To develop optimized protocols for automated glycan assembly using the Glyconeer synthesizer

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

Tanistha Gupta

2020

This work was performed between July 2015 and June 2019 in GlycoUniverse GmbH & Co KgaA and in the department of Biomolecular Systems of Max Planck Institute for Colloids and Interfaces under the guidance of Prof. Dr. Peter Seeberger.

1st Reviewer: Prof. Dr. Peter Seeberger

2nd Reviewer: Prof. Dr. Rainer Haag

Date of Oral Defense: 27.11.2020

Statement of Authorship

“I herewith declare that I have composed the present thesis myself and without use of any other than the cited sources and aids. Sentences or parts of sentences quoted literally are marked as such; other references with regard to the statement and scope are indicated by full details of the publications concerned. The thesis in the same or similar form has not been submitted to any examination body and has not been published. This thesis was not yet, even in part, used in another examination or as a course performance.”

Berlin, 20.12.2020

(Place, Date)

To my father

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my Ph.D. supervisor Prof. Dr. Peter Seeberger for providing me an opportunity to pursue my doctoral studies in an excellent research environment. His constant guidance and motivation helped me learn and improve a lot during the time of research.

Besides my advisor, I would like to thank Prof. Rainer Haag for agreeing to review my thesis.

Thereafter, I would like to express my sincere thanks to Dr. Mario Salwiczek for his motivation and enthusiasm during my PhD studies at GlycoUniverse. I would also like to acknowledge Mr. Sascha Meyer for the lively discussions and encouragement during the initial days of my PhD.

I would like to thank Prof. Sabine Flitsch for providing me with an internship (secondment) opportunity in her group at University of Manchester, within the framework of Immunoshape Initial Training Network (EU research grant) and guiding me to learn enzymatic synthesis.

I am deeply thankful to Joao Loucano Gomes for his constant help and support during my thesis work. The continuous sharing of thoughts, tasks, ideas, responsibilities, joys and sorrows, was a major help in overcoming the challenges of these past four years.

I am thankful to all my colleagues at Max Planck Institute for Colloids and Interfaces for their help and cooperation. I would like to thank Dr. Christian Roth for a fruitful collaboration and for proof reading my thesis chapter. I would especially like to acknowledge Ms. Eva Settels for her help in understanding and performing HPLC analysis. I would also like to thank Dr. Sourav Chatterjee for proof reading my thesis chapters. Additionally, I would like to thank Ms. Dorothee Böhme for availing the bureaucratic work at the Max Planck Institute. I am very grateful to Dr. Maria Bräutigam for her prompt availability to assist me in writing the Zussamenfassung as well as the exchange of scientific ideas during my Ph.D.

I am deeply grateful to the Immunoshape Initial Training Network (Marie Curie Actions, EU Horizon 2020 Programme, grant agreement no. 642870) for funding my Ph.D. studies and providing the opportunity to work in an interdisciplinary European research

network. I would particularly like to thank Dr. Anna Cioce and Dr. Antonio di Maio for their support and friendship. Finally, I am deeply thankful to my family and friends for their unfailing support. This work is also their achievement.

Publications

Scientific Publications

T.Gupta, M. Salwiczek. Automated glycan assembly of Lewis^x and Lewis^y epitopes: Does a Lactose spacer affect the synthesis? **(in preparation)**

Scientific Conferences and Symposia

Streamlining and optimisation of automated glycan synthesis. **(Poster)** 19th European Carbohydrate Symposium (EUROCARB), Barcelona, July 2017

Towards automated glycan assembly of immunologically relevant glycans using the Glyconeer. **(Poster)** International GlycoBiotec Symposium, Berlin, February 2017.

Developing optimized protocols for automated glycan assembly of complex glycans using the Glyconeer. **(Poster)** International GlycoBiotec Symposium, Berlin, February 2019.

Abstract

The introduction of automation in carbohydrate synthesis has paved the way towards getting faster access to complex, immunologically relevant glycans. Automated assembly enables the synthesis of oligomers and polymers (as long as 50 mers) to be accomplished in weeks, compared to months or even years when performed via conventional solution phase synthesis. However, the optimization of the entire process still lies in its infancy, and standard protocols for the automated synthesis of oligosaccharides are not yet available. The work in this thesis aims to develop optimized protocols for the automated assembly of complex glycans using the Glyconeer synthesizer, which was accomplished by firstly, optimizing the synthesis of building blocks. Since building block synthesis is very often the most laborious and time consuming step in the entire process of automation, ready availability of standard protocols for procuring multi-gram quantities of these monomers further reduces the time required to synthesize complex glycans via automated synthesis.

Thereafter, with multi-gram quantities of building blocks in hand, the automated synthesis of glycans using the Glyconeer synthesizer were streamlined. Optimized protocols for automated glycan assembly of complex glycans were established by streamlining the variables associated with the glycosylation reaction, namely, temperature, number of glycosylation cycles and concentration of donor (in this case building blocks). Therefore, the AGA of Lewis^{x} epitope was optimized to establish optimal glycosylation conditions for the building blocks (Chapter 3) on a 0.0125 mmol scale. With these optimized protocols, the synthesis was then scaled up to a 0.025 mmol scale to procure milligram quantities of the Le^{x} epitope.

Secondly, in order to establish the reproducibility of these protocols, the optimized synthetic protocols were applied to procure a library of Lewis antigens, namely, Lewis^{a}, Lewis^{y} and Lewis^{b} antigens (Chapter 4). The successful synthesis of these antigens further confirmed the reproducibility of the established synthetic protocols. Thereafter, these synthetic protocols were then validated for synthesizing longer structures, which was achieved by synthesizing a library of poly-N-acetyl glucosamine oligomers, such as, tetramer, pentamer and hexamer (Chapter 5). The oligomers synthesized were then used to determine the substrate specificity of the glucosamine hydrolase from

Pseudomonas aeruginosa. Finally, the boundaries of AGA were further pushed by synthesizing one of the most difficult linkage, namely, the beta mannose linkage via automation (Chapter 6), which makes the synthesis of complex N-glycans possible by automated synthesis.

In a nutshell, the work performed in this thesis aims to overcome the challenge of gaining quick access to pure oligosaccharides by establishing standard protocols for synthesizing immunologically relevant glycans via automated glycan assembly, thereby significantly reducing the time required to synthesize these glycans.

Zusammenfassung

Die Einführung von automatisierten Prozessen in die Kohlenhydratsynthese hat den Weg für einen schnelleren Zugang zu komplexen, immunologisch relevanten Glykanen geebnet. Automatisierte Festphasensynthese (*automated glycan assembly*, AGA) ermöglicht die Synthese von Oligo- und Polymeren (bis zu einer Länge von 50 Zuckerbausteinen) innerhalb weniger Wochen, während die entsprechenden konventionellen Flüssigphasen-Synthesen Monate oder sogar Jahre in Anspruch nehmen. Jedoch steckt die Optimierung des gesamten Prozesses noch in den Kinderschuhen und es gibt keine standardisierten Protokolle für die automatisierte Synthese von Oligosacchariden. Das Ziel dieser Arbeit ist es, optimierte Protokolle für die automatisierte Festphasensynthese komplexer Glykane unter Benutzung des Glyconeers zu entwickeln. Der erste Schritt dahin war die Optimierung der Baustein-Synthesen. Da die Synthese der Bausteine meist der arbeitsintensivste und zeitbestimmende Schritt während der gesamten Automation ist, ist das Vorhandensein von Standardprotokollen für die Herstellung von mehreren Gramm dieser Monomere entscheidend, um den Zeitaufwand für die Synthese komplexer Glykane mittels automatisierter Festphasensynthese weiter zu reduzieren.

Mit mehreren Gramm der Bausteine in der Hand, wurde nun die automatisierte Festphasensynthese der Glykane mittels Glyconeer optimiert. Durch die Rationalisierung aller Variablen, die während der Glykosylierung eine Rolle spielen, namentlich Temperatur, Anzahl der Glykosylierungszyklen und Konzentration des Donors (in diesem Fall der Bausteine), wurden optimierte Protokolle für die automatisierte Festphasensynthese komplexer Glykane entwickelt. Für das Lewis^{x}-Epitop wurden optimale Glykosylierungsbedingungen für die einzelnen Bausteine bei einer Ansatzgröße von 0.0125 mmol ermittelt (Kapitel 3). Mithilfe dieser optimierten Protokolle wurde die Synthese dann in einer Ansatzgröße von 0.025 mmol durchgeführt, um mehrere Milligramm Lewis^{x}-Epitop zu produzieren.

Im nächsten Schritt, um die Reproduzierbarkeit dieser Ergebnisse zu zeigen, wurde unter Anwendung der optimierten synthetischen Protokolle eine Reihe von Lewis-Antigenen dargestellt, darunter Lewis^{a}, Lewis^{y} und Lewis^{b} (Kapitel 4). Die erfolgreiche Synthese dieser Antigene belegt weiter die Reproduzierbarkeit der entwickelten

Synthese-Protokolle. Darauffolgend wurden die Protokolle für die Synthese längerer Strukturen validiert, was durch die Darstellung mehrerer poly-N-acetyl-glucosamin-Oligomere, namentlich einem Tetramer, einem Pentamer und einem Hexamer, erfolgt ist. The synthetischen Oligemere wurden dann eingesetzt, um die Substrat-Spezifität der Glucosamin-Hydrolase aus *Pseudomonas aeruginosa* zu bestimmen. Durch die Darstellung einer synthetisch sehr komplexen beta-Mannose-Verbindung mittels Automation (Kapitel 6), wurden die Anwendungsmöglichkeiten des AGA noch einmal erweitert und die Synthese von komplexen N-Glykanen in der Automation ermöglicht.

Zusammenfassend ist das Ziel der vorgelegten Arbeit, das Problem des schnellen Zugangs zu reinen Oligosacchariden durch die Einführung von standardisierten Protokollen für die Synthese von immunlogisch-relevanten Glykanen via AGA zu lösen und damit den Zeitaufwand für die Synthese dieser Glykane deutlich zu verringern.

Table of Contents

List of Abbreviations

Ac	Acetyl
Ac_2O	Acetic anhydride
AcOH	Acetic acid
ACN	Acetonitrile
AGA	Automated glycan assembly
BB	Building block
$BF_3.Et_2O$	Boron trifluoride diethyl etherate
Bn	Benzyl
BnBr	Benzyl bromide
Bz	Benzoyl
Bz_2O	Benzoic anhydride
$PhCH(OMe)_2$	Benzaldehyde dimethyl acetal
MsOH	Methane sulfonic acid
$CHCl_3$	Chloroform
$CDCl_3$	Deuterated Chloroform
Cs_2CO_3	Cesium carbonate
CSA	Camphor sulfonic acid
δ	Chemical shift
d	doublet
DBU	1,8-diazabicycloundec-7-ene
DCM	Dichloromethane
DMF	N,N-Dimethylformamide
DMSO	Dimethyl Sulfoxide
D_2O	Deuterium Oxide
EA	Ethyl acetate
Et_2O	Diethyl ether
Et_3N	Triethylamine
ESI	Electrospray ionization

EtOH	Ethanol
Eq	Equivalent
FMOC	9-fluorenylmethoxycarbonyl
GlcNAc	N-acetylglucosamine
HPLC	High-performance liquid chromatography
N_2H_4.AcOH	Hydrazine acetate
HRMS	High resolution mass spectroscopy
Lev	Levulinoyl
MALDI	Matrix assisted laser desorption/ionization
m	Multiplet
MeOH	Methanol
NaOMe	Sodium methoxide
NP	Normal phase
NIS	N-iodosuccinimide
NMR	Nuclear magnetic resonance
Pd/C	Palladium on activated charcoal
ppm	Parts per million
Py	Pyridine
RT	Room Temperature
s	Singlet
TCA	Trichloroacetyl
Tol	Toluene
THF	Tetrahydrofuran
TfOH	Triflic Acid
TLC	Thin Layer Chromatography
TMSOTf	Trimethylsilyltrifluoromethanesulfonate

List of Figures

List of Schemes

List of Tables

189

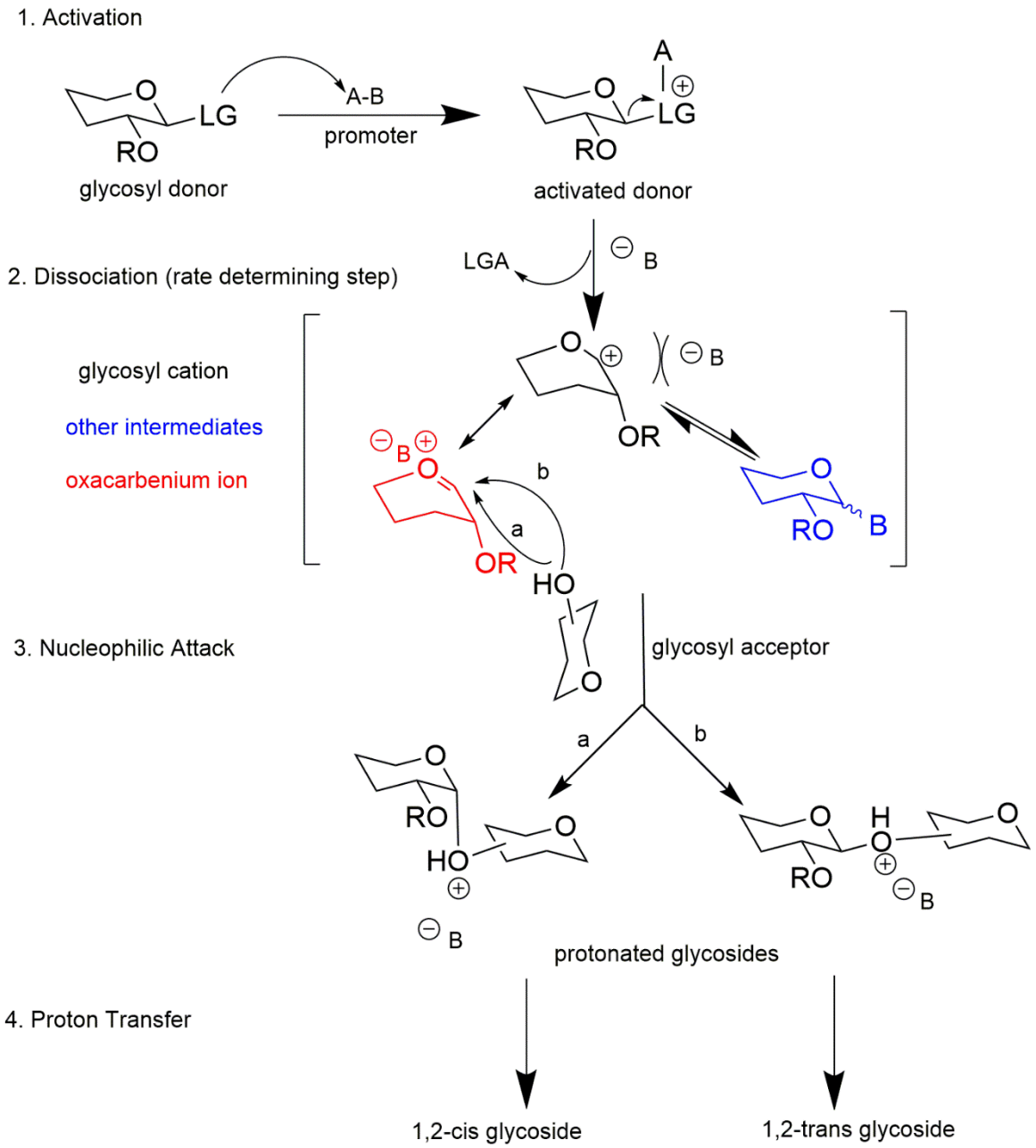
Chapter 1

Automation in carbohydrate synthesis: Developments made so far

1.1 Brief account of developments made in carbohydrate chemistry

Carbohydrates are one of the most diverse and abundant biomolecules on earth. They play important roles in various biological processes, such as cell growth, facilitating joint lubrication, inflammatory and immune responses.[8] Decades of research has also revealed the involvement of carbohydrates in pathogen-host interactions. The pathogenesis of diabetes, septicemia, cancer, pneumonia, malaria, AIDS, and hepatitis are all mediated by carbohydrates.[8] Therefore, understanding the role of carbohydrates in these processes has prompted the investigation of carbohydrate composition, conformation, and their interaction with other molecules and with themselves. Although, isolation of carbohydrates from natural sources is a plausible approach for providing samples for the biological testing of these molecules, it is chemical synthesis that allows access to both natural carbohydrates and their mimetics, which are often of interest because of their therapeutic and diagnostic potential.[8]

A majority of complex sugars are oligomers in which the monomer units are connected via glycosidic linkages. These linkages are obtained by glycosylation, a reaction of the nucleophilic displacement of an anomeric leaving group (LG) on the glycosyl donor by a hydroxyl group of the glycosyl acceptor. The remaining groups on the donor and acceptor are modified with different functional groups in order to minimize the formation of by-products. A general outline of the chemical glycosylation reaction is depicted in Scheme 1.[31]



Scheme 1: General outline of the chemical glycosylation reaction[31]

The earliest reactions performed by Michael, Fischer and Koenigs and Knorr at the turn of the twentieth century showed the complexity of glycosylation reaction.[6] At this stage, the glycosylation of sugar acceptors was quite inefficient and the synthesis of even a disaccharide was extremely challenging.

The improvements thereon led to the expansion of leaving groups beyond the halides, and hemiacetals. Thus, in the 1970's to early 1980's, new classes of glycosyl donors were introduced, namely, thioglycosides, O-imidates, thioimidates and glycosyl fluorides.[31] The list of new leaving groups grew further in the late 1980's, with the introduction of glycosyl carbonates, thiocyanates, glycals, sulfoxides, and heteroaryl glycosides. Apart from studying the anomeric leaving groups, protecting group effects were also investigated. The crucial findings of Lemeiux and Fletcher led to the recognition that the stereoselectivity of glycosylation is directly correlated to the nature of protecting groups, especially at the neighbouring C2 position.[9,31] For instance, 2-O-acyl substituents typically lead to the formation of 1,2-trans glycosidic linkages, albeit with the formation of 1,2-cis glycosides or 1,2-orthoesters as by-products. Demchenko and co-workers introduced donors equipped with a 2-O-picolinyl ether participating group that ensures complete 1,2-trans stereoselectivity. The presence of a non-participating group at C2 such as benzyl ether is necessary for the synthesis of 1,2-cis glycosides. In order to achieve more stereocontrol for the formation of cis glycosides, Boon et.al. introduced chiral auxiliaries capable of producing trans-decalin like intermediates, promoting "opposite face of the ring" type participation, thereby enabling higher 1,2-cis stereoselectivity.[9] The work of Crich et. al. on the synthesis of β-mannosides explored the deactivating and stereodirecting effect of 4,6-O-benzylidene substituent on the stereoselectivity of glycosylation.[30]

Several other factors, such as temperature, solvent, and type of promoter used were found to influence the outcome of glycosylation by affecting its stereoselectivity and yield. The solvent effect on the stereoselectivity of glycosylation has been widely studied. While nitrile solvents favour the formation of β-glycosides by forming axial nitrilium cation intermediates, diethyl ether, THF and dioxane have a tendency to form α-linked glycosides by forming the equatorial O-linked intermediate.[9]

The developments made in the glycosylation methods were followed by advances in the strategies used to assemble oligosaccharides. The synthesis of oligosaccharides are multistep reactions, which are repeated again and again until the desired oligosaccharide is obtained. Many advance strategies that streamline the oligosaccharide assembly are based on chemoselective or on selective activation of leaving groups. One-pot strategies have expedited oligosaccharide synthesis further. In this method, all the sequential reactions are performed in a single flask, and

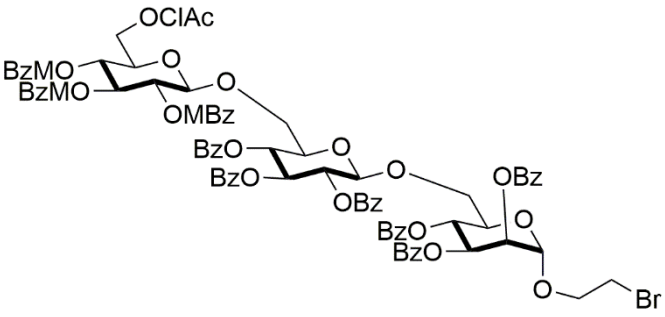
purification is only performed at the stage of the final product and purification of intermediates is not performed. Demchenko et.al. reported the synthesis of the blood group determinant H-type II pentasaccharide using the one-pot glycosylation strategy, which afforded the target oligosaccharide in 60% overall yield.[5] The convergent block synthesis strategy enables accessing complex oligosaccharides faster by pre-synthesizing the oligosaccharide fragments and then converging them by means of a glycosylation reaction. A relevant example was the synthesis of ganglioside GP3, developed by Kiso and co-workers,[13] wherein the target octasaccharide was synthesized by glycosylating pre-synthesized tetrasaccharide building blocks. Further breakthroughs in carbohydrate chemistry came with the development of supported synthesis techniques. Solid phase synthesis using insoluble polymer supports has been extensively used to synthesize various classes of biomolecules. Merrifield was the first to report the synthesis of polypeptides using polystyrene beads.[31] The introduction of solid phase synthesis to carbohydrates is credited to Frechet and Schuerch who reported the first oligosaccharide synthesis on solid support.[31] One of the examples for the synthesis of oligosaccharides on solid support was reported by Schmidt et. al. of a branched saccharide.[5,31]

1.2 Automated synthesis of carbohydrates

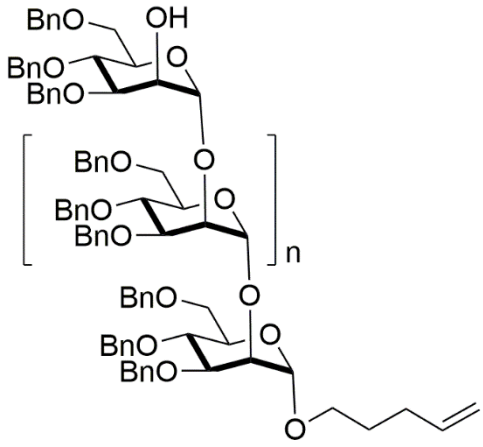
The developments made over the course of conventional synthesis of oligosaccharides, wherein all the protecting group manipulations were performed manually, have laid the basis for considering their automation in a bid to quicken synthesis manipulations. Automation enables operational simplicity, development of accessible methods for glycan synthesis. It offers a potential to revolutionize the way glycans are synthesized.[17]

Many automation platforms utilize a computer interface and a liquid handling equipment, which helps in minimizing human error and therefore, increases the reproducibility of results. The underlying idea of automation is that the synthesis is recorded as a computer program, which is then executed with a "click of a button". Takahashi et. al.[18] investigated a number of platforms for the automation of solution-based synthesis of oligosaccharides In 2000, they adapted a semi-automated instrument Quest 210 to synthesize a trisaccharide (Figure1) by selective activation of leaving groups.[18] They further extended their efforts to a number of automated platforms, such as L-COS by Moritex, that allows to automate temperature control, stirring and rate of reagent addition for glycosylation and deprotection steps.[17,18]

Thereafter, Seeberger and co-workers[21] introduced the first, fully automated oligosaccharide synthesizer by re-engineering a peptide synthesizer. To begin with, a 7-mer oligomannoside (Figure 1) was synthesized with 42% overall yield using Merrifield resin as solid support and an olefin type linker. The high promise of the automated approach was evident with the successful synthesis of the oligomannoside in a considerably short time compared to conventional synthesis. After this first milestone, the subsequent efforts made by Seeberger et. al.[21,22] focused on the synthesis of oligosaccharides containing various challenging linkages, which included sialic acids, furanosides, 1,2-cis glycosides, glycopeptides, and branched oligosaccharides.



Trisaccharide synthesized by solution phase automation of the oligosaccharide synthesis in one pot using Sythesizer Quest 210[18]



Oligomannoside synthesized using a modified peptide syntheizer

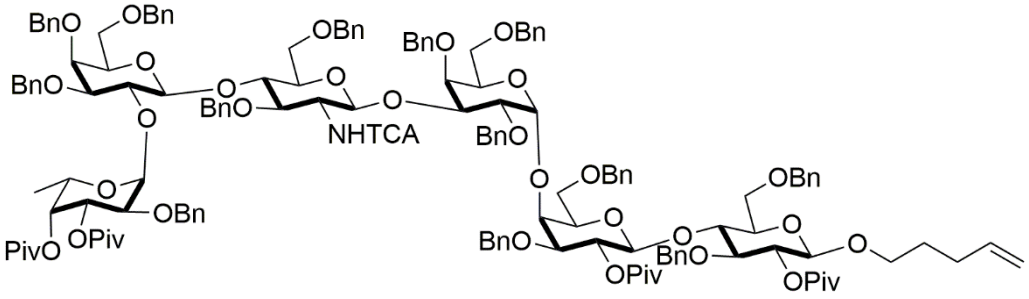


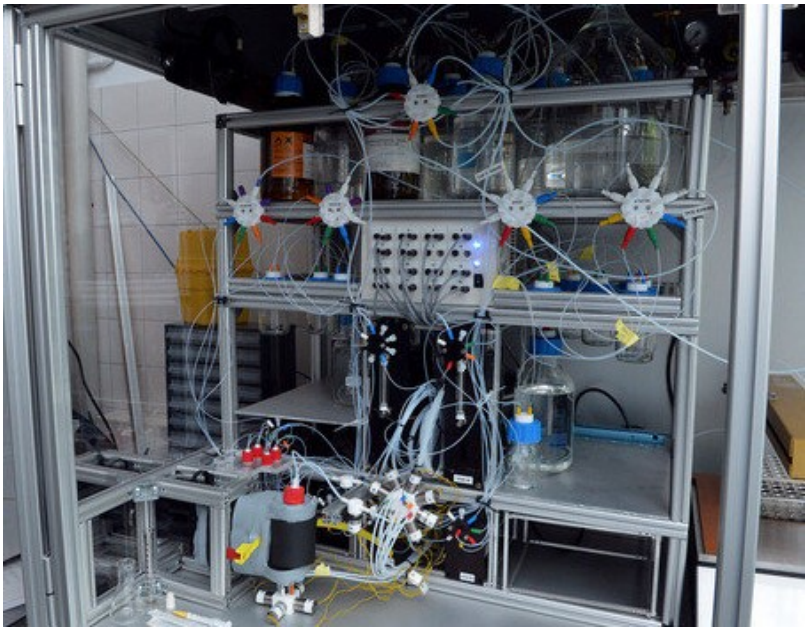
Figure 1: Complex glycans synthesized via automated synthesis of carbohydrates[31]

1.3 History of the synthesizer

The earliest version of the automated oligosaccharide synthesizer was developed by Seeberger and co-workers in 2001 by re-engineering an automated peptide synthesizer.[22] The custom-made reaction vessel was constructed of jacketed glass to allow for cooling. The building blocks were placed in cartridges to be delivered sequentially. The synthesizer allowed for use of nine different solvents and reagents. A systematic study was then carried out to ascertain the performance of the modified synthesizer by synthesizing a series of α-mannosides. With the successful synthesis of the mannosides, a more complicated phytoalexin elicitor (PE) fungal β-glucan was synthesized successfully synthesized using the synthesizer.[24] A series of complex glycans, such as tumor associated carbohydrate antigens, hexasaccharide malarial toxin, core N-linked pentasaccharide, common to all N-linked glycoproteins were successfully synthesized using the modified synthesizer.

Thereafter, a new, more versatile and less expensive platform based on syringe pumps was developed by Seeberger and co-workers.[21] The programming of the instrument could be modified easily to accommodate different cycles of glycosylations, deprotections as well as different washing procedures. Several complex glycans such as Tumor-Associated Carbohydrate Antigens Gb-3 and Globo-H, lipomannan backbone α-(1-6) oligomannoside, and a 50-mer polymannoside were synthesized with satisfactory yields.[21,22] Finally, a commercial version of the automated glycan assembly synthesizer, called the “Glyconeer” was developed.[31] The delivery of reagents/solvents are performed under argon atmosphere, and is equipped with flexible hardware and software to allow for complex glycosylation reaction to be accomplished via automated glycan assembly.

