2,3-di-O-benzoyl-1-thio-β-D-galactopyranoside **2.2b**



2.2a **2.2b**

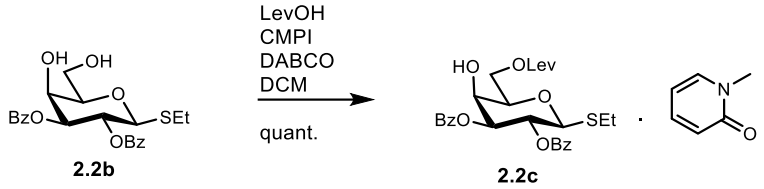
Ethyl 2,3-di-O-benzyl-4,6-O-benzylidene-1-thio-β-D-galactopyranoside (48 g, 92 mmol) was dissolved in DCM (230 mL) and the resulting solution stirred at rt. EtSH (69 mL, 918 mmol) and CSA (4.28 g, 18.4 mmol) were sequentially added and the reaction mixture was vigorously stirred overnight. The resulting white suspension was cooled to 4 °C. The white solid was filtered and washed with cold hexane. After drying, the solid was identified as the pure compound **2.2b** (29.8 g, 88%).

^{1}H NMR (500 MHz, DMSO-d6) δ 7.91 – 7.85 (m, 4H), 7.62 – 7.57 (m, 2H), 7.49 – 7.43 (m, 4H), 5.58 (t, J = 9.9 Hz, 1H, H-2), 5.38 (d, J = 5.6 Hz, 1H, 4-OH), 5.29 (dd, J = 9.8, 3.1 Hz, 1H, H-3), 4.94 (d, J = 9.9 Hz, 1H, H-1), 4.76 (dd, J = 6.1, 5.1 Hz, 1H, 6-

OH), 4.20 (ddd, J = 5.7, 3.1, 1.0 Hz, 1H, H-4), 3.79 (t, J = 6.3 Hz, 1H, H-5), 3.62 – 3.53 (m, 2H, H-6a, H-6b), 2.75 – 2.60 (m, 2H, SCH_2), 1.18 (t, J = 7.4 Hz, 3H, SCH_2CH_3).

^{13}C NMR (176 MHz, DMSO) δ 165.17 (C=O), 164.99 (C=O), 133.53, 133.43, 129.39, 129.22, 129.17, 129.14, 128.76, 128.61, 82.22 (C1), 78.76, 75.80, 68.64, 65.68, 59.89, 23.21 (CH_2), 15.05 (CH_3).

Ethyl 2,3-di-O-benzoyl-6-O-levulinoyl-2,3-di-O-benzoyl-1-thio-β-D-galacto-pyranoside **2.2c**



Ethyl 2,3-di-O-benzoyl-1-thio-β-D-galactopyranoside (808 mg, 1.87 mmol), DABCO (505 mg, 4.50 mmol), and 2-chloro-1-methylpyridium iodide (CMPI, 570 mg, 2.23 mmol) were combined with DCM (anhydrous, 6 mL) under a positive pressure of Ar and the resulting suspension was cooled in an ice bath. LevOH (8.40 g, 72.3 mmol) was added and the mixture was allowed to react for 6 h, while the temperature rose to rt. The mixture was then diluted with DCM (10 mL) and washed with sat. at. $NaHCO_3$ (15 mL). The aqueous layer was extracted with DCM (15 mL) and the combined organic layers were dried over $MgSO_4$, concentrated under reduced pressure and dried under high vacuum. The resulting oil was identified as a mixture of the product and N-methyl-2-pyridone (1.15 g, 1:1 mixture, quant.).

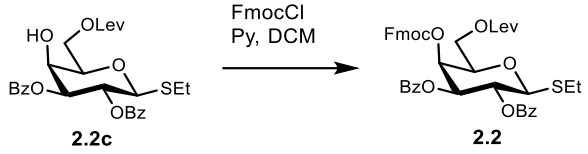
When procedure was scaled up to 28 g, a water contamination in DABCO led to an incomplete reaction. After column chromatography (hexane/DCM/EtOAc 10:1:10), the pure product was obtained (5.7 g, 17%).

^{1}H NMR (700 MHz, DMSO-d 6) δ 7.89 – 7.86 (m, 4H), 7.67 (ddd, J = 6.7, 2.1, 0.7 Hz, 1H, H-3 pyridone), 7.63 – 7.54 (m, 2H), 7.51 – 7.43 (m, 4H), 7.39 (ddd, J = 8.9, 6.6, 2.1 Hz, 1H, H-5 pyridone), 6.36 (ddd, J = 9.1, 1.4, 0.7 Hz, 1H, H-6 pyridone), 6.18 (td, J = 6.7, 1.4 Hz, 1H, H-4 pyridone), 5.65 (d, J = 5.7 Hz, 1H, 4-OH), 5.56 (t, J = 9.9 Hz, 1H, H-2), 5.36 (dd, J = 9.9, 3.2 Hz, 1H, H-3), 5.00 (d, J = 10.0 Hz, 1H, H-1), 4.20 (dd, J

= 11.4, 7.5 Hz, 1H, H-6a), 4.18 – 4.17 (m, 1H, H-4), 4.15 (dd, J = 11.4, 4.6 Hz, 1H, H-6b), 4.07 (dd, J = 7.6, 4.5 Hz, 1H, H-5), 3.41 (s, 3H, CH_3 pyridone), 2.73 (t, J = 6.4 Hz, 2H, CH_2 Lev), 2.71 – 2.68 (m, 1H, SCH*H*), 2.65 (dq, J = 12.8, 7.4 Hz, 1H, SC*H*H), 2.52 – 2.50 (m, 2H, CH_2 Lev), 2.12 (s, 3H, CH_3 Lev), 1.19 (t, J = 7.4 Hz, 3H, $SCH_2C\mathit{H_3}$).

^{13}C NMR (176 MHz, DMSO) δ 206.68 (C=O ketone), 172.16 (C=O Lev ester), 165.08 (C=O Bz), 164.96 (C=O Bz), 161.86 (C=O pyridone), 139.82, 139.78, 133.56, 133.48, 129.25, 129.19, 129.12, 128.76, 128.62, 119.13, 104.94, 82.24 (C1), 75.56 (C5), 75.09 (C3), 68.39 (C2), 66.17 (C4), 63.43 (C6), 54.89, 37.36 (CH_2 Lev), 36.69 (CH_3 pyridone), 29.56 (CH_3 Lev), 27.54 (CH_2 Lev), 23.53 (SCH_2), 15.18 ($SCH_2\mathit{C}H_3$).

Ethyl 2,3-di-O-benzoyl-4-*O*-(9-fluorenylmethoxy-carbonyl)-6-O-levulinoyl-2,3-di-O-benzoyl-1-thio-β-D-galactopyranoside **2.2**



Compound **2.2c** (5.60 g, mmol) was dissolved in DCM (anhydrous, 50 mL) under Ar. FmocCl (4.16 g, 1.5 equiv) and pyridine (2.6 mL, 3 equiv) were sequentially added and the resulting solution was stirred at rt overnight. The reaction mixture was diluted with DCM (50 mL), extracted with HCl 1M (2 X 100 mL), and brine (100 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude material was purified by column chromatography (Hexane:DCM:EtOAc 4:1:1 to 4:1:5) to afford the pure product as a white solid (6.96 g, 88%).

^{1}H NMR (700 MHz, Chloroform-d) δ 8.00 (dd, J = 8.0, 1.4 Hz, 2H), 7.82 – 7.75 (m, 4H), 7.64 (d, J = 7.4 Hz, 1H), 7.62 (d, J = 7.4 Hz, 1H), 7.54 – 7.50 (m, 1H), 7.45 – 7.31 (m, 7H), 7.08 (t, J = 7.7 Hz, 2H), 5.88 (t, J = 9.8 Hz, 1H, H-2), 5.52 (d, J = 7.9 Hz, 2H, H-3, H-4), 4.80 (d, J = 9.9 Hz, 1H, H-1), 4.40 (dd, J = 11.4, 6.5 Hz, 1H, H-6a), 4.29 – 4.24 (m, 2H, H-6b, FmocCH), 4.20 – 4.12 (m, 3H, H-5, Fmoc CH_2), 2.87 (dq, J = 12.3, 7.4 Hz, 1H, SC*H*H), 2.81 (dq, J = 12.4, 7.5 Hz, 1H, SCH*H*), 2.75 (td, J = 6.4, 1.6 Hz, 2H,

OLev), 2.59 (t, J = 6.5 Hz, 2H, OLev), 2.17 (s, 3H, OLev), 1.33 (t, J = 7.4 Hz, 3H, $SCH_2C\mathit{H_3}$).

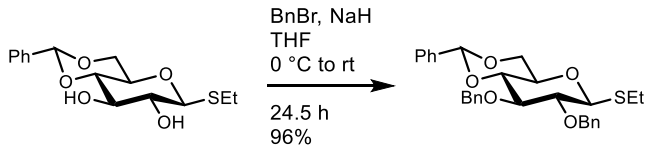
^{13}C NMR (176 MHz, Chloroform-d) δ 206.51 (C=O Lev ketone), 172.35 (C=O Lev ester), 165.56 (C=O Bz), 165.34 (C=O Bz), 154.83 (C=O carbonate), 143.50, 143.21, 141.36, 141.31, 133.44, 133.41, 129.97, 129.80, 129.47, 128.85, 128.52, 128.37, 128.06, 127.59, 127.45, 125.55, 125.44, 120.13, 120.10, 84.26 (C1), 74.51 (C5), 72.86, 71.82, 70.74 (C6), 67.85 (CH_2 Fmoc), 61.79 (C6), 46.56 (CH Fmoc), 38.02 (CH_2 Lev), 29.90 (CH_3 Lev), 27.97 (CH_2 Lev), 24.47 (SCH_2), 15.08 ($SCH_2\mathit{C}H_3$).

HRMS (ESI) m/z calcd. for $[M+K]^+$ 791.1923. Found 791.1898.

Synthesis of BB 2.3

General procedure for the optimization of the benzylation

A solution of starting material in the respective anhydrous solvent was stirred in an ice bath under Ar. NaH (60% in mineral oil) was added and after 10 min, BnBr (1.5 equiv) was dropwise added over 10 to 30 min. After 30 min, the cooling bath was removed, and the mixture was stirred at rt for the time stated for each experiment. For large scale reactions (≥ 1 g) an exhaust tube connected to a gas bubbler was attached to the reaction vessel to avoid accumulation of hydrogen. Crystallization led to the isolation of 50% of the starting material. [b] TLC showed the reaction was incomplete and side products were formed. [c]For safety reasons, in this reaction the cooling bath was kept for longer (1 h).



2.3a **2.3b**

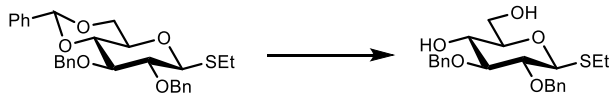
A solution of ethyl 4,6-*O*-benzylidene-1-thioglucopyranoside (39.9 g, 128 mmol) in DMF (anhydrous, 335 mL) was stirred under Ar and cooled in an ice bath for 5 to 10 min. NaH (60% in mineral oil, 16.0 g, 400 mmol) was added and after another 5 min BnBr (33.0 mL, 278 mmol) was added. After 30 min the cooling bath

was removed. After another 4.5 h, TLC showed complete conversion. The reaction was diluted with DCM (200 mL) and quenched by portion wise addition of sat. aq. NH_4Cl (400 mL). The aqueous layer was isolated and extracted with DCM (250 mL). The combined organic layers were washed with brine (500 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The resulting yellow solution was transferred to a 2 L flask. Ethanol (1.4 L) was dropwise added while stirring vigorously. Then H_2O (200 mL) was dropwise added. The resulting white suspension was kept at 4 °C overnight. The white solid was filtered and washed with cold EtOH/H_2O 3:1 (ca. 300 mL) and with cold hexane (ca. 200 mL). After drying under vacuum, the white solid was identified as the pure product (60.6 g, 96%).

^{1}H NMR (700 MHz, Chloroform-d) δ 7.52 – 7.48 (m, 2H), 7.42 – 7.32 (m, 9H), 7.33 – 7.26 (m, 4H), 5.59 (s, 1H, PhC*H*), 4.96 (d, J = 11.3 Hz, 1H, PhCH_2), 4.90 (d, J = 10.2 Hz, 1H, PhCH_2), 4.83 (d, J = 10.3 Hz, 1H, PhCH_2), 4.82 (d, J = 11.2 Hz, 1H, PhCH_2), 4.58 (d, J = 9.8 Hz, 1H, H-1), 4.37 (dd, J = 10.5, 5.0 Hz, 1H, H-6a), 3.82 (dd, J = 9.4, 8.4 Hz, 1H, H-3), 3.79 (t, J = 10.3 Hz, 1H, H-6b), 3.73 (t, J = 9.4 Hz, 1H,), 3.48 (dd, J = 9.8, 8.3 Hz, 1H, H-2), 3.47 (td, J = 9.7, 5.1 Hz, 1H, H-5), 2.80 (dq, J = 13.2, 7.8 Hz, 1H, SCH_2), 2.75 (dq, J = 12.6, 7.7 Hz, 1H, SCH_2), 1.33 (t, J = 7.4 Hz, 3H, CH_3).

^{13}C NMR (176 MHz, Chloroform-d) δ 138.54, 138.10, 137.42, 129.08, 128.49, 128.44, 128.38, 128.18, 128.02, 127.99, 127.83, 126.12, 101.25 (Ph*C*H), 85.99 (C1), 82.95 (C3), 81.75 (C4), 81.43 (C2), 76.10 (CH_2Ph), 75.36 (CH_2Ph), 70.37 (C5), 68.86 (C6), 25.31 (S*C*H_2), 15.26 (*C*H_3).

Ethyl 2,3-di-O-benzyl-1-thio-β-D-glucopyranoside **2.3c**



2.3b **2.3c**

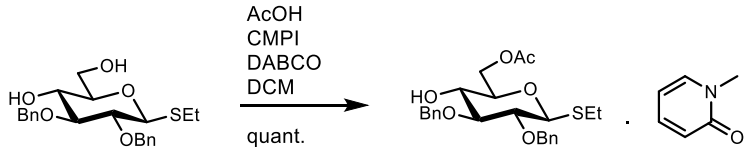
Compound **2.3b** (27.8 g, 56.4 mmol) was dissolved in DCM (140 mL) and the resulting colourless solution was stirred at rt. EtSH (43 mL, 580 mL) camphorsulfonic acid (2.65 g, 11.4 mmol) were sequentially added and the resulting solution was stirred overnight. The reaction mixture was concentrated under

reduced pressure and purified by a short column chromatography (Hex:EA 80:20 to 25:75). Compound **2.3c** was collected, concentrated, and dried under high vacuum and obtained as a white solid (22.6 g, 99%).

^{1}H NMR (700 MHz, Chloroform-d) δ 7.44 – 7.38 (m, 2H), 7.38 – 7.29 (m, 8H), 4.98 – 4.95 (m, 2H, $PhCH_2$), 4.74 – 4.71 (m, 2H, $PhCH_2$), 4.52 (d, J = 9.7 Hz, 1H, H-1), 3.87 (dd, J = 11.9, 3.5 Hz, 1H, H-6a), 3.75 (dd, J = 11.9, 5.2 Hz, 1H, H-6b), 3.58 (t, J = 9.3 Hz, 1H, H-4), 3.50 (t, J = 8.9 Hz, 1H, H-3), 3.40 (dd, J = 9.7, 8.7 Hz, 1H, H-2), 3.34 (ddd, J = 9.5, 5.2, 3.5 Hz, 1H, H-5), 2.83 – 2.70 (m, 2H, S CH_2), 2.36 (s, 1H, OH), 2.11 (br s, 1H, OH), 1.33 (t, J = 7.4 Hz, 1H, CH_3).

^{13}C NMR (176 MHz, Chloroform-d) δ 138.53, 137.94, 128.84, 128.58, 128.47, 128.18, 128.09, 128.02, 86.07 (C3), 85.50 (C1), 81.65 (C2), 79.19 (C5), 75.56, 75.52 (2x $PhCH_2$), 70.71 (C4), 62.93 (C6), 25.38 (SCH_2), 15.29 (CH_3).

Ethyl 6-O-acetyl-2,3-di-O-benzyl-1-thio-β-D-glucopyranoside **2.3d**



2.3c **2.3d**

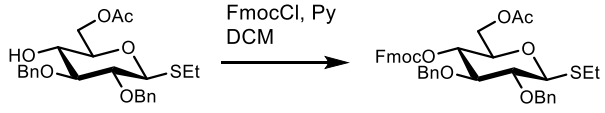
Ethyl 2,3-di-O-benzyl-1-thio-β-D-glucopyranoside (3.07 g, 7.59 mmol), DABCO (3.38 g, 30.1 mmol), and 2-chloro-1-methylpyridium iodide (CMPI, 4.74 g, 18.6 mmol) were dissolved in DCM (anhydrous, 65 mL) with pre-activated 4 Å MS (7 g) and the resulting mixture was cooled to -15 °C under a positive pressure of Ar. AcOH (470 µL) was portion wise added and the mixture was allowed to react for 4h, while the temperature rose to -10 °C. The mixture was then diluted with DCM (35 mL) and washed with sat. at. $NaHCO_3$ (100 mL). The aqueous layer was extracted with DCM (100 mL) and the combined organic layers were dried over $MgSO_4$, concentrated under reduced pressure and dried under high vacuum to afford the product in a 1:0.74 mixture with N-methyl-2-pyridone (3.94 g, 98%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.67 (dd, J = 6.6, 2.1 Hz, 1H, Pyridone), 7.40 (ddd, J = 8.9, 6.6, 2.2 Hz, 1H, Pyridone), 7.37 – 7.24 (m, 10H), 6.37 (dt, J = 9.2, 1.0 Hz,

1H, Pyridone), 6.19 (td, J = 6.7, 1.4 Hz, 1H, Pyridone), 5.59 (d, J = 6.5 Hz, 1H, 4-OH), 4.89 (d, J = 11.4 Hz, 1H, 3-OC*H*HPh), 4.75 (d, J = 10.9 Hz, 1H, 2-OC*H*HPh), 4.74 (d, J = 11.4 Hz, 1H, 3-OCH*H*Ph), 4.67 (d, J = 10.8 Hz, 1H, 2-OC*H*HPh), 4.58 (d, J = 9.8 Hz, 1H, H-1), 4.29 (dd, J = 11.9, 2.0 Hz, 1H, H-6a), 4.08 (dd, J = 11.9, 6.6 Hz, 1H, H-6b), 3.50 (ddd, J = 9.7, 6.7, 2.0 Hz, 1H, H-5), 3.49 (t, J = 8.8 Hz, 1H, H-3), 3.44 – 3.35 (m, 4H, H-4, NCH_3 pyridone), 3.26 (dd, J = 9.8, 8.8 Hz, 1H, H-2), 2.74 – 2.60 (m, 2H, SCH_2), 2.03 (s, 3H, 6-OCOCH_3), 1.23 (t, J = 7.4 Hz, 3H, SCH_2CH_3).

^{13}C NMR (126 MHz, DMSO-d$_6$) δ 170.25 (C=O Ac), 161.89 (C=O pyridone), 139.83, 138.96, 138.31, 128.08, 128.03, 127.67, 127.51, 127.45, 127.24, 119.14, 104.97, 85.68 (C3), 83.69 (C1), 80.68 (C2), 77.26 (C5), 74.32 (3-OCH_2Ph), 74.13 (2-OCH_2Ph), 70.20 (C4), 63.50 (C6), 36.69 (NCH_3 pyridone), 23.94 (SCH_2), 20.66 (6-OCOCH_3), 15.22 (SCH_2CH_3).

Ethyl 6-O-acetyl-2,3-di-O-benzyl-4-*O*-(9-fluorenylmethoxy-carbonyl)-1-thio-β-D-glucopyranoside **2.3**



2.3d **2.3**

Crude ethyl 6-*O*-acetyl-2,3-di-*O*-benzyl-1-thio-β-D-glucopyranoside (7.32 mmol) was dissolved in DCM (anhydrous, 35 mL) under Ar. FmocCl (3.78 g, 14.6 mmol) was added followed by pyridine (1.8 mL, 22 mmol). The resulting solution was stirred at rt overnight. Reaction mixture was extracted with HCl 1M (2X 40 mL), sat. $NaHCO_3$ (2X 40 mL) and brine (40 mL). The organic layer was dried over $MgSO_4$, concentrated under reduced pressure and purified by column (Hexane/DCM/EtOAc 10:1:0.5 to 10:1:2.5) to give the pure product was a white solid (3.81 g, 78%).

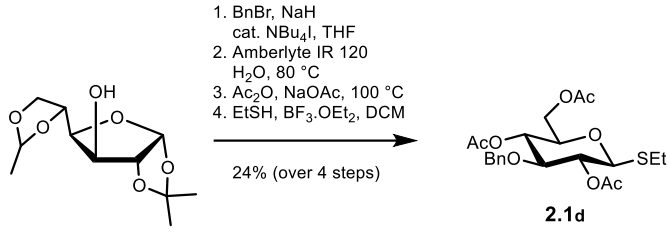
^{1}H NMR (500 MHz, Chloroform-d) δ 7.76 (dd, J = 7.6, 0.9 Hz, 2H), 7.59 (dt, J = 7.5, 0.9 Hz, 1H), 7.55 (dt, J = 7.5, 0.9 Hz, 1H), 7.40 – 7.17 (m, 14H), 4.96 – 4.86 (m, 2H, H-4, PhC*H*H), 4.83 (d, J = 11.2 Hz, 1H, PhCH*H*), 4.73 (d, J = 10.2 Hz, 1H, PhC*H*H), 4.67 (d, J = 11.2 Hz, 1H, PhCH*H*), 4.50 (d, J = 9.8 Hz, 1H, H-1), 4.45 (dd, J = 10.4, 7.0 Hz, 1H, Fmoc C*H*H), 4.37 – 4.24 (m, 2H, H-6a, Fmoc CH*H*), 4.23 – 4.12 (m, 2H, H-6b,

Fmoc CH), 3.72 (t, J = 9.1 Hz, 1H, H-3), 3.66 (ddd, J = 10.0, 5.4, 2.6 Hz, 1H, H-5), 3.50 (dd, J = 9.9, 8.7 Hz, 1H, H-2), 2.86 – 2.67 (m, 2H, SC*H*$_2$), 2.06 (s, 3H, COC*H*$_3$), 1.34 (t, J = 7.4 Hz, 3H, SCH$_2$C*H*$_3$).

^{13}C NMR (126 MHz, Chloroform-d) δ 170.81 (C=O Ac), 154.47 (C=O Fmoc), 143.39, 143.27, 141.41, 137.99, 128.57, 128.52, 128.44, 128.13, 128.05, 128.03, 127.84, 127.82, 127.33, 125.23, 125.10, 120.21, 120.19, 85.49 (C1), 83.82 (C3), 81.35 (C2), 75.75, 75.73, 75.57, 74.64, 70.38 (CH$_2$ Fmoc), 62.78 (C6), 46.82 (Fmoc CH), 25.33 (SCH$_2$), 20.91 (CO*C*H$_3$), 15.23 (SCH$_2$*C*H$_3$).