Modified peptide synthesizer used for automated synthesis of oligosaccharides[24]



Home-built synthesizer for automated glycan assembly of complex glycans[21]

Figure 2: Different versions of the automated oligosaccharide synthesizers[24,21]

1.4 Glyconeer 2.1

Figure 3: Glyconeer 2.1: The commercially available, automated solid phase oligosaccharide synthesizer

Glyconeer 2.1 is a commercially available, automated solid phase oligosaccharide synthesizer. It is designed with flexible hardware and software to accommodate the complexities of synthesizing complex glycans. In order to maintain an inert atmosphere for glycosylation, the entire synthesizer is kept under argon pressure. The synthesizer consists of separate “chambers” for solvents and reagents. The solvent chamber consists of eight positions, providing the flexibility to use eight different solvents for automation. The reagent chamber, on the other hand, is further divided into three blocks, namely, deprotection block, consisting of five positions to place the reagents to cleave the temporary protecting group, namely, FMOC, followed by the activator block (including the pre-cooled positions on a Peltier cooling block), further consisting of six positions to place the reagents needed to activate the building blocks, and finally, the aqueous block, containing five positions for miscellaneous reagents. Each of these blocks have a dedicated manifold through which the solvents/reagents flow before they reach the reaction vessel via the central manifold. The argon block situated on the right side of the Glyconeer synthesizer (Figure 3) distributes argon to

each of these manifolds, which further distributes argon on each of the solvent /reagent bottles.

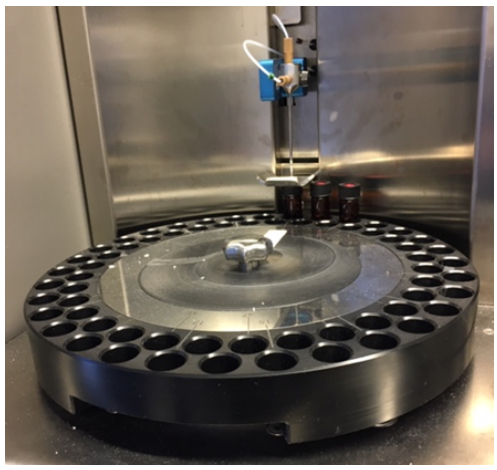
1.4.1 Components of the Glyconeer

The Glyconeer is equipped with different hardware units to accomplish complex glycosylation reactions. These hardware units allow for accurate and precise delivery of reagents as well as temperature control, which is essential for successful glycosylation reactions.

1. **Reaction vessel:**

The reaction vessel is triple jacketed to allow for accurate temperature control, which is a prerequisite for an efficient glycosylation reaction. It is fitted with a frit at the bottom to ensure efficient washing and filtration during automation.

2. **Building block carousel:**

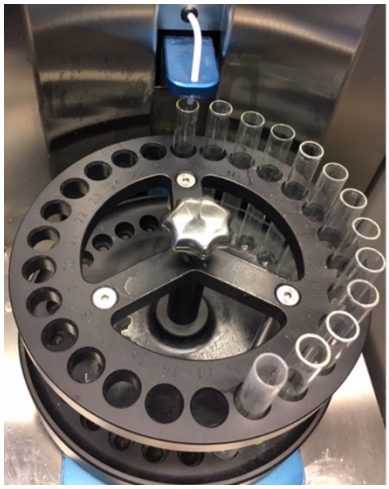


The building block carousel consists of 64 BB vial positions, which can be rotated to add the building blocks sequentially via a needle under argon pressure. Each BB vial can hold 3 mL of BB solution, and the BB needle can be programmed to take up the desired volume of BB solution to be delivered into the reaction vessel.

3. Cooling Unit: The Cryostat:

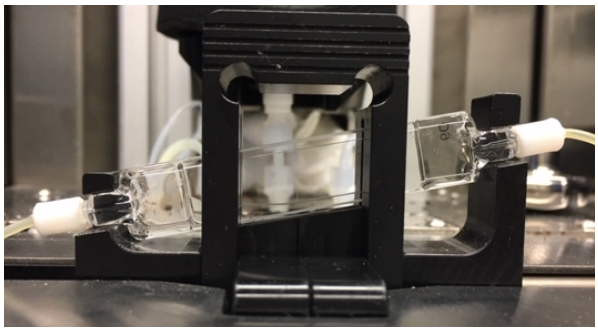
The cryostat is a commercial, table top cooling unit which is used to control the temperature for different processes in the Glyconeer. It is connected to the reaction vessel via specialized metal hoses to ensure uniform circulation of cooling fluid.

4. Fraction Collector:



The Glyconeer synthesizer is also equipped with a fraction collector consisting of 26 test tube positions. It can be rotated under argon pressure to the next position. The fraction collector can also be used to recover unreacted or hydrolysed building blocks.

5. UV lamp:



The UV lamp fitted in the Glyconeer measures the transmittance of the discharge solution upon FMOC cleavage, thus providing a real time feedback regarding the coupling efficiencies of the building blocks. The increasing amount of dibenzofulvene in the discharge solution leads to a sharp decrease in the transmittance values (see section 1.4.4 for more details), thereby giving a semi-quantitative estimate of the glycosylation efficiencies.

1.4.2 Operating the Glyconeer

The software used to operate the Glyconeer synthesizer is known as Glycosoft. It enables the user to program synthetic cycles in order to assemble complex glycans via automation. The software is equipped with crucial, user-friendly features that allow for smooth operation of the Glyconeer. For instance, the software provides a description of the different solvents and reagents which are placed in different positions in the solvent and reagent “chambers”.

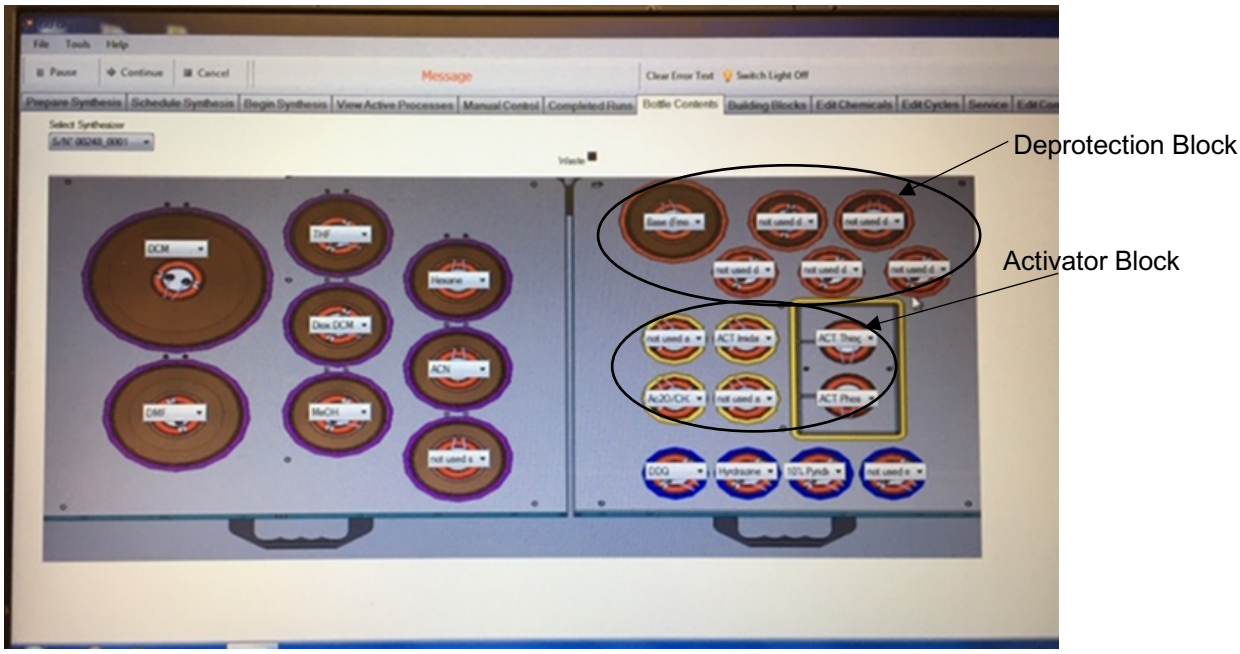


Figure 4: Description of solvents and reagents occupying different positions in the Gyconeer

This information provided by the software prevents any ambiguity regarding the solvents and reagents which occupy specific positions.

The synthetic cycles used to assemble complex glycans are programmed in the “Prepare synthesis” tab, which consists of the synthetic modules. These modules in turn comprise of “variables”, or reaction parameters which enable the users to define them, according to the desired synthesis.

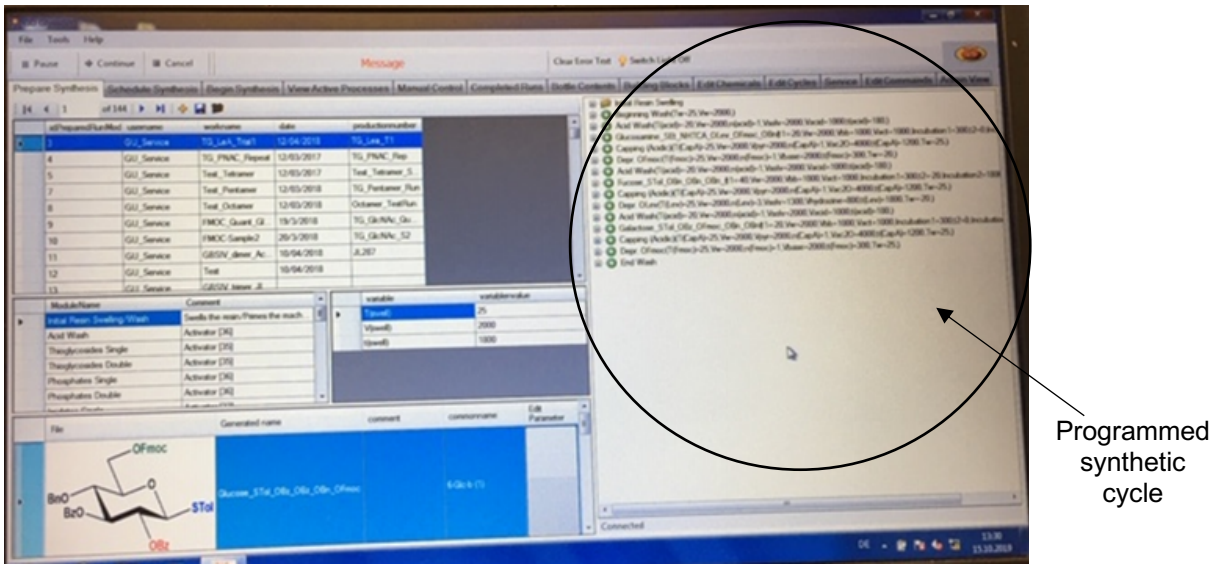


Figure 5: The “prepare Synthesis” tab to program synthetic cycles using the Glyconeer

These modules can then be dragged and dropped to the right panel according to the desired coupling cycles required to assemble the target molecule. This tab also enlists building blocks that the users can choose, and define the parameters for glycosylation (activation temperatures, reaction times etc.). These synthetic cycles then act as a set of instructions that are executed by the synthesizer. Once the synthesis is executed by the synthesizer, the completed runs can be viewed in the “Completed Runs” tab. This tab enables the user to also generate a report highlighting the details of the synthesis performed by the synthesizer. Additionally, the FMOC traces can also be viewed, which also gives a semi-quantitative estimate of the coupling efficiencies.

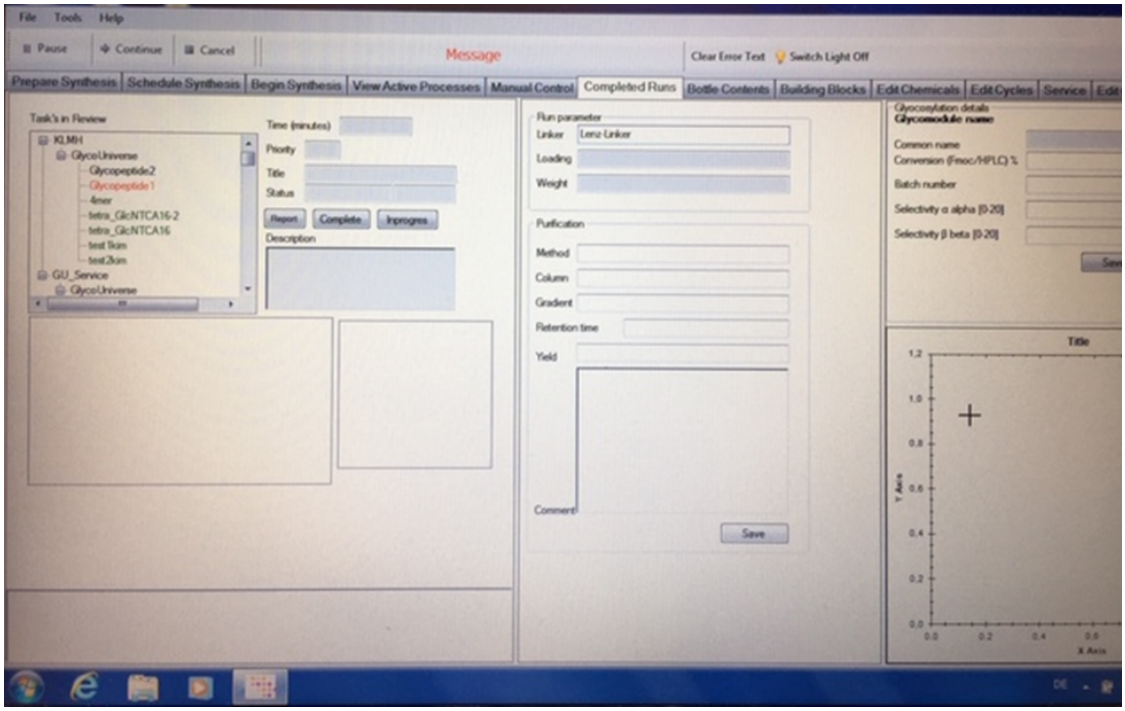


Figure 6: The “Completed Runs” tab to check the FMOC traces of the finished synthesis

Next, the software also enables the user to monitor the argon pressure on the various “chambers”, or “blocks”, namely, the solvent, activator, deprotection and aqueous blocks, which provides an advantage of detecting pressure leaks while the synthesizer is performing synthesis, or while refilling reagents/solvents in the reagent/solvent bottles.

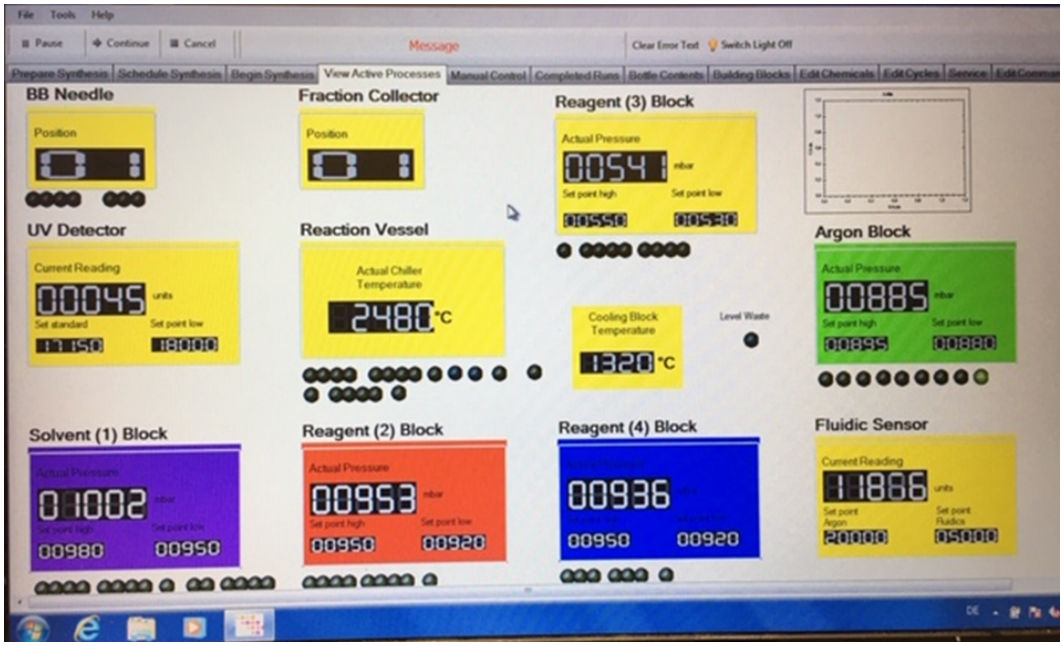


Figure 7: Argon Pressure reading on various blocks, namely, solvent, deprotection, activator and aqueous blocks

1.4.3 Synthetic modules

The automated assembly of glycans using the Glyconeer synthesizer is accomplished by using a set of commands (more details in supplementary information) programmed to allow for the glycosylation of building blocks to the solid support. Each command is comprised of a set of variables, also known as parameters, which are used to define the reaction conditions, such as temperature, reaction times, volume of reagents or solvents to be added during reaction, and the number of times a particular reaction needs to be performed. These parameters provide flexibility to the users to define the reaction conditions required for automated glycan assembly. The arrangement of these commands to accomplish each of the reaction steps are known as synthetic modules.

Each module begins by setting the desired temperature, followed by washing the resin with DCM or DMF to ensure that the resin is sufficiently swollen for effective diffusion on the solid support. This is followed by adding the reagent, and then incubating the resin with the reagent for a specified amount of time depending on the reaction being performed. After the incubation period is over, the reagent is drained out of the reaction vessel, and the resin is further washed with the desired solvent. With the design of synthetic modules in hand, they are then arranged stepwise in order to accomplish a coupling cycle (Figure 8).

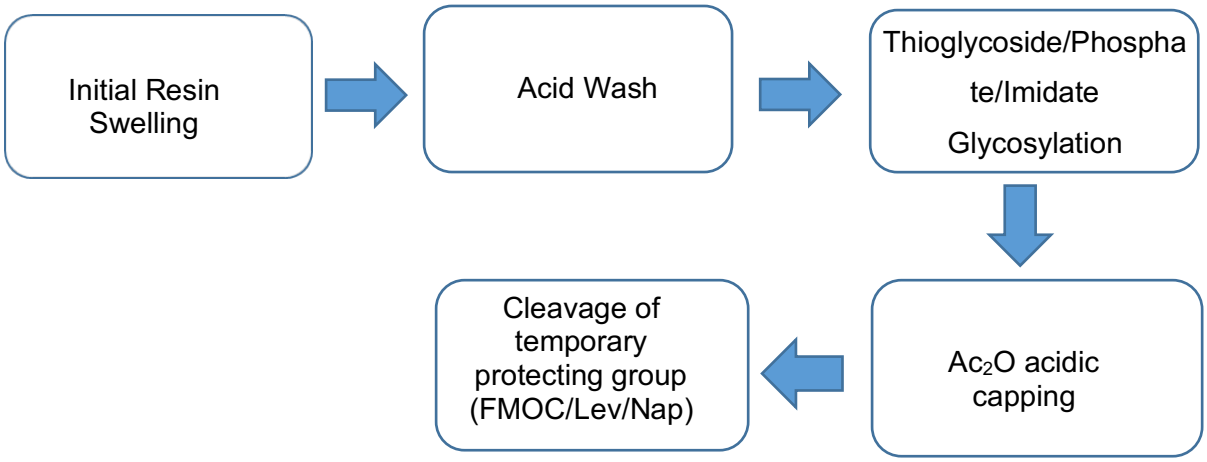


Figure 8: Step-wise arrangement of synthetic modules to accomplish a coupling cycle

All automated synthesis commenced by swelling the resin with dichloromethane, to allow for effective diffusion of reagents on the solid support, which is followed by washing the resin with trimethylsilyl trifluoromenthane sulfonate (TMSOTf) in order to coat the reaction vessel in a bid to prevent the resin from sticking to the walls of the reaction vessel. The building block is then glycosylated, which is followed by capping the deletion sequences, to render the by-products unreactive and prevent them from interfering in further reactions. Finally, the temporary protecting groups are cleaved in order to propagate the oligosaccharide chain.

1.4.4 FMOC trace: a semi-quantitative handle to assess the coupling efficiencies

With the synthetic modules in hand, a test synthesis was performed in order to validate the synthetic modules described in section 1.4.3. The test system chosen was a tetramer of α-1,6-Man. The parameters which were studied to validate the synthetic modules as well as the performance of the Glyconeer were the yield of the target oligomer, time required for assembling it on the solid phase as well as the HPLC purity of the oligosaccharide obtained upon automation.

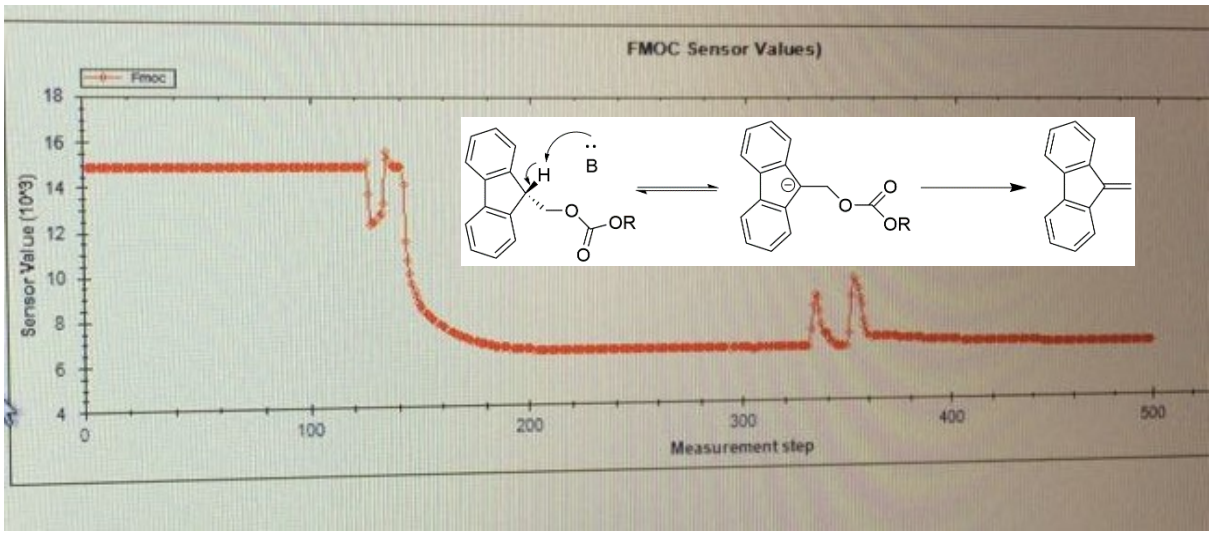


Figure 9: Transmission curve of discharge solution upon FMOC cleavage

In order to perform the test synthesis, the building blocks were glycosylated one by one to the solid phase, followed by capping the deletion sequences. The temporary protecting group, such as the FMOC was then cleaved to propagate the chain. Upon removal of the FMOC carbonate, the discharge solution passes through the UV lamp (see section 1.4.1 for more details), which measures the transmittance of the solution containing the 9-methylenefluorene (formed during cleavage of FMOC carbonate with a base) intermediate. As the amount of 9-methylenefluorene passing through the UV lamp increases, a sharp decrease in the transmittance value is observed. The decrease in the transmittance value over a period of time is indicative of the increase in the coupling efficiencies of the building blocks. Therefore, higher is the coupling efficiency of the building block, the bigger is the dip in the transmittance curve. In other

words, the direct feedback provided by the FMOC sensor provides the advantage of assessing coupling efficiencies “real time”, and thereby identify weak or inefficient glycosylations. Additionally, the real time monitoring further reduces time and resources by identifying inefficient couplings at the stage of automated assembly itself, thereby preventing the need for HPLC analysis.

Test System:

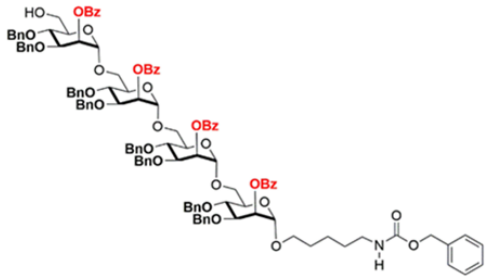
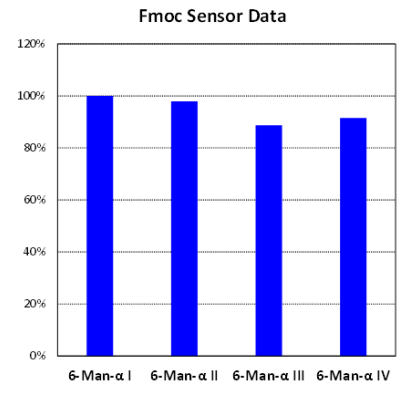
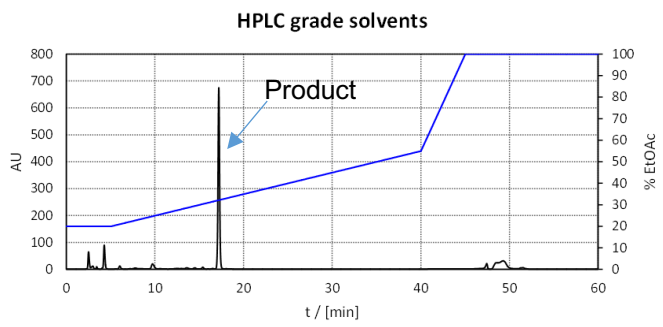
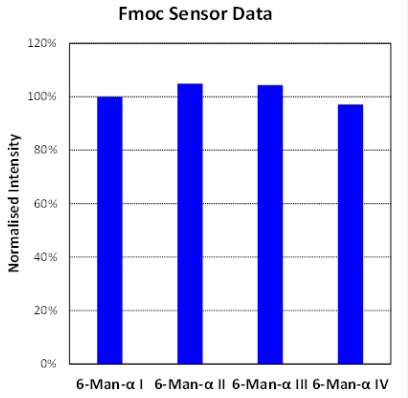
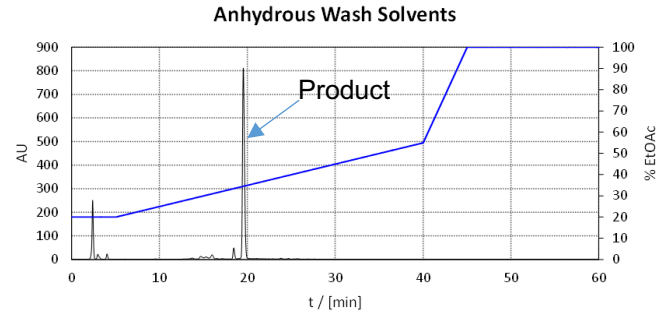
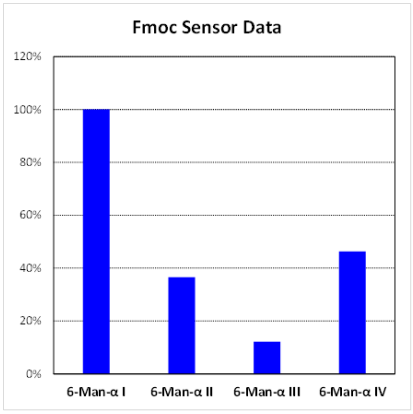
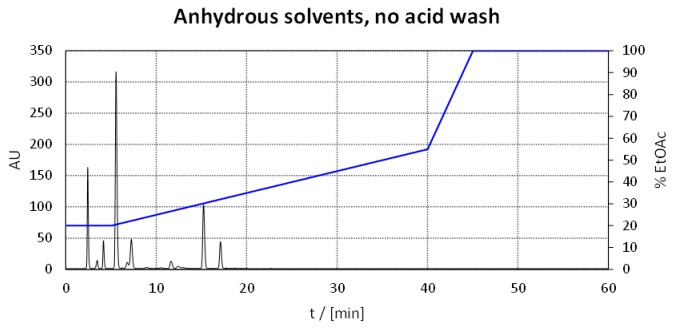


Table 1a: FMOC traces and the corresponding HPLC traces obtained for AGA of alpha-1,6-Man tetramer

Preliminary analysis (FMOC trace)	**Final Analysis (HPLC trace)**
	
	





Glycosylation Conditions: Man (I-IV): -20°C (5 min) to 0°C (20 min), 6.5 eq, 1x

The data in Table 1a indicates that the FMOC traces obtained showed excellent correlation with the HPLC traces. In other words, the excellent coupling efficiencies shown by the FMOC traces are confirmed by the clean HPLC traces obtained. These results further established the reliability of the data obtained from the FMOC traces, which could be used for “preliminary analysis” of the target structures obtained upon automation, which in turn implies that the inefficient couplings could be identified during automated synthesis using the Glyconeer, and HPLC analysis could be avoided in case the FMOC trace indicated a failed synthesis, thereby saving time and resources.

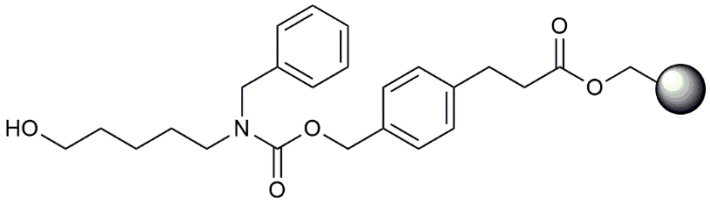
1.5 Solid support and linkers used for automated glycan assembly

A wide variety of resins have been utilized successfully in solid phase peptide synthesis to synthesize a plethora of peptides and proteins, such as polystyrene based resins, and PEG based resins.[31] Taking inspiration from solid phase peptide synthesis, Merrifield resin and Controlled pore glass type resin have been most extensively used for automated glycan assembly. Since the assembly of glycans involves a series of complex reactions using harsh conditions, the Merrifield resin was reported to be the most suitable choice as a solid support due to its resistance to the conditions used for assembling complex glycans.[21] Additionally, it has reasonable swelling properties in the most commonly used solvents for automated glycan assembly such as DCM, DMF and THF, which further allows for efficient diffusion of reagents and solvents at the solid phase.