Synthesis of BB 2.1

Ethyl 2,4,6-tri-O-acetyl-3-*O*-benzyl-1-thio-β-D-glucopyranoside **2.1d**



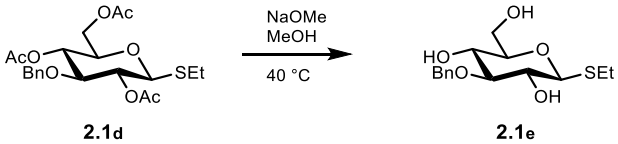
A solution of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (10 g, 39 mmol) in THF (anhydrous, 67 mL) under Ar was cooled in an ice bath. NaH (60% in mineral oil, 1.69 g, 70.4 mmol) was added and, after 5 min, BnBr (5.0 mL, 42 mmol) and INBu$_4$ (284 mg, 0.769 mmol) were added. After 45 min, the cooling bath was removed, and the reaction was stirred at rt. After 3 h, TLC showed complete conversion. The reaction was diluted with EtOAc (70 mL) and quenched with sat. aq. NH$_4$Cl (140 mL). The aqueous layer was extracted with EtOAc (70 mL). The combined organic layers were dried over MgSO$_4$, concentrated under reduced pressure and dried under high vacuum. The crude 3-*O*-benzyl-1:2,5:6-di-*O*-isopropylideneglucofuranose was combined with water (120 mL). Amberlyte IR 120 (16 g, pre-washed with MeOH and H$_2$O) was added and the resulting mixture was vigorously stirred under an 80 °C heating bath. After 6 h, TLC showed absence of starting material. The reaction mixture was filtered, evaporated under reduced pressure, and dried under high vacuum. The resulting material was combined with

Ac_2O (61.0 mL, 645 mmol) and NaOAc (1.3 g, 16 mmol), and stirred under a 100 °C heating bath for 1.5 h. The reaction mixture was poured into a beaker with ice to induce crystallization without success. The resulting solution was extracted with EtOAc. The organic layer was washed with sat. aq. $NaHCO_3$, concentrated under reduced pressure and dried under high vacuum. NMR analysis of the resulting oil showed a 1:4 α/β mixture of the target tetraacetate. The crude material was combined with DCM (anhydrous, 154 mL) and EtSH (3.9 mL, 54 mmol) under Ar, and the resulting solution was cooled in an ice bath. $BF_3{\cdot}OEt_2$ (7.5 mL, 61 mL) was dropwise added over 10 min. After 30 min, the cooling bath was removed, and the mixture was stirred at rt for 5 h. The reaction was quenched with H_2O (150 mL). The organic layer washed with sat. aq. $NaHCO_3$ (150 mL) and brine (150 mL), dried over $MgSO_4$, concentrated under reduced pressure, and dried in high vacuum overnight. The resulting material was purified by column chromatography (EtOAc in Hexane, 20% to 35%). Compound **2.1d** was isolated as a white solid (4.32 g, 24% over 4 steps).

^{1}H NMR (500 MHz, Chloroform-d) δ 7.37 – 7.23 (m, 3H), 7.27 – 7.18 (m, 2H), 5.10 (t, J = 9.7 Hz, 1H, H-2), 5.08 (t, J = 9.6 Hz, 1H, H-4), 4.67 – 4.54 (AB q, 2H, $PhCH_2$), 4.40 (d, J = 10.3 Hz, 1H, H-1), 4.18 (dd, J = 12.3, 5.3 Hz, 1H, H-6a), 4.11 (dd, J = 12.3, 2.5 Hz, 1H, H-6b), 3.70 (t, J = 9.3 Hz, 1H, H-3), 3.60 (ddd, J = 10.0, 5.3, 2.5 Hz, 1H, H-5), 2.79 – 2.60 (m, 2H, SCH_2), 2.07 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.96 (s, 3H, Ac), 1.25 (t, J = 7.5 Hz, 3H, $SCH_2C\mathit{H_3}$).

^{13}C NMR (126 MHz, DMSO-d$_6$) δ 170.86 (C=O), 169.43 (C=O), 169.39 (C=O), 137.86, 128.54, 127.94, 127.82, 83.77 (C1), 81.60 (C3), 76.29 (C5), 74.27 ($Ph\mathit{C}H_2$), 71.32, 69.73 (C2, C4), 62.59 (C6), 24.05 (SCH_2), 21.01, 20.86, 20.83 (3x $C{=}O\mathit{C}H_3$), 14.87 ($SCH_2\mathit{C}H_3$).

Ethyl 3-*O*-benzyl-1-thio-β-D-glucopyranoside **2.1e**



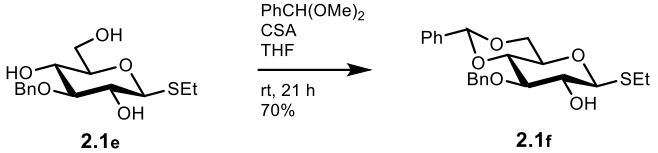
2.1d **2.1e**

Ethyl 2,4,6-tri-O-acetyl-3-*O*-benzyl-1-thio-β-D-glucopyranoside **2.1d** (3.98 g, 9.03 mmol) was suspended in MeOH (anhydrous, 30 mL). NaOMe (5.4 M in MeOH, 300 µL, 1.62 mmol) was added and the resulting suspension was stirred at rt for 2 h. The resulting solution was neutralized with Amberlyte IR 120, concentrated under reduced pressure and dried under high vacuum. NMR analysis showed a mixture of the target trial and a monoacetyl intermediate. The crude material was dissolved in MeOH (anhydrous, 30 mL) and NaOMe (5.4 M in MeOH, 1 mL, 5.4 mmol). The resulting solution was stirred under a 40 °C bath for 6h. The reaction mixture was neutralized with Amberlyte IR 120, concentrated under reduced pressure and dried under high vacuum. The resulting oil was identified as the pure compound **2.1e** (2.80 mg, 99%).

^{1}H NMR (500 MHz, DMSO-d$_6$) δ 7.42 – 7.40 (m, 2H), 7.33 – 7.29 (m, 2H), 7.28 – 7.21 (m, 1H), 5.31 (brs, 1H, OH), 5.14 (brs, 1H, OH), 4.85 – 4.74 (ABq, 2H, PhC*H$_2$*), 4.32 (d, J = 9.5 Hz, 1H, H-1), 3.68 (dd, J = 11.9, 2.1 Hz, 1H, H-6a), 3.45 (dd, J = 11.9, 5.9 Hz, 1H, H-6a), 3.28 – 3.22 (m, 2H, H-3, H-4), 3.20 – 3.14 (m, 2H, H-2, H-5), 2.72 – 2.58 (m, 2H, SCH$_2$), 1.20 (t, J = 7.4 Hz, 3H, SCH$_2$C*H$_3$*).

^{13}C NMR (126 MHz, CDCl$_3$) δ 139.54, 127.91, 127.47, 127.04, 86.63 (C1), 85.02, 81.02, 73.88 (Ph*C*H$_2$), 72.81, 69.71, 61.06, 22.96 (SCH$_2$), 14.97 (SCH$_2$*C*H$_3$).

Ethyl 3-*O*-benzyl-4,6-O-benzylidene-1-thio-β-D-glucopyranoside **2.1f**



2.1e **2.1f**

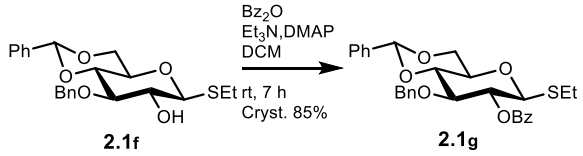
Ethyl 3-*O*-benzyl-1-thio-β-D-glucopyranoside (2.74 g, 8.72 mmol) was taken in a round-bottom flask and purged with Ar. CSA (373 mg, 1.61 mmol) was added

followed by anhydrous THF (19 mL). The resulting mixture was stirred at rt and after a few minutes a pale orange solution was formed. Benzaldehyde dimethyl acetal (2.9 mL, 19 mmol) was then added and the resulting solution stirred at rt. After 21 h, TLC (Hex/ EtOAc 2:1) showed the product (rf = 0.36) as major component. Reaction mixture was diluted with EtOAc (20 mL), extracted with aq. sat. $NaHCO_3$ (2x 40 mL), brine (40 mL), dried over $MgSO_4$ and concentrated to dryness under reduced pressure to give a white to pale yellow solid (4.39 g). The crude material was crystallized from EtOAc (11 mL, 70 °C) and hexane (50 mL) to give the pure product as a white solid (2.37 g, 68%). The mother liquor was concentrated and further subjected to crystallization from EtOAc (2.5 mL, 70 °C) and hexane (12.5 mL) to give a second portion of the pure product as white crystals (105 mg, 3%).

^{1}H NMR (700 MHz, DMSO-*d*$_6$) δ 7.43 – 7.36 (m, 7H, Ar-H), 7.31 – 7.28 (m, 2H, Ar-H), 7.27 – 7.24 (m, 1H, Ar-H), 5.67 (br s, 1H, 2-O*H*), 5.67 (s, 1H, PhC*H*), 4.82 (d, J = 12.0 Hz, 1H, PhC*H*H), 4.79 (d, J = 12.1 Hz, 1H, PhCH*H*), 4.56 (d, J = 9.7 Hz, 1H, H-1), 4.22 (dd, J = 10.1, 5.0 Hz, 1H, H-6a), 3.71 (t, J = 10.2 Hz, 1H, H-6b), 3.65 (t, J = 9.4 Hz, 1H, H-4), 3.56 (dd, J = 9.4, 8.3 Hz, 1H, H-3), 3.49 (ddd, J = 10.1, 9.3, 5.0 Hz, 1H, H-5), 3.37 – 3.34 (m, 1H, H-2), 2.67 (dq, J = 12.7, 7.4 Hz, 1H, SC*H*H), 2.63 (dq, J = 12.8, 7.5 Hz, 1H, SCH*H*), 1.22 (t, J = 7.4 Hz, 3H, C*H*$_3$).

^{13}C NMR (176 MHz, DMSO-*d*$_6$) δ 139.50, 138.14, 129.18, 128.53, 128.44, 127.94, 127.67, 126.41 (8X Ar-C), 100.54 (Ph*C*H), 86.29 (C1), 82.70 (C3), 80.78 (C4), 73.99 (Ph*C*H$_2$), 73.47 (C2), 70.02 (C5), 68.29 (C6), 23.71 (S*C*H$_2$), 15.63 (*C*H$_3$).

Ethyl 2-O-benzoyl-3-*O*-benzyl-4,6-O-benzylidene-1-thio-β-D-glucopyranoside **2.1g**



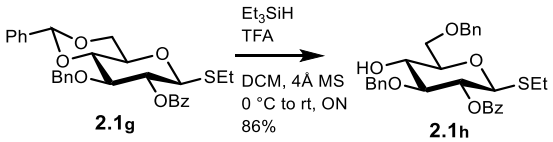
Ethyl 3-*O*-benzyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside (2.32 g, 5.76 mmol) and benzoic anhydride (19.8 g, 8.75 mmol) were taken in a 3-neck round-

bottom flask and purged with Ar (ca. 5 min). Anhydrous DCM (18 mL) resulting in a colourless solution that was stirred at rt under a positive pressure of Ar. Triethylamine (1.6 mL, 11 mmol) and DMAP (21 mg, 3 mol%) were subsequently added. After 7 h, TLC (hexane/EtOAc 4:1) showed complete conversion (Rf = 0.32). The reaction mixture was extracted with 1 M HCl (2X 20 mL), sat. aq. $NaHCO_3$ (2X 20 mL), dried over $MgSO_4$ and concentrated under reduced pressure and further dried under high vacuum to give a white solid (3.21 g). The crude product was dissolved in EtOAc (4 mL, 70 °C) and hexane (32 mL) was dropwise added while stirring vigourously. The resulting suspension was kept at 4 °C overnight and filtered. The white solid powder residue was dried in the desiccator under vacuum overnight and identified as the pure product (2.49, 85%).

^{1}H NMR (700 MHz, $CDCl_3$) δ 8.06 – 8.01 (m, 2H), 7.65 – 7.60 (m, 1H), 7.56 – 7.52 (m, 2H), 7.51 – 7.46 (m, 2H), 7.46 – 7.38 (m, 3H), 7.19 – 7.14 (m, 3H), 7.14 – 7.08 (m, 2H), 5.65 (s, 1H, PhC*H*), 5.37 (dd, J = 10.1, 8.7 Hz, 1H, H-2), 4.86 (d, J = 11.9 Hz, 1H, 3-OC*H*H), 4.73 (d, J = 12.0 Hz, 1H, 3-OC*H*H), 4.65 (d, J = 10.1 Hz, 1H, H-1), 4.44 (dd, J = 10.5, 5.0 Hz, 1H, H-6a), 3.93 (dd, J = 9.3, 8.6 Hz, 1H, H-3), 3.88 (t, J = 9.3 Hz, 1H, H-4), 3.86 (t, J = 10.3 Hz, 1H, H-6b), 3.59 (ddd, J = 10.0, 9.2, 5.0 Hz, 1H, H-5), 2.80 – 2.70 (m, 2H, SC*H*$_2$), 1.25 (t, J = 7.4 Hz, 3H, C*H*$_3$).

^{13}C NMR (126 MHz, $CDCl_3$) δ 165.25 (C=O), 137.86, 137.28, 133.28, 130.01, 129.84, 129.14, 128.46, 128.38, 128.25, 128.14, 127.66, 126.10, 101.37 (Ph*C*H), 84.40 (C1), 81.76 (C4), 79.31 (C3), 74.31 (Ph*C*H$_2$), 71.96 (C2), 70.81 (C5), 68.74 (C6), 24.11(S*C*H*2*), 14.90 (*C*H$_3$).

Ethyl 2-O-benzoyl-3-*O*-benzyl-4,6-O-benzylidene-1-thio-β-D-glucopyranoside **2.1h**



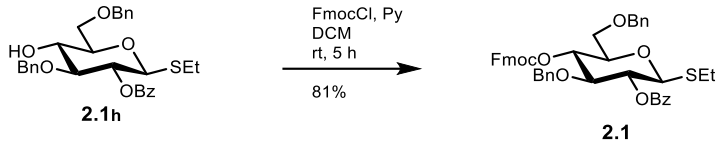
Ethyl 2-*O*-benzoyl-3-*O*-benzyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside was taken in a 100 mL 3-neck round-bottom flask and flushed with Ar (ca. 5 min).

DCM (anhydrous, 9.5 mL) was added resulting in a slightly viscous colourless solution to which activated 4 Å MS (1.5 g) were added. The mixture was stirred in an ice bath under Ar for 5 min. Et_3SiH (3.0 mL, 19 mmol) was added and after 20 min, TFA (1 450 µL, 19 mmol) was dropwise added over 1 min. After stirring overnight, the cooling bath had warmed up to rt and TLC (hexane/EtOAc 2:1) showed absence of the starting material and the product at Rf = 0.30. The reaction was diluted with MeOH (10 mL) and quenched by dropwise addition of Et_3N (2.6 mL). After stirring for ca. 15 min with the flask open, the reaction mixture was concentrated under reduced pressure and purified by column (hexane/EtOAc 80:20 to 70:30) to give the pure product as a colorless oil (2.08 g, 86%).

^{1}H NMR (700 MHz, $CDCl_3$) δ 8.08 – 8.06 (m, 2H), 7.62 – 7.59 (m, 1H), 7.50 – 7.47 (m, 2H), 7.40 – 7.36 (m, 4H), 7.34 – 7.31 (m, 1H), 7.23 – 7.20 (m, 5H), 5.31 (dd, J = 10.0, 9.1 Hz, 1H, PhC*H*), 4.76 (d, J = 11.5 Hz, 1H, 3-OC*H*H), 4.72 (d, J = 11.5 Hz, 1H, 3-OCH*H*), 4.65 (d, J = 12.0 Hz, 1H, 6-OC*H*H), 4.61 (d, J = 12.0 Hz, 1H, 6-OCH*H*), 4.58 (d, J = 10.0 Hz, 1H, H-1), 3.85 – 3.80 (m, 3H, H-4, H-6a, H-6b), 3.73 (t, J = 9.0 Hz, 1H, H-3), 3.60 (ddd, J = 9.6, 5.1, 4.5 Hz, 1H, H-4), 2.83 (d, J = 2.4 Hz, 1H, 4-OH), 2.79 – 2.67 (m, 2H, SC*H*$_2$), 1.25 (t, J = 7.5 Hz, 1H, CH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ 165.27 (C=O), 137.95, 137.71, 133.19, 129.86, 128.48, 128.42, 128.40, 127.98, 127.85, 127.78, 83.64 (C1), 83.56 (C3), 78.21 (C5), 74.67 (3-O*C*H_2), 73.78 (6-O*C*H_2), 72.33 (C4), 72.02 (C2), 70.55 (C6),23.97 (S*C*H2), 14.90 (*C*H_3).

Ethyl 2-O-benzoyl-3,6-di-*O*-benzyl-4-*O*-(9-fluorenylmethoxy-carbonyl)-1-thio-β-D-glucopyranoside **2.1**



Ethyl 2-*O*-benzoyl-3,6-di-*O*-benzyl-1-thio-β-D-glucopyranoside (2.01, 3.95 mmol) was taken in a 100 mL round-bottom flask and purged with Ar (ca. 5 min). Anhydrous DCM (20 mL) was added and the resulting colorless solution stirred at

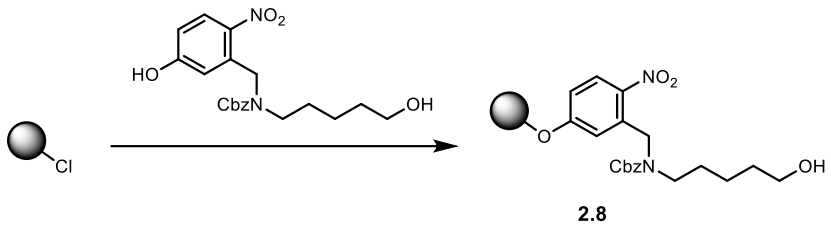
rt under a positive pressure of Ar. FmocCl (1.53 g, 5.88 mmol) was added followed by anhydrous pyridine (0.95 mL, 12 mmol). The solution turned yellow and within a few minutes turned reddish. After 5 h, TLC showed complete conversion (Rf = 0.33). Reaction mixture was diluted with DCM (20 mL), extracted with HCl 1M (2X 40 mL), sat. $NaHCO_3$ (2X 40 mL) and brine (40 mL). The organic layer was dried over $MgSO_4$, concentrated under reduced pressure and further dried in high vacuum to afford a yellow oil (3.04 g). The crude product was purified by column (Hexane/DCM/EtOAc 10:1:0.5 to 10:1:3) to give the pure product was a white solid (2.35 g, 81%).

^{1}H NMR (700 MHz, $CDCl_3$) δ 8.06 – 8.04 (m, 2H), 7.78 (ddt, J = 7.6, 7.0, 0.9 Hz, 2H), 7.63 – 7.60 (m, 2H), 7.57 (dq, J = 7.5, 0.9 Hz, 1H), 7.50 – 7.47 (m, 2H), 7.42 (tddd, J = 7.5, 4.9, 1.1, 0.6 Hz, 2H), 7.37 – 7.35 (m, 2H), 7.34 – 7.29 (m, 4H), 7.28 – 7.23 (m, 1H), 7.12 – 7.06 (m, 5H), 5.37 (dd, J = 10.0, 9.1 Hz, 1H, H-2), 5.03 (dd, J = 9.9, 9.2 Hz, 1H, H-4), 4.63 (d, J = 11.5 Hz, 1H, 3-OC*H*HPh), 4.60 (d, J = 10.0 Hz, 1H, H-1), 4.59 (d, J = 11.5 Hz, 1H, 3-OCH*H*Ph), 4.58 (s, 2H, 6-OC*H*$_2$Ph), 4.39 – 4.33 (m, 2H, Fmoc-C*H*$_2$), 4.16 (t, J = 7.2 Hz, 1H, fluorene H-9), 3.95 (t, J = 9.1 Hz, 1H, H-3), 3.79 (ddd, J = 10.0, 5.2, 3.9 Hz, 1H, H-5), 3.73 – 3.68 (m, 2H, H-6a, H-6b), 2.80 – 2.70 (m, 2H, SC*H*$_2$), 1.27 (t, J = 7.4 Hz, 3H, C*H*$_3$).

^{13}C NMR (176 MHz, $CDCl_3$) δ 165.03 (C=O Bz), 154.24 (C=O Fmoc), 143.30, 143.14, 141.32, 141.29, 137.90, 137.39, 133.25, 129.89, 129.70, 128.42, 128.33, 128.15, 127.92, 127.83, 127.62, 127.18, 125.10, 125.01, 120.08, 83.70 (C1), 81.13 (C3), 77.44 (C5), 75.58 (C4), 74.39 (3-O*C*H_2Ph), 73.62 (6-O*C*H_2Ph), 71.92 (C2), 70.05 (CH_2 Fmoc), 69.73 (C6), 46.73 (CH Fmoc), 24.07 (S*C*H_2), 14.91 (SCH_2*C*H_3).

HRMS (ESI/QTOF) m/z: $[M+Na]^+$ 753.2493. Found 753.2505.

Functionalization of the solid phase



2.8

5g batch

Merrifield Resin (0.46 mmol/g, 5.06 g, 2.33 mmol) was washed swollen in DMF (anhydrous, 20 mL) for ca. 20 min. Benzyl (5-hydroxy-2-nitrobenzyl)-(5-hydroxypentyl)carbamate (7.6 g, 8 equiv), Cs_2CO_2 (2.35 g, 3 equiv) and TBAI (482 mg, 0.5 equiv) were added to the suspension. The resulting mixture was gently stirred at 60 °C overnight. Afterwards, the resin was allowed to cool, filtered, washed four times each with 1:1 THF:H_20, THF, DMF, DCM and MeOH. The resin was dried in a desiccator under vacuum and protected from light.

Loading determination

A sample of the dried resin (200 mg) was taken into a 20 mL filter syringe. The syringe with the resin was purged with Ar and anhydrous DCM (15 mL) was uptaken. The syringe was wrapped in aluminum foil and shaken in an orbital shaker. After 30 min, the solvent was dispensed. A solution of FmocCl (600 mg) and pyridine (400 µL) in anhydrous DCM (15 mL) was uptaken and the syringe placed back in the shaker. After 18 h, the syringe was drained, and the resin abundantly washed with DCM and MeOH. The resin was dried under vacuum, protected from light, and the loading was determined by cleavage of Fmoc with DBU, according to a reported protocol[51,52]. The loading was 0.40 mmol/g.

50g batch

Merrifield resin (50.3 g, 0.46 mmol/g) suspended in DMF (anhydrous, 100 mL) in a round-bottom flask. Benzyl (5-hydroxy-2-nitrobenzyl)-(5-hydroxypentyl)-carbamate (50.1 g), Cs_2CO_3 (50.0 g) and TBAI (10.1 g) were added resulting in a strong yellow coloured mixture. After swirling the mixture for a few minutes, most of the added solids were dissolved and the flask was attached to a rotavap with a

heating bath at 60 °C and slow rotation. The vacuum was set to 600 mbar and the vacuum pump was turned off after the set point was reached. The reaction mixture was protected from light with aluminium foil and allowed to react in the rotavap. After 22h, the resin was filtered through a pore 1 sintered funnel and washed four times each with THF, H_2O, THF, DMF, and then 4X alternating cycles of DCM and MeOH. A last portion of DCM was added, and the resin allowed to swell for a few minutes. The solvent was drained, and the resin transferred into a round-bottom flask. DMF (anhydrous, 150 mL) and CsOAc (24.0 g) were added and the mixture rotavaped for a few minutes to remove DCM. The vacuum pump was turned off, and the mixture allowed to react in the rotavap with a 60 °C heating bath and slow rotation, protected from light with aluminium foil. After 24 h, the mixture was removed from the rotavap. The resin was filtered through a pore 1 sintered funnel and washed four times each with THF, THF:H_2O 1:1, H_2O, THF, DMF, and then 4X alternating cycles of DCM (300 mL) and MeOH (300 mL). The resin was dried in a desiccator under vacuum and protected from light for 48h.

Loading determination

As described for the 5 g batch. The loading was 0.40 mmol/g.

3. Automated Glycan Assembly of Fragments of GBS Type IV Capsular Polysaccharide

3.1. Introduction

The capsular polysaccharide of GBS type IV has received little attention from synthetic groups because this serotype represents only 1.1% of GBS disease cases. However, the prevalence is rising both in neonate and adult disease cases.[2,5]

So far, no synthetic route has been described to prepare GBS type IV CPS oligosaccharides. The repeating unit of GBS type IV consists of a branched hexasaccharide that bears an α-glucoside residue and the Neu5Ac-α-2,3-Gal motif that is common to all GBS serotypes[54] (**Figure 3.1**). These are the most challenging linkages in the repeating unit structure.