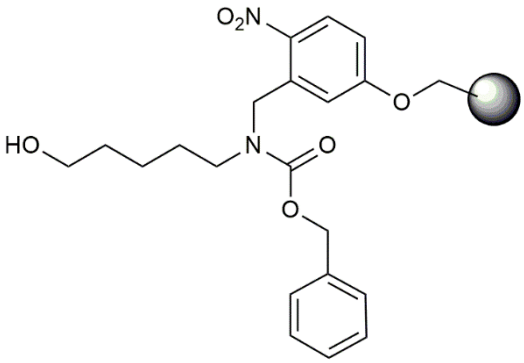
In order to anchor the growing oligosaccharide chain to the solid support, different linkers (which can also be viewed as a protecting group for the solid support) have been developed over the last decade. The choice of the linker is crucial, since it is required to be orthogonal to the reaction conditions used for the entire synthesis, and also to the cleavage conditions to cleave the final product off the solid support.

The earliest linker developed for automated glycan assembly is the octenediol linker, which is linked to the solid support via an ester or ether linkage. With this linker, the final oligosaccharide is released by Grubb's metathesis in the presence of ethylene or other alkenes, to afford an n-pentenyl moiety at the reducing end of the oligosaccharide upon cleavage from the resin.[24]

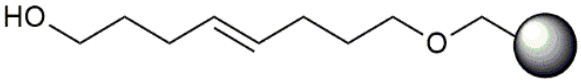
Among the other linkers used is Lenz linker, which is attached to the resin via an ester linkage.[31] It can be cleaved off the solid support by methanolysis to afford a pentenyl amine at the reducing end. The most recent and one of the most widely used linker for automated glycan assembly is the photocleavable linker. It is linked to the resin via an ether linkage. It is removed off the solid support by light induced cleavage. Similar to the Lenz linker, it affords a pentenyl amine at the reducing end.



Lenz Linker



Photocleavable Linker



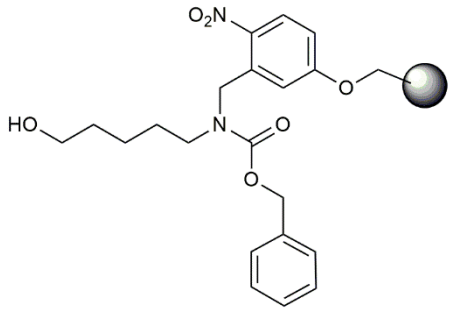
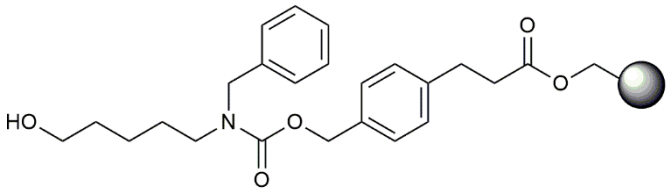
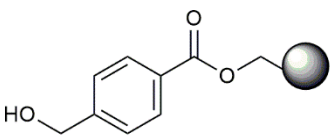
Octenediol Linker

Figure 10: Most commonly used linkers for automated glycan assembly of complex glycans

1.6 Stability of linkers towards acid wash

The linkers used to anchor the growing oligosaccharide chain to the solid support are required to be stable to all the conditions used for assembling complex glycans using AGA. As mentioned in section 1.4.3, every glycosylation step is preceded by an acid wash using TMSOTf. Therefore, it was hypothesized that the repeated use of acid wash might lead to the cleavage of the linkers during automated synthesis. Therefore, in order to prove the hypothesis, the linkers most commonly used, such as Lenz linker and photocleavable linker were subjected to successive acid washes, in order to study the stability of the linkers towards acid wash. The HMBA linker was used as a control to test whether the aromatic ester would survive the acid wash. FMOC carbonate was attached to the linkers, and upon each acid wash, the loading of the linker, or the number of active sites on the solid support occupied by the linker was determined (Table 1b).

Table 1b: Determining the stability of linkers towards successive acid washes with TMSOTf by measuring FMOC loading

Linker	Number of acid washes	Loading
	0	0.53
	3	0.3
	6	0.074
	9	0.024
	0	0.33
	3	0.098
	6	0.028
	9	0.002
	0	0.32
	3	0.31
	6	0.30
	9	0.32

The data shown in Table **1b** indicates a decreasing trend in the loading of the linkers upon increasing number of acid washes. These results further confirmed the speculation that successive acid washes lead to cleavage of the linker. More specifically, the Lenz linker was found to be particularly susceptible to cleavage upon acid wash compared to the photoclevable and HMBA linkers, which deemed it unfit to be used for automated glycan assembly involving extensive acid washes.

1.7 Aims of the Thesis

The objective of this work was to develop optimized synthetic protocols for the automated glycan assembly of complex, immunologically relevant glycans using the Glyconeer synthesizer. Firstly, building block synthesis was optimized in order to overcome the roadblock towards automated glycan assembly (Chapter 2). Thereafter, the established protocols were used to scale up the synthesis of these monomers to 40g batch size in order to procure multi-gram quantities of diverse, differentially protected building blocks. The ready availability of bulk quantities of building blocks further reduced the time required to access complex glycans via automation.

With the BBs in hand, efforts were then turned towards developing optimized protocols for the automated glycan assembly of the Lewis^{x} epitope using the Glyconeer synthesizer (Chapter 3). The reproducibility of the established synthetic protocols was then ascertained by synthesizing a library of Lewis antigens, such as Lewis^{a}, Lewis^{y} and Lewis^{b} antigens (Chapter4).

With the optimized protocols in hand, the Glyconeer was then validated to synthesize longer structures by synthesizing a library of Poly-N-acetyl-Glucosamine oligomers, such as tetramer, pentamer and hexamer. The rapid access to these oligomers helped to study their involvement in biofilm formation in *Pseudomonas aeruginosa*. Finally, the beta mannose linkage, considered to be one of the most challenging linkages to establish via synthetic carbohydrate chemistry was attempted via automation using the Glyconeer synthesizer, which opened up the possibility of synthesizing the complex N-glycans using the Glyconeer synthesizer.

Chapter 2

Streamlining Building Block Synthesis: Overcoming the Roadblock Towards Automated Glycan Assembly

2.1 Introduction

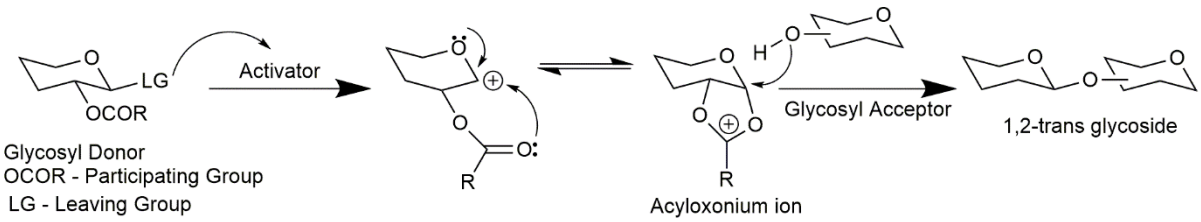
Recent attempts towards automating the synthesis of oligosachharides[21-24] have paved the way towards fast and efficient synthesis of biologically relevant, complex oligosaccharides. However, the success of automated synthesis relies heavily on the ready availability of a set of diverse differentially protected building blocks.[3] Therefore, it is of utmost importance to procure these building blocks in multi-gram quantities, which in turn necessitates the need to establish streamlined protocols for the synthesis of these building blocks.

The "approved building blocks" highlighted by Seeberger[21] in 2015 have laid the foundation towards overcoming the tedious task of synthesizing BBs for automation. This chapter highlights the strategies used to establish optimized protocols for the synthesis of these building blocks, which not only helps in procuring them in multi-gram quantities, but also helps in establishing a routine on the Glyconeer while synthesizing complex glycans. Additionally, having faster access to the differentially protected building blocks allows to forego the laborious and time consuming step of synthesizing the BBs, thereby further speeding up the automated glycan assembly of complex glycans using the Glyconeer.

This chapter is divided into three sections. The first section describes the selection of the most suitable aglycon to mask the anomeric centre, followed by identifying the most suitable thioglycoside derivative for GlcNac, Fuc, Man and Gal. The second section highlights the optimization of building block synthesis, with particular attention to the common synthetic steps required for the synthesis of all the building blocks. Finally, the third section establishes the scalability of the synthetic protocols developed for the GlcNAc BB to a 40g batch size.

2.1.1 Exploring the variability in building block synthesis

Carbohydrates are composed of monosaccharide building blocks joined together via a glycosidic linkage. Most commonly, the glycosylation reactions follow an S_N1 type mechanism,[4] wherein the leaving group at the anomeric centre of the glycosyl donor is substituted by the nucleophile (glycosyl acceptor) to establish the glycosidic linkage. The stereochemical outcome of the glycosylation reaction is influenced by the protecting group at the C2 position of the glycosyl donor as well as the solvent used for performing the glycosylation reaction. The participating protecting groups, such as acetyl (OAc) and benzoyl (OBz) esters promote the formation of 1,2-trans glycosidic linkages by forming an acyloxonium ion intermediate that blocks the α face of the oxocarbenium ion, thereby forcing the nucleophile to attack from the top face as depicted in Scheme1.



Scheme 1: General reaction mechanism of Glycosylation and the plausible intermediates formed

Some of the most commonly used monosaccharides are depicted in Figure 1

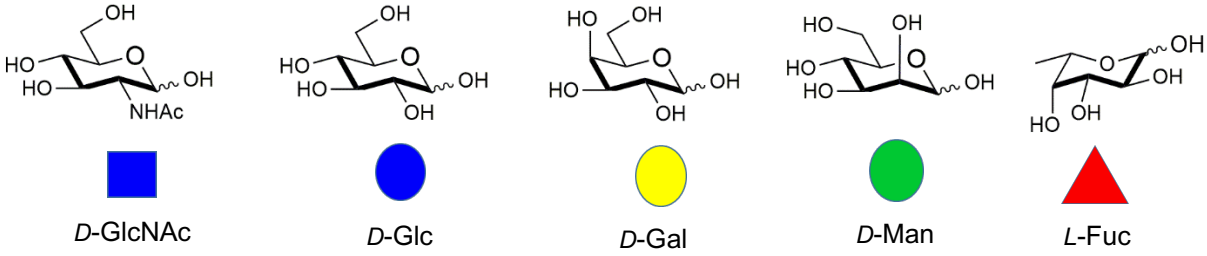
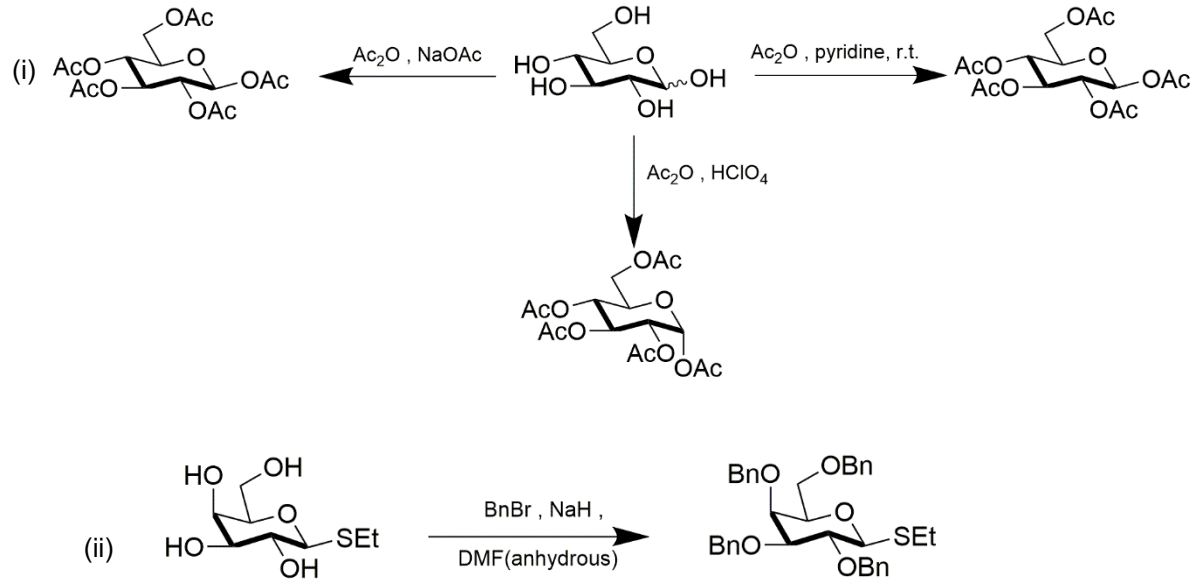


Figure 1: Symbolic representation of the most commonly used monosaccharides

As indicated by the structures in Figure 1, the presence of several hydroxyl groups with similar reactivity (O2, O3 and O4) necessitate the need to differentially modify them in order to achieve the desired linkage and stereochemistry in the final oligosaccharide structure.

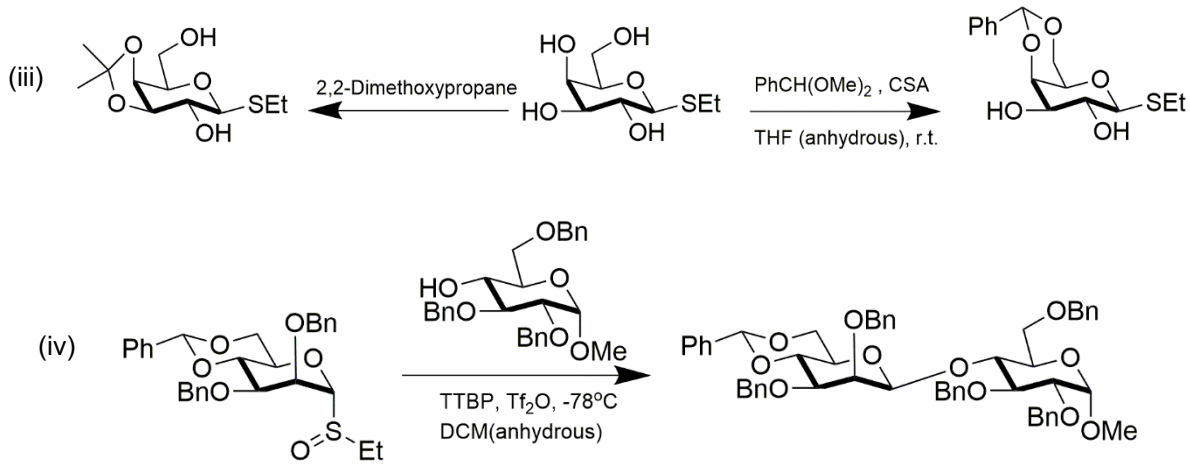
Most commonly, the hydroxyl groups are modified as esters, ethers or acetals. While the ester protecting groups such as acetates, benzoates, chloroacetates and pivaloates can be installed under mild acidic/basic or neutral conditions using acid anhydrides, or acid chlorides, the ether groups such as benzyl, silyl, allyl are installed under acidic, basic and neutral conditions using benzyl bromide, silyl chloride and allyl bromide/chloride respectively.



Scheme 2: Modifying the hydroxyl groups with ester and ether functional groups (i) Introduction of acetyl functional groups under acidic, basic and neutral conditions. (ii) Introduction of benzyl functional group

Since the ester type protecting groups can be easily cleaved under mild basic or acidic conditions, they are used as “temporary protecting groups”, which can be cleaved during automation in order to extend the oligosachharide chain. The ether protecting groups, on the other hand, are quite robust to the conditions of glycosylation, and can be installed in positions that do not require further extension.

The most commonly used acetal protecting groups are the benzylidene and isopropylidene acetals (cyclic acetals of benzaldehyde and acetone, 2,2-dimethoxyropane respectively). These acetal protecting groups simultaneously mask two hydroxyl groups (cis or trans). Additionally, the 4,6-O benzylidene modification provides a conformational advantage in establishing a cis glycosidic linkage, especially in the case of mannose. Crich et.al.[30] reported that the 4,6-O benzylidene mannose sulfoxide can be used to form β-mannosides in excellent yield and stereoselectivity.



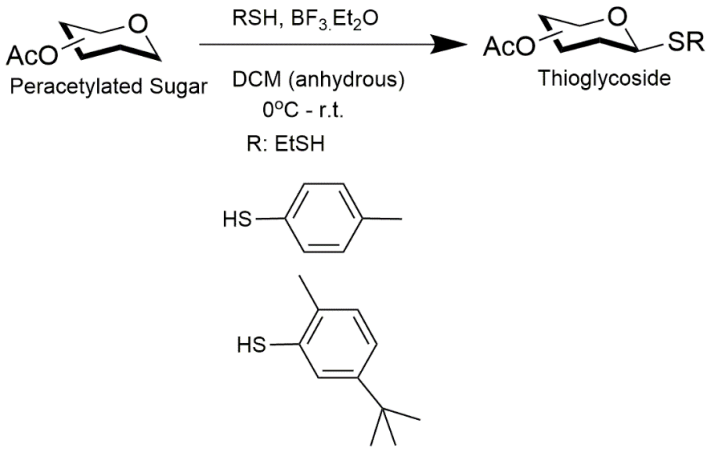
Scheme 3: Masking two hydroxyl groups via cyclic acetals (iii) Benzylidene and isopropylidene acetals to protect the O4 and O6 positions. (iv) Conformational advantage provided by benzylidene acetals in the generation of 1,2-cis linkage in mannose

2.1.2 Choosing an appropriate leaving group to mask the anomeric centre

The earliest class of glycosyl donor, such as the glycosyl halides were developed by Koenigs and Knorr in the nineteenth century.[31] These halides could be reacted with alcohols in the presence of Ag_2CO_3, Ag_2O or heavy salts of Hg^{2+}. Since then, there have been tremendous advancements towards developing new anomeric leaving groups. In the 1970s and 1980s, new classes of glycosyl donors such as thioglycosides, O-imidates and thioimidates were developed.[29] These developments were followed by the use of glycosyl phosphates, selenium-glycosides as well as tellurium-glycosides in the last decade.[29]

Among the various classes of glycosyl donors, the thioglycosides, phosphates and O-imidates remain the most popular choices to modify the anomeric centre. The glycosyl imidates and phosphates most often result in a mixture of stereoisomers that are difficult to separate. On the other hand, the introduction of the thiol moiety proceeds with excellent stereoselectivity. Additionally, the thioglycoside derivatives are highly stable and can be stored for long periods of time, unlike the O-imidate and phosphate derivatives, which degrade readily. Therefore, keeping all these factors in mind, the thiolate was chosen as the anomeric leaving group of choice for all the building blocks synthesized.

2.2 Selecting the most suitable thioglycoside leaving group

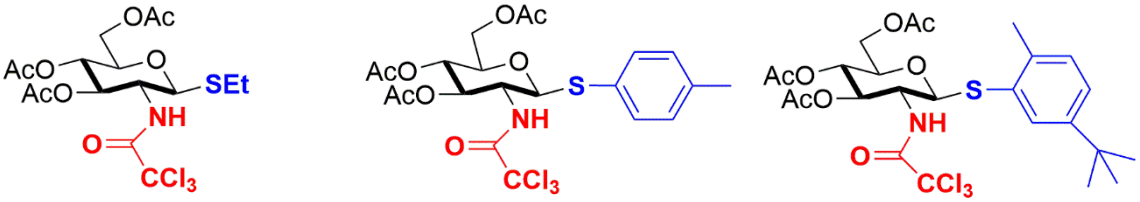


Scheme 5: General reaction scheme for synthesizing thioglycoside derivatives for GlcNAc, Man, Fuc and Gal monosaccharides

There are three most commonly used thioglycoside leaving groups reported in the literature, namely, the ethanethiol (-SEt), p-toluylthiol (-STol) and the 5-tert-butyl-2-

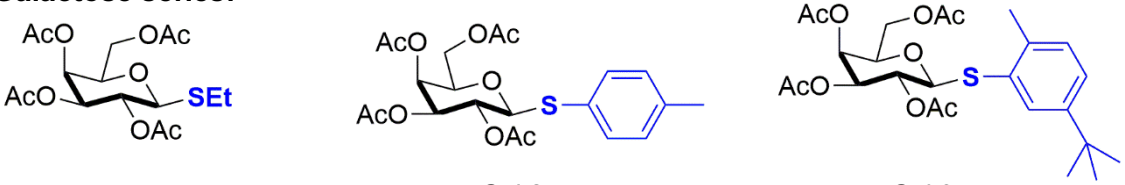
methyl-phenyl thiol (-SAr). However, the rationale behind choosing a particular thioglycoside leaving group for a specific monosaccharide remains unclear. Therefore, in order to reduce the ambiguity, the three thioglycosides were synthesized for GlcNAc, Fuc, Gal and Man.

Glucosamine series:



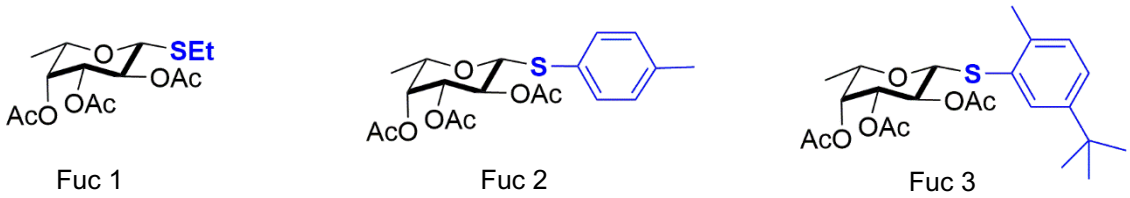
GlcN 1 GlcN 2 GlcN 3

Galactose series:



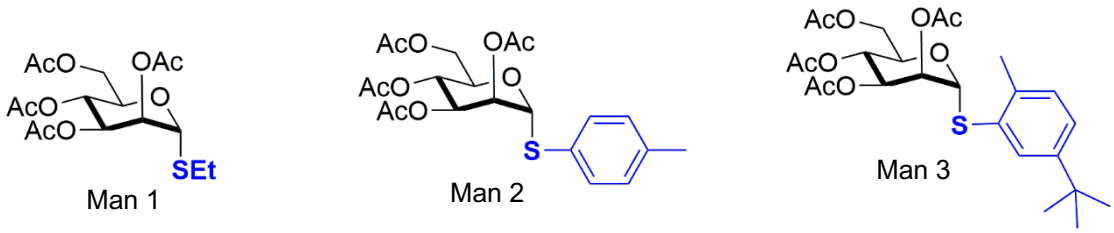
Gal 1 Gal 2 Gal 3

Fucose series:



Fuc 1 Fuc 2 Fuc 3

Mannose series:



Man 1 Man 2 Man 3

Figure 2: Library of thioglycoside derivatives synthesized for GlcNAc, Gal, Fuc, Man and Gal in order to select the most suitable derivative

The most suitable thioglycoside derivative was chosen by taking into account, firstly, the ease of purification and secondly, the toxicity of the respective thiol. The derivatives that crystallized were preferred over the ones that needed column

purification, because of ease in scaling up the synthesis to a 40g batch size. This is because, crystalline derivatives prevented the use of column chromatography for higher batch size, thereby saving resources and reducing the cost.

Table 2a: Thioglycoside derivatives synthesized for GlcNAc, and the yields and crystallinity of the derivatives

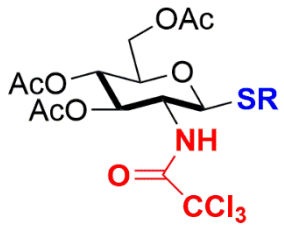
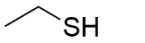
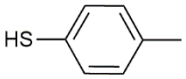
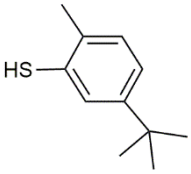
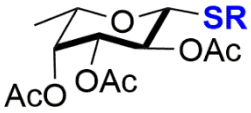
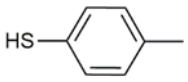
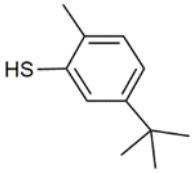
Thioglycoside	Thiol moiety (R)	% Yield	Crystallization
		88%	✓
		80%	✓
		58%	✓

Table 2a indicates that the ethanethiol and p-toluyl thiol afford the respective thioglycoside derivatives in excellent yields. On the other hand, the 5-tert-butyl-2-methyl-phenyl thiol gives the product in poor yields. Therefore, based on the results in Table 2a, the ethanethiol protecting group was found to be the most suitable thiol derivative for GlcNAc.

Table 2b: Yield and crystallinity of the Fucose thioglycoside derivatives synthesized

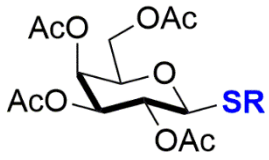
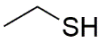
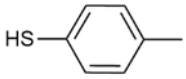
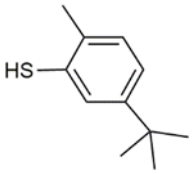
Thioglycoside	Thiol moiety(R)	Stereoselectivity	%Yield	Crystallization
		α/β, 1:1*	60%	×
		Exclusively β*	50%	✓
		α/β, 0.5:1*	65%	×

Note - *ratio determined by NMR

From Table 2b, it is evident that only the p-toluyl thiol derivative for Fuc affords a crystalline derivative. Although the yield for this particular derivative was observed to be the lowest of the three thioglycosides, the low yield was compensated by firstly, the

time saved in getting access to the thioglycoside, and also by the excellent stereoselectivity observed.

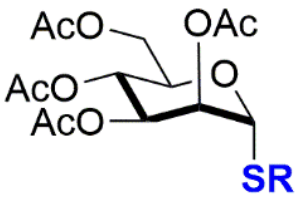
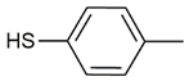
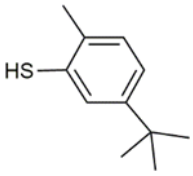
Table 2c: Thioglycoside derivatives synthesized for Gal

Monosaccharide	Thiol moiety(R)	Stereoselectivity	% Yield	Crystallization
		α/β, 1:1*	72%	×
		Exclusively β*	85%	✓
		α/β, 0.5:1*	69%	×

Note - *ratio determined by NMR

The results obtained in Table 2c confirm the p-toluyl thiol derivative as the obvious choice, since it is a crystalline derivative, provides excellent stereoselectivity and affords the respective thioglycoside in satisfactory yield. On the other hand, the ethanethiol and 5-tert-butyl-2-methyl-phenyl thiol derivatives result in poor stereoselectivity, as well as poor yields.

Table 2d: the different thioglycoside derivatives synthesized for Mannose to select the most suitable derivative

Thioglycoside	Thiol moiety(R)	%Yield	Crystallization
		50%	✓
		60%	×
		55%	×

Since mannose did not pose any challenge with regards to stereoselectivity because of no participation from C2 position, the only criteria which governed the choice of a suitable thiol moiety were the yield and crystallinity of the thioglycoside. The results (Table 2c) proved that the ethanethiol moiety afforded a crystalline derivative, thereby making it the preferred choice.

In a nutshell, the different thioglycoside derivatives, ethanethiol, p-toluyl as well as 5-tert-butyl-2-methyl-phenyl thiol synthesized for GlcNAc, Fuc, Man and Gal helped in determining the optimal thioglycoside for each of these monosachharides. Therefore, from the results obtained, it could be concluded that while intermediate GlcN1 and Man1 were found to be the favoured thioglycoside derivative for GlcNAc as well as Man, Fuc2 and Gal2 was the preferred derivative for Fuc and Gal. With the most suitable thioglycoside derivative in hand, efforts were then turned towards optimizing the individual synthetic steps leading to the synthesis of the differentially protected building blocks for automation.

2.3 Optimizing building block synthesis: Establishing streamlined synthetic protocols

The success of automated glycan assembly heavily relies on the availability of differentially protected building blocks. Therefore, in order to procure multi-gram quantities of BBs, it is of utmost importance to optimize their synthesis. Additionally, having easy access to multi-gram quantities of building blocks is also helpful in establishing a routine on the Glyconeer. This is further highlighted in Chapters 3, 4 and 5, wherein procuring multi-gram quantities of building blocks proved to be advantageous in gaining rapid access to the target glycan structure. Also, it assisted in conducting multiple synthetic trials using the Glyconeer synthesizer in order to obtain optimized protocols for automated glycan assembly of the target structure, thereby establishing a streamlined protocol.