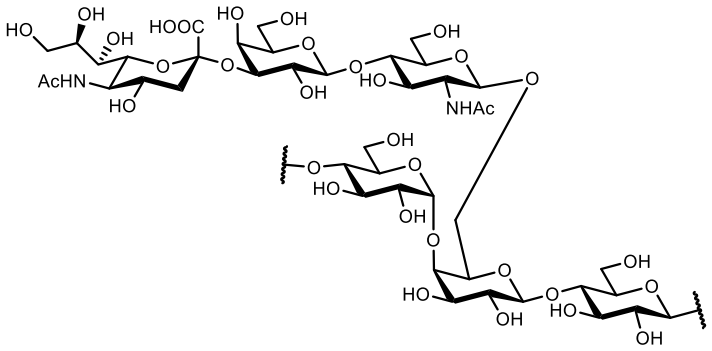
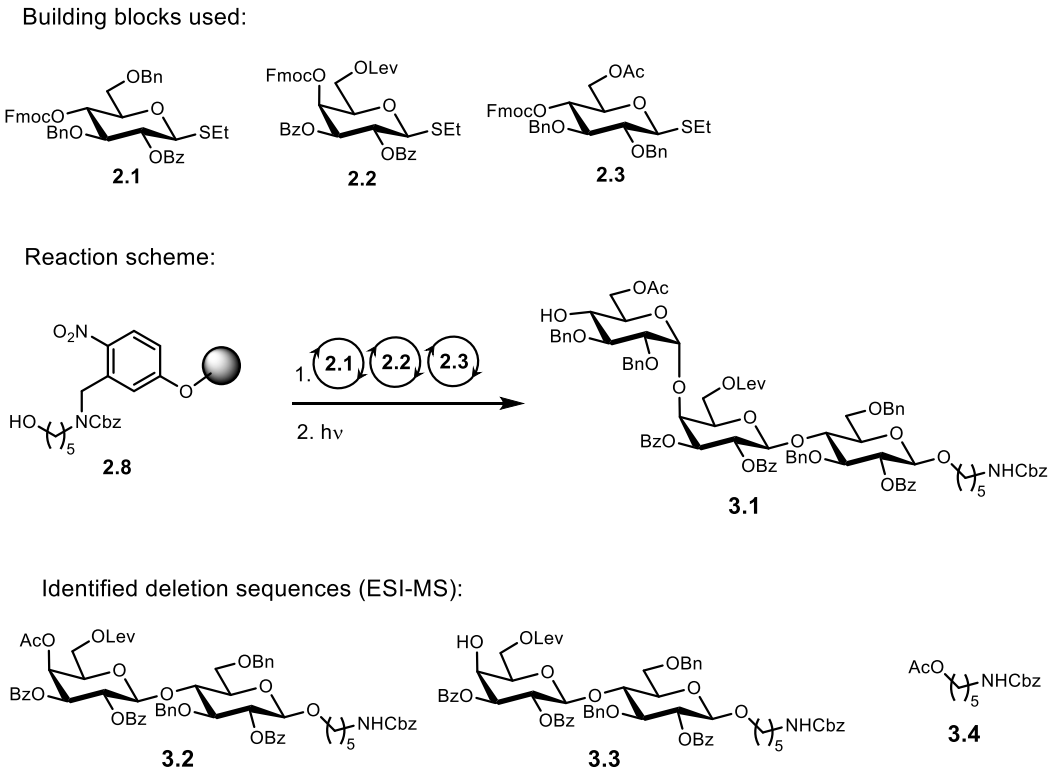


Figure 3.1 Repeating unit of GBS type IV CPS.

This chapter describes a strategy to assemble the backbone trimer of GBS type IV repeating unit using the Glyconeer. The synthesis of oligosaccharides using the Glyconeer is based on the optimization of an elongation cycle that incorporates each building block (BB) into the growing oligosaccharide chain. Each cycle is comprised of four modules: acid wash, glycosylation, capping and deprotection. Based on data from the Fmoc sensor and HPLC analysis of the crude material obtained after photocleavage, key parameters related to the glycosylation module were optimized: temperature of glycosylation, number of coupling repetitions, and concentration of donor.

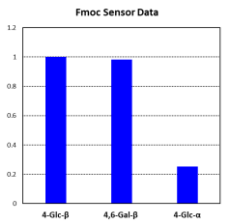
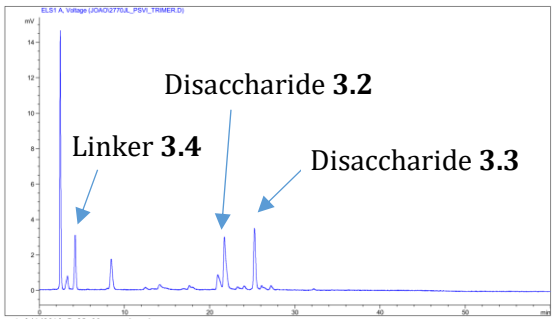
3.2. Results

Initially, all the building blocks were coupled using the same conditions: single coupling with 6.5 equiv of donor, and temperature -20 to 0 °C (**Scheme 3.1**, **Table 3.1**). The Fmoc sensor data (normalized for the first coupling), showed a very low efficiency for the coupling of BB **2.3**. The corresponding HPLC showed the formation of the disaccharide deletion sequence but not the product.



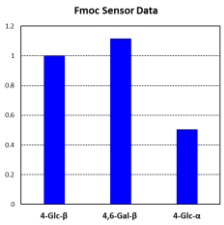
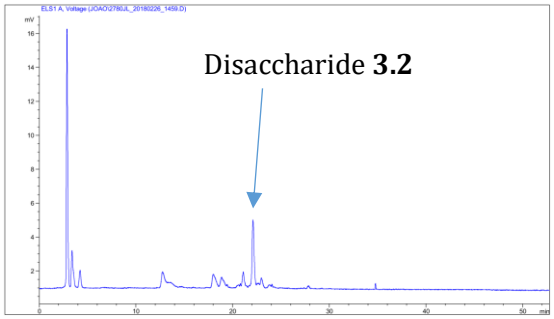
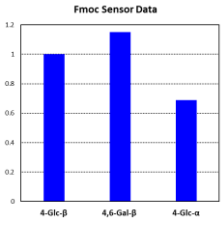
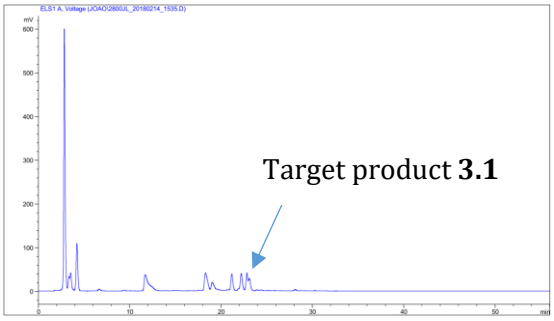
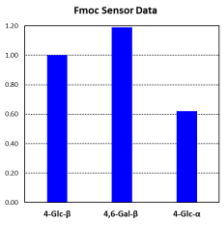
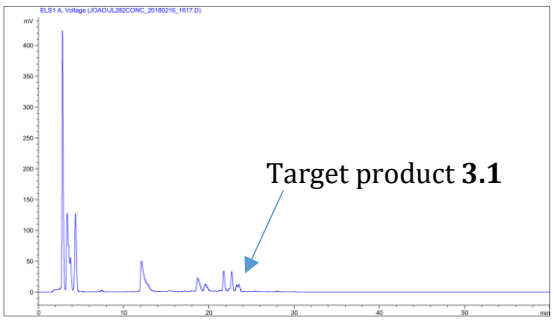
Scheme 3.1 AGA trisaccharide **3.1** using BBs **2.1**, **2.2** and **2.3**. Circular arrows represent the elongation cycle (acid wash, glycosylation, capping, deprotection) for each BB.

Table 3.1 AGA of GBS type IV backbone trisaccharide **3.1**.

Entry	Conditions	UV Sensor Data	HPLC of crude material
1	**2.1** 1x 6.5 equiv -20 to 0 °C **2.2** 1x 6.5 equiv -20 to 0 °C **2.3** 1x 6.5 equiv -20 to 0 °C		

Judging by the UV data and the HPLC data, the major bottleneck is the coupling of glucoside **2.3**. With that in mind, a series of experiments were conducted to optimize this coupling (**Table 3.2**).

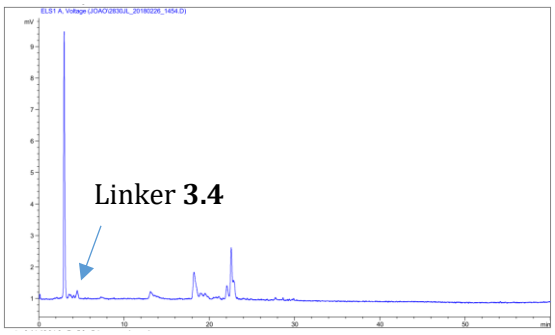
Table 3.2 Optimization of the coupling of the third BB (glucoside **2.3**).

Entry	Conditions	UV Sensor Data	HPLC of crude material
1	**2.1** 1x 6.5 equiv -20 to 0 °C **2.2** 1x 6.5 equiv -20 to 0 °C **2.3** 2x 6.5 equiv -20 to 0 °C		
2	**2.1** 1x 6.5 equiv -20 to 0 °C **2.2** 1x 6.5 equiv -20 to 0 °C **2.3** 2x 6.5 equiv -30 to -10 °C		
3	**2.1** 1x 6.5 equiv -20 to 0 °C **2.2** 1x 6.5 equiv -20 to 0 °C **2.3** 2x 6.5 equiv -40 to -20 °C		

When BB **2.3** was used in a double coupling (**Table 3.2**, entry 1), a marked increase in the glycosylation efficiency was observed (according to the UV sensor). Next, the temperature effect was probed. When **2.3** was coupled at -30 to -10 °C there was a further increase in the coupling efficiency (**Table 3.2**, entry 2). When the temperature was decreased to -40 to -20 °C, no change was observed in the coupling efficiency compared with -30 to -10 °C (**Table 3.2**, entry 3).

A common peak in all the HPLC traces was identified as the capped linker **3.4** (retention time 4 min). When the first BB was used in a double coupling, this peak was negligible (**Table 3.3**).

Table 3.3 Optimization of the coupling of the first BB (glucoside **2.1**).

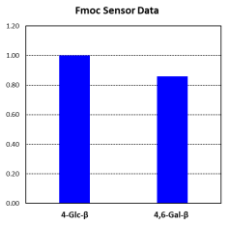
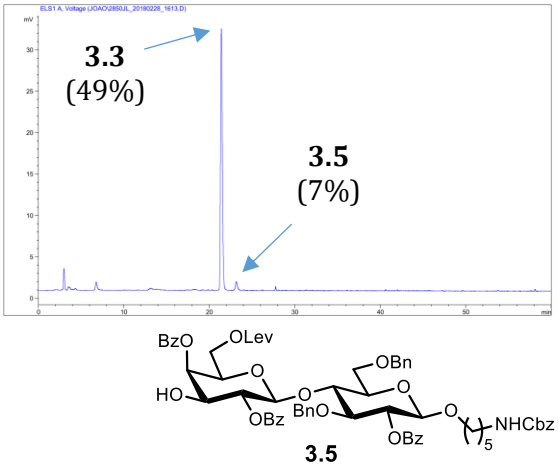
Entry	Conditions	UV Sensor Data	HPLC of crude material
1	**2.1** 2x 6.5 equiv -20 to 0 °C **2.2** 1x 6.5 equiv -20 to 0 °C **2.3** 2x 6.5 equiv -30 to -10 °C	(Not available)	

At this point, the conditions for the first and second coupling were satisfactory. Fmoc sensor data shows that glucoside **2.3** performs best when used in a double coupling and with temperature -30 to -10 °C. However, HPLC data is difficult to interpret owing to the existence of several peaks. Some of the peaks could not be assigned due to low resolution in the HPLC or low abundance. The deletion sequences **3.2** and **3.3** are present in all HPLC traces. The existence of these deletion sequences, together with the possible formation of α and β linkages with BB **2.3** justify the low abundance of the analytes. To try to understand better what happens in the course of the assembly of trisaccharide **3.1**, a synthesis of the precursor disaccharide **3.3** was set up (**Table 3.4**). In this experiment, the crude HPLC showed a dominant peak. Interestingly, the major peak and a minor peak at 23 min showed an m/z value consistent with the target disaccharide **3.3**. NMR analysis confirmed the major peak was disaccharide **3.3** (49% yield). The minor peak was identified as the isomer **3.5**, which arose from Bz migrated to O4 (7% yield).

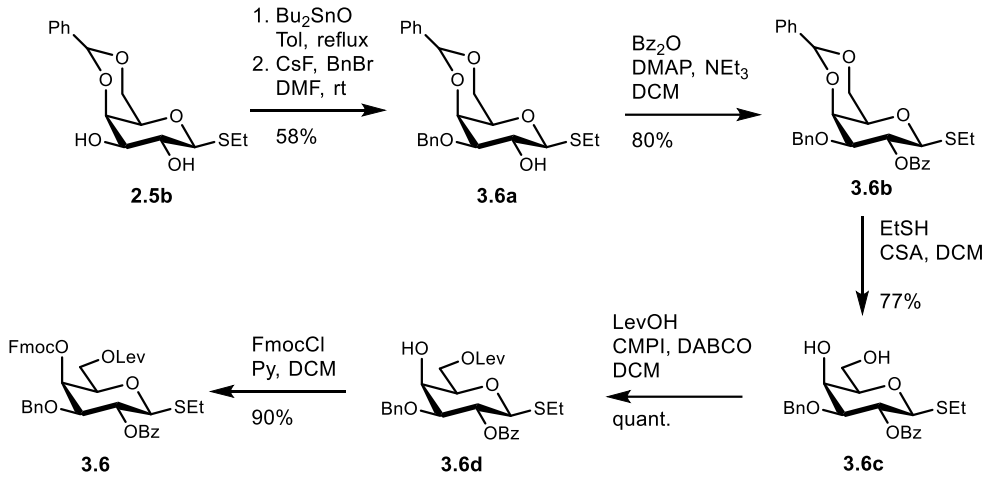
The synthesis of **3.3** proved that there was Bz migration when Fmoc was cleaved from the galactose unit. The extent of Bz migration was relatively low. However, the migration can be further enhanced by the acid wash treatment at the beginning of the following elongation cycle. More Bz migration would lead to a higher proportion of disaccharide **3.5** which would elongate from O3 to form unwanted chains. This effect decreases the yield of the target product and, on the

other hand, generates similar size by-products that are generally more difficult to separate chromatographically. Using a galactose BB with an ether at O3 could be a solution to avoid this problem. Hence, a new BB was synthesized.

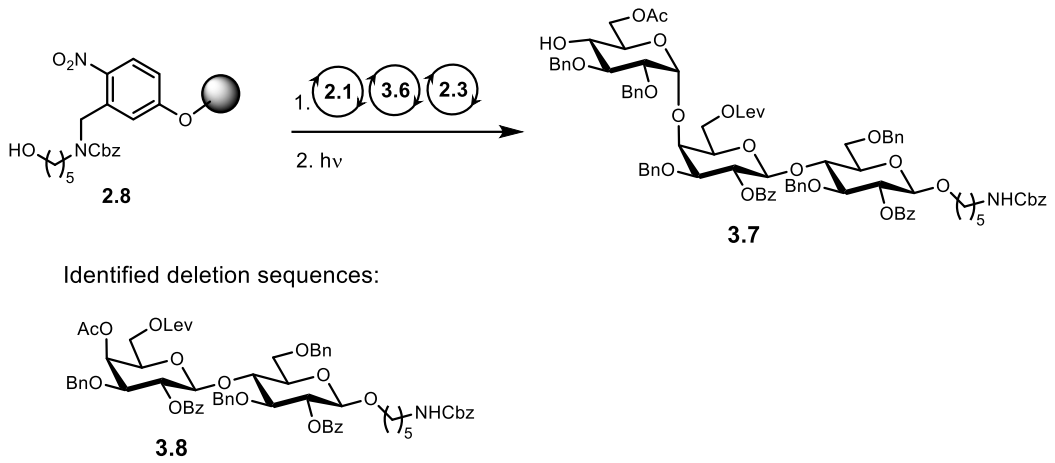
Table 3.4 AGA of disaccharide **3.3**.

Entry	Conditions	UV Sensor Data	HPLC of crude material
1	**2.1** 2x 6.5 equiv -20 to 0 °C **2.2** 1x 6.5 equiv -20 to 0 °C		

Galactoside **3.6** was prepared from intermediate **2.5b** as described in **Scheme 3.2**. Condensation with dibutyltin oxide and subsequent alkylation with BnBr in the presence of CsF led the regioselective benzylation at O3. The resulting compound **3.6a** was crystallized from EtOAc/hexane in 58% yield (over two steps). Subsequent transformations were performed using optimized procedures described in chapter 2. The final BB **3.5** was obtained in 32% over 6 steps.



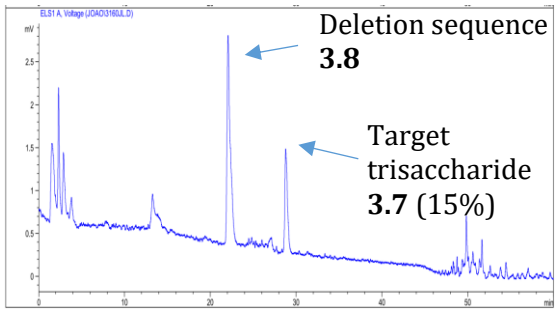
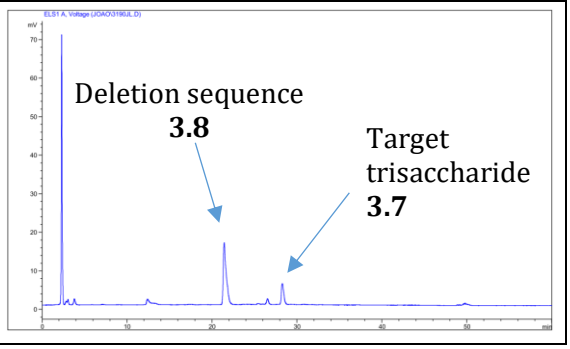
Scheme 3.2 Synthesis of galactose building block **3.6**.

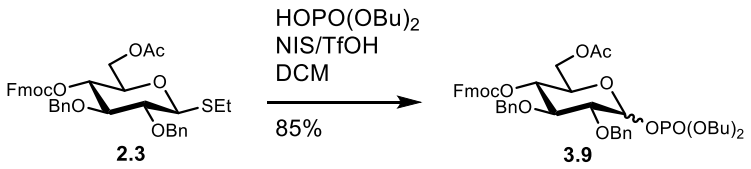


Scheme 3.3 AGA of GBS type IV backbone trisaccharide with galactoside **3.6**.

With the new BB **3.6**, the synthesis of the trisaccharide led to a more conlsusive HPLC trace (**Scheme 3.3**, **Table 3.5**, entry 1). However, the coupling of the last BB, glucoside **2.3**, was not efficient and the major product was the disaccharide deletion sequence. Since phosphate donors tend to be more reactive than thioglycosides, BB **2.3** was converted into the corresponding phosphate **3.9** (**Scheme 3.4**). The use of **3.9** (**Table 3.5**, entry 2) did not lead to a complete glycosylation of the O4 position of galactose and again the disaccharide was the major product.

Table 3.5 AGA of GBS type IV backbone trisaccharide using galactoside **3.5**.

Entry	Conditions	UV Sensor Data	HPLC of crude material
1	**2.1** 2x 6.5 equiv -20 to 0 °C **3.6** 2x 6.5 equiv -20 to 0 °C **2.3** 2x 6.5 equiv -30 to -10 °C	(Not available)	
2	**2.1** 2x 6.5 equiv -20 to 0 °C **3.5** 2x 6.5 equiv -20 to 0 °C **3.9** 1x 3.8 equiv -30 to -15 °C	(Not available)	



Scheme 3.4 Synthesis of the phosphate building block **3.9**.

3.3. Conclusion

The synthesis of oligosaccharides using the Glyconeer is based on the optimized elongation cycles that introduce each BB in the growing oligosaccharide chain. The work here described shows the process of optimizing conditions for the synthesis of GBS type IV backbone trisaccharide.

Benzoyl migration was observed between oxygens in vicinal positions during the deprotection of the Fmoc group from one of the oxygen atoms. Replacement of the benzoyl for a benzyl prevented the migration. This finding shapes the retrosynthetic analysis for future building blocks.

The major bottle neck in the synthesis of target trisaccharide was the Glc-α-1,4-Gal cis linkage. Using thioglycoside as a donor (BB **2.3**) led to low yields even when using a double coupling and after adjusting the glycosylation temperature. The use of a phosphate donor (BB **3.9**) in a single coupling led to a similar result. However, further improvements in the glycosylation conditions can lead to a satisfactory coupling yield. Another possible strategy to introduce the cis linkage is to assemble the Glc-α-1,4-Gal disaccharide in solution and use it as a building block in the Glyconeer.

The optimization of the assembly of trisaccharide **3.7** is crucial to the assemble of the whole repeating unit. The work in this chapter can be used as a novel starting point that is one step ahead in achieving the repeating unit of GBS type IV.

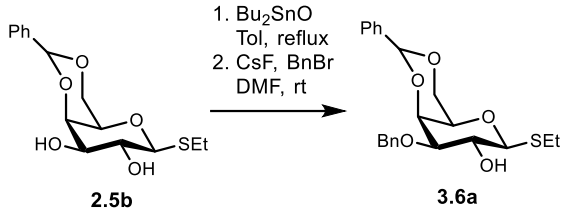
3.4. Experimental Section

General Materials and Methods

All reagents and solvents were acquired from commercial sources, unless stated otherwise. The synthesis of the building blocks and functionalization of resin are described in Chapter 2. Anhydrous solvents were obtained from a Solvent Dispensing System (J.C. Meyer). Amberlite IR-120 (Across Organics) protonic exchange resin was rinsed with THF, water, methanol and dichloromethane before use. NMR spectra were obtained using Ascend 400 (Bruker) and Agilent 400 MHz NMR Magnet (Agilent Technologies) spectrometers at 400 MHz (^{1}H) and 100 MHz (^{13}C) or a Varian 600 (Agilent) at 600 MHz (^{1}H) and 150 MHz (^{13}C), or a Ascend 700 (Bruker) at 700 MHz (^{1}H) and 176 MHz (^{13}C). $CDCl_3$ or D_2O were used as solvents and chemical shifts (δ) referenced to residual non-deuterated solvent peak unless stated otherwise. Splitting patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet for 1 H-NMR data. NMR chemical shifts (δ) are reported in ppm and coupling constants (J) are reported in Hz. Assignments were supported by COSY and HSQC experiments and compared with literature data when available. MALDI-TOF spectra were obtained with a Daltonics Autoflex Speed spectrometer (Bruker) using 2,5-dihydroxybenzoic acid (DHB) as matrix. All MALDI measurements were done in reflectron mode except for the protected octamers whose data was acquired using the linear mode. ESI-HRMS were performed with a Xevo G2-XS Q-Tof (Waters). HPLCs were performed on Agilent 1200 Series systems.

Synthesis of galactose BB 3.6

Synthesis of compound **3.6a**

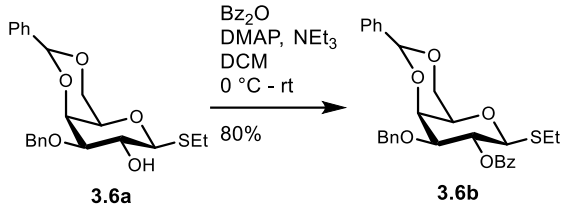


A mixture of **2.5b** (19.7 g, 63.0 mmol) and Bu_2SnO (19.5 g, 78.3 mmol) in toluene (anhydrous 50 mL) and 4 Å MS (25 g) was refluxed for 6 h. The solvent was evaporated under reduced pressure. The residue was combined with CsF (29.3 g, 193 mmol) and BnBr (11.5 mL, 96.7 mmol) in DMF (100 mL) and stirred at rt. After 18 h, TLC showed complete conversion. The reaction mixture was filtered through a pad of celite. The resulting solution was diluted with DCM (200 mL), washed with sat. aq. $NaHCO_3$, and 1 M HCl. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The resulting material was crystallized from EtOAc/hexane 1:3 to give the pure product as a white solid (14.7 g, 58%).

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.52 – 7.49 (m, 2H), 7.40 (d, J = 7.1 Hz, 2H), 7.38 – 7.28 (m, 6H), 5.45 (s, 1H, PhCH), 4.81 – 4.74 (m, 2H, $PhCH_2$), 4.35 (d, J = 9.6 Hz, 1H, H-1), 4.31 (dd, J = 12.4, 1.6 Hz, 1H, H-6a), 4.18 (d, J = 3.5 Hz, 1H, H-4), 4.07 (td, J = 9.3, 1.7 Hz, 1H, H-2), 3.97 (dd, J = 12.4, 1.9 Hz, 1H, H-6b), 3.50 (dd, J = 9.2, 3.4 Hz, 1H, H-3), 3.44 – 3.37 (m, 1H, H-5), 2.84 (dq, J = 12.5, 7.5 Hz, 1H, SCH*H*), 2.75 (dq, J = 12.5, 7.5 Hz, 1H, SC*H*H), 2.57 (d, J = 1.9 Hz, 1H, 2-OH), 1.33 (t, J = 7.5 Hz, 3H, CH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ 138.09, 137.86, 129.01, 128.48, 128.18, 127.89, 126.41, 101.28 (Ph*C*H), 85.33 (C1), 80.35 (C3), 73.55 (C4), 71.56 (Ph*C*H_2), 70.16 (C5), 69.46 (C6), 68.05 (C2), 22.97 (SCH_2), 15.31 (CH_3).

Synthesis of compound **3.6b**[55]



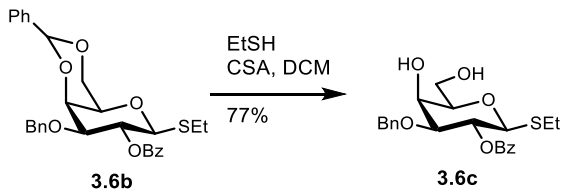
3.6a **3.6b**

A solution of **3.6a** (14.7 g, 36.6 mmol) and Bz_2O (12.3 g, 1.5 equiv) in DCM (anhydrous, 115 mL) was stirred under Ar. Et_3N (11 mL, 2 equiv) and DMAP (126 mg, 3 mol%) were sequentially added and the reaction was stirred under a 35 °C bath. After 4 h, the mixture was diluted with DCM (50 mL), washed with HCl 1 M (2 X 160 mL), sat. aq. $NaHCO_3$ (160 mL), and brine (160 mL). The organic layer was dried over MgSO4, concentrated under reduced pressure and dried under high vacuum. The resulting solid was crystallized from EtOAc/Hex 1:2 to give the pure product **3.5b** as a white solid (14.8 g, 80%).

^{1}H NMR (700 MHz, $CDCl_3$) δ 8.04 (d, J = 7.8 Hz, 2H), 7.59 (t, J = 7.5 Hz, 1H), 7.56 (d, J = 7.2 Hz, 2H), 7.46 (t, J = 7.7 Hz, 2H), 7.38 (q, J = 9.9, 8.4 Hz, 3H), 7.21 (dt, J = 22.8, 7.4 Hz, 5H), 5.74 (t, J = 9.7 Hz, 1H, H-2), 5.52 (s, 1H, PhCH), 4.68 (d, J = 12.8 Hz, 1H, PhC*H*H), 4.62 (d, J = 12.8 Hz, 1H, PhCH*H*), 4.54 (d, J = 9.9 Hz, 1H, H-1), 4.36 (dd, J = 12.2, 1.6 Hz, 1H, H-6a), 4.27 (d, J = 3.4 Hz, 1H, H-4), 4.02 (dd, J = 12.2, 1.7 Hz, 1H, H-6b), 3.75 (dd, J = 9.6, 3.4 Hz, 1H, H-3), 3.49 – 3.46 (m, 1H, H-5), 2.92 (dq, J = 14.9, 7.5 Hz, 1H, SC*H*H), 2.77 (dq, J = 14.2, 7.5 Hz, 1H, SCH*H*), 1.27 (t, J = 7.5 Hz, 3H, CH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ 165.26 (C=O), 137.83, 137.75, 133.01, 130.15, 129.88, 129.07, 128.33, 128.23, 127.74, 127.69, 126.49, 101.38 (Ph*C*H), 82.91 (C1), 78.15 (C3), 73.46 (C4), 71.03, 70.17, 69.40, 68.76, 22.72 (SCH_2), 14.88 (CH_3).

Synthesis of compound **3.6c**



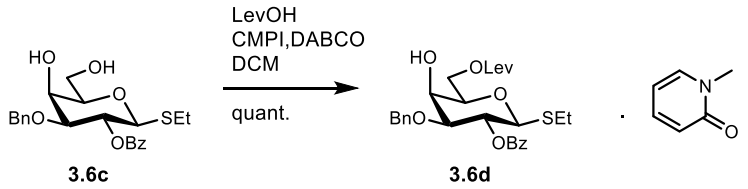
3.6b **3.6c**

Compound **3.6b** (13.0 g, 25.7 mmol) was dissolved in DCM (50 mL) and the resulting solution was stirred at rt. CSA (1.21 g, 0.2 equiv) and EtSH (19 mL, 10 equiv) were sequentially added and the resulting solution was allowed to react overnight. Then, the mixture was concentrated under reduced pressure and purified by column chromatography from Hexane/EtOAc 5:1 to 5:20. The product was isolated as a white solid (8.29 g, 77%).

^{1}H NMR (700 MHz, Chloroform-d) δ 8.04 (dd, J = 8.1, 1.4 Hz, 2H), 7.62 (t, J = 7.5 Hz, 1H), 7.48 (t, J = 7.7 Hz, 2H), 7.23 (ddt, J = 8.8, 7.4, 2.4 Hz, 1H), 7.19 (d, J = 5.7 Hz, 4H), 5.56 (t, J = 9.7 Hz, 1H, H-2), 4.69 (d, J = 12.3 Hz, 1H, PhC*H*H), 4.57 (d, J = 12.3 Hz, 1H, PhCH*H*), 4.53 (d, J = 10.0 Hz, 1H, H-1), 4.19 (d, J = 3.3 Hz, 1H, H-4), 4.03 (dd, J = 11.8, 6.7 Hz, 1H, H-6a), 3.87 (dd, J = 11.7, 4.7 Hz, 1H, H-6b), 3.70 (dd, J = 9.3, 3.3 Hz, 1H, H-3), 3.63 (td, J = 5.0, 2.5 Hz, 1H, H-5), 2.79 (dq, J = 12.3, 7.4 Hz, 1H, SCH*H*), 2.73 (dq, J = 12.4, 7.5 Hz, 1H, , SC*H*H), 2.58 (s, 2H, 2x OH), 1.25 (t, J = 7.5 Hz, 3H, CH_3).

^{13}C NMR (176 MHz, Chloroform-d) δ 165.46 (C=O), 137.03, 133.16, 129.89, 128.47, 128.39, 128.04, 127.87, 83.53 (C1), 79.22 (C3), 78.42 (C5), 71.46 (Ph*C*H_2), 69.49 (C2), 66.88 (C4), 62.52 (C6), 23.69 (SCH_2), 14.86 (CH_3).

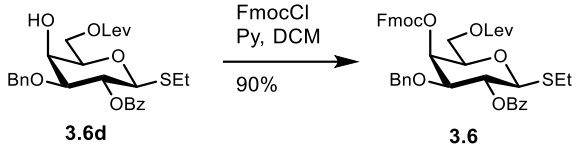
Synthesis of compound **3.6d**



Compound **3.6c** (7.03 g, 13.6 mmol), DABCO (6.20 g, 55.3 mmol), and 2-chloro-1-methylpyridium iodide (8.80 g, 34.4 mmol) were combined with DCM (anhydrous, 43 mL) under a positive pressure of Ar and the resulting suspension was cooled in an ice bath. LevOH (1.94 g, 16.7 mmol) was added and the mixture was allowed to react for 6 h, while the temperature rose to 0 °C. The mixture was then diluted with DCM (40 mL) and washed with H_2O (80 mL) and brine (80 mL). The organic layer was dried over $MgSO_4$, concentrated under reduced pressure and dried under high

vacuum. The resulting oil was identified as a mixture of the product and N-methyl-2-pyridone (9.33 g, 1:1 mixture, quant.).

Synthesis of compound **3.6**



Crude **3.6d** (7.75 g, 13.6 mmol) was dissolved in DCM (anhydrous, 74 mL) and the resulting solution was stirred at rt under Ar. FmocCl (7.00 g, 2 equiv) and pyridine (2.4 mL, 3 equiv) were sequentially and the mixture was allowed to react for 24h. The mixture was diluted with DCM (70 mL) washed with HCl 1 M (2x 140 mL), and brine (140 mL), dried over $MgSO_4$, amd concentrated under reduced pressure. The crude material was purified by column chromatography from hexane/DCM/EtOAc 10:1:0.5 to 10:1:5 to give the pure compound **3.6** as a white powder (9.00g, 90%).

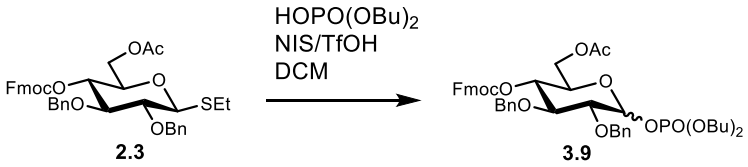
^{1}H NMR (700 MHz, Chloroform-d) δ 8.02 – 7.98 (m, 2H), 7.78 – 7.75 (m, 2H), 7.70 (dd, J = 7.4, 1.0 Hz, 1H), 7.64 (dt, J = 7.5, 0.9 Hz, 1H), 7.60 (tt, J = 7.4, 1.3 Hz, 1H), 7.48 – 7.45 (m, 2H), 7.44 – 7.36 (m, 3H), 7.32 (td, J = 7.5, 1.1 Hz, 1H), 7.13 (ddd, J = 14.5, 7.6, 1.3 Hz, 3H), 7.03 (dd, J = 8.3, 6.9 Hz, 2H), 5.59 (t, J = 9.8 Hz, 1H, H-2), 5.49 (dd, J = 3.3, 1.2 Hz, 1H, H-4), 4.68 (d, J = 12.5 Hz, 1H, PhC*H*H), 4.57 (d, J = 10.0 Hz, 1H, H-1), 4.51 (d, J = 12.6 Hz, 1H, PhCH*H*), 4.50 – 4.45 (m, 1H, Fmoc CH), 4.39 (dd, J = 11.2, 6.5 Hz, 1H, H-6a), 4.31 – 4.27 (m, 2H, Fmoc CH_2), 4.24 (dd, J = 11.3, 6.9 Hz, 1H, H-6b), 3.93 (td, J = 6.7, 1.2 Hz, 1H, H-5), 3.76 (dd, J = 9.6, 3.2 Hz, 1H, H-3), 2.84 – 2.70 (m, 4H, SCH_2, Lev CH_2), 2.64 – 2.53 (m, 2H, Lev CH_2), 2.19 (s, 3H, Lev CH_3), 1.27 (t, J = 7.5 Hz, 3H, $SCH_2C\mathit{H_3}$).

^{13}C NMR (176 MHz, Chloroform-d) δ 206.77 (C=O ketone), 172.49 (C=O Lev ester), 165.29 (C=O Bz), 155.14 (C=O Fmoc), 143.74, 143.29, 141.42, 141.31, 137.29, 133.23, 130.08, 130.01, 128.46, 128.32, 128.17, 127.96, 127.93, 127.81, 127.46, 127.41, 125.80, 125.45, 120.05, 84.11 (C1), 77.34 (C2), 74.33 (C5), 71.16, 70.53,

70.44 (C4, PhCH2, Fmoc CH_2), 69.30 (C2), 62.09 (C6), 46.67 (Fmoc CH), 38.15 (Lev CH_2), 29.92, 28.08 (S*C*H_2, Lev CH_2), 24.23 (Lev CH_3), 15.03 (SCH_2*C*H_3).

HRMS (ESI/QTOF) m/z: $[M+Na]^+$ Calcd 761.2391. Found 761.2400.

Synthesis of glucose BB 3.9



A solution of SM (1.00 g, 1.50 mmol) in DCM (anhydrous, 9 mL) cooled to 0 °C. A 1.26 M solution of dibutyl phosphate in DCM was dried with 4 Å MS one hour prior to use. From this solution, dibutyl phosphate (2.4 mL, 3.02 mmol) was added to the reaction mixture, then NIS (444 mg, 1.97 mmol) and then TfOH (40 µL, 0.45 mmol). The reaction mixture was stirred at 0 °C for one hour and TLC showed complete conversion. The reaction was diluted with DCM and washed with a 1:1 mixture of sat. aq. $NaHCO_3$ and 10% $Na_2S_2O_5$. The organic layer was dried over $MgSO_4$, concentrated and purified by column chromatography to give the product as a 1:1 anomeric mixture (1.04 g, 85%).

HRMS (ESI/QTOF) m/z: $[M+Na]^+$ Calcd for C13H17NO3Na 839.3172 ; Found 839.3196.

General procedure for setting up the Glyconeer

All solvents were loaded in the respective bottle and attached in the corresponding position of the Glyconeer prior to the synthesis. For each synthesis, the building blocks were co-evaporated with toluene, dried under high vacuum for 2h, dissolved in anhydrous DCM (1 mL) and transferred to oven-dried building block vials. The vials were then placed in the appropriate position in the building block

carousel. The reagent solutions were prepared as described below. A polystyrene base resin functionalized with 0.40 mmol/g of a photocleavable linker was used. The resin (12.5 μmol of hydroxyl groups, unless otherwise stated) was weighed and transferred into the reaction vessel and the synthesis programme was initiated.

Preparation of reagent solutions:

TMSOTf solution: Anhydrous DCM (40 mL) was transferred into an oven-dried reagent bottle under Ar. TMSOTf (0.45 mL) was added and the bottle was placed in the predetermined acid-wash position and attached to the Glyconeer.

Activation solution: *N*-Iodosuccinimide (NIS, 1.35 g) was added to an oven-dried reagent bottle. Anhydrous dioxane (13 mL) and anhydrous DCM (26 mL) were added and the mixture gently stirred. Triflic acid (TfOH, 55 μL) was added and the bottle placed in the cooling block, in the predetermined position, and attached to the Glyconeer.

Pyridine solution: Pyridine (5 mL) was diluted in 45 mL of DMF and the resulting solution was transferred into a reagent bottle. The bottle was placed in the predetermined position and attached to the Glyconeer.

Capping solution: Anhydrous DCM (36 mL) was transferred into an oven-dried reagent bottle under Ar. Ac_2O (4 mL) was added followed by MsOH (0.8 mL). The bottle was placed in the predetermined position and attached to the Glyconeer.

Piperidine solution: Piperidine (50 mL) was diluted with 200 mL of DMF and the resulting solution was transferred into the reagent bottle. The bottle was placed in the predetermined position and attached to the Glyconeer.

Hydrazine solution: Hydrazine acetate (550 mg) was transferred into a reagent bottle and pyridine (32 mL) was added. AcOH (8 mL) was carefully added followed by H_2O (2 mL). The resulting mixture was gently stirred for a few minutes and the bottle was then placed in the predetermined position and attached to the Glyconeer.

All experiments in the Glyconeer start with a “Resin swelling” module. During this module, DCM (2 mL) is delivered in the reaction vessel and the resin is incubated at 25 °C for 30 min. During the incubation time, the machine rinses the manifolds. Afterwards syntheses were programmed by combining the pre-defined modules described below. For each monosaccharide to be added to the target glycan, the cycle Acid Wash, Glycosylation, Capping, Deprotection was repeated.

Module 1 – Acid Wash: the temperature is set to -20 °C and the resin is washed with the TMSOTf solution (1 mL).

Module 2 – Glycosylation: The temperature is adjusted to the addition temperature T1 (-20 °C unless otherwise stated), the building block solution (1 mL) is delivered and then the activator solution (1 mL). An incubation period t1 = 5 min is followed. The temperature is then adjusted to T2 (0 °C unless otherwise stated) and an incubation period t2 = 20 min is followed. The resin is then washed with a 1:1 solution of DCM/Dioxane, and then with DCM. When a double coupling was required, the procedure was repeated. When a quadruple couple was required, the procedure was repeated to a total of four times.

Module 3 – Capping: The temperature is set to 25 °C. The resin is washed with DMF, then with the pyridine solution (2 mL), then with DCM. The capping solution is added (4 mL) and resin is incubated for 20 min. The resin is finally washed with DCM.

Module 4 – Fmoc Deprotection: The temperature is set to 25 °C. The resin is washed with DMF and then the piperidine solution (2 mL) is added. The resin is incubated for 5 min and afterwards washed with DMF and DCM.

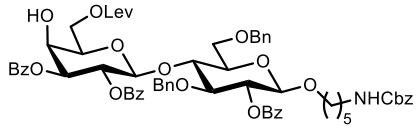
Module 5 – Lev Deprotection: The temperature is set to 25 °C. The resin is washed with DCM and drained. DCM (1.3 mL) is added followed by the hydrazine solution (0.8 mL). The resin is incubated for 30 min and the procedure is repeated another two times. The resin is finally washed with DMF and DCM.

General procedure for cleavage from the solid support and purification

After automated synthesis in the Glyconeer, the solid-support was suspended in DCM and injected into a continuous-flow photoreactor as described previously[51] to release the generated oligosaccharides. The resulting crude material was analysed by analytical HPLC using a YMC-Diol-300 column (150 x 4.6 mm) and the following elution method: flow rate 1 mL/min, elution started with 20% EtOAc in hexane for 5 min (isocratic), then linear gradient to 55% EtOAc in hexane for 35 min, then linear gradient to 100% EtOAc for 5 min, then 100% EtOAc for 5 min (isocratic). The target oligosaccharide was purified using a preparative HPLC using a YMC-Diol-300 column (150 x 20 mm) and the following elution method: flow rate 15 mL/min, elution started with 20% EtOAc in hexane for 5 min (isocratic), then linear gradient to 55% EtOAc in hexane for 35 min, then linear gradient to 100% EtOAc for 5 min, then 100% EtOAc for 5 min (isocratic). The product fractions were collected, evaporated under reduced pressure and dried under high vacuum overnight.

Analytical data

Disaccharide **3.3**



3.3

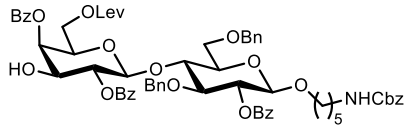
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 – 7.91 (m, 4H), 7.94 – 7.85 (m, 2H), 7.58 – 7.47 (m, 3H), 7.48 – 7.28 (m, 16H), 7.24 – 7.17 (m, 2H), 7.11 (dp, J = 4.7, 1.8 Hz, 3H), 5.70 (dd, J = 10.4, 8.0 Hz, 1H, H-2 Gal), 5.19 (dd, J = 9.4, 8.0 Hz, 1H, H-2 Glc), 5.13 (dd, J = 10.4, 3.2 Hz, 1H, H-3 Gal), 5.05 (s, 2H), 4.90 (d, J = 11.4 Hz, 1H), 4.79 (d, J = 8.0 Hz, 1H, H-1 Gal), 4.71 (d, J = 12.1 Hz, 1H), 4.67 (d, J = 11.4 Hz, 1H), 4.53 (t, J = 6.1 Hz, 1H, NH), 4.36 (d, J = 12.1 Hz, 1H), 4.36 (d, J = 7.8 Hz, 1H, H-1 Glc), 4.26 (dd, J = 11.3, 7.0

Hz, 1H, H-6a), 4.17 (d, J = 3.3 Hz, 1H, H-4 Gal), 4.13 (dd, J = 9.7, 8.7 Hz, 1H, H-4 Glc), 4.09 (dd, J = 11.3, 6.3 Hz, 1H, H-6b), 3.79 (dd, J = 9.7, 5.9 Hz, 1H, OC*H*H pentanylene), 3.76 (t, J = 9.1 Hz, 1H, H-3 Glc), 3.71 – 3.61 (m, 2H, H-6a, H-5), 3.55 (dd, J = 10.9, 1.8 Hz, 1H, H-6b), 3.36 – 3.25 (m, 2H, H-5, OCH*H* pentanylene), 2.87 (q, J = 6.6 Hz, 2H, CH_2 pentanylene), 2.73 (dd, J = 7.1, 5.8 Hz, 2H, CH_2 Lev), 2.54 (t, J = 6.2 Hz, 2H, CH_2 Lev), 2.17 (s, 3H, CH_3 Lev), 1.53 – 1.33 (m, 2H, CH_2 pentanylene), 1.32 – 1.20 (m, 2H, CH_2 pentanylene), 1.13 (q, J = 7.6 Hz, 2H, CH_2 pentanylene).

^{13}C NMR (101 MHz, $CDCl_3$) δ 206.87 (C=O, Lev ketone), 172.80 (C=O, Lev ester), 165.79, 165.22, 165.18 (3x C=O, Bz), 156.36 (C=O Cbz) 138.54, 138.11, 130.10, 129.32, 129.15, 128.59, 128.26, 128.21, 128.17, 128.08, 101.32 (C1), 100.53 (C1), 80.39, 76.84, 74.82, 74.66, 74.05, 73.66, 73.38, 72.02, 70.15 (C2 Gal), 69.58, 67.72, 66.92, 66.61, 61.97, 40.90 (NH*C*H_2, pentenylene), 38.05 (CH_2, Lev), 29.98 (CH_3, Lev), 29.47 (CH_2, pentenylene), 28.95 (CH_2, pentenylene), 27.99 (CH_2, Lev), 23.17 (CH_2, pentenylene).

ESI-MS $[M+Na]^+$ calcd for $C_{65}H_{69}NO_{18}Na$ 1174.4 Found 1174.2.

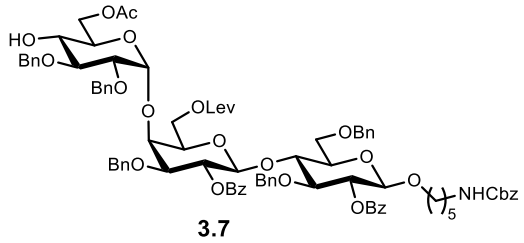
Disaccharide **3.5**



3.5

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 – 7.91 (m, 6H), 7.64 – 7.30 (m, 19H), 7.21 – 7.14 (m, 2H), 7.08 – 6.98 (m, 3H), 5.55 (d, *J* = 3.6 Hz, 1H, H-4 Gal), 5.33 (dd, *J* = 10.0, 7.9 Hz, 1H, H-2 Gal), 5.21 (dd, *J* = 9.4, 8.0 Hz, 1H, H-2 Glc), 4.94 (d, *J* = 11.5 Hz, 1H), 4.82 (d, *J* = 8.0 Hz, 1H, H-1 Gal), 4.74 (d, *J* = 12.2 Hz, 1H), 4.73 (d, *J* = 11.5 Hz, 1H), 4.53 (br s, 1H, NH), 4.41 (d, *J* = 12.2 Hz, 1H), 4.39 (d, *J* = 8.0 Hz, 1H, H-1 Glc), 4.03 (dd, *J* = 11.3, 6.4 Hz, 1H), 3.89 (dd, *J* = 10.1, 3.4 Hz, 1H, H-3 Gal), 2.88 (q, *J* = 6.6 Hz, 2H, CH_2 pentanylene), 2.74 – 2.67 (m, 2H, CH_2 Lev), 2.52 (t, *J* = 6.5 Hz, 2H, CH_2 Lev), 2.17 (s, 3H, CH_3 Lev), 1.46 – 1.35 (m, 2H, CH_2 pentanylene), 1.28 – 1.23 (m, 2H, CH_2 pentanylene), 1.20 – 1.12 (m, 2H, CH_2 pentanylene).