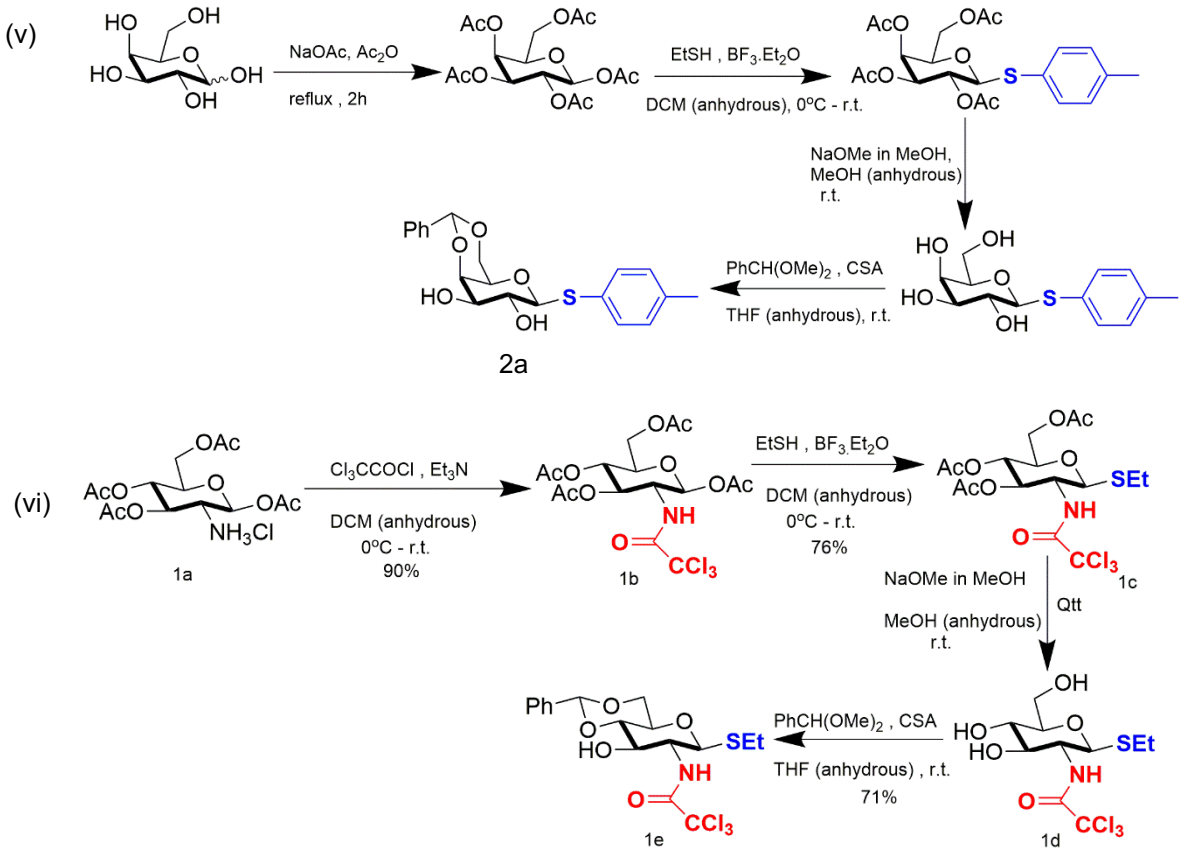
The first step towards achieving streamlined synthetic protocols was to design the differentially protected building blocks required to assemble the target glycans using the Glyconeer synthesizer. In other words, the protecting group manipulations needed in the monosachharides were chosen upon retrosynthetic analysis of the target structures.

Firstly, the positions that required to be extended during automation were modified with temporary protecting groups, such as levulinate ester (Lev) and and 9-fluorenylmethyl carbonate (FMOC), because they are stable to the glycosylation conditions for thioglycosides, and are orthogonal to each other, which enables them to be removed selectively during automation. Secondly, the remaining positions that didn't need to be extended were modified with permanent protecting groups, namely, benzyl ether (OBn).

Another very important factor that needs to be taken into account while designing building blocks is the desired stereoselectivity in the target oligosaccharide structure. For instance, in order to establish trans glycosidic linkages, participating protecting groups such as benzoyl (OBz) and trichloacetamide (TCA) were installed in the C2 positions. The design of all building blocks required to assemble the target structures using the Glyconeer is further highlighted in the following chapters using this strategy.

With the design of building blocks, as well as the optimal thioglycoside derivative for each of them in hand, efforts were then turned towards establishing optimized reaction conditions for each synthetic step, affording satisfactory yields, and ensuring minimum loss of material along the synthetic scheme. Thus, for each reaction step, parameters such as concentration of reagents, temperature and time were studied to establish optimized protocols, and obtain satisfactory yields.

The synthesis of building blocks commenced from the free sugar, except in the case of glucosamine, wherein, the synthesis started from commercially available intermediate **1a**. The first four synthetic steps for synthesis of Gal and GlcNAc are highlighted in Scheme 5.



Scheme 5: The first four steps of the synthetic scheme for synthesizing (v) Gal intermediate 2a (vi) GlcNAc intermediate 1e

The reactions in Scheme 5 further highlight that the first four synthetic steps, such as peracetylation, introduction of the thiol moiety, removal of acetyl groups as well as installation of the benzylidene protecting group serve as the reaction steps involved in the synthesis of all the building blocks. Therefore, efforts were turned towards

streamlining these four synthetic steps, to help in establishing standard operating protocols to get faster access to differentially protected building blocks.

The peracetylation of the monosaccharides was optimized by establishing the most suitable method among the procedures reported in the literature.[3] Thus, based on the experiments conducted for galactose, fucose, and glucose, it was observed that the NaOAc in Ac_2O method of peracetylation afforded the pentaaacetate intermediate in satisfactory yields, as well as the more reactive β anomer. Additionally, the reaction time for this method is the minimum, ranging from 1h - 2h, thereby providing faster access to the peracetylated intermediates.

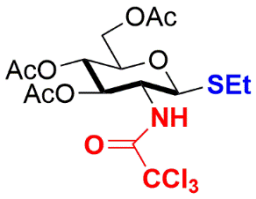
Table 2e: Peracetylation of Glc, Gal and Fuc, using the NaOAc method and the stereoselectivity and yields obtained for each of these derivatives

Reaction Conditions	Monosaccharide	Stereoselectivity	% Yield
NaOAc, Ac_2O, reflux	*D*-Glucose	Exclusively β	72%
	D-Galactose	Exclusively β	77%
	L-Fucose	Exclusively β	Quant.

The next step, which is the introduction of the thiol moiety to afford a thioglycoside was optimized as described in section 2.4. Thereafter, particular attention was paid to streamline the crystallization or precipitation of these thioglycoside derivatives. This was achieved by studying the most suitable solvent mixtures and precipitation conditions (temperature), to afford thioglycoside derivative with excellent yields. The solvent system chosen for precipitation of the product was governed by the polarity of the product as well as impurities present in the crude mixture. It was observed that ethyl acetate (EA) was the most suitable solvent. However, no precipitation was observed with only EA as the solvent. Therefore, it was speculated that because of the presence of unreacted thiol moieties, such as ethanethiol and p-toluylthiol as impurities, a co-solvent might be required to dissolve these impurities, and thereby help in precipitating the product.

Thus, precipitation of the thioglycoside derivatives was performed with a mixture of Hex/EA. This afforded the product in excellent yields, which in turn proved the hypothesis of using a solvent mixture instead of only one solvent. The detailed conditions of the solvent mixtures are indicated in Table 2f.

Table 2f: Yields obtained upon crystallizing the thioglycoside derivatives at different temperatures

Thioglycoside	Precipitation conditions	% Yield
	Hex/EA, 4:1, room temperature	56%
	Hex/EA, 4:1, 35°C	72%
	Hex/EA, 4:1, 50°C	88%

The results obtained in Table 2f confirm that the temperature played a pivotal role in achieving successful precipitation of the product, which is proved by the excellent yield obtained for the thioglycoside derivatives at higher temperatures. With the optimized reaction and purification conditions for the synthesis of thioglycoside derivatives in hand, the next step, the removal of the acetate groups, was found to be straightforward, and the deacetylated derivative **1d** (Scheme 5) did not require to be purified and used for further steps without any purification.

Next, the optimization of the introduction of the benzylidene protecting group was of particular interest. The benzylidene intermediates **1e**, **2a** (Scheme 5) could be selectively decorated with various protecting groups to provide access to a plethora of differentially protected building blocks for automation. An example to further highlight the importance of the benzylidene intermediate is shown in Figure 4.

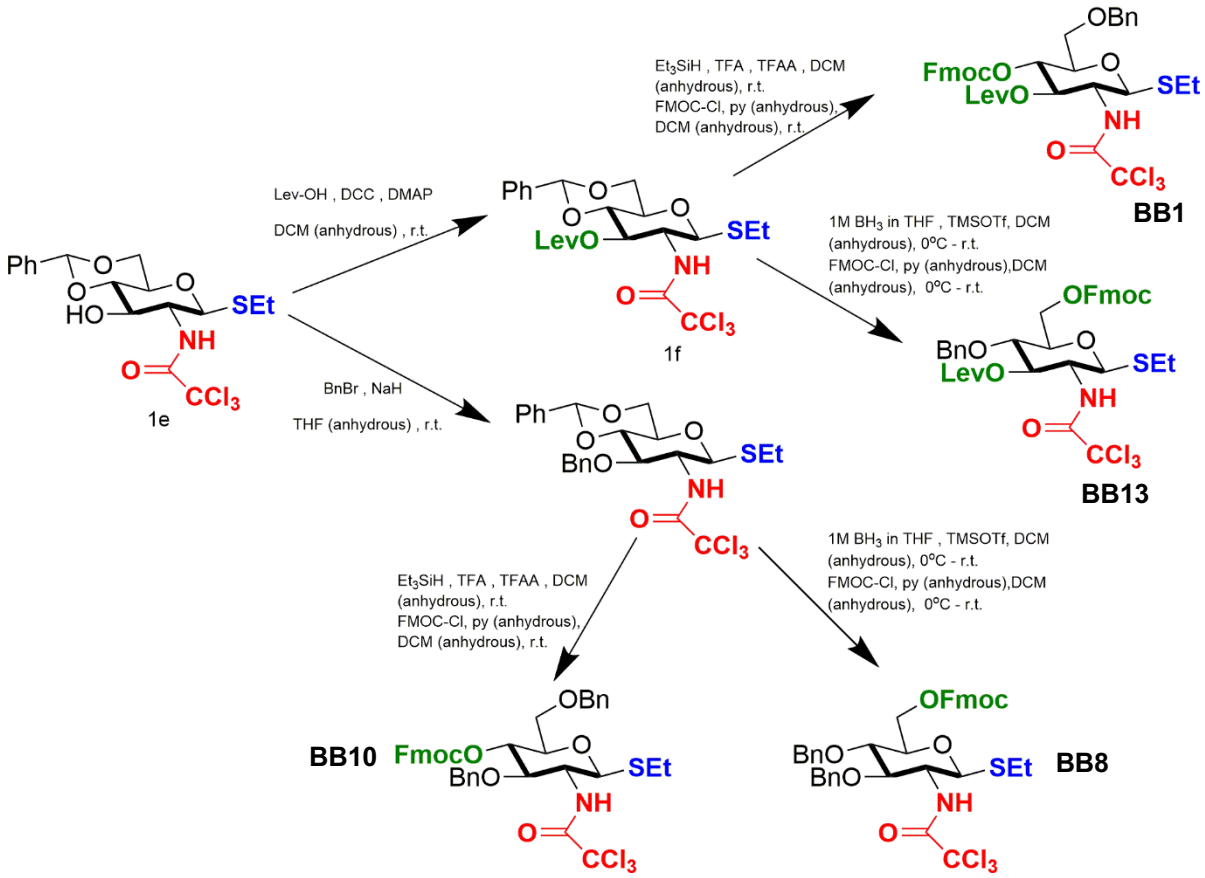
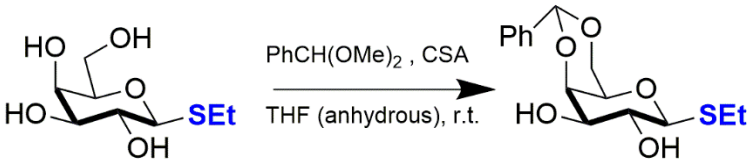


Figure 4: Utilizing the benzylidene intermediate to synthesize a plethora of differentially protected building blocks

The scheme depicted in Figure 4 further confirms the versatility of the benzylidene intermediate **1e**.The benzylidene intermediates **1e**, **2a** will be referred to as a "common intermediate" in the succeeding chapters, since they could be adorned with various protecting group patterns affording diverse building blocks as depicted in Figure 4. Intermediate **1e** also ensured faster access to the differentially protected building blocks in fewer synthetic steps. In a nutshell, this intermediate provided twofold advantage, since firstly, it provided access to a repertoire of diverse building blocks, and secondly, it expedited the synthesis of diverse building blocks BB1, BB9, BB10, BB11 (Figure 4) by allowing access to them in three or less synthetic steps.

Literature reports[8] indicate that N,N-dimethylformamide and tetrahydrofuran are most commonly used to synthesize the benzylidene intermediate. Since DMF is known to be carcinogenic as well as mutagenic, THF was opted as the solvent of choice in order to synthesize the benzylidene derivatives. Secondly, the concentration of the reagents, namely, benzaldehyde dimethyl acetal $PhCH(OMe)_2$ and camphorsulfonic acid were optimized. To begin with, the reaction conditions reported in literature were used, and the reproducibility of these conditions was studied by conducting a series of reaction trials and observing the yields obtained.

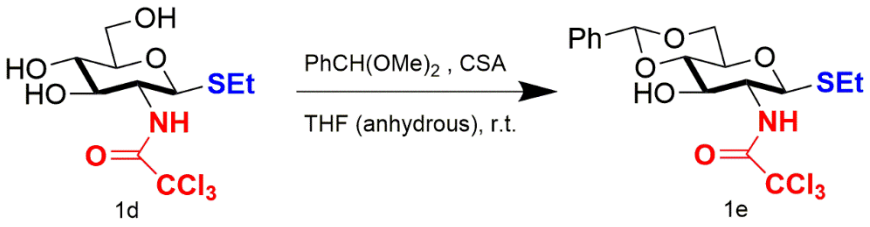


2a

Table 2g: Optimizing the introduction of the benzylidene intermediate **2a**

$PhCH(OMe)_2$ (Eq. based on starting material)	**CSA** (Eq. based on starting material)	**% Yield**
2.0	0.175	75%
		77%
		77%

The reaction conditions were reproducible (Table 2g), which was confirmed by the consistent yields obtained for the successive trials conducted for the synthesis of intermediate **2a**. With the optimized conditions in hand, these protocols were then applied to synthesize the benzylidene intermediate **1e** for the glucosamine building block.

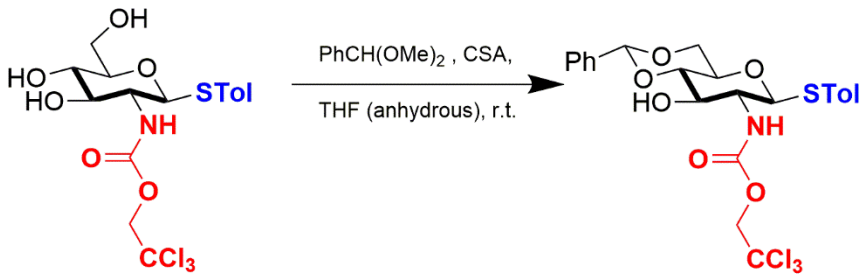


1d 1e

Table 2h: Optimizing the introduction of the benzylidene intermediate for synthesizing intermediate **1e**

Reaction Conditions (equivalents based on starting material 1d)	**% Yield**
$PhCH(OMe)_2$ (2.0 eq), CSA (0.175 eq)	71%
	72%
	72%

The reaction conditions established for the synthesis of the benzylidene intermediate were reproducible, and afforded the product **1e** in consistent as well was satisfactory yields (Table 2h). Similar to the intermediate **2a**, the glucosamine intermediate **1e** could be recrystallized using the same solvent system and temperature, such as DCM/Hexane, 4:1 at 50°C. Next, with these reproducible reaction conditions in hand, it was further applied to another building block, namely, the GlcNac intermediate 4b.



4a 4b

Table 2i: Optimizing the synthesis of intermediate 4a using the optimized reaction conditions

Reaction Conditions	**% Yield**
$PhCH(OMe)_2$ (2.0 eq), Camphorsulfonic acid (0.175 eq)	No Product
$PhCH(OMe)_2$ (2.0 eq), p-toluenesulfonic acid (0.175 eq)	No Product

The results obtained in Table 2i indicate that the reaction conditions for synthesizing intermediate 4b required further optimization. Therefore, the focus was now shifted towards other solvents that are reported in literature in order to synthesize the intermediate 4b. The reaction was then performed in acetonitrile, which resulted in the formation of product in 76% yield.

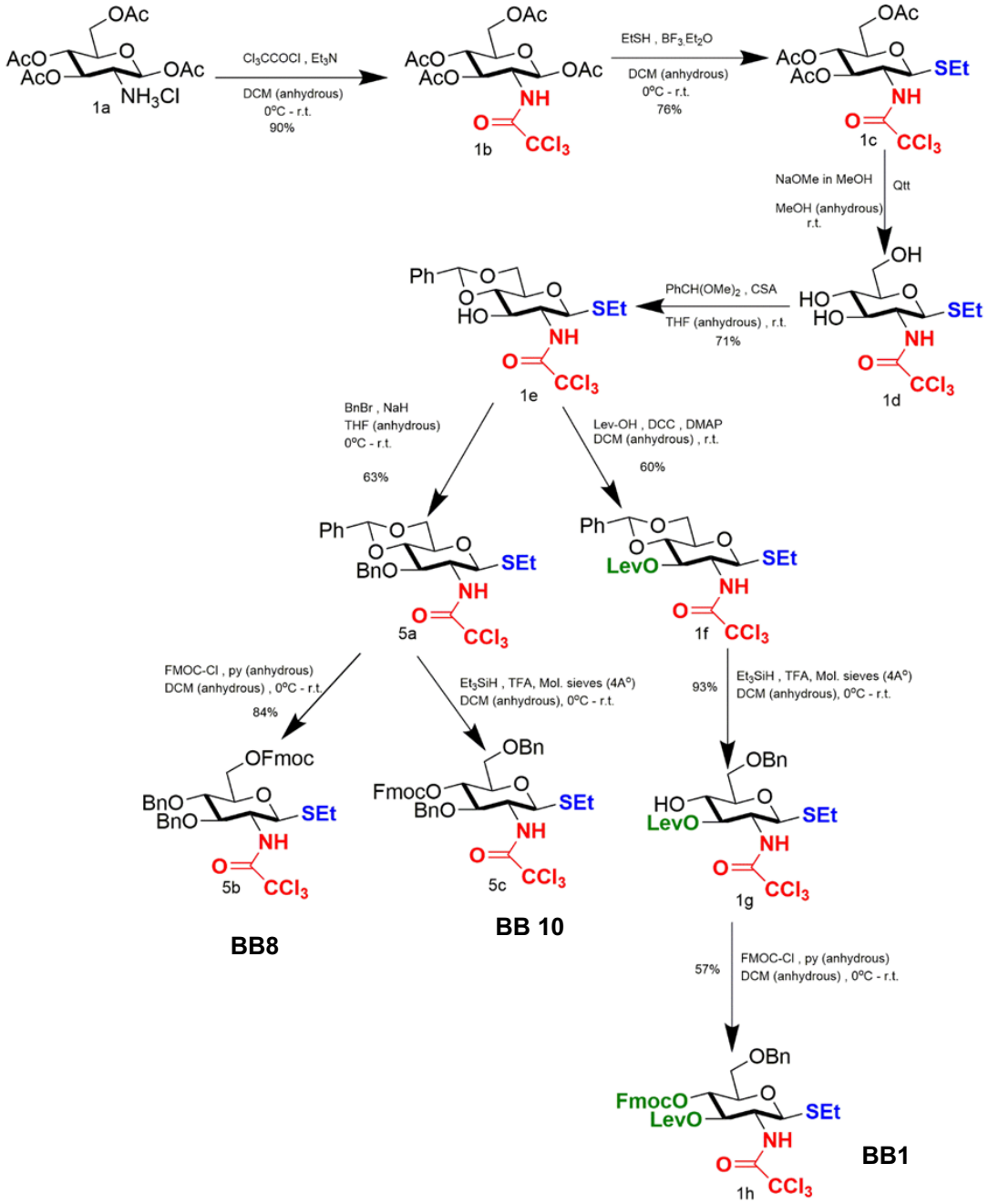
In a nutshell, the reaction conditions for the synthesis of the benzylidene intermediates **2a**, **1e** and **4b** were optimized and the reproducibility of these conditions were established. Additionally, the purification of these intermediates was streamlined, thereby affording the products in excellent yields.

2.4 Establishing the robustness and scalability of established synthetic protocols

With the optimized reaction conditions for the common synthetic steps in hand, the next step was to establish their robustness and applying these protocols to the 40g batch sizes. The aim for performing the synthetic steps in a large batch size was to procure multi-gram quantities of the differentially protected building blocks in order to establish a routine on the Glyconeer synthesizer. The first step towards achieving robust and scalable protocols was to determine the impact of the various reaction parameters, such as concentration of reagents, temperature, mixing, and rate or order of addition of reagents on the 40g batch size reactions.

Another important factor which required optimization was the purification of the intermediates. In this regard, the glucosamine intermediates (Figure 4) posed an advantage since these derivatives could either be precipitated or used for further synthetic steps without any purification. A particular observation made while inducing precipitation in the 40g batch sizes was the formation of large agglomerates. The NMR of these lumps confirmed that they trapped impurities and solvents, thereby reducing the purity of the products. Therefore, in order to avoid the formation of these macroscopic structures, the stirring speed was increased gradually with increasing precipitate formation, which led to the precipitate being finely suspended in the solvent, thereby affording a highly pure product. For example, in case of the intermediate 2a, the formation of agglomerates could be avoided by gradually increasing the stirring speed upon adding hexane to induce precipitation giving a highly pure product. Secondly, the solvent system used for washing the precipitated product was optimized to minimize loss of product during filtration.

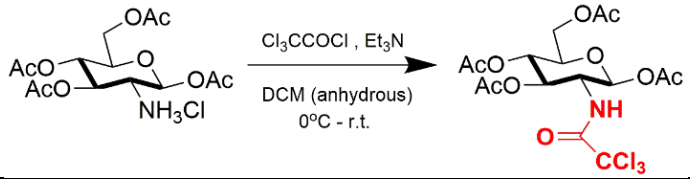
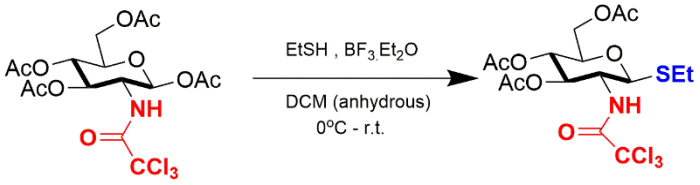
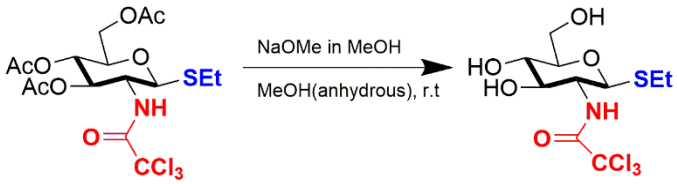
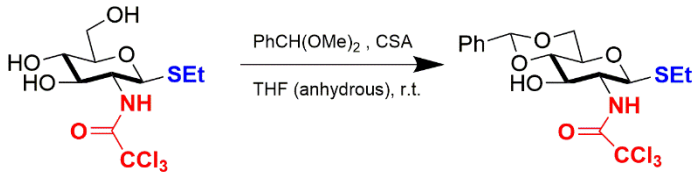
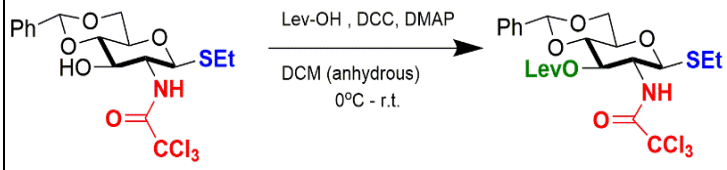
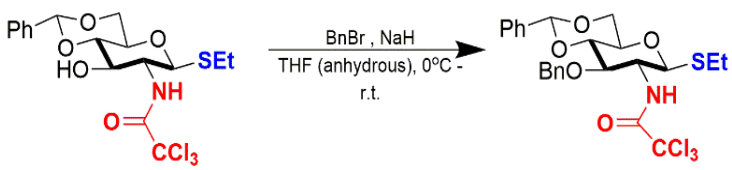
Since the glucosamine building blocks (BB1, BB9 and BB10) were utilized to procure the target oligosaccharides mentioned in the succeeding chapters, the synthetic steps leading to these building blocks were optimized and scaled up to 40g batch sizes in order to obtain multi-gram quantities of these differentially protected building blocks. As a result, standard operating protocols could be established for the synthesis of these BBs which enabled batch production of the final building blocks. A reaction scheme depicting the synthesis of all the GlcNAc BBs synthesized is shown in Scheme 6.



Scheme 6: Reaction scheme for the synthesis of diverse differentially protected GlcNAc BBs for automation, also highlighting the versatility of intermediate **1e**

The scalability of the protocols to synthesize the glucosamine building blocks was established by performing the synthesis on 10g, 20g and 40g batch sizes. The yields obtained in the 40g batch size helped to ascertain the robustness of these protocols.

Table 2j: Establishing the scalability of each synthetic step leading to the common intermediates **1e**, **5a** by applying the established protocols in a 40g batch size

Reaction Step	%Yield		
	10g	**20g**	**40g**
	91%	83%	94%
	57%	88%	80%
	75%	97%	95%
	64%	71%	81%
	42%	13%	60%
	60%	71%	75%

The results in Table 2j indicate that the protocols established for each of the synthetic steps are scalable, as confirmed by the excellent yields obtained for the 40g batch size.

A particular step which needs mention is the introduction of the Lev protecting group to obtain intermediate **1f**. As shown in Table 2j, the yields in the 10g and 20g batch sizes were low, which necessitated the need to further optimize the synthetic protocol. Therefore, taking inspiration from the procedure used to introduce the Lev protecting group in peptides, the DCC and intermediate **1e** were pre-mixed, followed by adding a solution of levulinic acid in DCM. A marked increase in yield was observed in the 40g batch size using the altered procedure. This improved yield could be attributed to the fact that by changing the order of reagent addition, the rate of reaction was faster owing to better mass transfer.

With the optimized and scalable protocols for each synthetic step in hand, the building blocks could then be procured in multi-gram quantities, and the benzylidene intermediates **1e**, **2a** could be stocked up and used to synthesize a library of differentially protected building blocks for automation, which is highlighted in the subsequent chapters. Additionally, the ready availability of these building blocks also helped in conducting multiple synthetic trials on the Glyconeer.

2.5 Conclusion

The synthesis of building blocks was optimized by firstly, selecting the thioglycosides as the most suitable aglycon to mask the anomeric centre owing to their stability and excellent stereoselectivity. Thereafter, the most suitable thioglycoside derivative was chosen for GlcNAc, Fuc, Man and Gal. The results obtained indicated that while GlcN 1, Fuc2 were found to be the most suitable derivatives for GlcNAc and Fuc, Man1 and Gal 1 were the optimal derivatives for Man and Gal. With these results in hand, the common synthetic steps leading to the synthesis of the building blocks, such as peracetylation, introduction of the thiol moiety, removal of the acetate groups , followed by formation of the benzylidene intermediate (Scheme 5) were optimized to obtain excellent yields.

Thereafter, the benzylidene intermediates **1e**, **2a** were identified as common intermediates which provided access to a plethora of differentially protected building blocks (Figure 4). Therefore, this intermediate was procured in multi-gram quantities in order to get quicker access to diverse building blocks.

Finally, the scalability of the protocols developed for the synthetic steps leading to the GlcNAc BBs (BB1, BB9, BB10) were established by performing the synthesis in 10g, 20g and 40g batch sizes. The results indicated that the synthesis in the 40g batch sizes afforded the intermediates in excellent yield, thereby confirming the scalability of the protocols established. The availability of the optimized protocols established pave the way for developing standard operating protocols, which also enable the batch production of the building blocks, and represent one step further to overcome the roadblock towards automated glycan assembly of complex glycans.