ESI-MS $[M+Na]^+$ calcd for $C_{65}H_{69}NO_{18}Na$ 1174.4 Found 1174.2.



3.7

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 – 7.92 (m, 2H), 7.90 – 7.83 (m, 2H), 7.65 – 7.57 (m, 1H), 7.55 – 7.43 (m, 3H), 7.40 – 7.29 (m, 7H), 7.32 – 7.09 (m, 20H), 7.09 – 7.02 (m, 2H), 7.02 – 6.90 (m, 3H), 5.49 (dd, J = 10.4, 7.8 Hz, 1H, H-2 Gal), 5.12 (dd, J = 9.5, 8.0 Hz, 1H, H-2 βGlc), 5.04 (s, 2H), 4.99 (d, J = 3.4 Hz, 1H, H-1 αGlc), 4.89 (d, J = 11.8 Hz, 1H), 4.78 – 4.62 (m, 5H, H-1 βGlc), 4.61 – 4.40 (m, 8H), 4.28 (m, 3H), 4.17 (d, J = 2.7 Hz, 1H, H-4 Gal), 4.08 (t, J = 9.2 Hz, 1H, H-4 βGlc), 3.84 (t, J = 9.3 Hz, 1H, H-3 αGlc), 3.78 – 3.60 (m, 4H, OC*H*H pentanylene, H-3 βGlc), 3.57 (t, J = 6.9 Hz, 1H), 3.51 (d, J = 10.7 Hz, 1H), 3.46 (dd, J = 9.8, 3.4 Hz, 1H, H-2 , αGlc), 3.43 (t, J = 9.5, 8.7 Hz, 1H), 3.37 (dd, J = 10.4, 2.6 Hz, 1H, H-3 Gal), 3.33 – 3.20 (m, 2H, H-5 βGlc, OCH*H* pentanylene), 2.84 (qd, J = 8.5, 4.9 Hz, 3H), 2.76 – 2.64 (m, 2H, CH_2 Lev), 2.56 (ddd, J = 16.9, 8.3, 4.9 Hz, 1H, C*H*H Lev), 2.47 (dt, J = 17.0, 5.9 Hz, 1H, CH*H* Lev), 2.20 (s, 3H, CH_3 Lev), 2.00 (s, 3H, CH_3 Ac), 1.50 – 1.29 (m, 4H, pentanylene), 1.16 – 1.03 (m, 2H, pentanylene).

ESI-MS $[M+Na]^+$ calcd for $C_{87}H_{95}NO_{23}Na$ 1544.6. Found 1544.4.

4. Automated Glycan Assembly of Fragments of GBS Type III Capsular Polysaccharide

4.1. Introduction

Serotype III is the most prevalent Group B Streptococcus (GBS) strain accounting for 62% of the cases of invasive disease in young infants.[5] Understanding the structure-immunogenicity relationship of type III GBS capsular polysaccharide (CPS) can lead to important advances in the process of developing a highly efficient and safe GBS vaccine.

The repeating unit of GBS type III CPS (**Figure 4.1**) is a pentasaccharide with a terminal Neu5Ac-α-2,3-Gal moiety[56] and is structurally related to the CPS of *S. pneumoniae* type 14 (Sp14)[57]. In the conceptualization of the synthesis of GBS type III repeating unit, a combined approach was envisioned where the core structure, corresponding to Sp14, could be obtained by AGA and converted into a GBSIII CPS fragment by enzymatic sialylation. *S. pneumoniae* is a leading cause of serious invasive diseases such as bacterial pneumonia, septicaemia, and meningitis in young children worldwide, and is listed in the WHO global priority list of antibiotic-resistant bacteria.[58] Rising levels of antibiotic resistance reinforce the need for alternative treatments and prevention strategies. Thus, combining enzymatic sialylation with AGA could lead to a streamlined strategy for obtaining glycans from two important pathogens.

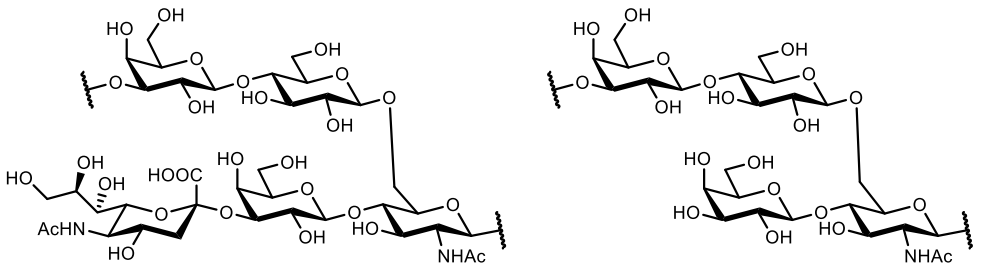


Figure 4.1 Capsular polysaccharide repeating unit of Sp14 and GBSIII.

Sp14 fragments have been previously prepared by a block-coupling approach where the repeating unit was synthesized as a thioglycoside and used both as a donor and a protected acceptor. The repeating unit block was first coupled to a spacer. Afterwards, removal of an isopropylidene acetal yielded an acceptor, which was elongated with the donor block to give a protected dimer of the repeating unit.

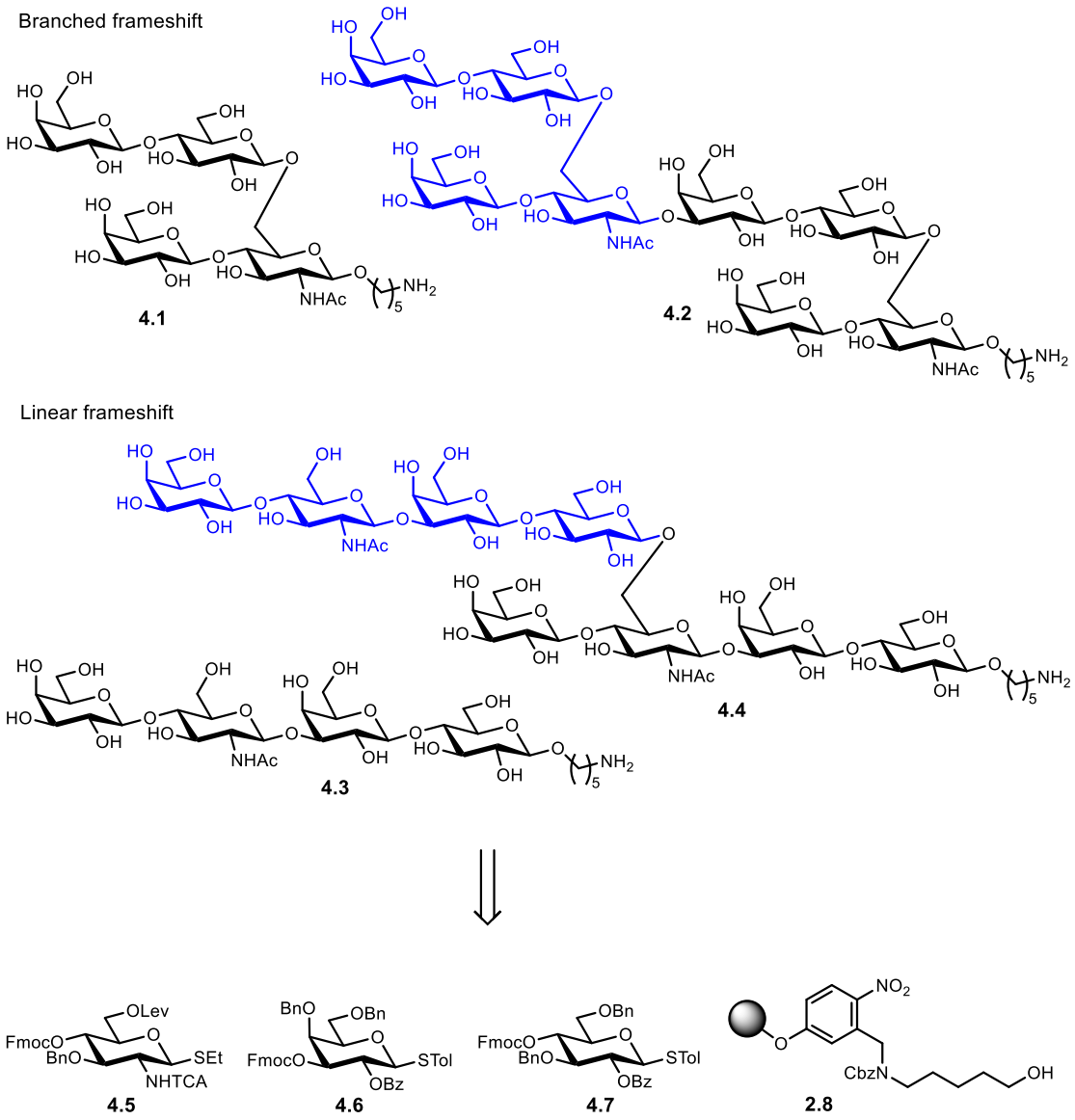
Iteration of this methodology yielded the trimer. In this strategy, the assembly of the tetrasaccharidic block was achieved in six synthetic steps starting from semi-protected mono or disaccharides, which represents at least two weeks of highly-specialized labour-intensive work.[59] Other strategies include the use of differentially protected lactose and lactosamine building blocks that are coupled together to generate up to octasaccharide fragments.[60] The synthesis of GBSIII has been achieved by enzymatic introduction of the sialic acid in Sp14 structures.[61,62] To avoid sialylation in the lactose arm of the structure, the appropriate galactose was blocked with a methyl ether in position 3 that could not be removed in the end of the synthesis. Boons *et al.* described a fully chemical synthesis of a heptasaccharide fragment of GBS type III based on a convergent approach. The incorporation of sialic acid was based on α-NeuNAc-(2-3)-Gal*p* methylthioglycoside dimer which had been assembled by coupling a methylthiosialoside and a galactose acceptor.[63]

This chapter describes the synthesis of Sp14 fragments via automated glycan assembly using the Glyconeer, and its transformation into GBS type III glycans by enzymatic sialylation. The optimization of AGA is based on data from the Fmoc sensor and HPLC analysis of the crude material obtained after photocleavage. Key parameters related to the glycosylation module were studied: temperature of glycosylation, number of coupling repetitions, and concentration of donor. A reproducible procedure for the AGA of Sp14 is described.

4.2. Retrosynthetic Analysis of Sp14 Glycans

The target structures for AGA (**Scheme 4.1**) represent two different frameshifts of the repeating unit (monomer) and the corresponding dimer of Sp14 CPS. In the branched frameshift (monomer **4.1** and dimer **4.2**), the reducing terminus is comprised of a glucosamine unit. In the linear frameshift (monomer **4.3** and dimer **4.4**), glucose lies at the reducing terminus. The retrosynthetic analysis of the target structures led to three thioglycoside building blocks that can be used to make both frameshifts. Fmoc serves as the main temporary protecting group whereas Lev is used as an orthogonal temporary protecting to enable branching

(glucosamine). Benzyl ethers serve as permanent protecting groups. Benzoyl ester at O2 and trichloroacetyl (TCA) at N2 were used as participating protecting groups to ensure selective trans-glycosylation. One galactose building block (**4.6**) was used both for terminal positions and for positions with elongation along O3. In the first case, a capping step was used right after deprotecting the Fmoc group giving rise to a terminal 3-O-acetylated galactose residue. In all other cases, the elongation cycle consisted of acid wash, glycosylation, capping, and deprotection.

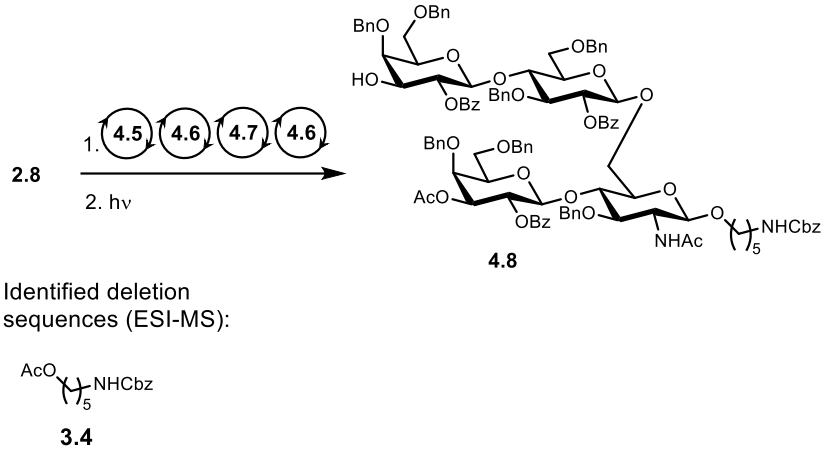


Scheme 4.1 Retrosynthesis of oligosaccharides **4.1**-**4.4** using building blocks **4.5**-**4.7** and the functionalized Merrifield resin **2.8**.

4.3. Automated Glycan Assembly

4.3.1. Branched frameshift

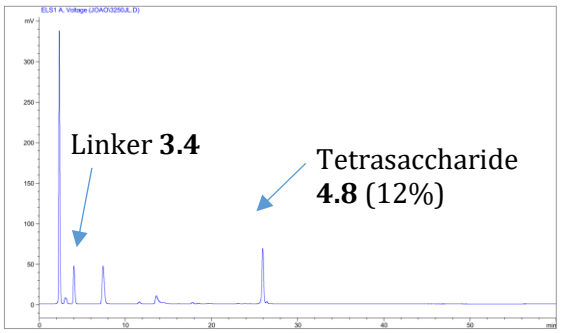
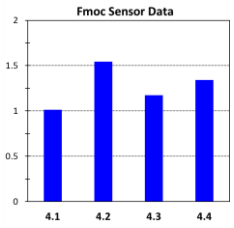
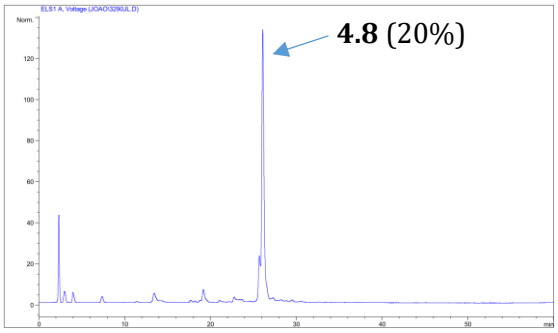
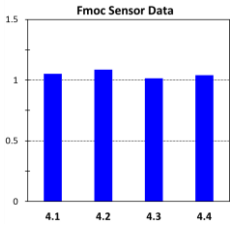
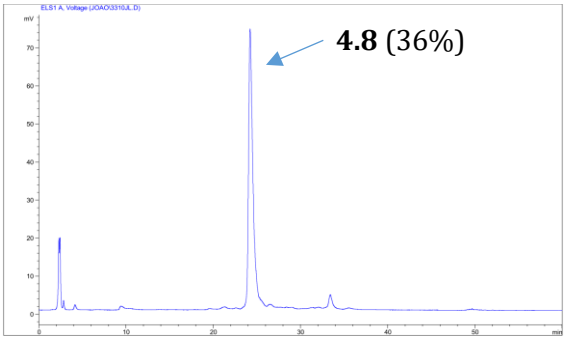
To assemble the branched frameshift of Sp14 monomer (**Scheme 4.2**), an initial trial was performed where all the BBs where coupled using 2x 6.5 equiv (41 mM) at -20 to 0 °C (**Table 4.1**, entry 1). With these conditions, the target tetrasaccharide **4.8** was obtained in 12% yield. The HPLC of the crude product showed a significant peak for the capped linker.



Scheme 4.2 AGA of the branched frameshift of Sp14 monomer.

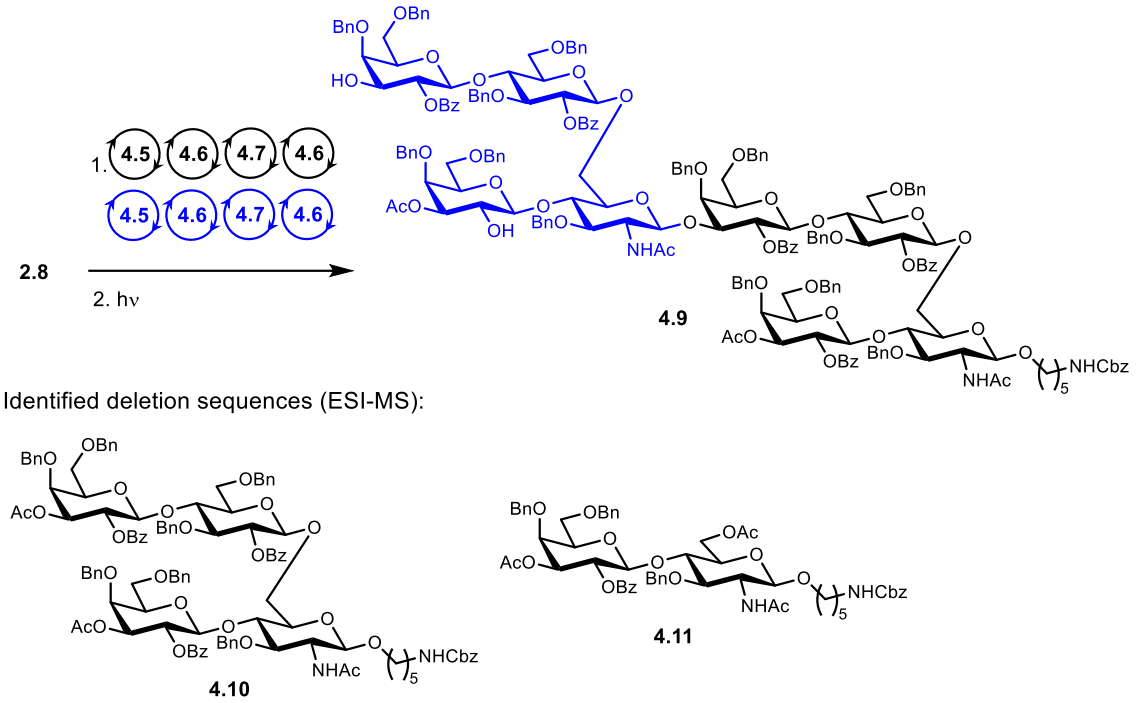
When the first building block **4.1** was coupled using ten equivalents (62 mM), the signal for the capped linker was negligible and tetrasaccharide **4.8** was isolated in 20% yield (**Table 4.1**, entry 2). This finding proved that the glycosylation efficiency increased upon increasing the concentration of glucosamine **4.1**. To probe the effect of concentration for the other BBs, the synthesis of **4.8** was repeated using ten equivalents of donor in one single coupling for building blocks **4.2** and **4.3** (**Table 4.1**, entry 3). The crude material obtained after photocleavage showed negligible deletion sequences and the product was isolated in 31% yield. With these conditions, the synthesis time was reduced from 14 to 11 h and 2.5 equivalents per coupling of BBs **4.2** and **4.3** were saved.

Table 4.1 AGA of tetrasaccharide **4.8**.

Entry	Conditions	UV Sensor Data	HPLC of crude material
1	**4.5** 2x 6.5 equiv -20 to 0 °C **4.6** 2x 6.5 equiv -20 to 0 °C **4.7** 2x 6.5 equiv -20 to 0 °C **4.6** 2x 6.5 equiv -20 to 0 °C	(Not available)	
2	**4.5** 2x 10 equiv -20 to 0 °C **4.6** 2x 6.5 equiv -20 to 0 °C **4.7** 2x 6.5 equiv -20 to 0 °C **4.6** 2x 6.5 equiv -20 to 0 °C		
3	**4.5** 2x 10 equiv -20 to 0 °C **4.6** 1x 10 equiv -20 to 0 °C **4.7** 1x 10 equiv -20 to 0 °C **4.6** 1x 10 equiv -20 to 0 °C		

The branched dimer **4.9** was assembled according to **Scheme 4.3**. In the first attempt, the best conditions for monomer **4.8** were applied. However, the expected octasaccharide was a minor product. The major product was tetrasaccharide **14** (**Table 4.2**, entry 1) which arises from a low coupling efficiency of glucosamine building block **5** to the O3 position of galactose. The effect of the glycosylation temperature in the formation of this linkage was probed: -10 to 10 °C led to an increase in the formation of the target octasaccharide (**Table 4.2**, entry 2); 0 to 20 °C led to a decrease in the formation of the target octasaccharide (**Table 4.2**, entry 3). Even with the best temperature set, deletion sequence **14** was the major product. When glucosamine **5** was coupled at -10 °C to 10 °C and repeated in a quadruple

coupling, the synthesis proceeded with the formation of **10** as the major product (**Table 4.2**, entry 4, 8% yield). Building block **4.7** was used again in a double coupling to prevent the formation of deletion sequence **4.11** and the amount of building block **6** was reduced to 6.5 equiv without formation of deletion sequences.



Scheme 4.3 AGA of octasaccharide **4.9**.

Table 4.2 (Part I/II) AGA of tetrasaccharide **4.9**.