Chapter 3

Combining Automated Glycan Assembly and Enzymatic Sialylation to prepare a Lewis^x epitope

3.1 Introduction

Lewis^x is a tumor associated carbohydrate antigen TACA, that is overexpressed on the surface of several types of cancers.[1] As a result, it has been an attractive target for developing an anti-cancer vaccine.[1] The biological relevance of Lewis^x has turned it into a subject of thorough examination.[24] Therefore, there have been several attempts to synthesize the Lewis^x antigen by solution phase synthesis.[32-34] The solid phase synthesis of Lewis^x was reported by Seeberger in 2004,[23] wherein the Lewis^x antigen was assembled via automation to get rapid access to the target oligosaccharide. This work paved the way for further optimizations in order to improve the yield as well further reduce the time required to access the final oligosaccharide. The work in this chapter highlights the step-by-step approach adopted to establish optimized protocols for automated glycan assembly of Lewis^x on the Glyconeer synthesizer to obtain satisfactory yields of the target oligosaccharide.

This chapter is divided into four sections. The first section describes the identification of building blocks required for automated glycan assembly of the Lewis^x epitope structure followed by optimization of the building block synthesis, as well as scaling them up to a 40g batch to procure multi-gram quantities of the building block. The second section describes the assembly of these building blocks on the Glyconeer, as well as the strategy adopted to establish an optimized protocol for automated solid phase synthesis of Lewis^x epitope structure, followed by synthesis on the larger scale (0.025 mmol). The third section details the optimization of global deprotection of the Lewis^x epitope. Finally, the fourth section focusses on the enzymatic sialylation of the fully deprotected Lewis^x using bacterial α-2,3 sialyltransferases which was performed in collaboration with Prof. Sabine Flistch at University of Manchester, as part of the EU ITN Immunoshape.

3.2 Retrosynthetic analysis of target structure and identification of building blocks

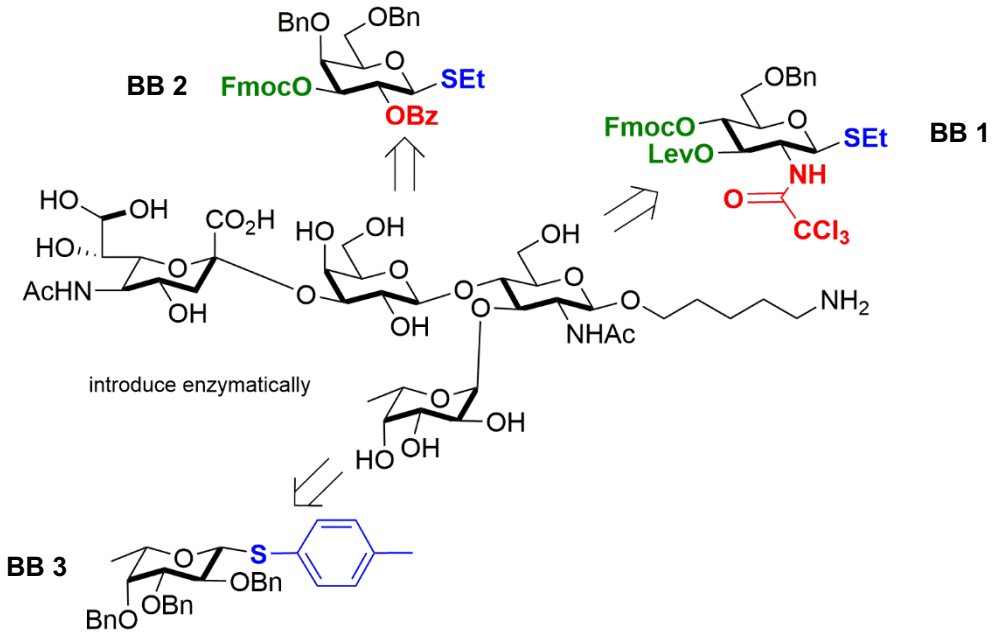


Figure 1: Retrosynthetic analysis of target structure and identification of building blocks

The first step towards obtaining the target structure was the identification of building blocks upon retrosynthetic analysis of the Lewis^x epitope structure. This was achieved by taking into account the specific positions to be glycosylated and the stereochemistry required in the target structure. Therefore, the C3 and C4 hydroxyl groups of GlcNAc (BB1) were modified as Levulinic ester (Lev) and 9-fluorenylmethoxycarbonyl (Fmoc) carbonates respectively, in order to extend the chain during automated synthesis. Secondly, in order to establish β-glycosidic linkages, the GlcNAc (BB1) and Gal (BB2) building blocks were modified at C2 position with N-trichloroacetyl (TCA) and benzoyl (OBz) functional groups respectively. These functional groups, also known as participating protecting groups promote the formation of 1,2-trans linkages by selectively blocking the bottom face of the oxocarbenium ion intermediate formed during glycosylation, thus prompting the incoming nucleophile to approach via the top face, thus forming β-glycosidic linkage.[4] On the contrary, the benzyl ether (OBn) type protecting groups do not provide such a participation, thereby allowing for α connectivity. Therefore, the Fuc BB (BB 3) was synthesized as a perbenzylated derivative in order to establish α glycosidic linkage.

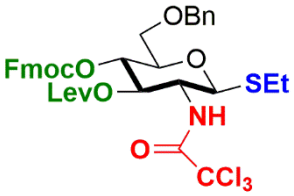
3.3 Optimization and bulk synthesis of building blocks

With the design of building blocks in hand, efforts were turned towards developing optimized synthetic protocols and scale up synthesis. The ready availability of differentially protected building blocks is a prerequisite for the success of automated glycan assembly, and required to establish a routine on the Glyconeer.[1,3]

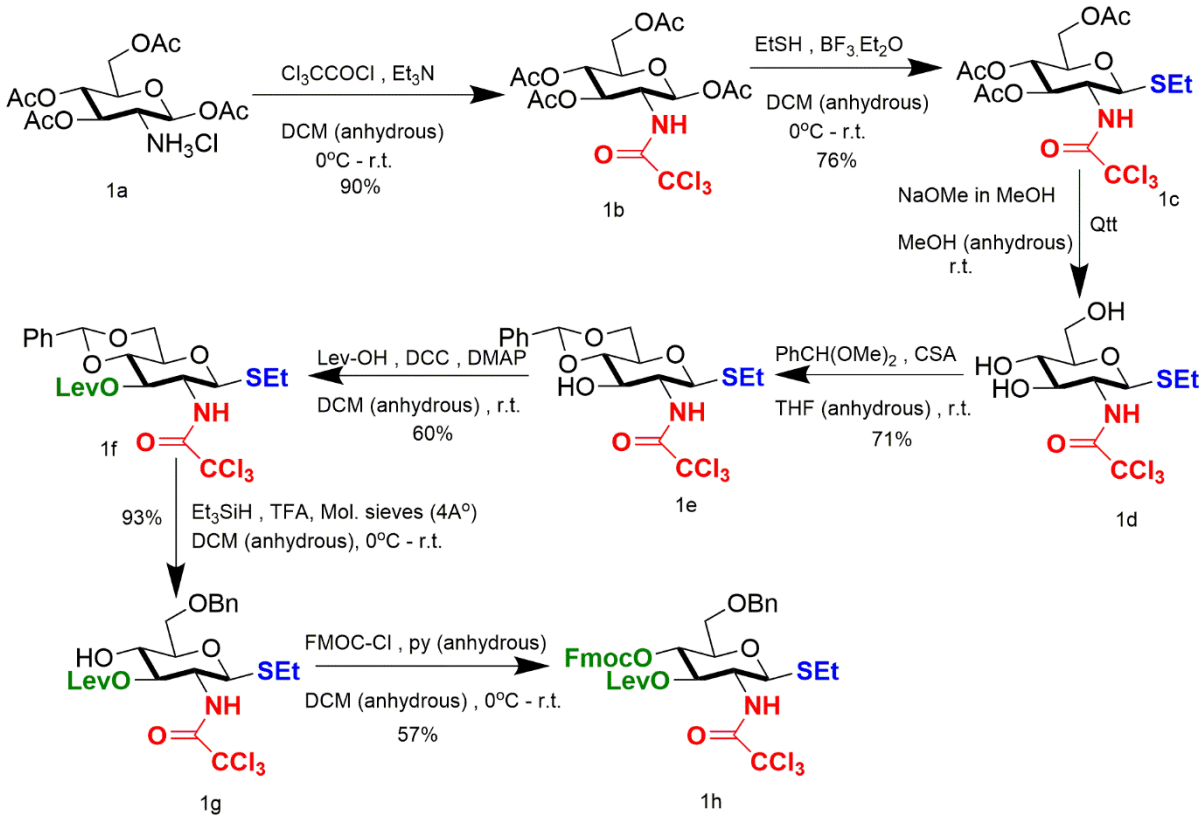
In the process of optimizing the synthesis of building blocks, particular attention was paid to minimize the column chromatography purifications required after each synthetic step. Firstly, it ensured faster access to the final building block, because, column chromatography purification is time consuming. Secondly, reducing the column purifications avoids the use of enormous amounts of solvents on large scale, thereby reducing the waste and saving resources.

The synthetic intermediates shown below could either be crystallized, or used for the next synthetic step without any purification. For example, the synthesis of GlcNAc BB (BB1) and Fuc BB (BB3) as shown below, could be synthesized without any column purification, thereby allowing the synthesis to be completed in 2 weeks. Finally, GlcNAc (13g), Fuc (9g) and Gal (8g) building blocks were obtained respectively, which could be utilized for multiple synthetic trials on the Glyconeer.

3.3.1 Glucosamine building block

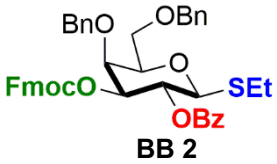


BB 1

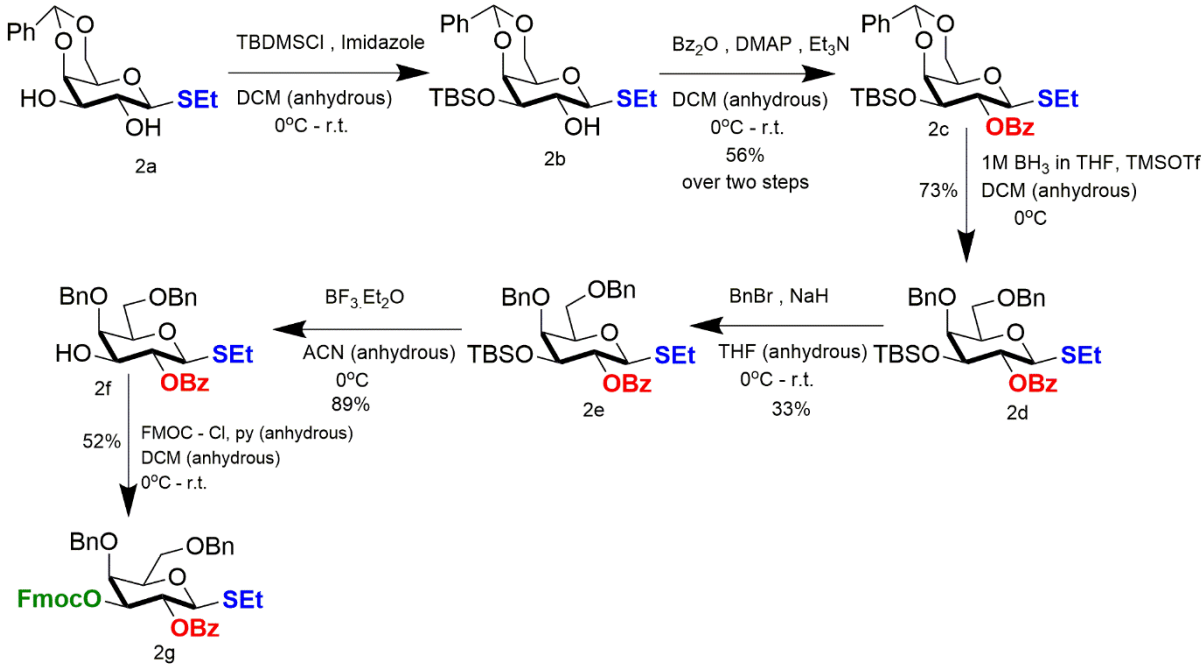


The synthesis commenced from commercially available intermediate **1a**. The amine group was then modified as the trichloroacetamide (TCA) **1b**, followed by introduction of the thiol moiety in order to obtain thioglycoside **1c**. Subsequently, the acetate groups were hydrolysed **1d** and the C4 and C6 positions were functionalized as the benzylidene intermediate **1e**. The 3-OH group was then modified as the levulinic ester (1f). Thereafter, the benzylidene ring was regioselectively opened to obtain the C4 hydroxyl group **1g**, which was finally converted to an FMOC carbonate **1h**. Upon scale-up synthesis (40g batch size) for this building block, 13g of the final building block was procured.

3.3.2 Galactose building block



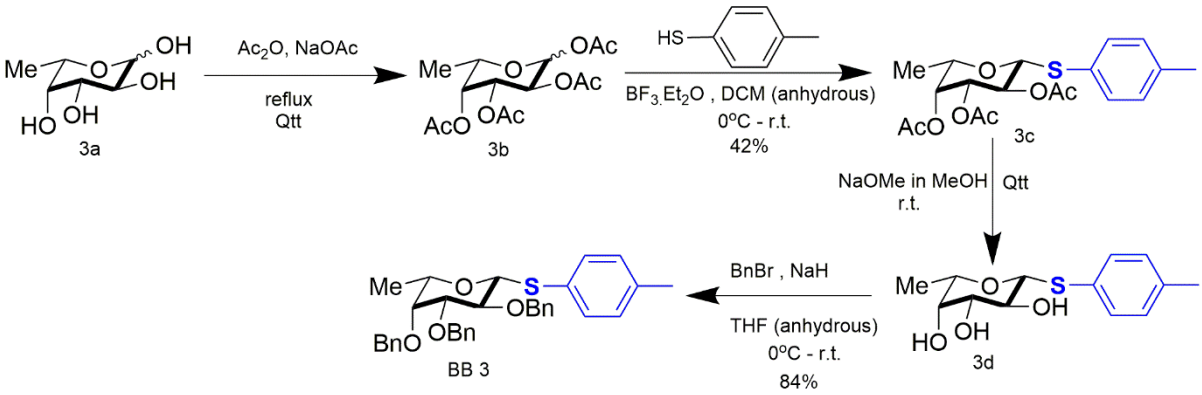
BB 2



The synthesis of this building block commenced from the common intermediate **2a**, synthesized in bulk quantities in-house, and the 3-OH was selectively modified as a silyl ether **2b**, followed by the introduction of a benzoyl ester at C2 position **2c**, in order to serve as a participating protecting group, thereby favouring the formation of the β-glycosidic linkage. Thereafter, the benzylidene ring was regioselectively opened to afford a free hydroxyl at C6 position **2d**, which was then modified as a benzyl ether (2e). Subsequently, the silyl group was selectively cleaved (2f), which was then modified as an FMOC carbonate to obtain the target building block (2g). Scale up synthesis to 40g batch afforded 8g of the final Gal BB.

3.3.3 Fucose building block

BB 3



The building block synthesis started from commercially available L-fucose **3a**, which was then converted into the peracetylated derivative **3b**. Thereafter, the p-toluyl thiol moiety was used to convert it into a thioglycoside **3c**. The acetate groups were subsequently removed (3d), followed by perbenzylation to afford the final building block. The final building block was obtained in 9g upon scale-up synthesis starting with 40g **3a**.

3.4 Assembly of building blocks to obtain target structure

With multi-gram quantities of BBs in hand, efforts were turned towards assembling them on the Glyconeer, and establish optimized glycosylation conditions for each of them in order to obtain maximum purity of the final oligosaccharide. The first step towards achieving this was to develop a workflow on the synthesizer to tether the building blocks one-by-one on to the solid phase depending on the linkage and the compatibility of the temporary protecting groups, the synthetic modules on the Glyconeer (see Chapter 1 for more details) were programmed accordingly (Table 3a, 3b). Therefore, for the Lewis^x epitope structure, the synthesis was started by first attaching the GlcNAc Building Block to the linker loaded resin, followed by cleaving the levulinoyl ester protecting group. Any deletion sequences were then capped,[12] and the fucose moiety was glycosylated to the C3-OH position of GlcNAc. Thereafter, the FMOC protecting group was removed from the C4-OH of GlcNAc, and the galactose unit was attached, followed by capping of deletion sequences, and finally removing the FMOC protecting group from galactose to obtain the final Lewis^x trisaccharide.

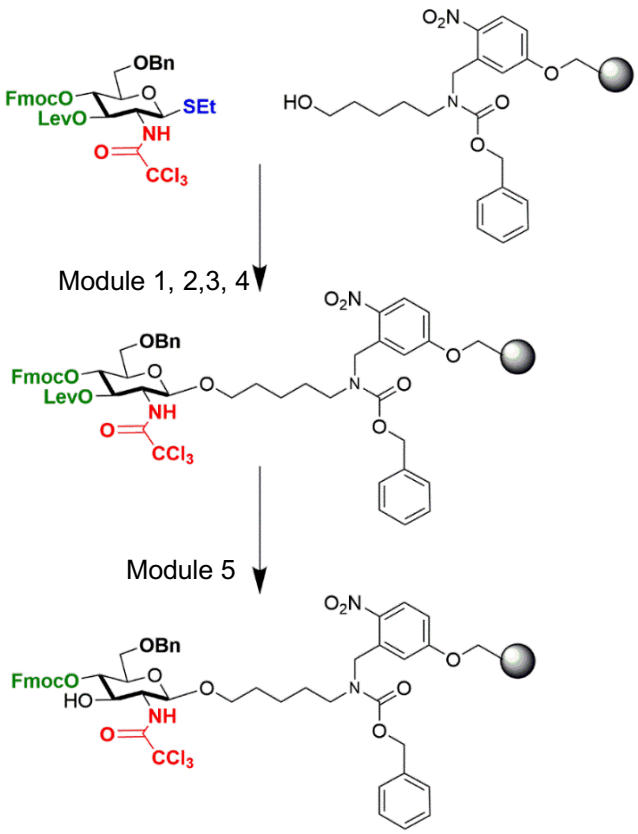


Table 3a: Synthetic modules required to synthesize Lewis^x epitope on the Glyconeer

S.No.	Module	Conditions
1.	Resin preparation for synthesis	DCM, 25°C , 30 min
2.	Acid Wash	TMSOTf in DCM (anhydrous), -20°C
3.	Thioglycoside Glycosylation Activator – NIS/TfOH in DCM (anhydrous) :Dioxane (anhydrous) 2:1	GlcNAc-20°C (5 min) , 0° C (20 min)
4.	Ac_2O acidic capping, preceded by Pyridine wash	10% Ac_2O , 2% Methanesulfonic acid in DCM
5.	Lev Deprotection	N_2H_4.HOAc in Py/AcOH/H_2O 4:1:0.5

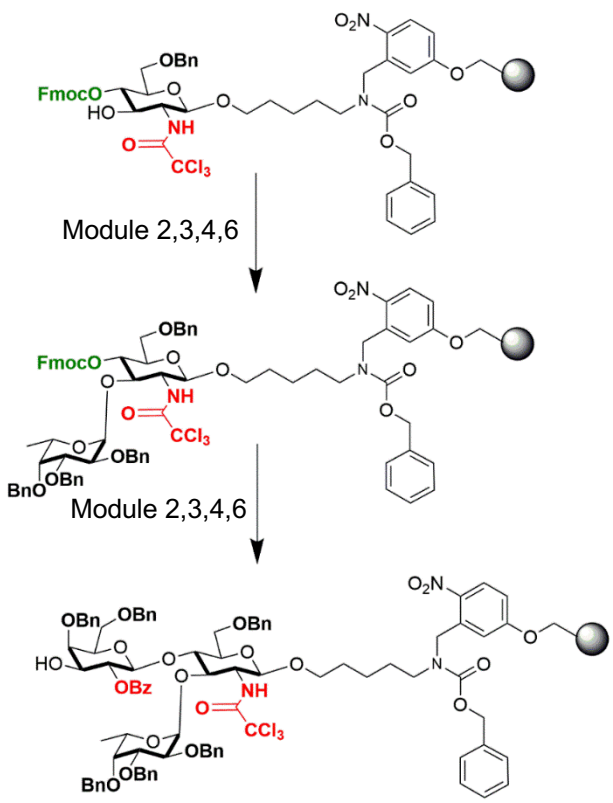


Table 3b: Description of synthetic modules on the Glyconeer

S.No.	Module	Conditions
2.	Acid Wash	TMSOTf in DCM (anhydrous) , -20°C
3.	Thioglycoside Glycosylation Activator – NIS/TfOH in DCM (anhydrous) : Dioxane (anhydrous)	Fuc in DCM (anhydrous): -40°C (5 min) , -20° C (20 min), Gal in DCM (anhydrous): -20°C (5 min) , 0°C (20 min)
4.	Ac_2O acidic capping	10% Ac_2O , 2% Methanesulfonic acid in DCM (anhydrous) (v/v), 25°C
6.	FMOC Deprotection	20% Piperidine in DMF (v/v), 25°C

Post-automation steps

Cleavage from solid support

After automated synthesis, the oligosaccharides were cleaved from the solid support using a continuous flow photoreactor.[12] (See SI for detailed procedure)

Purification and HPLC analysis

The crudes were dissolved in 1:1 Hex:EA and analysed using Analytical HPLC (YMC-Diol-300 column, 150 X 4.6 mm, ELSD Detector and DAAD, 280 nm). The product was then isolated using Preparative HPLC. The detailed solvent gradients used for analysis as well as purification of the Lewis^x epitope is shown in Table 3c.

Method – (Stop Time – 60.0 mins)

Table 3c: Description of solvent gradient used to analyse crude samples by normal phase analytical HPLC

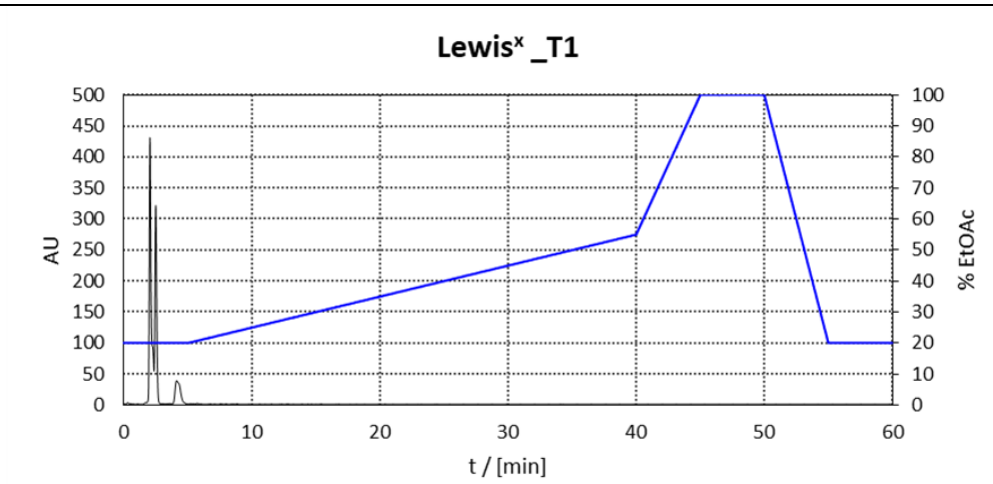
Time (min)	%Ethyl acetate	%Hexane	Flow rate (mL/min)
0.00	20	80	1.000
5.00	20	80	1.000
40.00	55	45	1.000
45.00	100	0	1.000
50.00	100	0	1.000

3.5 Establishing optimized glycosylation conditions

Glycosylation reactions usually follow an SN_1 type mechanism, wherein the glycosyl donor upon activation by a promotor/activator forms an oxocarbenium ion intermediate. The nucleophile, in this case, the glycosyl acceptor then attacks to form the glycosidic linkage.[4] Therefore, the glycosylation efficiencies are governed by the concentration of the glycosyl donor, in this case, the BBs. Thus, in order to obtain optimized conditions of glycosylation, these parameters were studied, and their impact on the yield and the purity of the reaction was thereby ascertained.

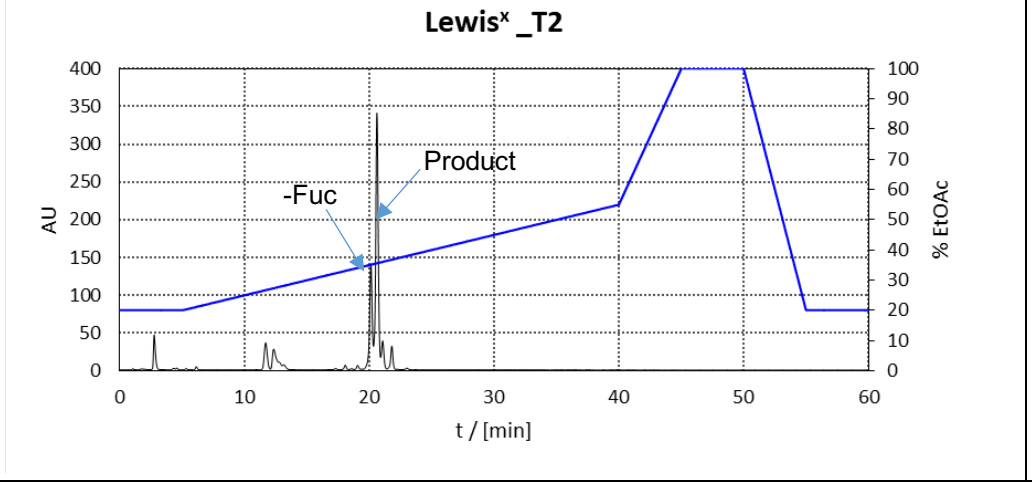
Number of Glycosylation Cycles – The glycosylation efficiencies of building blocks for each coupling cycle are dependent on the amount of BB added. Therefore, the number of glycosylation cycles, or the number of times a BB is added per coupling cycle, were varied to obtain the most optimized value to achieve maximum glycosylation efficiency. The standard conditions reported by Delbianco[7] (6.5 equivalents of BB) were implemented for the BBs 1-3, and the number of glycosylation cycles were further optimized depending on the results obtained.

Table 3d: Establishing optimized glycosylation conditions for BBs 1-3

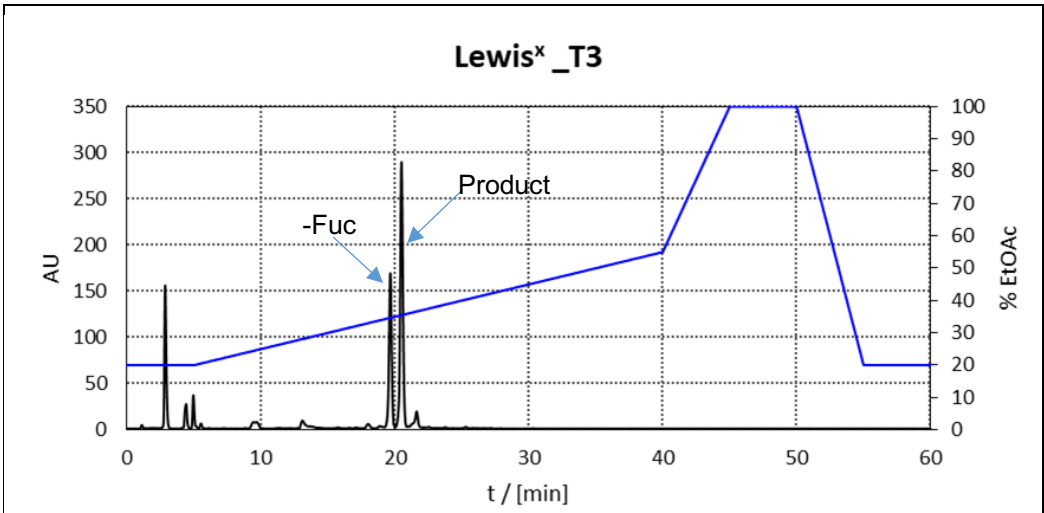
Final Analysis (HPLC Trace)	% Yield (Isolated)
	No Product

Glycosylation Conditions: GlcNAc: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 1x, Fuc: -40 °C (5 min) to -20 °C (20 min), 6.5 eq, 1x, Gal: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 1x

Table 3e: Optimizing glycosylation conditions by varying the number of glycosylation cycles for BBs 1-3

	21%

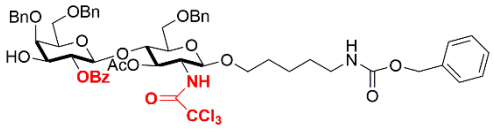
Glycosylation Conditions: GlcNAc: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 3x, Fuc: -40 °C (5 min) to -20 °C (20 min), 6.5 eq, 2x, Gal: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x



22%

Glycosylation Conditions: GlcNAc: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x, Fuc: -40 °C (5 min) to -20 °C (20 min), 6.5 eq, 2x, Gal: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x

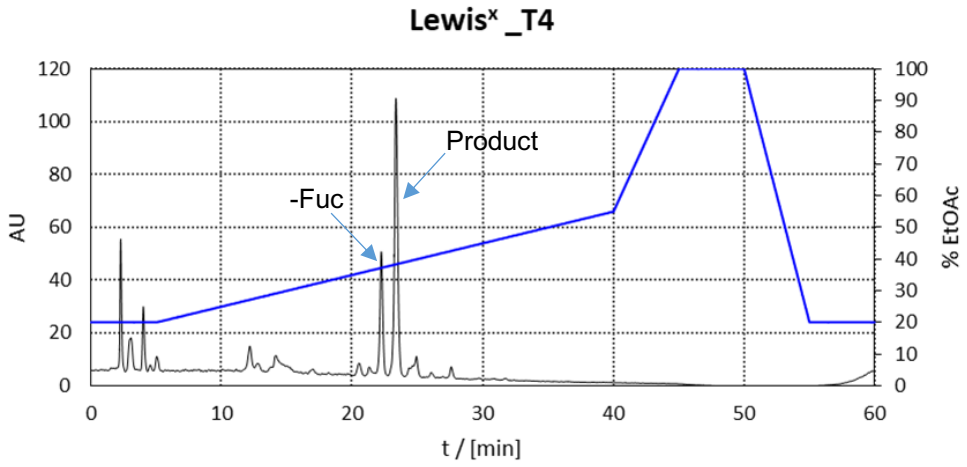
Structure of LacNAc deletion sequence (-Fuc):



When single glycosylation cycle was used for all the three building blocks, there was No product formation observed in the first trial (Lewis^x_T1) (Tables 3d and 3e). Adding the GlcNAc, Gal and Fuc BBs once was not sufficient to perform a successful synthesis. Taking inspiration from solid phase peptide chemistry where the first building block coupling is the most difficult glycosylation step, the number of coupling cycles were increased to three for GlcNAc, and two for Gal as well as Fuc BBs. However, the second trial (Lewis^x_T2) indicated the formation of a by-product, which was identified as LacNAc (structure shown above), in addition to the product. This trial also confirmed that the GlcNAc coupling was efficient, which was confirmed by the absence of capped linker. Hence, the number of cycles for this BB were then reduced to two, in a bid to use the GlcNAc BB judiciously, and thereby reduce the cost, while maintaining the same number of cycles for Fuc and Gal. HPLC analysis of the 3rd trial (Lewis^x _T3) indicated formation of product, and an identical deletion sequence as seen in the second trial. The formation of the LacNAc deletion sequence in both the

second and third trials indicated inefficient fucosylation, thereby prompting the need to further optimize the coupling efficiency of the Fuc BB, in order to remove the LacNAc deletion sequence.