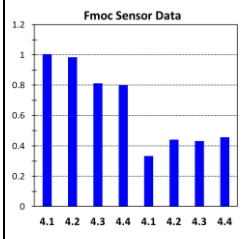
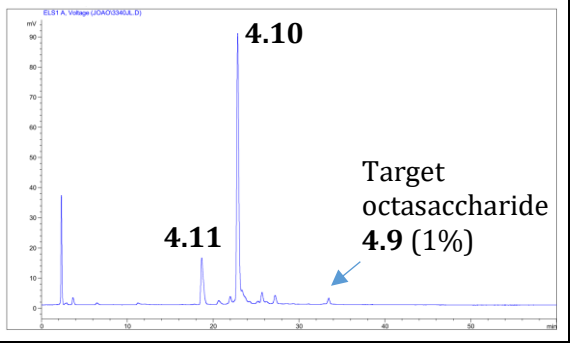
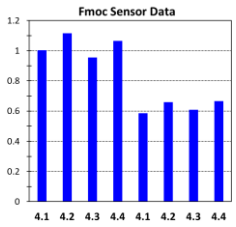
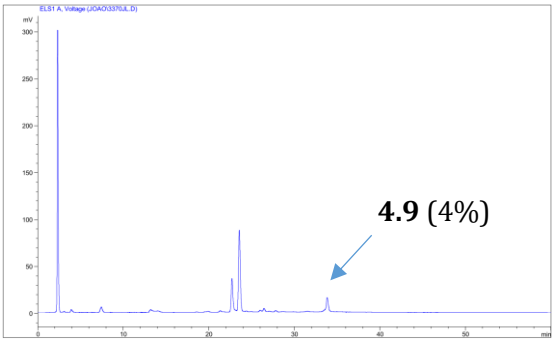
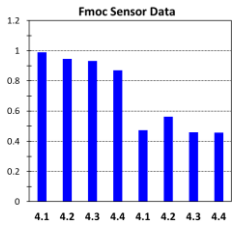
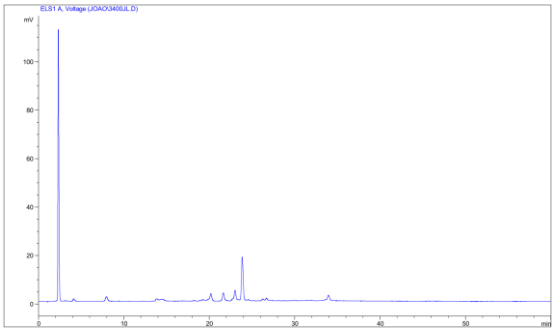
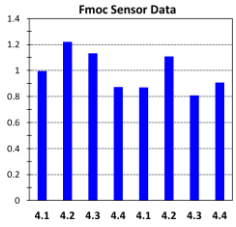
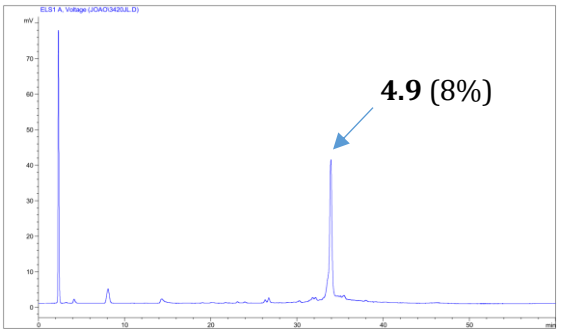
Entry	Conditions*	UV Sensor Data	HPLC of crude material
1	**4.5** 2x 10 equiv **4.6** 1x 10 equiv **4.7** 1x 10 equiv **4.6** 1x 10 equiv (2x)		

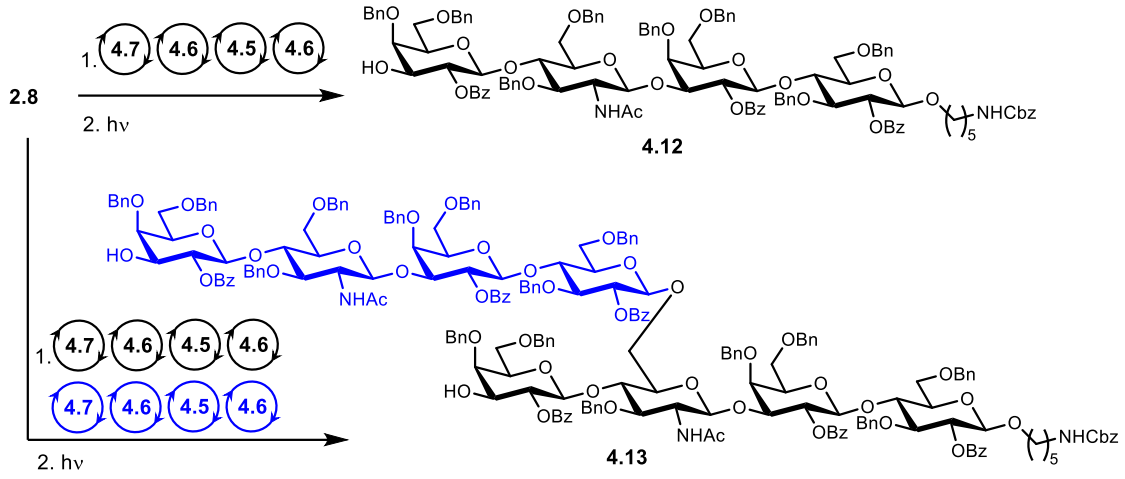
Table 4.2 (Part II/II) AGA of tetrasaccharide **4.9**.

Entry	Conditions*	UV Sensor Data	HPLC of crude material
2	**4.5** 2x 10 equiv **4.6** 1x 6.5 equiv **4.7** 2x 6.5 equiv **4.6** 1x 6.5 equiv **4.5** 2x 10 equiv -10 to 10 °C **4.6** 1x 6.5 equiv **4.7** 2x 6.5 equiv **4.6** 1x 6.5 equiv		
3	**4.5** 2x 10 equiv **4.6** 1x 6.5 equiv **4.7** 2x 6.5 equiv **4.6** 1x 6.5 equiv **4.5** 2x 10 equiv 0 to 20 °C **4.6** 1x 6.5 equiv **4.7** 2x 6.5 equiv **4.6** 1x 6.5 equiv		
4	**4.5** 2x 10 equiv **4.6** 1x 6.5 equiv **4.7** 2x 6.5 equiv **4.6** 1x 6.5 equiv **4.5** 4x 10 equiv -10 to 10 °C **4.6** 1x 6.5 equiv **4.7** 2x 6.5 equiv **4.6** 1x 6.5 equiv		

*Temperature of glycosylation -20 to 0 °C unless otherwise stated.

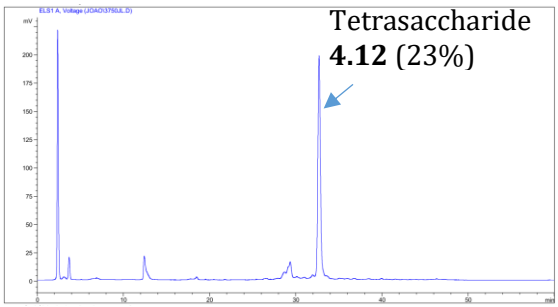
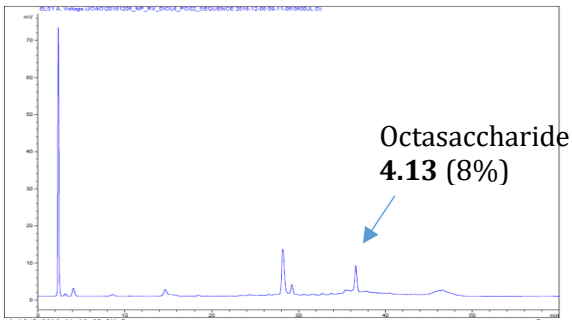
4.3.2. Linear frameshift

To generate the fragments of the linear frameshift, the best conditions for the synthesis of dimer **4.9** were used. The synthesis of tetrasaccharide **4.12** (**Table 4.3**, entry 1) was successful and led to 23% yield. The synthesis of **4.13** was performed by repeating the elongation cycles. A deletion sequence was identified as a tetrasaccharide with the Lev at glucosamine O6 indicating incomplete cleavage of this protecting group. The octasaccharide **4.3** was isolated in 8% yield.



Scheme 4.4 AGA of linear frameshift of Sp14 CPS.

Table 4.3 AGA of the linear tetrasaccharide **4.52**.

Entry	Conditions*	HPLC of crude material
1	**4.7** 2x 6.5 equiv **4.6** 4x 10 equiv **4.5** 1x 6.5 equiv -10 to 10 °C **4.6** 1x 6.5 equiv	
2	**4.7** 2x 6.5 equiv **4.6** 1x 6.5 equiv **4.5** 4x 10 equiv -10 to 10 °C **4.6** 1x 6.5 equiv (2x)	

*Temperature of glycosylation -20 to 0 °C unless otherwise stated.

4.3.3. Scale up experiments

The syntheses described so far were performed on a 12.5 μmol scale and afforded less than 10 milligrams of the protected oligosaccharides. In order to procure more material, a scale-up study was conducted (**Table 4.4**). The linear scale up to 25 μmol led to successful synthesis of both the branched and linear tetrasaccharides. The synthesis of tetrasaccharide **4.8** was further scaled up to 50 μmol. In this case, due to volume limitations, the amount of BBs was reduced to 2x 5 equivalents per coupling, keeping the concentration at 41 mM. Tetrasaccharide **9** was isolated in 33%.

Table 4.4 Yield comparison for 12.5 μmol experiments and the scale up experiments.

Entry	Conditions*	12.5 μmol	25 μmol	50 μmol
1	**4.5** 2x 10 equiv **4.6** 2x 6.5 equiv **4.7** 2x 6.5 equiv **4.6** 2x 6.5 equiv	**4.8** 20%	**4.8** 28%	**4.8** 33% (2x 5 equiv for all BBs)
2	**4.7** 2x 6.5 equiv **4.6** 1x 6.5 equiv **4.5** 4x 10 equiv -10 to 10 °C **4.6** 1x 6.5 equiv	**4.12** 23%	**4.12** 21%	-

*Temperature of glycosylation -20 to 0 °C unless otherwise stated.

4.4. Global deprotection

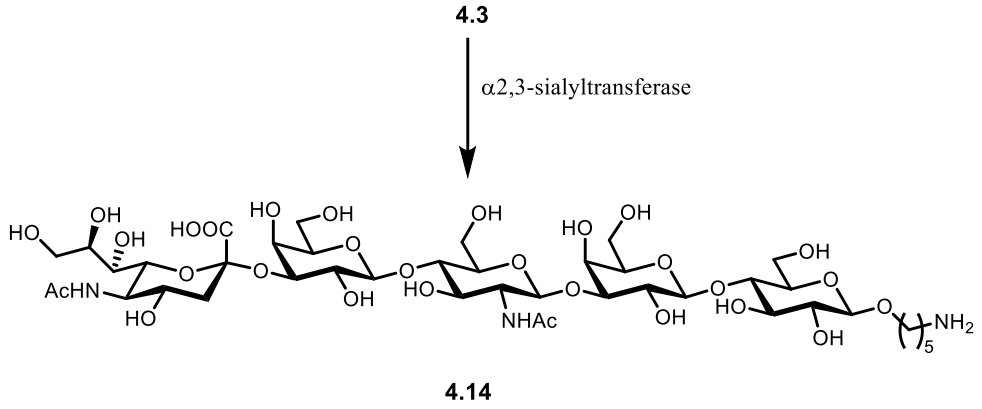
Following AGA, cleavage from the solid support, and HPLC purification, compounds **4.8**, **4.9**, **4.12** and **4.13** were deprotected in two steps. Ester groups (benzoyl, acetyl and levaloyl) were cleaved with sodium methoxide in 1:1 MeOH/DCM. In the second step, benzyl ethers, TCA and the carboxybenzyl group at the amino linker were removed by hydrogenation catalyzed by Pd/C. Methanolysis of tetrasaccharide **4.8** was complete after 24h using 25 mM NaOMe (by MALDI). The crude product was hydrogenated and purified using a C18 cartridge to give **4.1** in

93% yield. The linear tetrasaccharide **4.12** was subjected to the same methanolysis procedure but after 24h, MS spectra showed partially cleaved intermediates. The reaction was performed with an increase concentration of NaOMe (50 mM) but ester cleavage was not complete after 24 h. Upon addition of another portion of NaOMe (final concentration 0.1 M) and after another 24h, the conversion was complete. The crude product was then subjected to hydrogenation. Tetrasaccharide **4.3** was obtained after HPLC purification as the formate salt (71%).

Deprotection of octasaccharides **4.9** and **4.13** proved to be cumbersome as the attempts to follow the methanolysis by LC-MS or MALDI were unsuccessful. For branched structure **4.9**, the procedure used for methanolysis was the same as for the branched tetrasaccharide **4.8**. After hydrogenation, the crude product was subjected to RP-HPLC but was isolated with a triethylamine contamination, originating from the work up procedure. The linear structure **4.13** was deprotected using the same conditions as for linear tetrasaccharide **4.12**. After hydrogenation and RP-HPLC purification, the product was isolated in 22% yield.

4.5. Enzymatic sialylation

To convert the *S. pneumoniae* type 14 repeating unit (oligosaccharide **4.3**) into the GBS type III repeating unit (compound **4.14**), an α(2,3)-sialyltransferase from *Pasteurella multocida* (PmST1)[64] was used (**Scheme 4.5**). The reaction was performed using three equivalents of CMP-Neu5Ac (3 mM) as donor in the presence of an alkaline phosphatase to dephosphorylate the CMP by-product. The enzyme was used in a concentration of 6 µg/mL and after incubation at 37 °C with a Tris-HCl pH 9.3 buffer, the sialoside was filtered by a polyethersulfone membrane (MWCO 10 kDa) to remove the protein. A final purification by RP-HPLC gave **4.14** in 30% yield.



Scheme 4.5 Enzymatic synthesis of sialoside **4.14**. α-(2,3)-Sialyltransferase PmST1, CMP-Neu5Ac, alkaline phosphatase, Tris-HCl pH 9.3, $MgCl_2$.

4.6. Conclusion

The success of AGA relies on general and standardized procedures. The coupling of BBs **4.5**-**4.7** was explored with the best conditions leading to reproducible coupling yields in two different frameshifts of Sp14 CPS oligosaccharides. The glucosamine BB **4.5** showed a low coupling efficiency, particularly when the acceptor was O3 of galactose. Using a quadruple coupling with a high concentration of BB led to the formation of the desired compounds. An investigation of the reasons for the low efficiency can lead to solutions that do not require such a large excess of donor. The use of only three BBs to assemble four different structures showcases the importance of the optimization work here described and proves the versatility the BBs, setting the scene for their use in different structures. The combination of enzymatic sialylation with AGA proved to be an expeditious approach to the preparation of complex glycans for vaccine development. The glycans produced can be used in immunologic assays such as ELISA or conjugated to carrier proteins and injected in animal models.

4.7. Experimental Section

All reagents and solvents were acquired from commercial sources, unless stated otherwise. All building blocks were obtained from GlycoUniverse stock. The resin equipped with a photocleavable linker (loading of 0.40 mmol/g) was also obtained from GlycoUniverse. Anhydrous solvents were obtained from a Solvent Dispensing System (J.C. Meyer). Amberlite IR-120 (Across Organics) protonic exchange resin was rinsed with THF, water, methanol and dichloromethane before use. NMR spectra were obtained using Ascend 400 (Bruker) and Agilent 400 MHz NMR Magnet (Agilent Technologies) spectrometers at 400 MHz (^{1}H) and 100 MHz (^{13}C) or a Varian 600 (Agilent) at 600 MHz (^{1}H) and 150 MHz (^{13}C), or a Ascend 700 (Bruker) at 700 MHz (^{1}H) and 176 MHz (^{13}C). $CDCl_3$ or D_2O were used as solvents and chemical shifts (δ) referenced to residual non-deuterated solvent peak unless stated otherwise. Splitting patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet for 1 H-NMR data. NMR chemical shifts (δ) are reported in ppm and coupling constants (J) are reported in Hz. Assignments were supported by COSY and HSQC experiments and compared with literature data when available. MALDI-TOF spectra were obtained with a Daltonics Autoflex Speed spectrometer (Bruker) using 2,5-dihydroxybenzoic acid (DHB) as matrix. All MALDI measurements were done in reflectron mode except for the protected octamers whose data was acquired using the linear mode. ESI-HRMS were performed with a Xevo G2-XS Q-Tof (Waters). HPLCs were performed on Agilent 1200 Series systems.

General procedure for setting up the Glyconeer

(See **Chapter 3.4**, page 97)

Experiments at 25 μmol scale

The resin (25 μmol of hydroxyl groups) was weighed and transferred into the reaction vessel. The building blocks were prepared as described above dissolved in

2 mL of anhydrous DCM instead of 1 mL. The parameters of the synthesis were adjusted for the glycosylation, Fmoc deprotection, and Lev deprotection modules, as follows:

Module 2 – Glycosylation: The temperature is adjusted to the addition temperature T1 (-20 °C unless otherwise stated), the building block solution (2 mL) is delivered and then the activator solution (2 mL). An incubation period t1 = 5 min is followed. The temperature is then adjusted to T2 (0 °C unless otherwise stated) and an incubation period t2 = 20 min is followed. The resin is then washed with a 1:1 solution of DCM/Dioxane, and then with DCM. When a double coupling was required, the procedure was repeated. When a quadruple couple was required, the procedure was repeated to a total of four times.

Module 4 – Fmoc Deprotection: The temperature is set to 25 °C. The resin is washed with DMF and then the piperidine solution (4 mL) is added. The resin is incubated for 5 min and afterwards washed with DMF and DCM.

Module 5 – Lev Deprotection: The temperature is set to 25 °C. The resin is washed with DCM and drained. DCM (2.5 mL) is added followed by the hydrazine solution (1.5 mL). The resin is incubated for 30 min and the procedure is repeated another two times. The resin is finally washed with DMF and DCM.

Experiment at 50 μmol scale

The resin (50 μmol of hydroxyl groups) was weighed and transferred into the reaction vessel. The building blocks were prepared as described above and dissolved in 2 mL of DCM mL. The parameters of the synthesis were adjusted for the glycosylation, Fmoc deprotection, and Lev deprotection modules, as follows:

Module 2 – Glycosylation: The temperature is adjusted to the addition temperature T1 (-20 °C unless otherwise stated), the building block solution (2 mL) is delivered and then the activator solution (4 mL). An incubation period t1 = 5 min is followed. The temperature is then adjusted to T2 (0 °C unless otherwise stated) and an incubation period t2 = 20 min is followed. The resin is then washed with a 1:1 solution of DCM/Dioxane, and then with DCM. When a double coupling was

required, the procedure was repeated. When a quadruple coupling was required, the procedure was repeated up to four times.

Module 4 – Fmoc Deprotection: The temperature is set to 25 °C. The resin is washed with DMF and then the piperidine solution (8 mL) is added. The resin is incubated for 5 min and afterwards washed with DMF and DCM.

Module 5 – Lev Deprotection: The temperature is set to 25 °C. The resin is washed with DCM and drained. DCM (5 mL) is added followed by the hydrazine solution (3 mL). The resin is incubated for 30 min and the procedure is repeated another two times. The resin is finally washed with DMF and DCM.

Fmoc sensor data

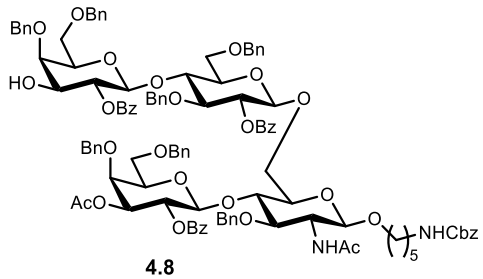
The output of the UV sensor in the Glyconeer is the transmittance of the Fmoc deprotection solution, discharged through the UV cell after incubation with the resin inside the RV. The obtained transmittance value is subtracted from DMF transmittance (blank) and normalized to the transmittance obtained after cleaving Fmoc group from 0.0125 mmol of Fmoc-Gly-Wang resin (commercially available).

General procedure for cleavage from the solid support and purification

After automated synthesis in the Glyconeer, the solid-support was suspended in DCM and injected into a continuous-flow photoreactor as described previously[51] to release the generated oligosaccharides. The resulting crude material was analysed by analytical HPLC using a YMC-Diol-300 column (150 x 4.6 mm) and the following elution method: flow rate 1 mL/min, elution started with 20% EtOAc in hexane for 5 min (isocratic), then linear gradient to 55% EtOAc in hexane for 35 min, then linear gradient to 100% EtOAc for 5 min, then 100% EtOAc for 5 min (isocratic). The target oligosaccharide was purified using a preparative HPLC using

a YMC-Diol-300 column (150 x 20 mm) and the following elution method: flow rate 15 mL/min, elution started with 20% EtOAc in hexane for 5 min (isocratic), then linear gradient to 55% EtOAc in hexane for 35 min, then linear gradient to 100% EtOAc for 5 min, then 100% EtOAc for 5 min (isocratic). The product fractions were collected, evaporated under reduced pressure and dried under high vacuum overnight.

Compound **4.8**



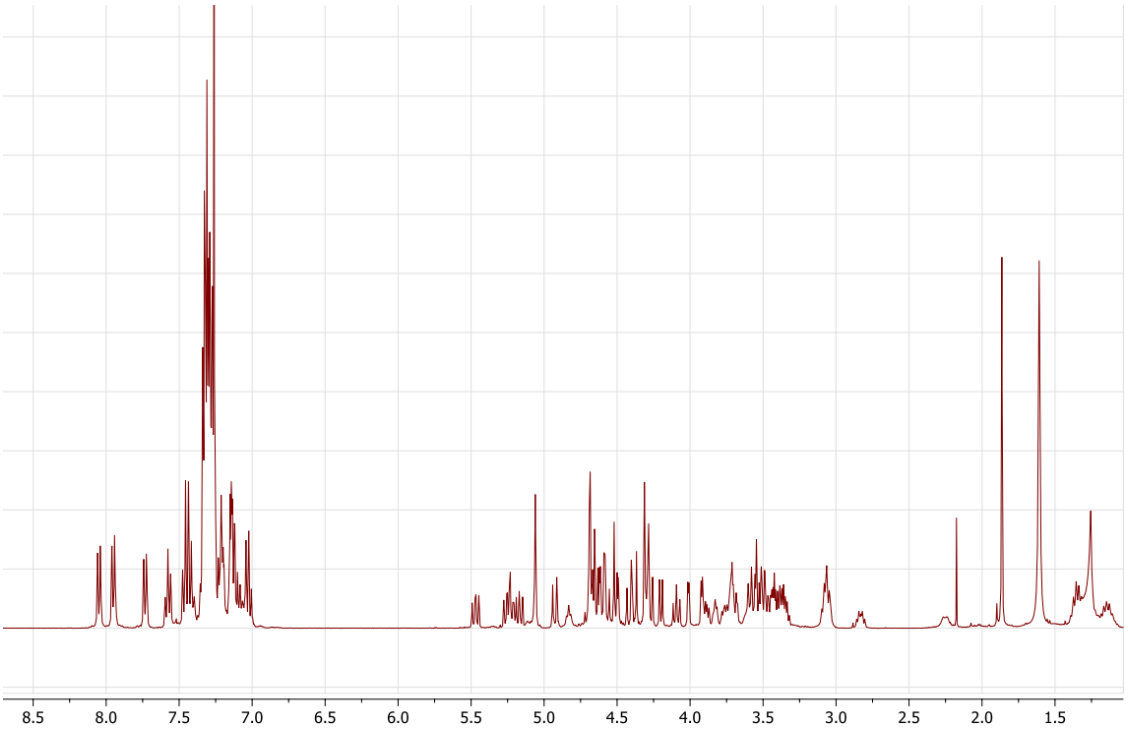
4.8

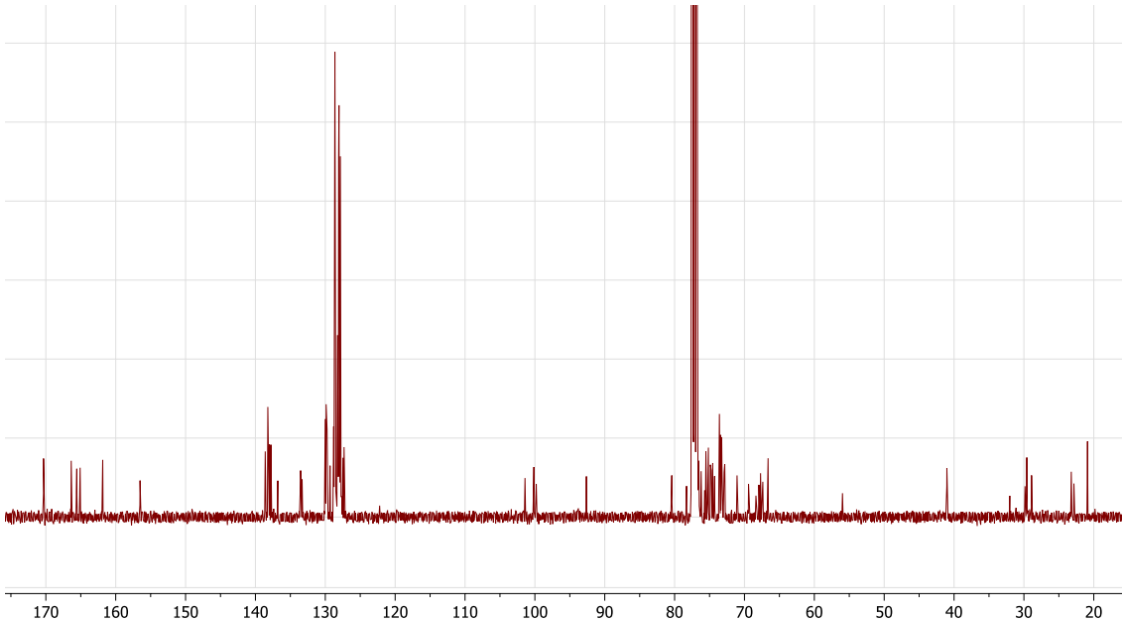
^{1}H NMR (400 MHz, Chloroform-d) δ 8.08 – 8.01 (m, 2H), 7.99 – 7.92 (m, 2H), 7.77 – 7.70 (m, 2H), 7.63 – 7.53 (m, 2H), 7.45 (q, J = 8.0 Hz, 5H), 7.38 – 7.17 (m, 33H), 7.18 – 6.97 (m, 10H), 5.47 (dd, J = 10.5, 7.8 Hz, 1H), 5.28 – 5.20 (m, 2H), 5.17 (dd, J = 9.5, 7.9 Hz, 1H), 5.06 (s, 2H), 4.93 (d, J = 11.2 Hz, 1H), 4.83 (t, J = 6.0 Hz, 1H), 4.73 – 4.48 (m, 10H), 4.42 (d, J = 11.9 Hz, 1H), 4.38 (d, J = 11.9 Hz, 1H), 4.32 – 4.24 (m, 4H), 4.20 (d, J = 7.9 Hz, 1H), 4.09 (t, J = 9.2 Hz, 1H), 4.01 (d, J = 3.2 Hz, 1H), 3.93 – 3.87 (m, 2H), 3.82 (d, J = 5.4 Hz, 1H), 3.79 – 3.67 (m, 4H), 3.63 – 3.31 (m, 11H), 3.13 – 3.01 (m, 3H), 2.83 (dt, J = 9.8, 6.2 Hz, 1H), 2.25 (d, J = 10.0 Hz, 1H, OH), 1.86 (s, 3H, COC*H*$_3$), 1.42 – 1.20 (m, 4H, pentanylene chain), 1.15 (p, J = 8.4, 7.7 Hz, 2H, pentanylene chain).