Table 3f: Optimizing the coupling efficiency of Fuc BB by varying the incubation time during glycosylation

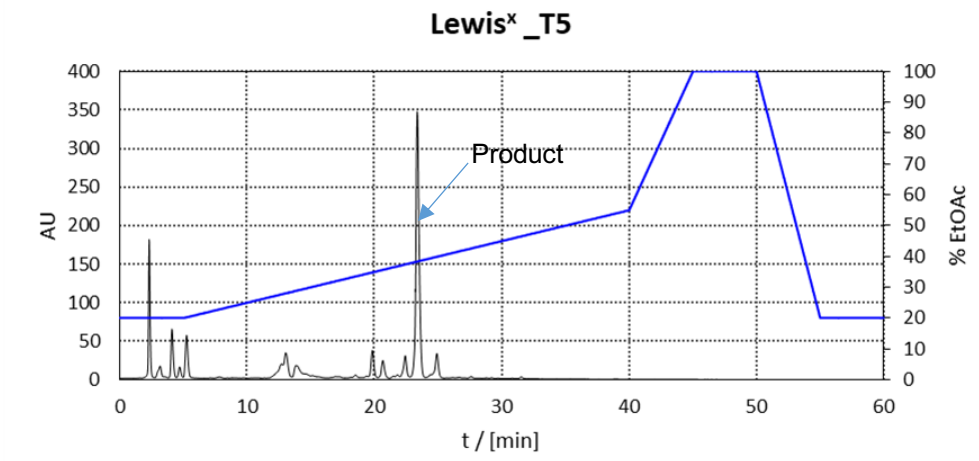
Final Analysis (HPLC Trace)	**% Yield (Isolated)**
	25%

Glycosylation Conditions: GlcNAc: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x, Fuc: -40 °C (5 min) to -20 °C (30 min), 6.5 eq, 2x, Gal: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x

In order to improve the coupling efficiency of the Fuc BB, the incubation time during glycosylation was increased to 30 min. However, the above trial (Table 3f) indicated that the increase in reaction time had no effect on the coupling efficiency of the BB, thereby leading to the formation of the LacNAc by-product. However, on the flip-side, the HPLC trace also confirmed that the coupling conditions for GlcNAc and Gal, i.e. 6.5 eq of BB with two glycosylation cycles were the best conditions to obtain high coupling efficiencies for these particular building blocks as confirmed by the absence of deletion sequence lacking the GlcNac BB (capped linker) or the Gal BB (capped GlcNAc). The coupling conditions for the Fuc BB required further optimizations in order to get rid of the deletion sequence lacking Fuc.

Building block concentration –The Fuc BB was found to be glycosylating inefficiently using 6.5 equivalents and two glycosylation cycles. Therefore, the concentration of the Fuc BB was increased to 10 eq and just one glycosylation cycle was used.

Table 3g: Optimizing coupling efficiency of Fuc BB by increasing the concentration of Fuc BB in the Glyconeer

Final Analysis (HPLC Trace)	**% Yield (Isolated)**
	35%

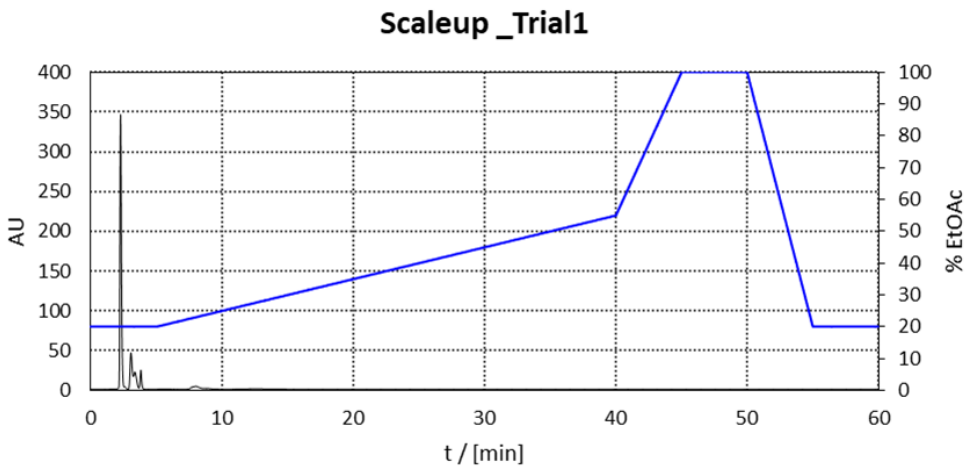
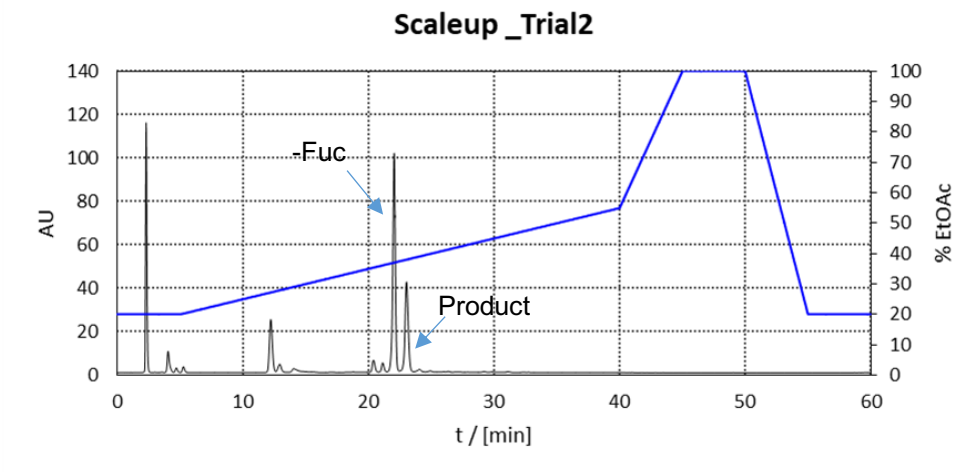
Glycosylation Conditions: GlcNAc: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x, Fuc: -40 °C (5 mins) to -20 °C (20 min), 10 eq, 1x, Gal: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x

From the HPLC trace of the above trial, it was evident that increasing the concentration of Fuc BB improved the coupling efficiency, and thus, no deletion sequence, i.e. LacNAc was observed in the HPLC anaylsis. Therefore, it was concluded that 6.5eq, 2x, -20°C (5 min) to 0°C (20 min) for GlcNAc and Gal BB, and 10 eq, 1x, -40°C (5 min) to -20°C (20 min) for Fuc were the optimal glycosylation conditions for these building blocks.

3.5.1 Scale-up of the Lewis^x epitope synthesis

With optimized glycosylation conditions for BBs **1-3** on the 0.0125 mmol scale in hand, the AGA of Lewis^x was scaled up to 0.025 mmol to obtain multi-milligram quantities of the final oligosaccharide, and to test the scalability of the established protocols and the results obtained were as follows:

Table 3h: Applying the optimized protocols developed on the 0.0125 mmol scale for scale up synthesis of Lewis^x epitope

Final Analysis (HPLC Trace)	%Yield (Isolated)
	No Product
	12%

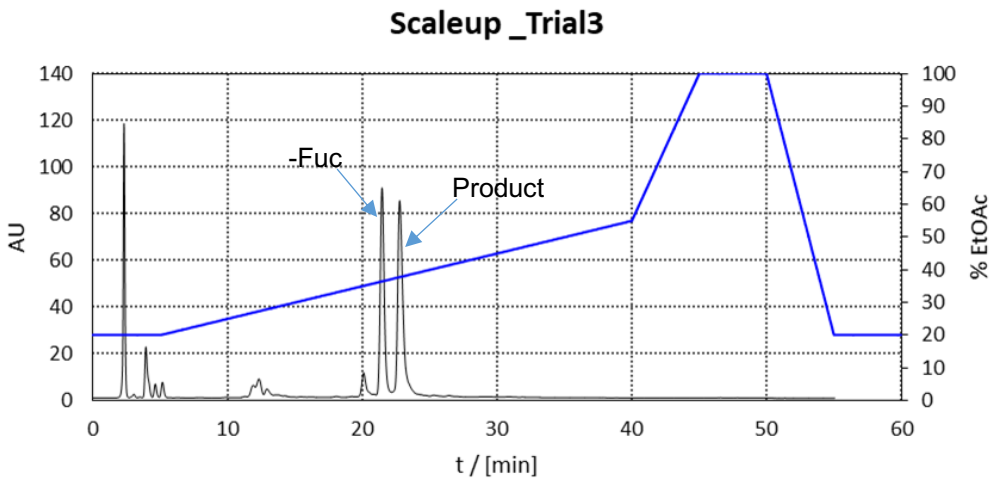
Glycosylation Conditions: GlcNAc: -20 °C (5 min) to 0 °C (20min), 6.5 eq, 2x, Fuc: -40 °C (5 min) to -20 °C (20 min), 10 eq, 1x, Gal: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x

The trials (Table 3h), indicated that the fucoslyation was inefficient, which was confirmed by the presence of deletion sequence (LacNAc) in the HPLC traces for trials

1 and 2. Further optimization of the reaction conditions for the assembly of the Lewis^x epitope on the 0.025 mmol scale. Since the glycosylation conditions for the building blocks of the scale-up trials were identical to that on smaller scale (0.0125 mmol), the focus was then shifted towards other parameters.

Acid Wash – Since the first two scale-up trials indicated inefficient fucosylation, it was speculated that two acid washes per glycosylation cycle might have over-exposed the Fuc BB to the TMSOTf acid, thereby promoting its cleavage. Therefore, the number of acid washes were reduced to one.

Table 3i: Optimizing the coupling efficiency of Fuc BB on 0.025 mmol scale by reducing the number of acid washes to 1.

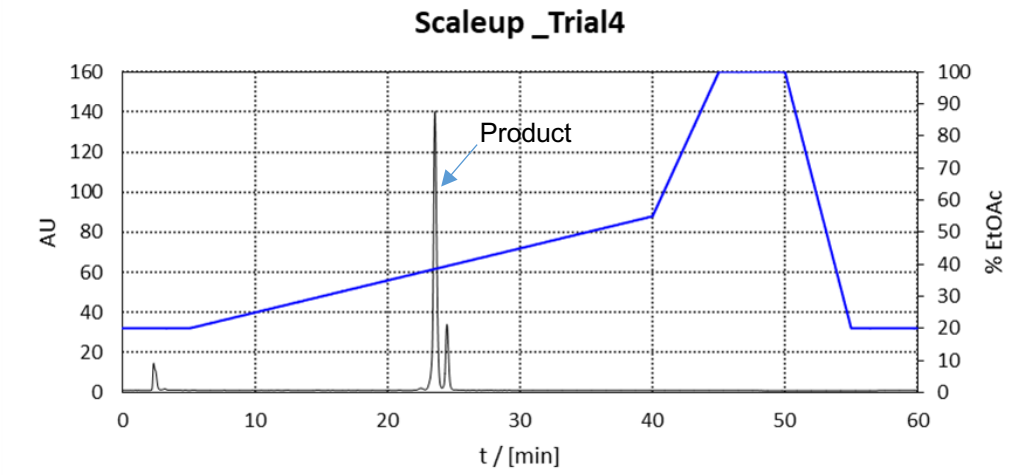
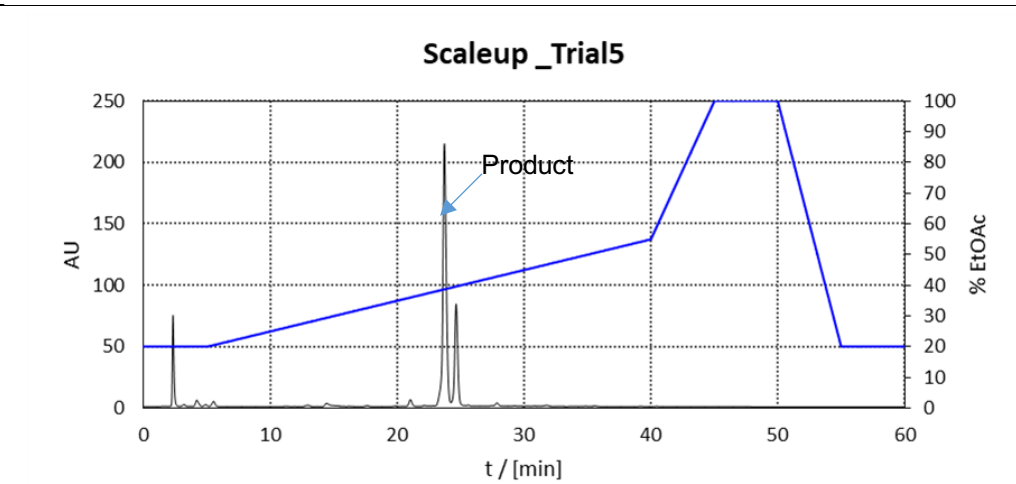
Final Analysis (HPLC Trace)	**% Yield (Isolated)**
	22%

Glycosylation Conditions: GlcNAc: -20 °C (5 min) to 0 °C (20min), 6.5 eq, 2x, Fuc: -40 °C (5 min) to -20 °C (20 min), 10 eq, 1x, Gal: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x

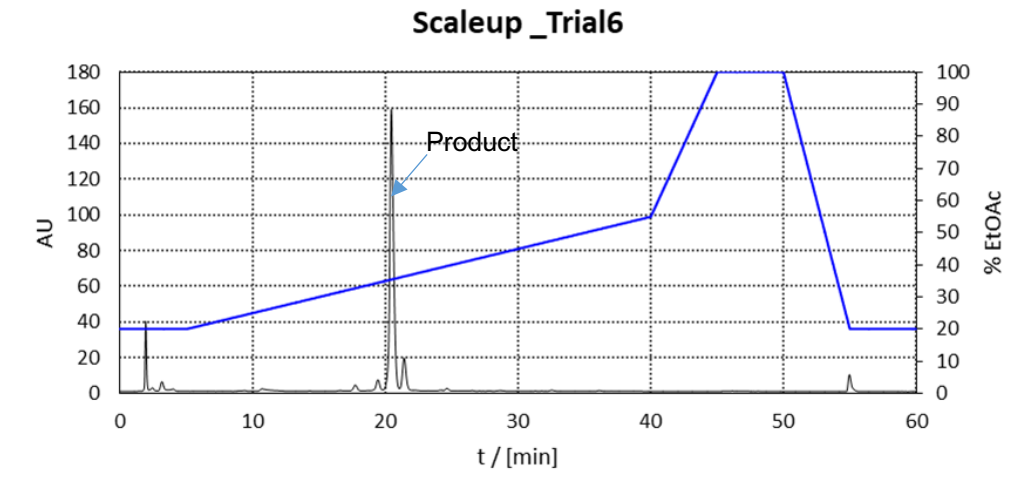
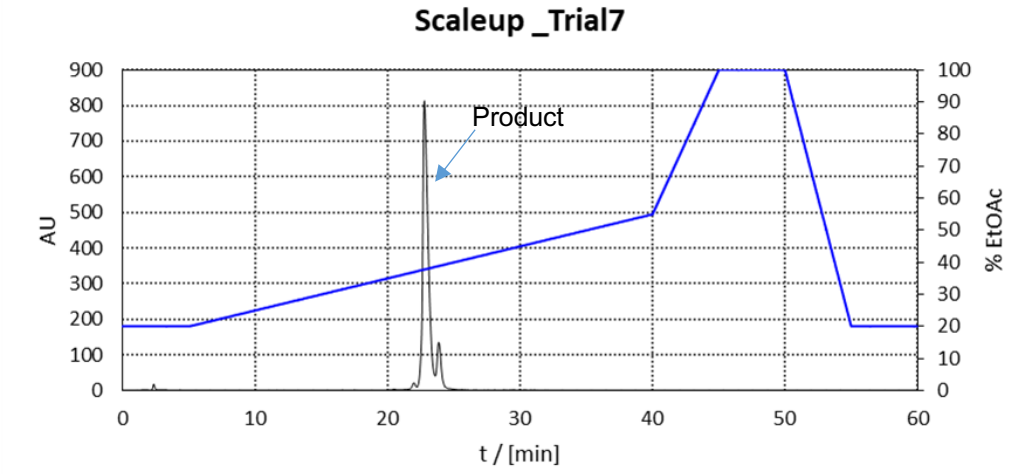
The HPLC trial (Table 3i), revealed that the reduced acid washes did not influence the outcome of the synthesis, as confirmed by the peak for LacNAc deletion sequence lacking Fuc. Now the focus was turned towards argon mixing.

Argon mixing – Mixing is known to play a fundamental role in synthesis scale up. It ensures homogeneous dispersion between multiple phases. Additionally, it also minimizes concentration/temperature gradient between two immiscible phases, thus improving mass transfer and increasing the rate of reaction. Therefore, it was speculated that increasing the argon mixing duration could be beneficial in improving the glycosylation efficiency of the Fuc BB on the 0.025 mmol scale. Thus, for the following scale up trials, the argon mixing was increased from 1s to 3s.

Table 3j: Optimizing the coupling efficiency of Fuc BB on the 0.025 mmol scale by increasing argon mixing

Final Analysis (HPLC Trace)	**% Yield (Isolated)**
	27%
	30%

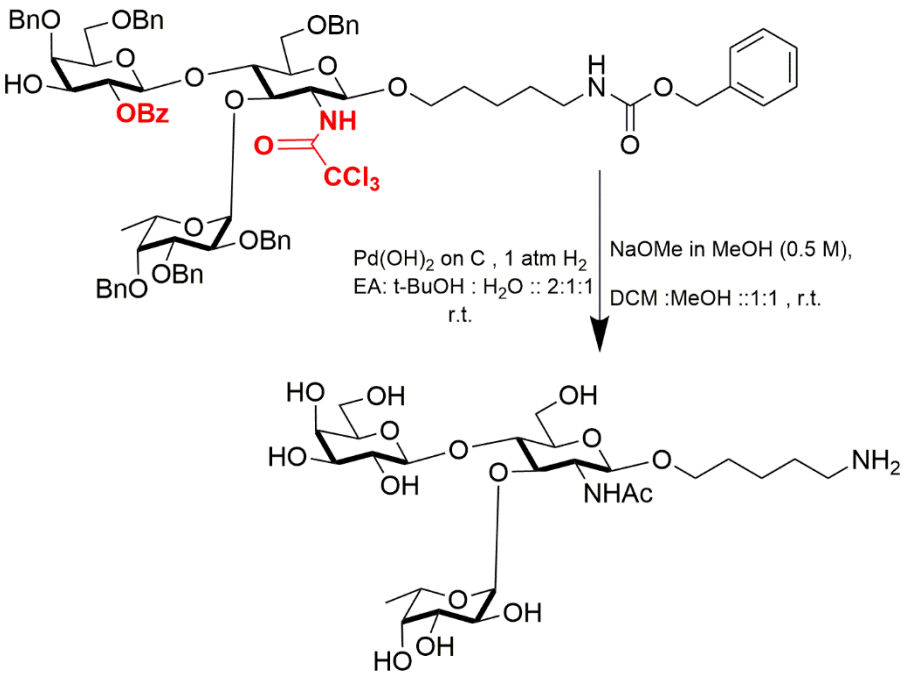
Glycosylation Conditions: GlcNAc: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x, Fuc: -40 °C (5 min) to -20 °C (20 min), 10 eq, 1x, Gal: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x

	56%
	52%

Glycosylation Conditions: GlcNAc: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x, Fuc: -40 °C (5 min) to -20 °C (20 min), 10 eq, 1x, Gal: -20 °C (5 min) to 0 °C (20 min), 6.5 eq, 2x

The HPLC trials above carried out with increased Argon mixing indicated no formation of LacNAc by-product, and thus, resulted in significantly improved yield and purity of the Lewis^x epitope. Increased argon mixing showed that inefficient mixing was leading to the formation of LacNAc deletion sequence. Thereafter, with multi-milligram quantities of the protected Lewis^x epitope structure in hand, efforts were then turned towards obtaining the deprotected trisaccharide.

3.6 Global deprotection of Lewis^x



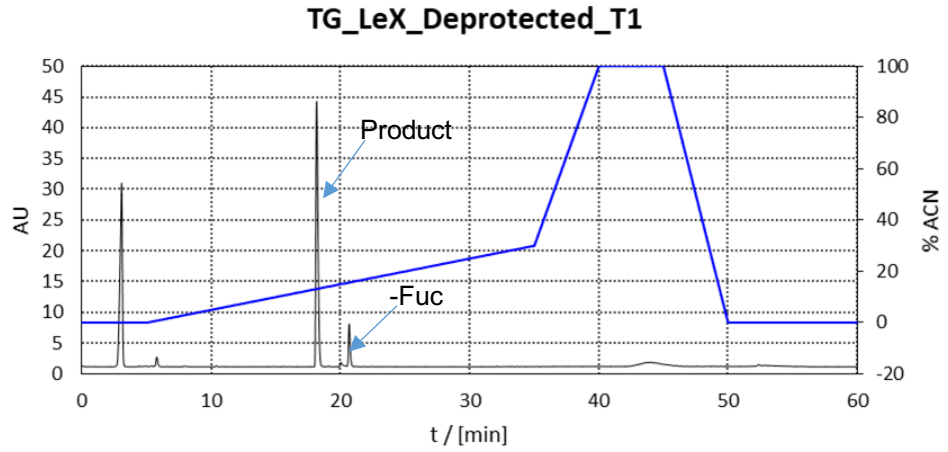
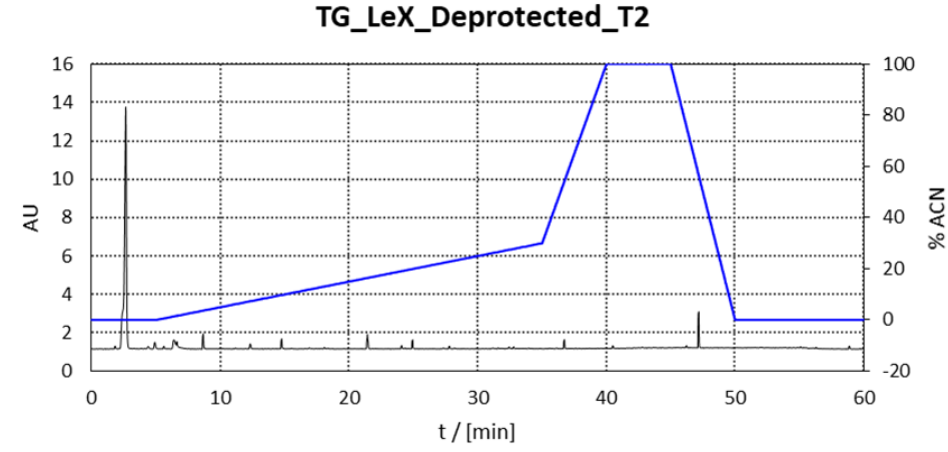
In order to obtain the fully deprotected Lewis^x epitope structure, the temporary protecting group, namely, -OBz protecting group was first removed via methanolysis. Thereafter, the permanent protecting group, i.e. –OBn functional group was cleaved by hydrogenation using Palladium catalyst under a H_2 atmsophere.[15] Additionally, the global deprotection also enabled the removal of the trichloroacetamide group (-NHTCA), as well as the Cbz group thereby yielding an amine spacer at the reducing end of the fully deprotected Lewis^x epitope structure.

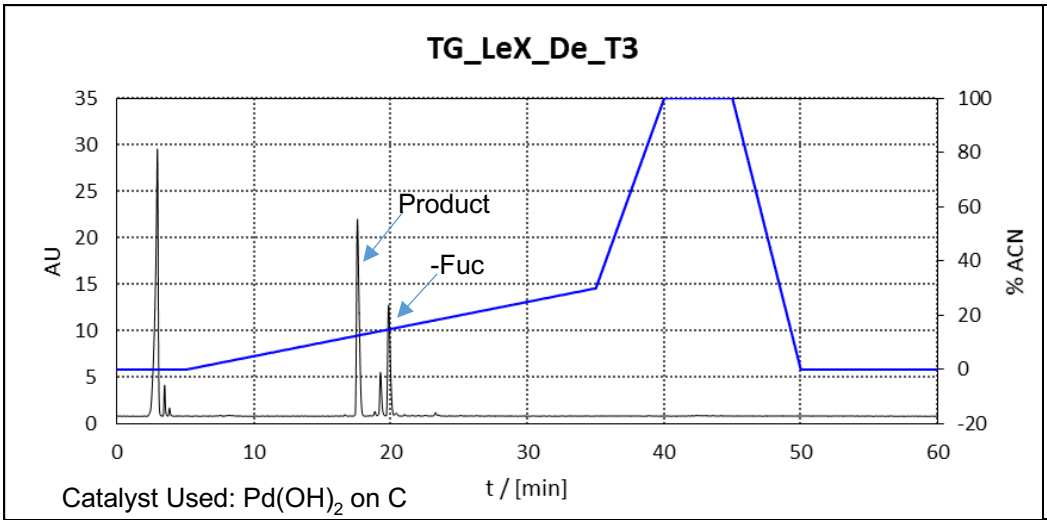
3.6.1 Establishing optimized conditions for global deprotection of Lewis^x

Different Palladium catalysts have been reported regarding the global deprotection of oligosaccharides. Some studies use only one type of Palladium catalyst, while others use a mixture of catalysts such as $Pd(OH)_2$ on C and Pd on C.[15,17] Optimization trials were conducted using different catalysts in order to select the best choice to procure a satisfactory yield of the final product.

Secondly, the work-up procedure, or the washing procedure of the Palladium catalyst after hydrogenation was also taken into consideration as an important parameter for obtaining high yields of the final deprotected glycan. Ineffective washing of the catalyst might lead to low yields of the final compound, owing to the compound getting trapped in the catalyst. With these factors in mind, optimization trials were carried out with different Pd catalysts, as well as rigorous work-up procedures.

Table 3k: Establishing Optimized conditions for Global deprotection of Lewis^x by varying the choice of catalyst

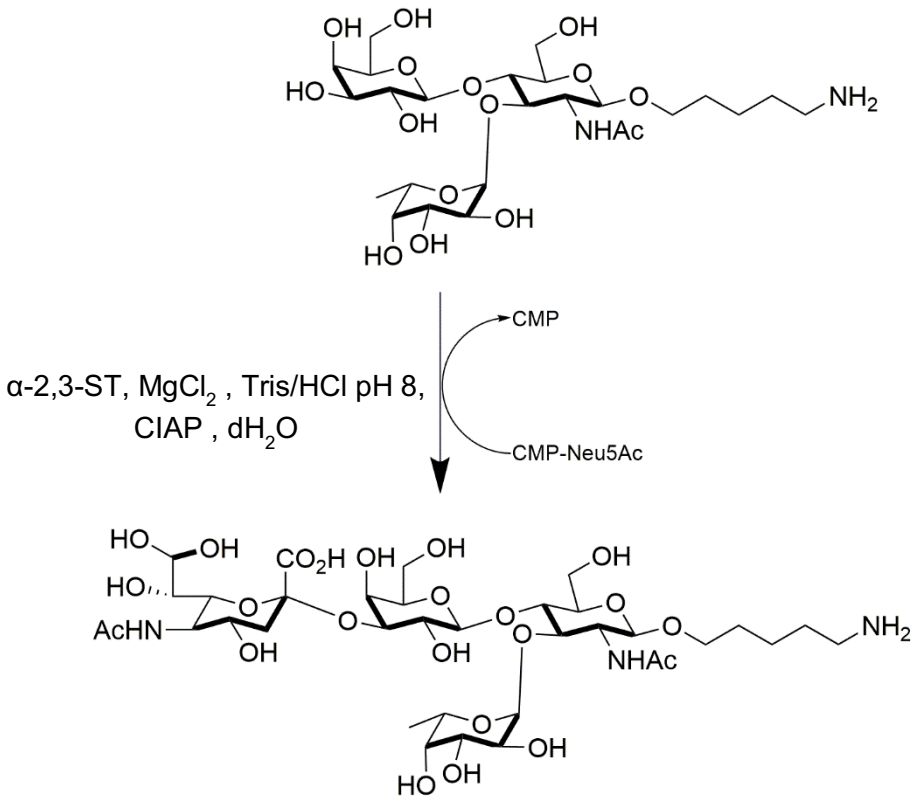
Final analysis (HPLC Trace)	**%Yield**
 Catalyst Used: $Pd(OH)_2$ on C	13%
 Catalyst Used: $Pd(OH)_2$ on C and Pd on C	No Product



39%
(Crude)

From the HPLC trials above, it is evident that while near complete conversion into deprotected Lewis^x could be seen with $Pd(OH)_2$ on C, a combination of $Pd(OH)_2$ on C and Pd on C did not yield any product. This proved beyond doubt that $Pd(OH)_2$ on C is the most optimal catalyst for the global deprotection of Lewis^x. It can be speculated that the rigorous washing procedures of the catalyst used in the first(TG_LeX_Deprotected_T1) and thrid (TG_LeX_Deprotected_T3) trials led to higher purity of the reaction as indicated by the HPLC traces.

3.7 Enzymatic sialylation of Lewis^x



The last step towards obtaining the Sialyl Lewis^x target structure was the attachment of sialic acid moiety to the Lewis^x trisaccharide via enzymatic sialylation.

The biosynthetic pathway to synthesize Sialyl Lewis^x comprises the attachment of Sialic acid to LacNAc, followed by fucosylation by an α-1,3 fucosyltransferase. This sequence is highly conserved since common human α-2,3 sialyltransferases cannot act on fucose containing Lewis^x as a substrate (Patent no. US 9,255,257 B2). However, a mutant α-2,3 sialyltransferase from Pasteurella multicoda was reported by Chen and co-workers[33] which could transfer sialic acid to the fucosylated Lewis^x epitope structure. They reported a significant advantage of adding sialic acid as the last synthetic step over normal sialyl Lewis^x biosynthetic pathway, since it reduced the number of synthetic steps thereby simplifying the reaction scheme. The purification process was much easier as the negatively charged Sialyl Lewis^x from the neutral Lewis^x compared to separating both negatively charged Sialyl Lewis^x and non-fucosylated sialosides. Therefore, drawing inspiration from the findings of Chen,[33] the Lewis^x epitope synthesized via automated glycan assembly was subjected to enzymatic sialylation using bacterial α-2,3 sialyltransferases in collaboration with Prof.

Sabine Flitsch's group at the University of Manchester. Six bacterial α-2,3 sialyltransferases along with five putative bacterial enzymes were screened, and the reaction mixtures were analysed by MALDI.

Table 3l: Bacterial α-2,3 sialyltransferases screened for enzymatic sialylation of Lewis^x epitope structure

α-2,3-sialyltransferase	Bacteria
Tt_86	Pasteurella multicoda
Tt_88	Photobacterium phosphoreum
Tt_89	Pasteurella dagmatis
Tt_190	Pasteurella dagmatis
Tt_192	Photobacterium sp. JT-ISH-224
Tt _193	Vibrio sp. JT-FAJ-1

Table 3m: Putative α-2,3 sialyltransferases screened for enzymatic sialylation of Lewis^x epitope structure

Enzyme	Bacteria
Tt_52	Campylobacter insulaenigrae
Tt_123	Neissera meningitides MC58
Tt_136	Haemophilus influenzae
Tt_159	Streptococcus mitis
Tt_127	Neisseria gonorrhoeae

Procedure – The bacterial α-2,3 sialyltransferases were procured commercially from Prozyme as a suspension in NH_4SO_4, and stored at -80^{o}C. The suspension was centrifuged and the supernatant was discarded. The pellet was then dissolved in 20 µL distilled H_2O. The concentration was then roughly estimated by Nanodrop. The stock solutions and reaction mixture were prepared as follows: 2 µL of Tris/HCl buffer, 4 µL of CMP-Neu5Ac, 1 µL CIAP and 7 µL distilled H_2O were added into an Eppendorf tip, and aliquoted into 6 vials, with 2 µL Le^x (acceptor) and 2 µL of the silayltransferase. The reaction mixtures were then thawed, and incubated overnight at 37^{o}C, and then analysed using MALDI.

The initial screenings were performed using the bacterial α-2,3 sialyltransferases (as described in Table 3l, 3m), and the MALDI data is as follows:

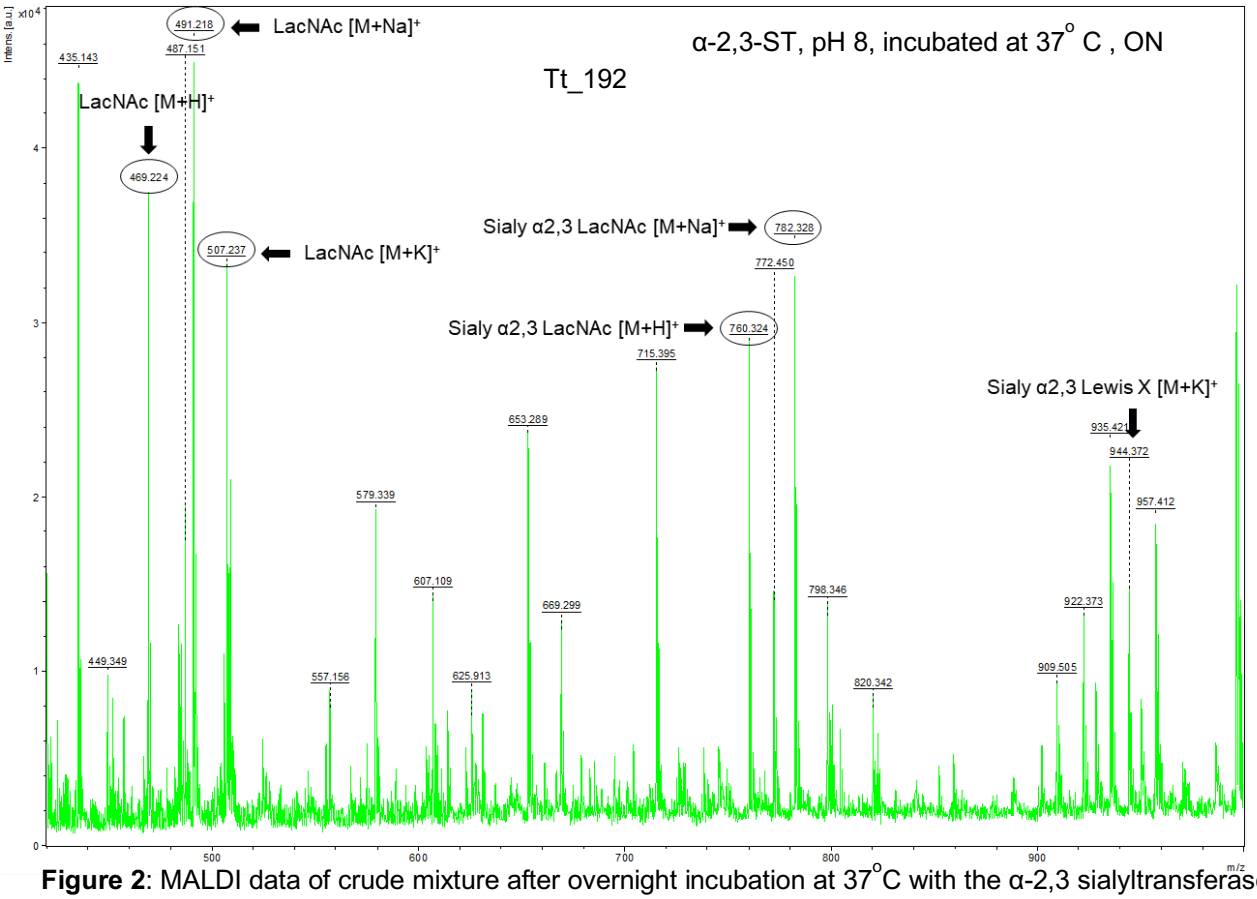


Figure 2: MALDI data of crude mixture after overnight incubation at 37°C with the α-2,3 sialyltransferase isolated from Photobacterium sp. JT-ISH-224 (Tt_192)

The MALDI trace shown above indicates that the attachment of Sialic acid moiety to the Lewis^x epitope structure could be accomplished using an α-2,3 sialyltransferase from Photobacterium sp. JT-ISH-224. The optimization of the enzymatic silalylation is currently underway.

3.8 Conclusion and Outlook

The synthesis of building blocks (BBs **1-4**) required for automated glycan assembly of Lewis^x epitope was optimized, and the monosaccharides were procured in multi-gram quantities. A noteworthy improvement made in the synthesis of building blocks was the minimal use of column chromatography, thereby reducing the time needed to access the final building block as well as solvents used for 40g batches. A workflow was established on the Glyconeer synthesizer to assemble these building blocks to obtain the Lewis^x epitope.

Next, inefficient fucosylation encountered during the AGA of the target structure, was overcome by increasing the concentration of the Fuc BB from 6.5 to 10 equivalents. Subsequently, a similar problem (inefficient fucosylation) was seen while scaling up the synthesis to 0.025 mmol scale, which could be overcome by increasing the time for argon mixing. Next, in order to obtain the fully deprotected Lewis^x epitope structure, the global deprotection was optimized by identifying the most suitable Palladium catalyst ($Pd(OH)_2$ on C) that resulted in minimal cleavage of Fuc in the final structure.

Last but not the least, enzymatic sialylation was attempted on deprotected Lewis^x using bacterial α-2,3 silayltransferases. However, these enzymes were unable to transfer sialic acid to the fucosylated Lewis^x efficiently. Therefore, the mutated α-2,3 silayltransferase from Pasteurella multicoda can be used to perform enzymatic sialylation on Lewis^x epitopes.

Chapter 4

Automated glycan assembly of Lewis^a, Lewis^y and Lewis^b epitopes using the Glyconeer

4.1 Introduction

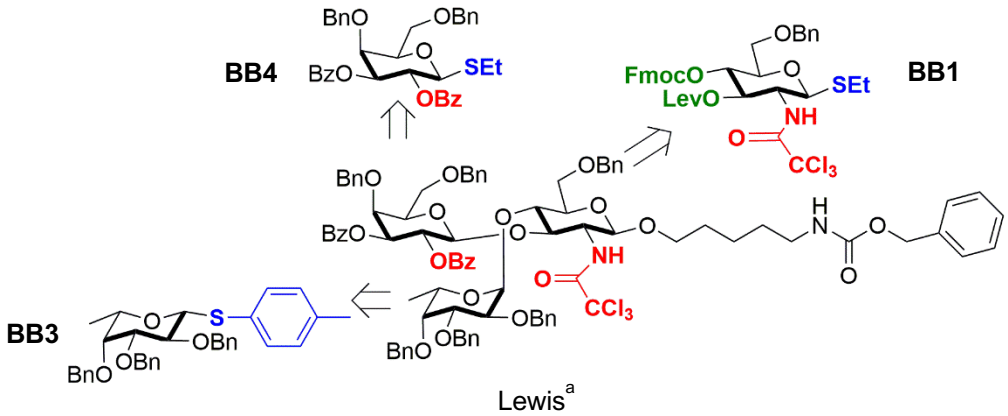
Lewis^a and Lewis^y are type I and type II blood group antigens that play an important role in tumor metastasis and signal transduction.[1] The overexpression of these glycans is a sign of tumor progression and can be used to distinguish normal cells from tumor cells. Therefore, these tumor associated carbohydrate antigens are considered promising targets for designing anti-cancer vaccines.[1] Lewis^b on the other hand is a receptor for the gram negative bacterium *Helicobacter pylori* that causes chronic gastritis and gastric carcinoma.[8] The biological relevance of these glycan structures have prompted several attempts to synthesize them by solution phase synthesis.[32,34]

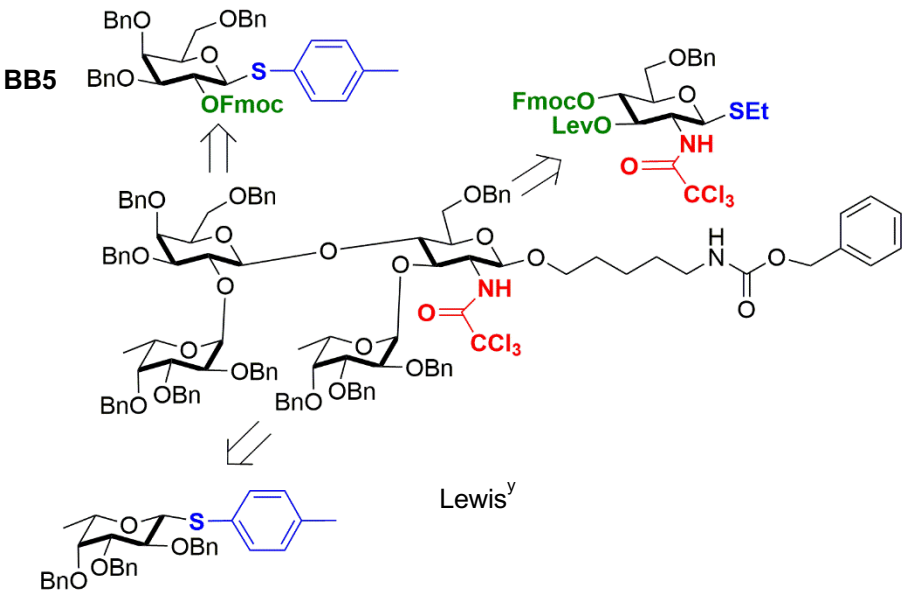
The optimization of automated glycan assembly of Lewis^x epitope using the Glyconeer (see Chapter 3 for more details) paved the way for extending these protocols in order to access the Lewis^a, Lewis^y and Lewis^b antigens by automation. The goal for synthesizing these molecules was not only to procure a repertoire of biologically relevant glycans, but also to prove the robustness of the synthetic protocols developed using the Glyconeer.

This chapter is divided into four sections. The first section discusses the identification of building blocks upon retrosynthetic analysis of the target glycans, followed by highlighting the importance of the benzylidene intermediate to get faster access to the final building block. The second section describes the workflow used to assemble the target structures using the Glyconeer. Thereafter, the third section details the automated glycan assembly of the Lewis^a epitope structure on the Glyconeer in order to consolidate the reproducibility of the established AGA protocols. Finally, the fourth section describes the special cases of Lewis^y and Lewis^b epitope structures, which proved to be challenging to assemble via automation on the Glyconeer synthesizer, and the strategy adopted to address this problem.

4.2 Retrosynthetic analysis of target structure and identification of building blocks

In continuation with the approach adopted for automated glycan assembly of Lewis^x using the Glyconeer synthesizer (described in Chapter 3), the building blocks required to assemble the Le^a, Le^y and Le^b epitope structures were identified upon retrosynthetic analysis as shown in Figure 3. The positions that required to be extended during automation were modified with temporary protecting groups, namely, levulinic ester (-Lev) and 9-fluorenylmethoxycarbonyl (Fmoc) carbonates. Secondly, in order to establish β-glycosidic linkage, participating protecting groups such as trichloroacetamide (TCA) for BB1 and benzoyl ester (OBz) for BB5 were installed in the C2 position. On the other hand, the Fuc BB was synthesized as a perbenzyl derivative to promote the formation of α-glycosidic linkage.





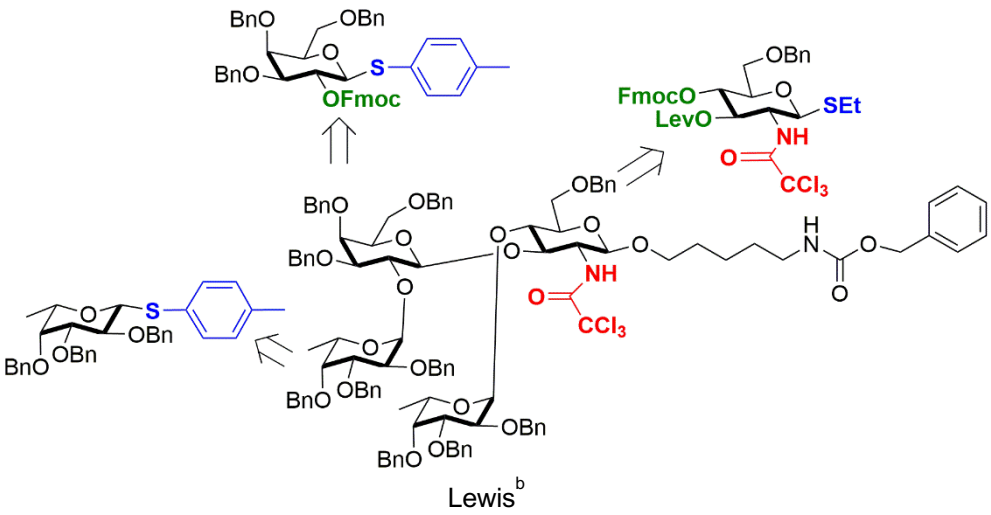
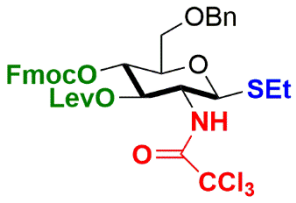


Figure 3: Retrosynthetic analysis of Lewis antigens and identification of building blocks

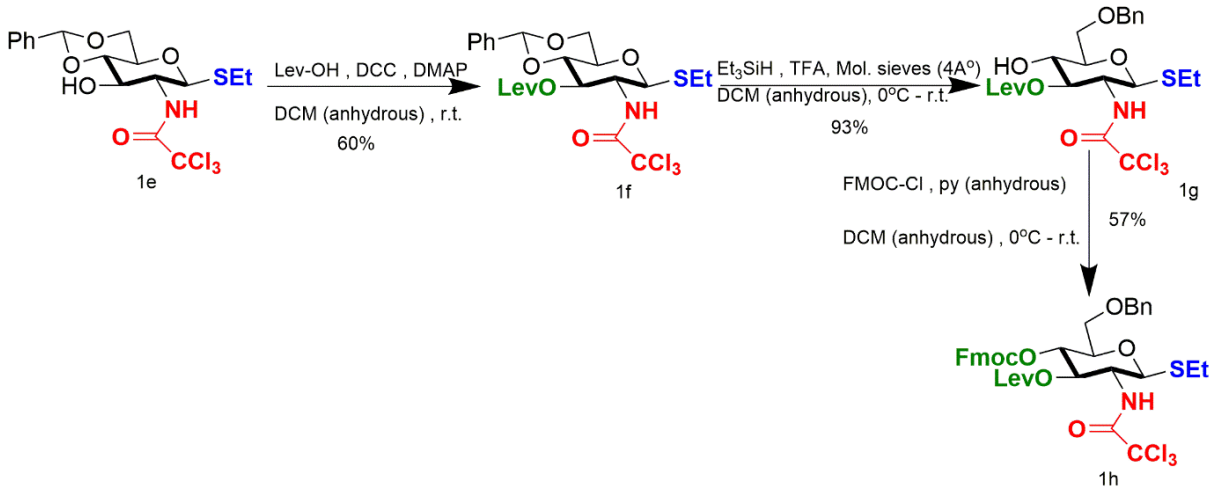
4.3 Utilizing the common intermediate to expedite the synthesis of building blocks

In the process of optimizing the synthesis of BB1 and BB4 for AGA of the Lewis^x epitope structure (see Chapter 3 for more details), the benzylidene derivatives **1e** and **2a** were identified as common intermediates which were synthesized in multi-gram quantities. This intermediate had two major advantages, firstly, it could be functionalized with a myriad of functional groups, thereby giving access to a plethora of differentially protected building blocks for automation. Secondly, it significantly reduced the number of synthetic steps to get quicker access to the final building block.

4.3.1 Glucosamine building block

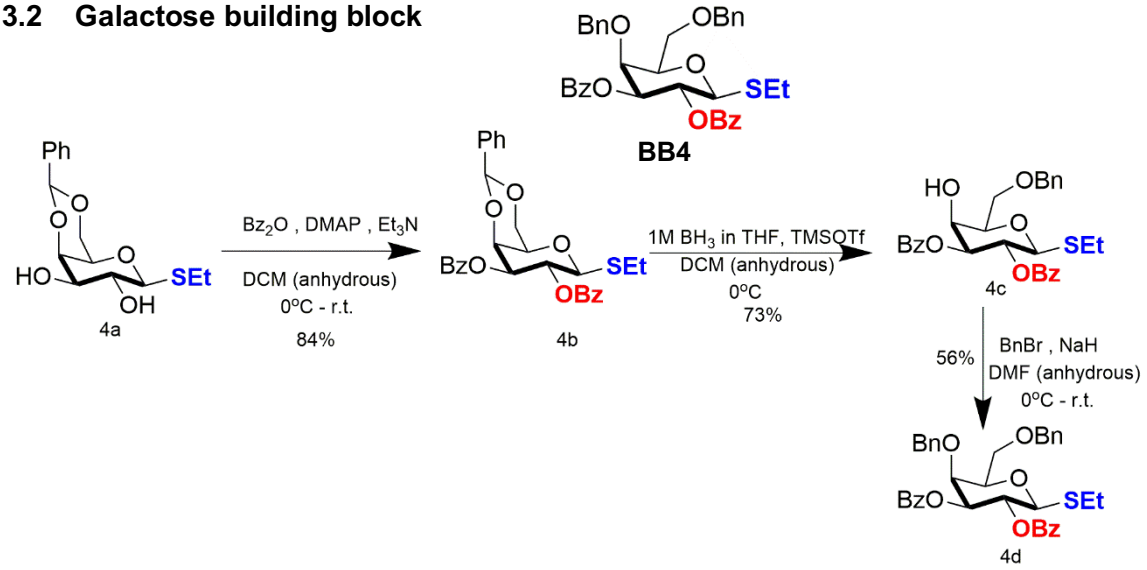


BB1



As discussed in Chapter 2, the first four steps of the reaction scheme such as, peracetylation, thioglycosylation, deacetylation and formation of benzylidene intermediate were optimized, and scaled up to a 40g batch. For e.g. in the case of GlcNAc BB above (BB 1), the final building block **1h** could be synthesized in 3 synthetic steps, in contrast to 6 steps when started from the intermediate **1a** (see Chapter 3).

4.3.2 Galactose building block



The synthesis of **BB4** commenced from the "common intermediate" **4a** (see Chapter 2 for more details). The C2 and C3 hydroxyl groups were then modified as benzoyl esters **4b**. Then, regioselective opening of the benzylidene intermediate **4c** was

performed to afford the C4 hydroxyl group free, which was finally derivatized as a benzyl ether **4d**.

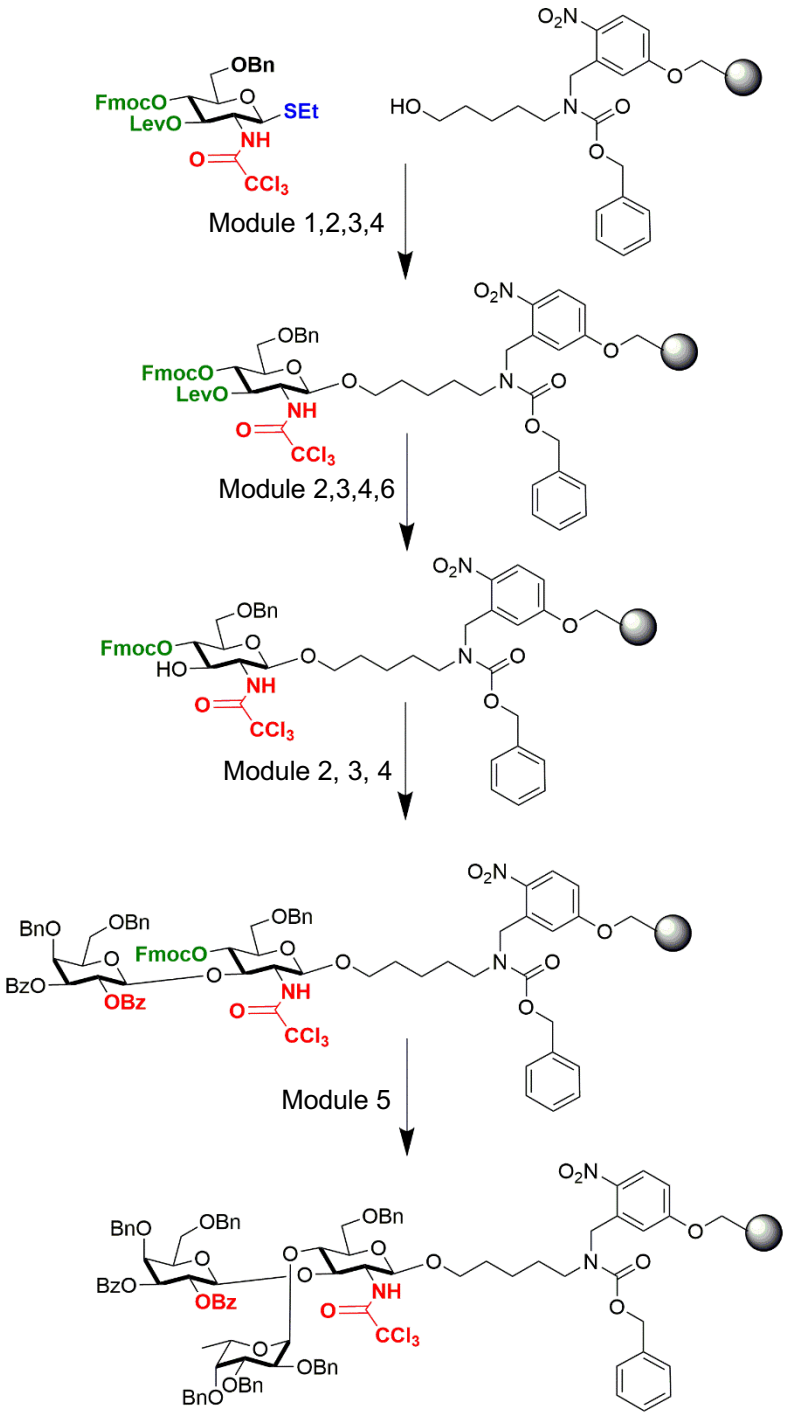
Table 4a: Synthetic modules required to synthesize the Le^a, Le^y and Le^b epitope structures using the Glyconeer. T_1 = Initial temperature, T_2 = Final Temperature

Module no.	Module name	Conditions
1.	Resin swelling	DCM, 25°C, 30 min
2.	Acid wash	TMSOTf in DCM(anhydrous), -20°C
3.	Thioglycoside Glycosylation	BB in DCM (anhydrous), T_1(5 min) to T_2 (20 min)
4.	Ac_2O acidic capping	10% Ac_2O, 2% Methanesulfonic acid in DCM(anhydrous)(v/v)
5.	Lev Deprotection	Hydrazine acetate in py, AcOH, H_2O, 25°C
6.	FMOC Deprotection	20% Piperidine in DMF(v/v), 25°C

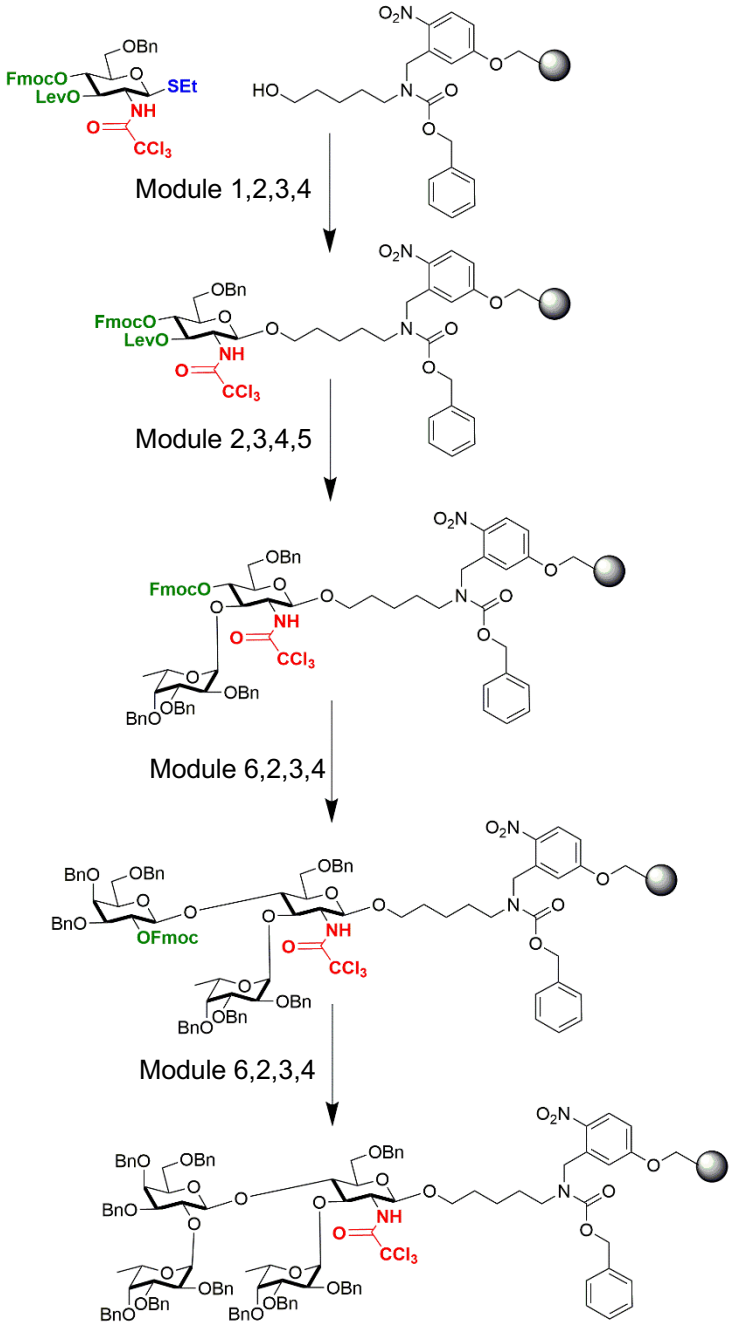
4.4 Assembly of building blocks to obtain target structure

With multi-gram quantities of building blocks in hand, the next step was to assemble them using the Glyconeer synthesizer to obtain the target oligosaccharides, which was accomplished by applying the optimized synthetic modules established during the synthesis of the $Lewis^x$ epitope structure. The application of these synthetic protocols to synthesize $Lewis^y$, $Lewis^a$ and $Lewis^b$ epitopes was also beneficial in determining the robustness as well as reproducibility of these protocols. For all automated glycan assemblies, Merrifield resin loaded with a photolabile linker was used as solid support.[9] A brief description of the synthetic modules used to assemble the Le^a, Le^y and Le^b epitope structures are shown in Table 4a.