^{13}C NMR (101 MHz, CDCl 3) δ 170.33, 166.37, 165.57, 165.09, 161.86, 156.51, 138.60, 138.23, 138.22, 138.21, 138.14, 137.99, 137.78, 136.80, 133.67, 133.53, 133.32, 130.01, 129.98, 129.88, 129.75, 129.66, 129.32, 128.80, 128.72, 128.71, 128.67, 128.63, 128.56, 128.45, 128.25, 128.21, 128.17, 128.09, 128.08, 128.05, 128.04, 128.02, 128.00, 127.87, 127.84, 127.80, 127.47, 127.31, 101.41, 100.15, 100.02, 99.80, 92.63, 80.40, 78.29, 77.48, 77.36, 77.16, 76.84, 76.53, 76.21, 75.70, 75.52, 75.17, 74.88, 74.76, 74.55, 74.28, 73.61, 73.59, 73.41, 73.27, 73.23, 73.17,

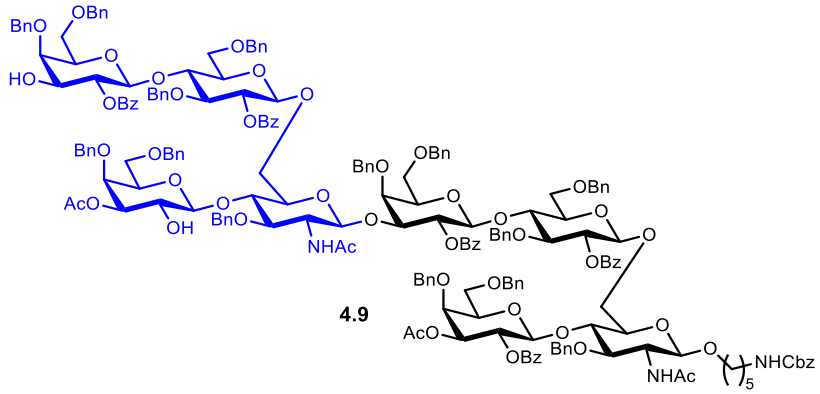
72.91, 72.82, 71.06, 69.41, 68.36, 67.93, 67.70, 67.38, 66.62, 55.97, 41.03, 29.59, 28.90, 23.22, 20.90.

MALDI: calcd for $C_{111}H_{115}Cl_3N_2NaO_{27}$ [M+Na] 2035.7. Found 2035.2.



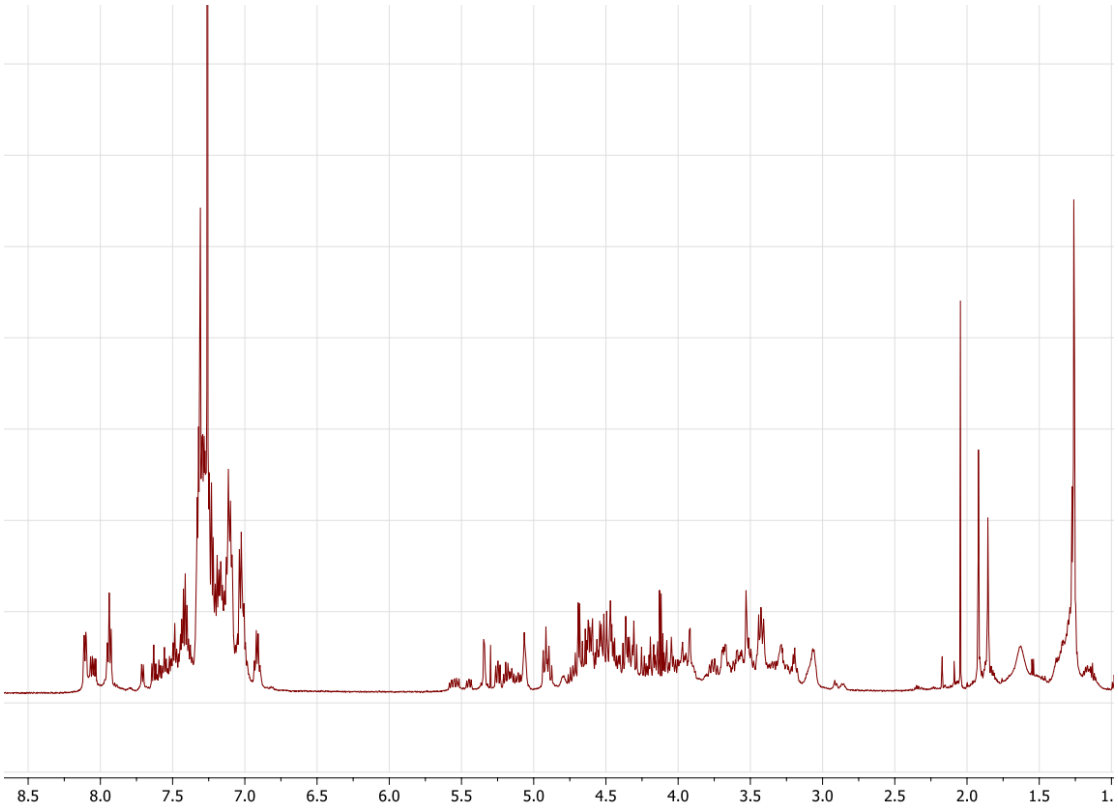


Compound **4.9**

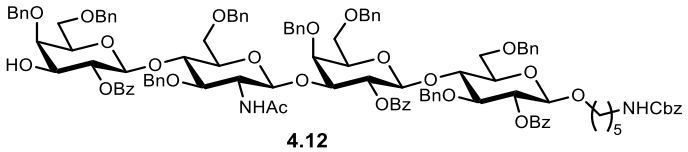


^{1}H NMR (600 MHz, Chloroform-d) δ 8.13 – 8.09 (m, 2H), 8.08 – 8.02 (m, 2H), 7.96 – 7.92 (m, 3H), 7.73 – 7.69 (m, 1H), 7.65 – 6.97 (m, 97H), 6.94 – 6.87 (m, 2H), 5.57 (dd, J = 10.2, 7.8 Hz, 1H), 5.53 (dd, J = 10.2, 8.0 Hz, 1H), 5.45 (dd, J = 10.5, 8.0 Hz, 1H), 5.37 – 5.31 (m, 2H), 5.25 (dd, J = 10.0, 7.9 Hz, 1H), 5.21 – 5.04 (m, 5H), 4.96 – 4.87 (m, 3H), 4.82 – 3.87 (m, 40H), 3.81 – 3.02 (m, 35H), 2.94 – 2.82 (m, 2H), 1.92 (s, 3H), 1.85 (s, 3H), 1.42 – 1.27 (m, 4H), 1.20 – 1.10 (m, 2H).

MALDI: calcd for $C_{209}H_{211}Cl_6N_3NaO_{51}$ [M+Na] 3811.2. Found 3813.0.



Compound **4.12**

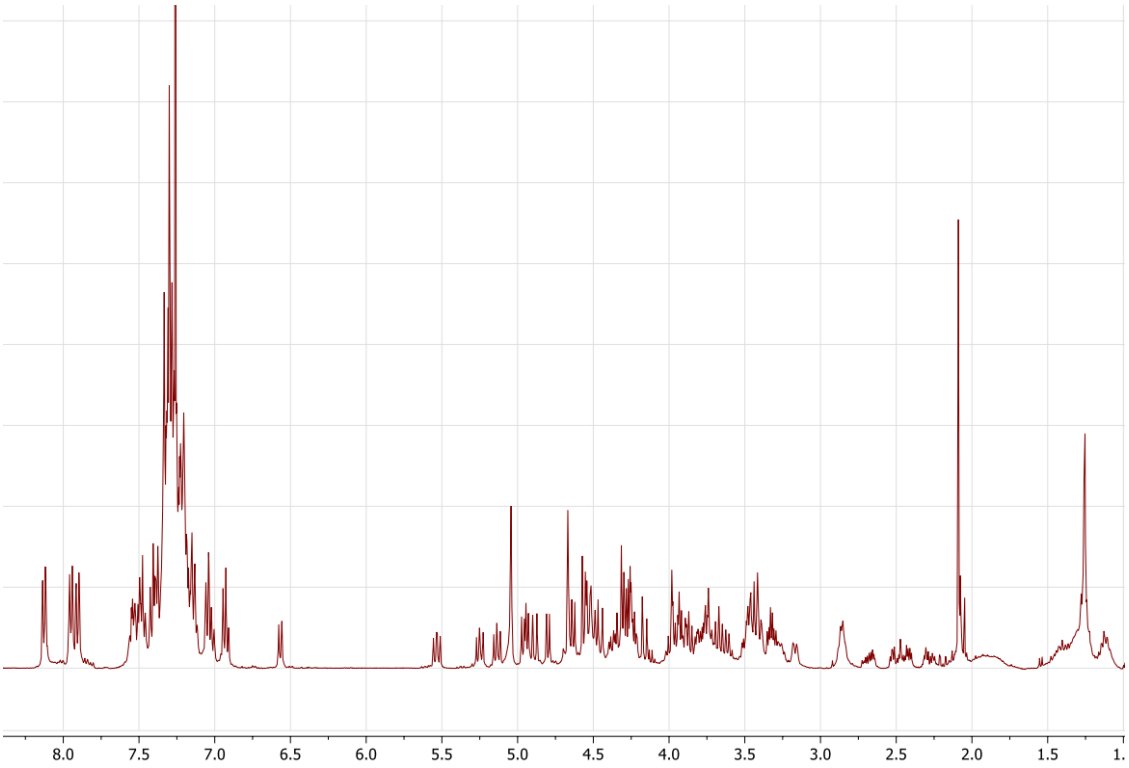


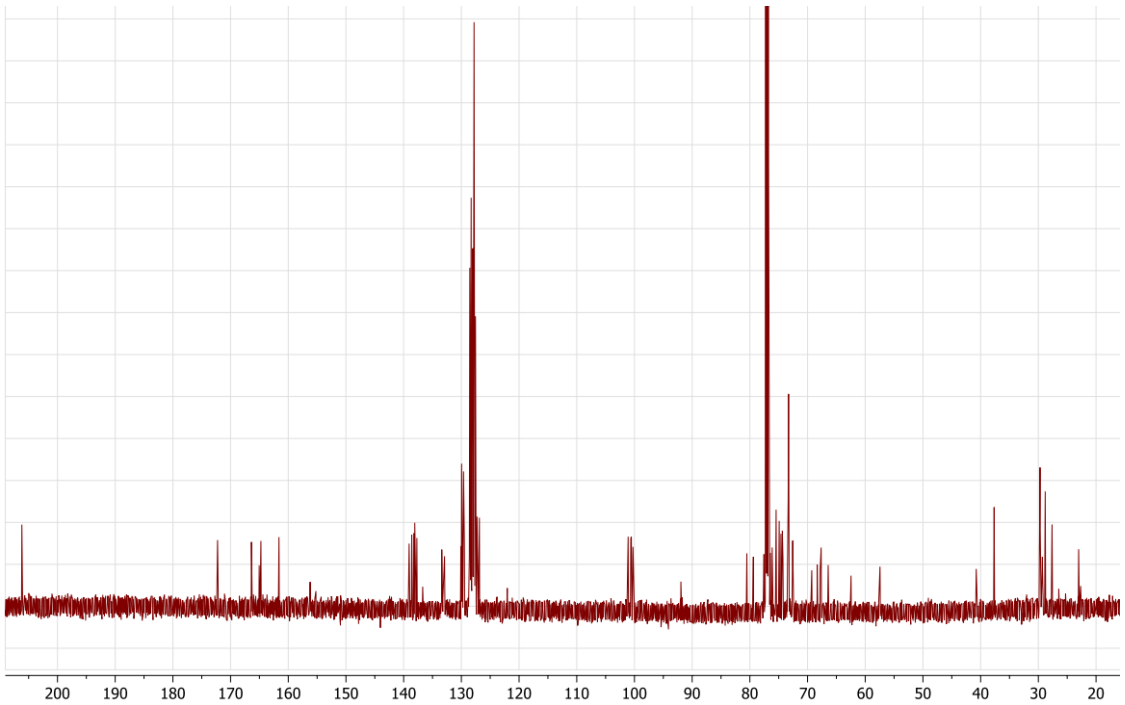
4.12

^{1}H NMR (400 MHz, Chloroform-d) δ 8.13 (dd, J = 8.4, 1.4 Hz, 2H), 7.95 (dd, J = 8.4, 1.4 Hz, 2H), 7.91 (dd, J = 8.4, 1.3 Hz, 2H), 7.59 – 7.09 (m, 44H), 7.08 – 6.98 (m, 3H), 6.97 – 6.89 (m, 2H), 6.57 (d, J = 8.2 Hz, 1H, N*H*TCA), 5.53 (dd, J = 10.2, 7.9 Hz, 1H), 5.25 (dd, J = 9.8, 7.9 Hz, 1H), 5.14 (dd, J = 9.4, 8.1 Hz, 1H), 5.04 (s, 2H), 4.96 (d, J = 11.6 Hz, 1H), 4.94 (d, J = 10.9 Hz, 1H), 4.89 (d, J = 11.1 Hz, 1H), 4.80 (d, J = 7.7 Hz, 1H), 4.69 – 4.64 (m, 2H), 4.63 (d, J = 7.8 Hz, 1H), 4.56 (d, J = 7.8 Hz, 1H), 4.57 – 4.42 (m, 5H), 4.38 (dd, J = 12.0, 4.5 Hz, 1H), 4.37 – 4.19 (m, 5H), 4.16 (d, J = 11.6 Hz, 7H), 4.03 – 3.58 (m, 9H), 3.52 – 3.23 (m, 7H), 3.20 – 3.14 (m, 1H), 2.86 (d, J = 6.6 Hz, 2H), 2.75 – 2.62 (m, 1H), 2.56 – 2.38 (m, 2H), 2.35 – 2.22 (m, 1H), 2.09 (s, 3H), 1.48 – 1.19 (m, 4H, pentanylene chain), 1.19 – 1.06 (m, 2H, pentanylene chain).

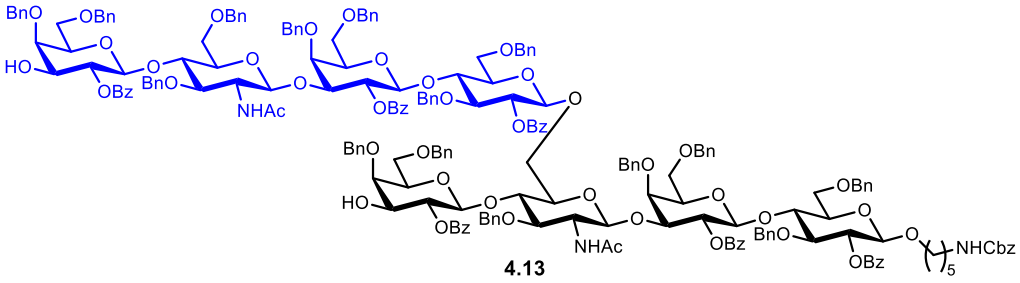
^{13}C NMR (151 MHz, Chloroform-d) δ 206.17, 172.25, 166.38, 165.04, 164.74, 161.61, 156.21, 139.05, 138.60, 138.25, 138.19, 138.16, 138.08, 137.71, 136.70, 133.40, 133.33, 132.92, 130.09, 129.96, 129.82, 129.62, 129.59, 129.47, 128.61, 128.55, 128.53, 128.52, 128.46, 128.40, 128.34, 128.30, 128.10, 128.05, 128.01, 127.92, 127.90, 127.88, 127.80, 127.78, 127.76, 127.72, 127.64, 127.60, 127.55, 127.53, 127.28, 127.25, 126.87, 126.85, 101.09, 100.64, 100.54, 100.26, 91.95, 80.52, 79.39, 77.56, 76.59, 76.44, 76.13, 75.45, 74.92, 74.88, 74.61, 74.37, 74.26, 73.49, 73.35, 73.34, 72.57, 72.55, 69.27, 68.32, 67.81, 67.64, 66.42, 62.46, 57.45, 40.77, 37.66, 29.71, 29.31, 28.81, 27.62, 23.01.

MALDI: calcd for $C_{114}H_{119}Cl_3N_2NaO_{28}$ [M+Na] 2091.6. Found 2091.5.


8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5


200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20

Compound **4.13**



MALDI: calcd for $C_{212}H_{215}Cl_6N_3KO_{52}$ $[M+K]^+$ 3883.2. Found 3886.4

Oligosaccharide deprotection

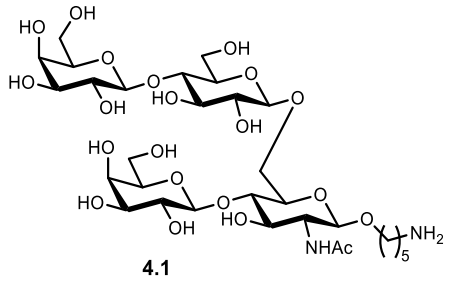
General procedure for deprotection and purification

The purified protected oligosaccharide was dissolved in a 1:1 mixture of MeOH/DCM (anhydrous) and NaOMe (0.5 M in MeOH) was added. The resulting solution was stirred at room temperature until completion of reaction (monitored by MALDI). The reaction was neutralized with Amberlyte IR 120, filtered and concentrated under reduced pressure. The resulting material was dissolved in EtOAc/t-BuOH/H_2O 2:1:1 and one drop of acetic acid. The resulting mixture was purged with H_2 for ca. 15 min. The reaction vessel was equipped with two H_2 balloons and the mixture was stirred at room temperature for 24h. Then the mixture was filtered through a filter syringe equipped with a reverse cellulose syringe filter (pore size: 0.45 µm). The reaction vessel and the catalyst were washed with EtOAc (1 mL), t-BuOH (2x 1 mL) and H_2O (4x 1 mL). The combined filtrates were concentrated under reduced pressure and lyophilised.

The crude material was purified using a pre-packed 6 mL C-18 CHROMABOND column. To the crude material was added H_2O (10 mL, first fraction) and the mixture was passed through the cartridge. Then 10 mL fractions of H_2O, 10% MeOH in H_2O, 50% MeOH in H_2O and MeOH were successively passed through the cartridge and analysed by MALDI. The fractions containing the product were gathered and dried in the lyophilizer. When needed, the product was further purified by HPLC using a

Synergi Hydro RP18 column (250 x 10 mm) using a flow rate of 4.0 mL/min and the following elution method: H_2O (0.1% formic acid) for 5 min (isocratic), then linear gradient to 10% ACN 30 min, then linear gradient to 100% ACN for 5 min. The product fractions were collected and lyophilised to obtain the pure product as formate salt. Analytical HPLC was done with a Synergi Hydro RP18 column (250 x 4.6 mm) using a flow rate of 1.0 mL/min and the following elution method: H_2O (0.1% formic acid) for 5 min (isocratic), then linear gradient to 10% ACN 30 min, then linear gradient to 100% ACN for 5 min. Alternatively, analytical HPLC was done using a Hypercarb column (150 x 4.6 mm) using a flow rate of 0.7 mL/min and the following elution method: H_2O (0.1% formic acid) for 5 min (isocratic), then linear gradient to 30% ACN 30 min, then linear gradient to 100% ACN for 5 min.

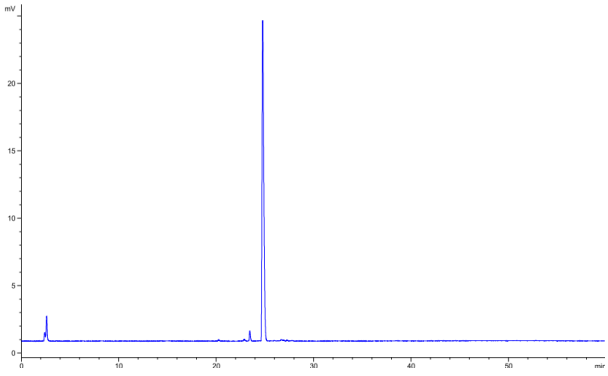
Compound **4.1**



4.1

Protected tetrasaccharide **4.8** (14 mg) was subjected to methanolysis with NaOMe (final concentration 25 mM) for 24 h, according to the general procedure. The resulting crude material was subjected to hydrogenation according to the general procedure. Purification by C18 cartridge gave compound **4.1** (5.0 mg, 93%).

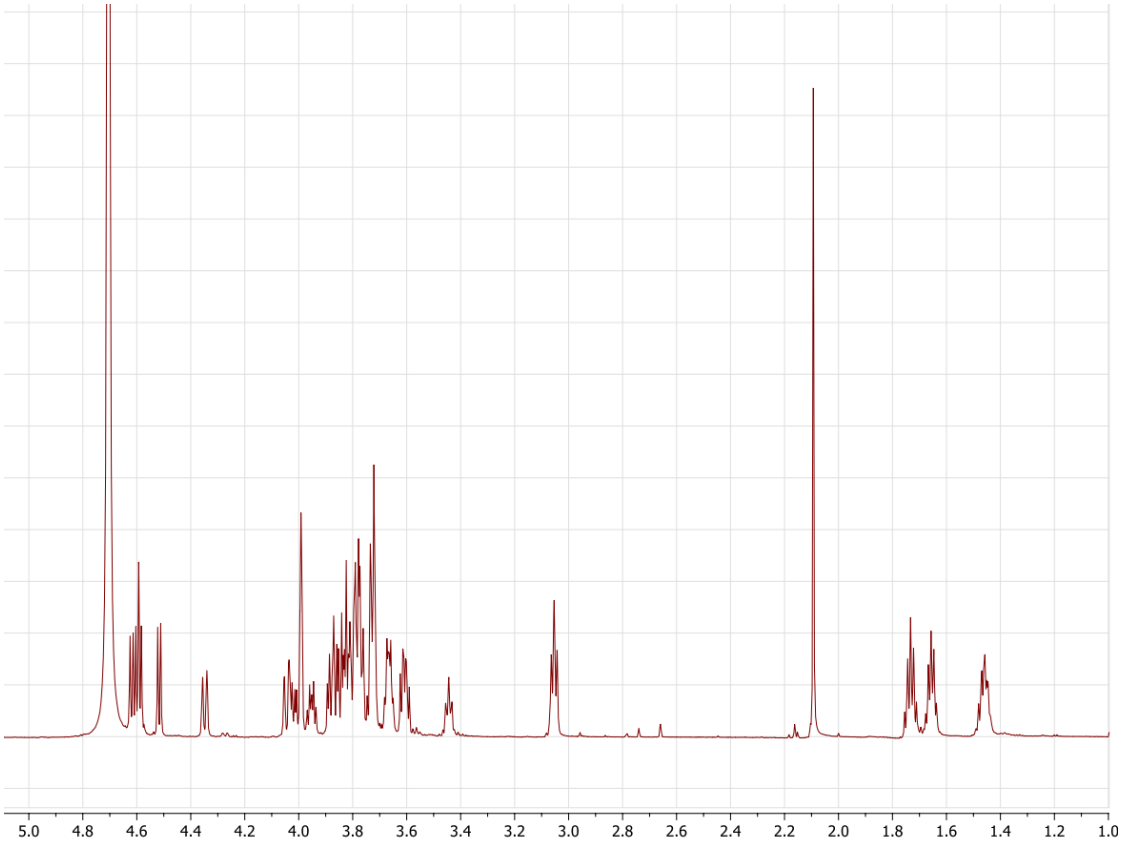
Analytical HPLC of pure product (Hypercarb column):



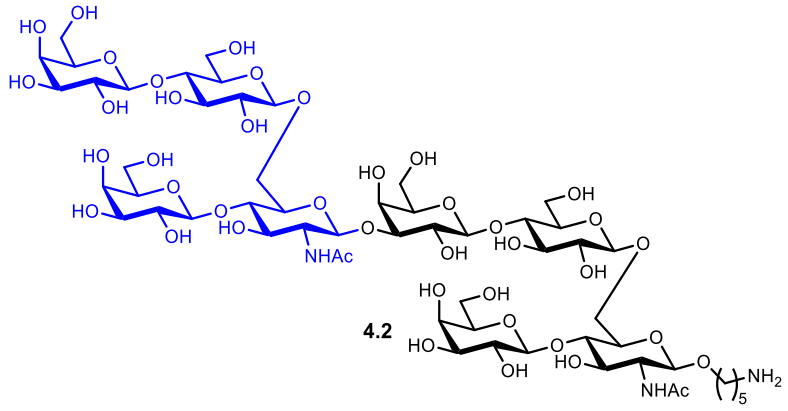
NMR data in agreement with the literature[65].

^{1}H NMR (700 MHz, Deuterium Oxide) δ 4.62 (d, J = 7.9 Hz, 1H, GlcNAc H-1), 4.62 – 4.57 (m, 2H, Glc H-1, Gal_b H-1), 4.52 (d, J = 7.8 Hz, 1H, Gal_a H-1), 4.35 (d, J = 11.2 Hz, 1H, GlcNAc H-6a), 4.05 (dd, J = 12.3, 2.2 Hz, 1H, Glc H-6a), 4.02 (dd, J = 11.4, 4.7 Hz, 1H, GlcNAc H-6b), 4.00 – 3.98 (m, 2H, Gal_a H-4, Gal_b H-4), 3.95 (dt, J = 10.6, 6.2 Hz, 1H, pentanylene chain, OC*H*H), 3.90 – 3.71 (m, 15H), 3.67 (dt, J = 10.2, 6.2 Hz, 1H, pentanylene chain, OCH*H*), 3.61 (dt, J = 9.4, 6.5 Hz, 2H, Gal_a H-2, Gal_b H-2), 3.44 (t, J = 8.4 Hz, 1H, Glc H-2), 3.05 (t, J = 7.6 Hz, 2H, pentanylene chain, C$H_2$$NH_2$), 2.09 (s, 3H, NHCOC$H_3$), 1.73 (p, J = 7.7 Hz, 2H, pentanylene chain), 1.66 (p, J = 6.7 Hz, 2H, pentanylene chain), 1.51 – 1.42 (m, 2H, pentanylene chain, C$H_2$$NH_2$). Gal_a refers to Gal-β-1,4-Glc. Gal_b refers to Gal-β-1,4-GlcNAc.

HRMS (ESI-QTOF): calcd for $C_{31}H_{57}N_2O_{21}$ $[M+H]^+$ 793.3448. Found: 793.3448.

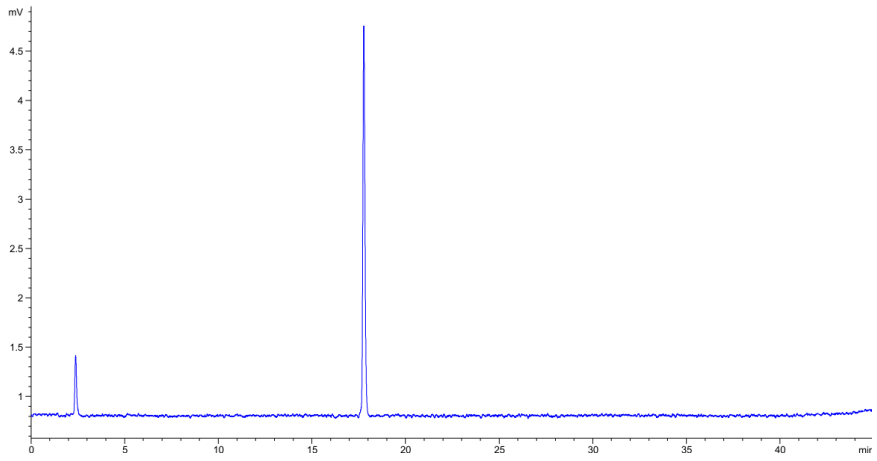


Compound **4.2**



Protected octasaccharide **4.9** (8 mg) was subjected to methanolysis with NaOMe (final concentration 25 mM) for 24 h, according to the general procedure. The resulting crude material was subjected to hydrogenation according to the general procedure. The mixture was neutralized with Et_3N (1.5 µL). After RP-HPLC purification, compound **4.2** was isolated as the formate salt (0.7 mg, 22%), containing traces of triethylammonium formate.

Analytical HPLC of purified product (Synergi column):

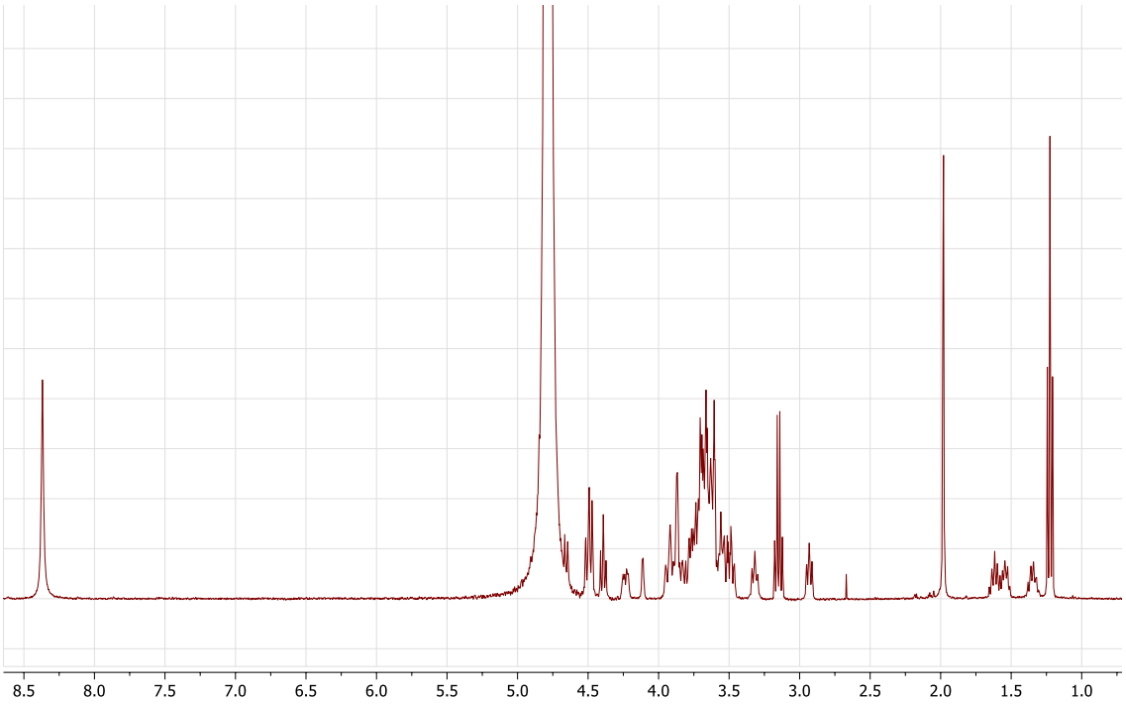


NMR data in agreement with the literature[59].

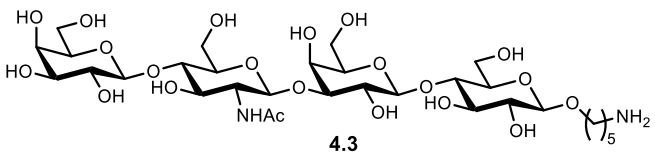
^{1}H NMR (400 MHz, Deuterium Oxide) δ 4.66 (d, J = 8.3 Hz, 1H, H-1), 4.54 – 4.45 (m, 5H, 5x H-1), 4.39 (m, 2H, 2x H-1), 4.23 (dd, J = 10.2, 4.6 Hz, 2H, 2x GlcNAc H-6a), 4.11 (d, J = 3.3 Hz, 1H), 3.96 – 3.46 (m, 45H), 3.32 (t, J = 8.1 Hz, 2H, 2x Glc H-2), 2.93 (t, J = 7.7 Hz, 2H, pentanylene chain, C*H*$_2$NH$_2$), 1.98 (s, 3H, NHCOC*H*$_3$), 1.98 (s, 3H,

NHCOC*H*$_3$), 1.66 – 1.50 (m, 4H, pentanylene chain), 1.40 – 1.30 (m, 2H, pentanylene chain).

HRMS (ESI-QTOF): calcd for $C_{57}H_{100}N_3O_{41}$ $[M+H]^+$ 1482.5827. Found: 1482.5850.



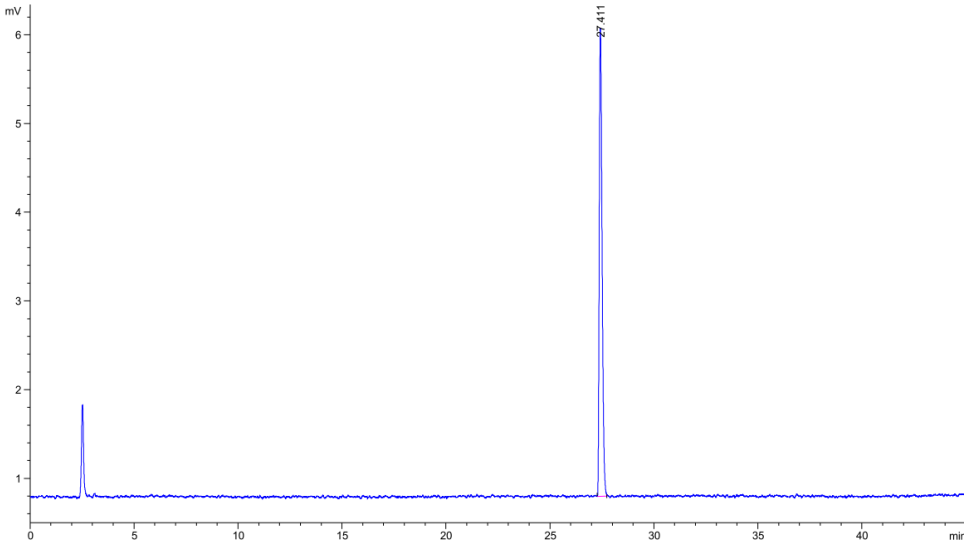
Compound **4.3**



4.3

Protected tetrasaccharide **4.12** (6 mg) was subjected to methanolysis with NaOMe (final concentration 50 mM). After 24h, another portion of NaOMe was added (final concentration 100 mM) and after another 24h the conversion was complete, and the reaction was worked up according to the general procedure. The resulting crude material was subjected to hydrogenation according to the general procedure. After RP-HPLC purification, compound **4.3** was isolated as the formate salt (1.6 mg, 71%).

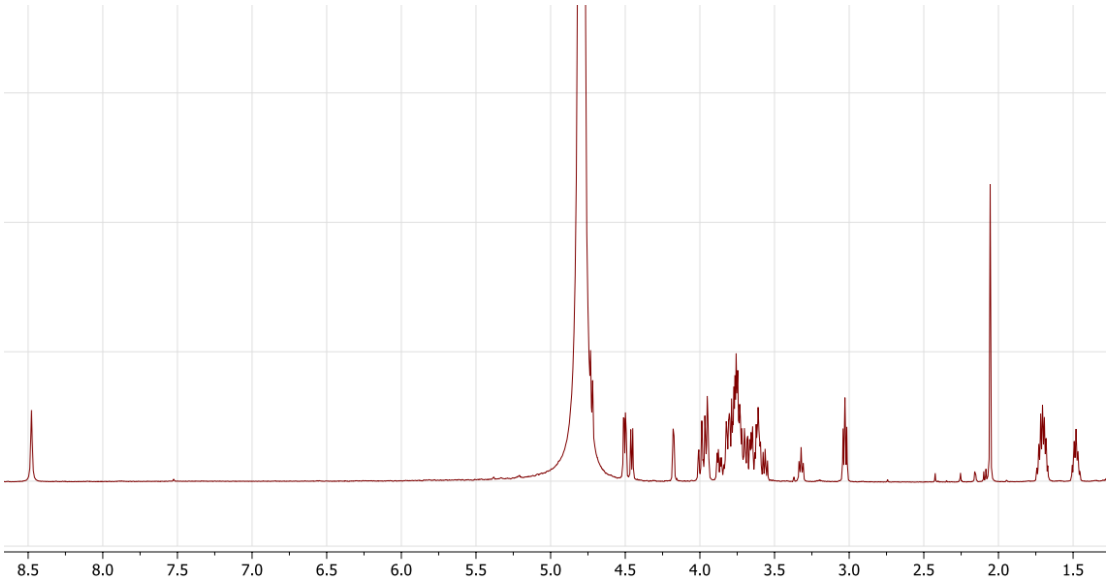
Analytical HPLC of pure product:



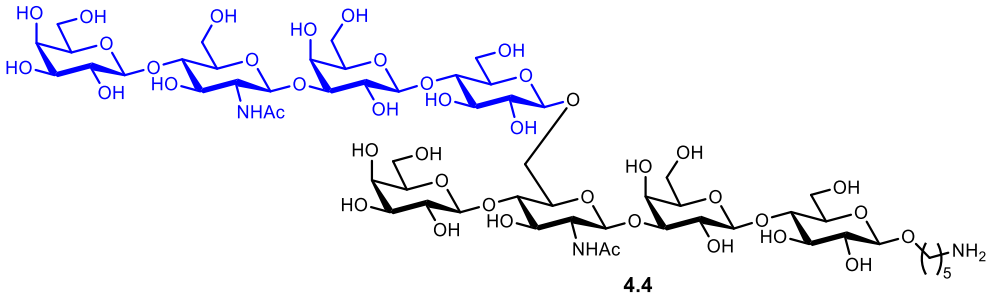
NMR in agreement with the literature[66].

^{1}H NMR (600 MHz, Deuterium Oxide) δ 4.73 (d, J = 8.3 Hz, 1H, H-1), 4.51 (d, J = 8.0 Hz, 1H, H-1), 4.50 (d, J = 7.8 Hz, 1H, H-1), 4.46 (d, J = 7.9 Hz, 1H, H-1), 4.18 (d, J = 3.3 Hz, 1H), 4.02 – 3.94 (m, 4H), 3.87 (dd, J = 12.3, 4.7 Hz, 1H), 3.84 – 3.59 (m, 18H), 3.56 (dd, J = 9.9, 7.8 Hz, 1H), 3.32 (t, J = 8.4 Hz, 1H), 3.03 (t, J = 7.6 Hz, 2H, pentanylene chain, C*H*$_2$NH$_2$), 2.06 (s, 3H, NHCOC*H*$_3$), 1.71 (dp, J = 13.8, 7.2, 6.7 Hz, 5H, pentanylene chain), 1.48 (p, J = 7.7 Hz, 2H, pentanylene chain).

HRMS (ESI-QTOF): calcd for $C_{31}H_{57}N_2O_{21}$ $[M+H]^+$ 793.3448. Found: 793.3449.



Compound **4.4**



4.4

Protected tetrasaccharide **4.13** (6 mg) was subjected to methanolysis with NaOMe (final concentration 50 mM). After 24h, another portion of NaOMe was added (final concentration 100 mM) and after another 24h the conversion was complete, and the reaction was worked up according to the general procedure. The resulting crude material was subjected to hydrogenation according to the general procedure. Purification by C18 cartridge gave compound **4.4** (0.5 mg, 22%).

MALDI: calcd for $C_{57}H_{99}N_3O_{41}Na$ $[M+Na]^+$ 1504.6. Found: 1504.9.

Enzymatic sialylation

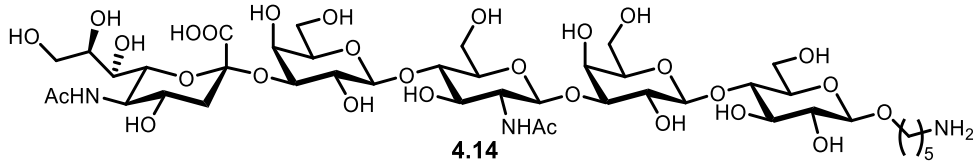
Tetrasaccharide **4.3** (1.0 mg, 1.3 µmol) was dissolved in 781 µL of H_2O and combined with:

- 12.6 µL of CIAP (calf-intestine alkaline phosphatase, Promega, 20 u/µL)
- 25 µL of CIAP buffer (buffer supplied with the CIAP, when diluted 1:10, it has a composition of 50 mM Tris-HCl (pH 9.3 at 25°C), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine)
- 12.6 µL of $MgCl_2$ 10 mM
- 75.6 µL of CMP-NANA 3 mM (3 equiv)
- 126 µL of α(2,3)-sialyltransferase from *Pasteurella multocida* (PmST1)[64]

The resulting mixture was incubated at 37 °C for 18 h. Then the mixture was centrifuged, and the supernatant was filtered through a polyethersulfone Vivaspin 500 membrane (MWCO 10 kDa). The resulting material was analysed and purified using preparative reverse-phase HPLC with a Hypercarb column (150 x 4.6 mm), flow 0.7 mL/min, starting with H_2O (0.1% formic acid) isocratic for 5 min, then linear gradient of 0 to 30% ACN for 15 min, then linear gradient of 30 to 90% ACN

for 15 min. The purified fractions were lyophilized to give the pure compound **4.14** (0.4 mg, 30%).

Compound **4.14**

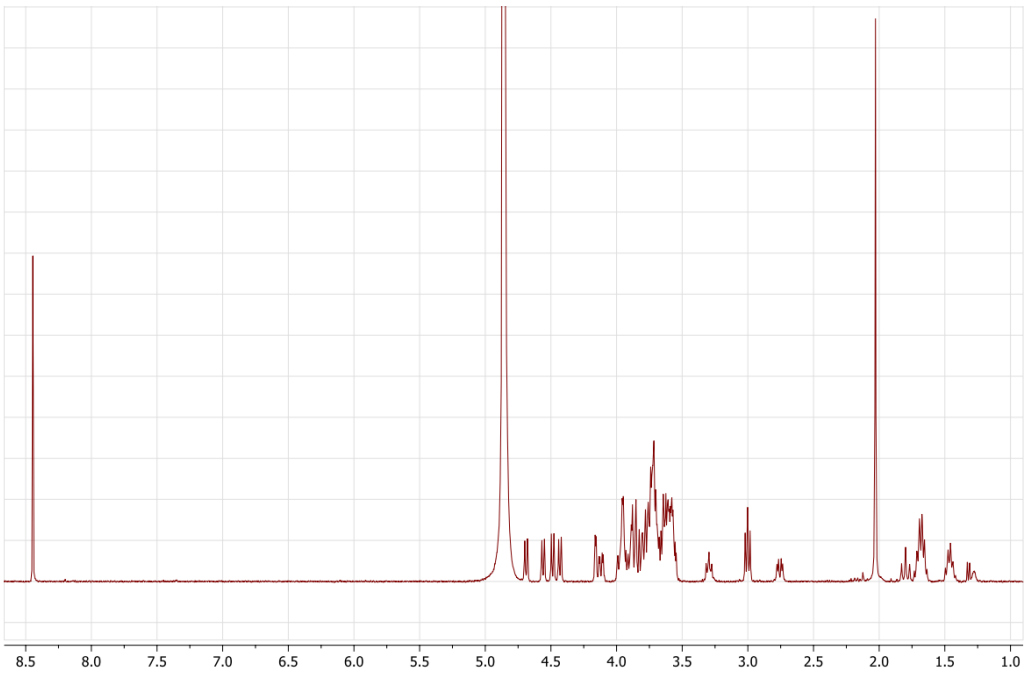


4.14

NMR in agreement with the literature[67].

^{1}H NMR (400 MHz, Deuterium Oxide) δ 8.45 (s, 1H, formate), 4.69 (d, J = 8.2 Hz, 1H, GlcNAc H-1), 4.56 (d, J = 7.9 Hz, 1H, Gal$_s$ H-1), 4.49 (d, J = 8.0 Hz, 1H, Glc H-1), 4.43 (d, J = 7.9 Hz, 1H, Gal H-1), 4.16 (d, J = 3.3 Hz, 1H, Gal H-4), 4.12 (dd, J = 9.9, 3.1 Hz, 1H, Gal$_s$ H-3), 4.00 – 3.54 (m, 30H), 3.30 (t, J = 8.4 Hz, 1H, Glc H-2), 3.00 (t, J = 7.5 Hz, 2H, pentanylene chain, C*H$_2$*NH$_2$), 2.76 (dd, J = 12.5, 4.6 Hz, 1H, Neu5Ac H-3e), 2.03 (s, 6H, 2x Ac), 1.80 (t, J = 12.2 Hz, 1H, Neu5Ac H-3a), 1.68 (m, 4H, pentanylene chain), 1.46 (p, J = 7.9 Hz, 2H, pentanylene chain). (Gal$_s$ refers to the galactose residue linked to Neu5Ac.)

HRMS (ESI-QTOF): calcd for $C_{31}H_{57}N_2O_{21}$ $[M+H]^+$ 1084.4402. Found: 1084.4414.



5. Conclusion

Carbohydrate based vaccines are the most promising solutions to tackle the burden of GBS disease in the world. The availability of chemically defined GBS oligosaccharides can have an extraordinary impact in understanding the molecular mechanisms in the immunological events. In turn, this knowledge can be used to procure safer and more efficient vaccines.

AGA is a powerful source of chemically defined glycans and despite the continuous advances in the field, it still requires great time and efforts to prepare libraries of oligosaccharides. The first bottle neck was identified as the availability of differentially protected "ready-to-use" building blocks. With the work in this thesis, a synthetic route to produce these BBs is standardized.

With the building blocks in hand, the synthesis of GBS type IV repeating unit was attempted. The major bottle neck was identified as the Glc-α-1,4-Gal cis linkage. This linkage was obtained in poor yield. Further developments can be achieved by controlling the stereochemistry using dioxane, by repeating the coupling with the phosphate donor, or simply by synthesizing the Glc-α-1,4-Gal dimer in solution and using it as a building block in the Glyconeer. The work in this chapter can be used as a starting point that is one step ahead in achieving the repeating unit of GBS type IV. The synthesis of the monomer and dimer of GBS type IV repeating unit can lead to the elucidation of the minimal epitope crucial for the immunogenic response.

In the fourth chapter, the synthesis of GBS type III glycans was achieved. Two different frameshifts of the repeating unit were synthesized as well as the corresponding dimers. The yield of the syntheses drops as the oligosaccharide chains grow, which reveals inefficient steps during the automated cycles. There is certainly room for improvement as the target oligosaccharides get longer. At the same time, the Glyconeer proved efficient in the synthesis of different molecules where the same linkages appeared in different positions. The combination of enzymatic sialylation with AGA proved to be an expeditious approach to the preparation of complex glycans for vaccine development. The glycans produced can be used in immunologic assays such as ELISA or conjugated to carrier proteins and injected in animal models.

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