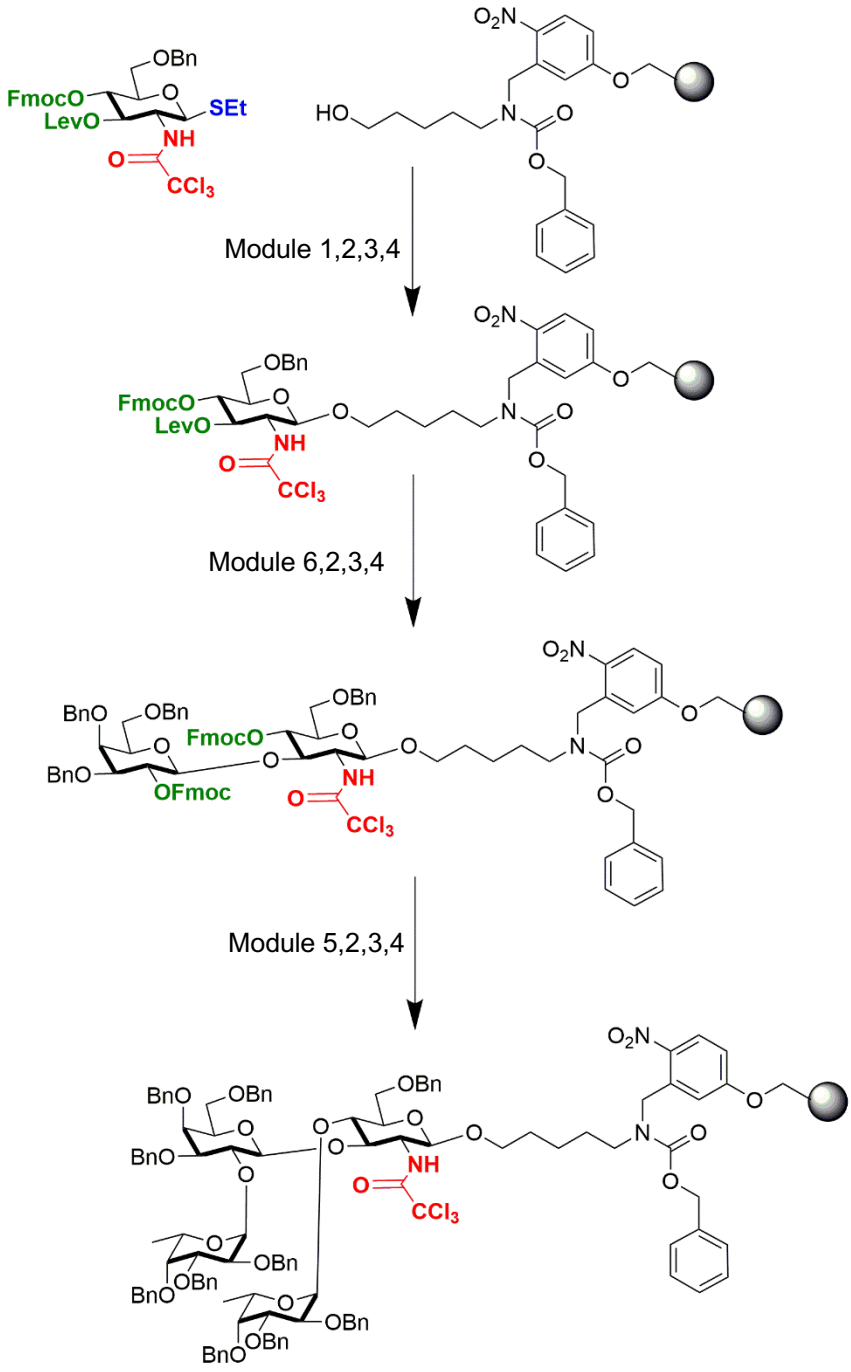
Le^a: The synthesis was initiated by glycosylating BB1 (Figure 3) to the solid support, followed by capping[7] the deletion sequences and cleaving the Levulinic ester protecting group. BB5 was then glycosylated to the C3 position, which was followed by removing the FMOC protecting group from C4 position of BB1. Finally, BB3 was glycosylated to this position and the deletion sequences were capped to afford the Le^a epitope structure.



Le^y: The first building block (BB1) was glycosylated to the solid support, and the deletion sequences were then capped. Then, the –Lev protecting group from C3 of BB1 was cleaved. Thereafter, BB3 was glycosylated to this position, the deletion sequences were capped, and the FMOC group was removed from the C4 position of BB1. In order to attach the second Fucose unit, a Gal BB bearing an FMOC protecting group at C2 position (BB5) was then glycosylated to the C4 position of BB1, followed by capping of the deletion sequences and removing the FMOC protecting group from C2 position of BB6. Finally, the second Fucose unit (BB3) was attached to this position to obtain the final Le^y epitope structure.



Le^b: The workflow used to assemble the Le^b epitope structure was similar to that of Le^y with the exception of cleaving the FMOC protecting groups from the C2 position and C4 position of BB6 and BB1 respectively. BB3 was glycosylated simultaneously to the C2 and C4 positions of BB to obtain the Le^b epitope structure.



Post automation steps

After assembling the building blocks on the Glyconeer to obtain the final glycan structures, the resin loaded with the final oligosaccharide was subjected to photocleavage. The crude mixture was then subjected to HPLC analysis and purification (see Chapter 3 for more details).

4.5 Determining the reproducibility of established glycosylation conditions

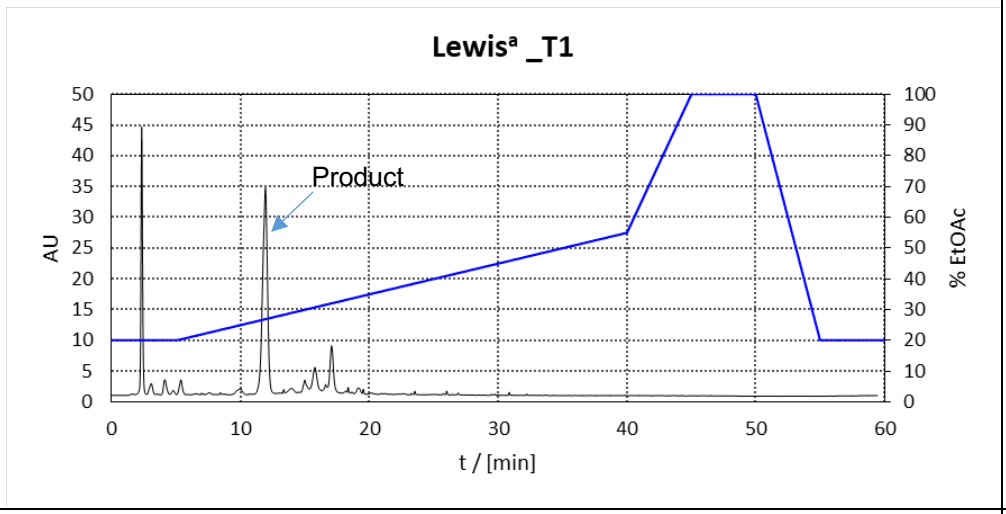
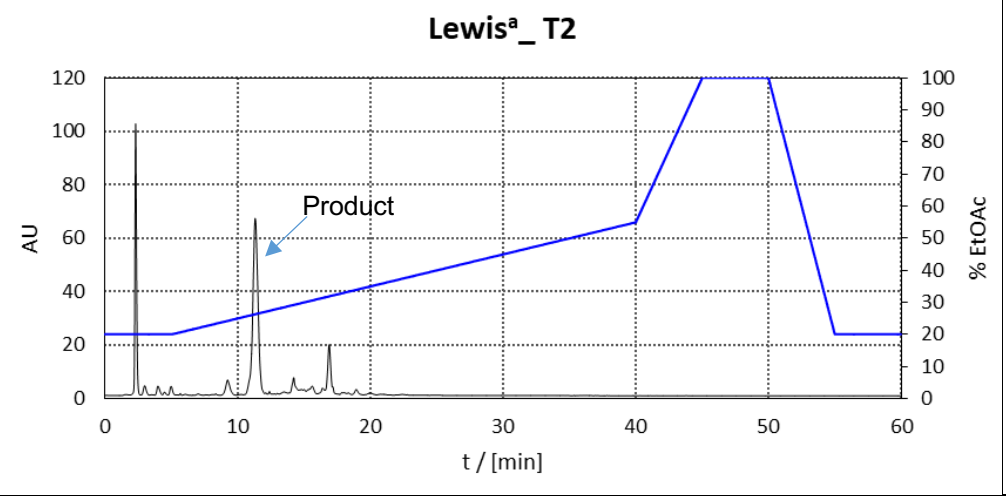
Lewis^a , Lewis^b and Lewis^y antigens bear two monosaccharide units in common (BB1 and BB3). Therefore, in order to get rapid access to these immunologically relevant glycans, it is of utmost importance to establish reproducible glycosylation conditions for these building blocks. Additionally, these monomers can also provide access to a plethora of other Lewis antigen derivatives, such as Lewis^x dimeric, H antigen, KH-1 antigen.[27]

The streamlined glycosylation conditions established for these two building blocks while synthesizing the Lewis^x epitope structure (see Chapter 3 for more details) were applied to assemble the Le^a, Le^y and Le^b epitope structures using the Glyconeer. For the remaining building blocks, i.e. BB5 and BB6 (Figure 3), the standard conditions reported by Delbianco[12] (6.5 equivalents of BB) were implemented, and further optimized depending on the results obtained.

4.5.1 Automated glycan assembly of Lewis^a epitope structure

After establishing a workflow for synthesizing the Lewis^a epitope structure, the building blocks, BB1, BB3 and BB5 were assembled, and the results are shown in Table 4b.

Table 4b: Automated glycan assembly of Lewis^a epitope structure using the Glyconeer on the 0.0125 mmol scale

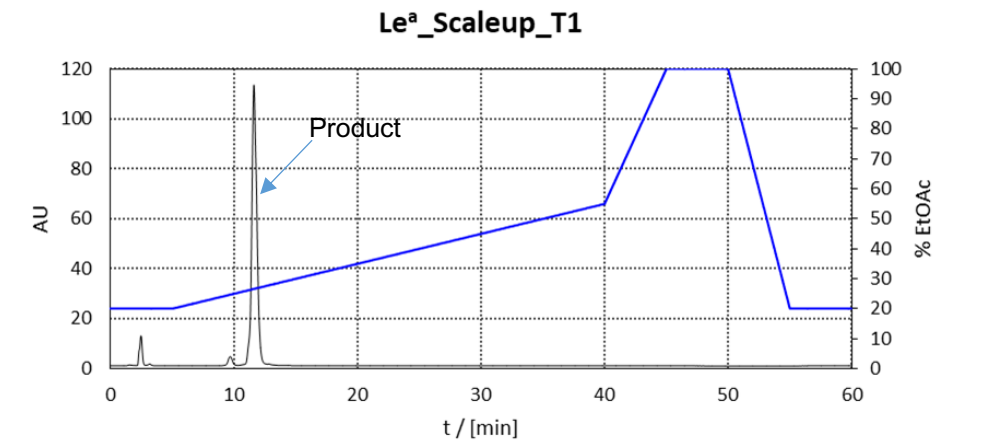
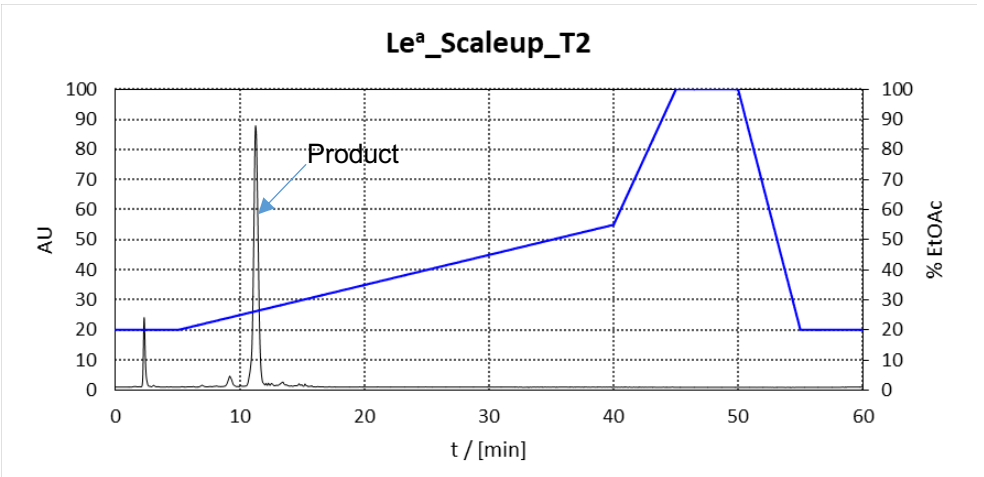
Final Analysis (HPLC Trace)	%Yield (Isolated)
	30%
	32%

Glycosylation Conditions: GlcNAc: -20^{o}C (5 min) to 0^{o}C (20 min), 6.5 eq, 2x, Fuc: -40^{o}C (5 min) to -20^{o}C (20 min), 10 eq,1x, Gal: -20^{o}C (5 min) to 0^{o}C (20 min), 6.5 eq, 2x

The HPLC traces (Table 4b) confirm that the glycosylation conditions established for BB1 and BB3 are reproducible as indicated by the formation of product and the absence of any deletion sequence. It also showed that 6.5 equivalents with two glycosylation cycles are the optimal glycosylation conditions for BB5.

In a nutshell, the successful synthesis of the Le^a epitope structure in the 0.0125 mmol scale indicated that the glycosylation conditions for BB1 and BB3 are highly reproducible, and afford the target structure in satisfactory yields (Table 4b). Therefore, with the optimized glycosylation conditions for BB1, BB3 and BB5 in hand, the synthesis of Le^a epitope structure was then scaled up to 0.025 mmol in order to procure more material. Thus, drawing inspiration from the effect of increased argon mixing on efficiency of fucosylation for the scaled up synthesis of Le^x epitope structure (see Chapter 3 for more details), the AGA of Le^a epitope structure on the 0.025 mmol scale was carried out with increased argon mixing (1s to 3s).

Table 4c: Scale up trials (0.025 mmol) of Lewis^a epitope structure using the Glyconeer

Final Analysis (HPLC Trace)	**% Yield (Isolated)**
	40%
	45%

Glycosylation Conditions: GlcNAc: -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x, Fuc: -40°C (5 min) to -20°C (20 min), 10 eq,1x, Gal: -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x

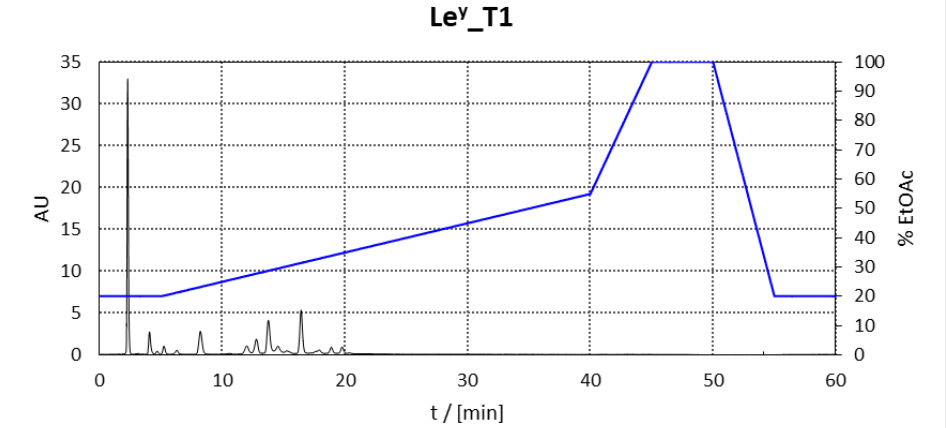
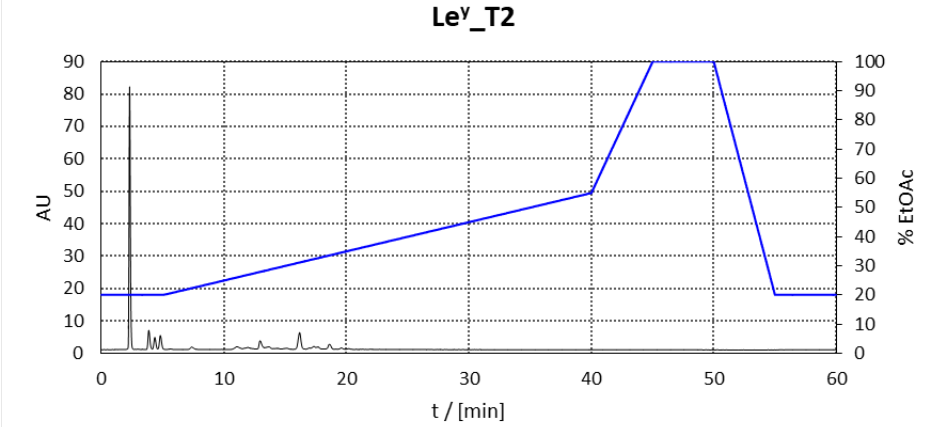
The HPLC trials (Table 4c) indicate that the scale up trials of the Le^a epitope structure was successful, which was proved by the formation of product. Additionally, it again established the importance of increased argon mixing in achieving efficient fucosylation on the 0.025 mmol scale.

4.6 Automated glycan assembly of Le^y and Le^b epitope structures: The curious case of difucosylation

The Le^y and Le^b epitope structures bear two fucose units. While in Le^y, one of the fucose moieties is attached to the C3 position of BB1, in Le^b, it is attached to the C4 position of BB1. Since the Le^y epitope structure is identical to the Le^x epitope, with the exception of an extra Fuc BB attached to the C2 position of Gal BB (Figure 3), a different Gal BB (BB6) having an FMOC carbonate in C2 position was chosen to extend the oligosaccharide chain during automation and thus obtain the target glycan. The optimized glycosylation conditions established for BB1 and BB3 (see Chapter 3 for more details) were applied to synthesize Le^y epitope via AGA.

BB5 was utilized for the first time for AGA. Therefore, the activation temperature was chosen as 0°C to 20°C. This is because, from previous AGA trials for BBs bearing the –Stol leaving group at the anomeric centre, it was observed that these BBs could be activated only at higher temperatures. Therefore, the initial trials were conducted using 6.5 equivalents and two glycosylation cycles of the BB5.

Table 4d: Automated glycan assembly of Lewis^{y} epitope structure using the Glyconeer on the 0.0125 mmol scale

Final Analysis (HPLC Trace)	% Yield (Isolated)
	No Product
	No Product

Glycosylation Conditions: GlcNAc: -20^{o}C (5 min) to 0^{o}C (20 min), 6.5 eq, 2x, Fuc: -40^{o}C (5 min) to -20^{o}C (20 min), 10 eq,1x, Gal: -20^{o}C (5 min) to 0^{o}C (20 min), 6.5 eq, 2x

The failed synthetic trials (Table 4d) indicated that the synthesis of the Lewis^{y} epitope required further optimization. The coupling conditions established for BB1 and BB3 were found to be optimal as well as reproducible, which was proved by the successful synthesis of Lewis^{a} epitope structure and efforts turned towards streamlining the glycosylation conditions for BB6.

4.6.1 Establishing optimized glycosylation conditions for BB5

BB5 bears an FMOC carbonate in C2 position (Figure 3) that may promote limited participation via anchimeric assistance, thereby resulting in poor stereoselectivity. Taking inspiration from the activation temperature reported by Guberman[11] for BB5, -30ºC to -10ºC was attempted in order to establish the optimized activation temperature for this BB.

Structure of BB5 containing LacNAc dimer:

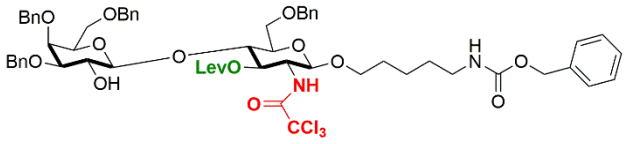
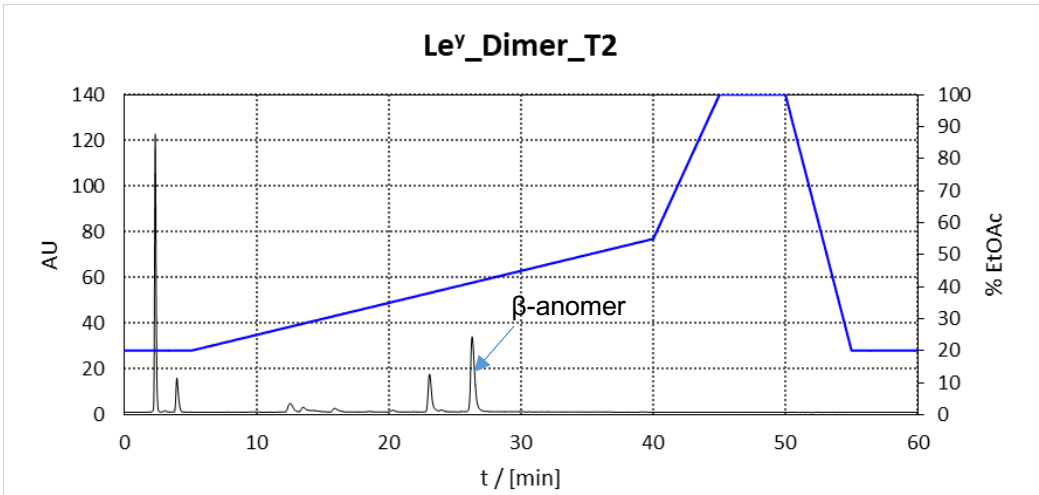


Table 4e: Automated glycan assembly of BB6 containing LacNAc dimer using the Glyconeer on the 0.0125 mmol scale

Final Analysis (HPLC Trace)



Glycosylation Conditions: GlcNAc: -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x, Gal: -30°C (5 min) to -10°C (20 min), 6.5 eq, 2x

The HPLC trace (Table 4d) showed that -30ºC to -10ºC yielded the most promising stereoselectivity. With these optimized glycosylation conditions in hand, the synthesis was taken one step further, and the trisachharide was attempted via AGA.

AGA of BB5 containing Le^x Trisaccharide:

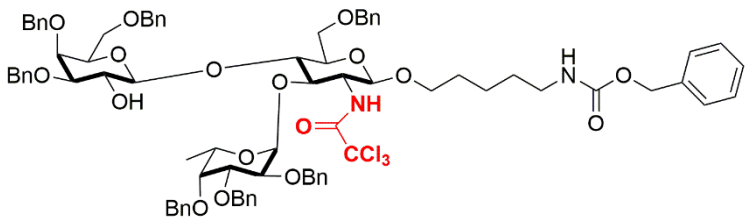
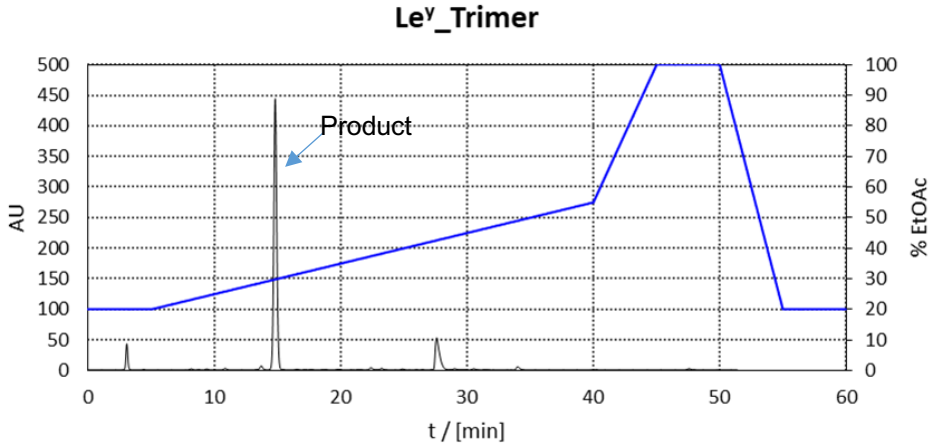


Table 4f: Automated glycan assembly of the BB6 containing Le^x trisaccharide on the Glyconeer on the 0.0125 mmol scale

Final Analysis (HPLC Trace)	**%Yield (Isolated)**
	35%

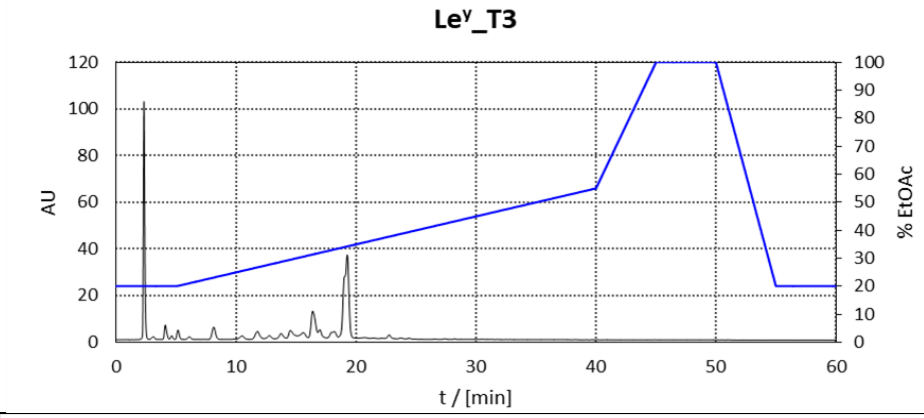
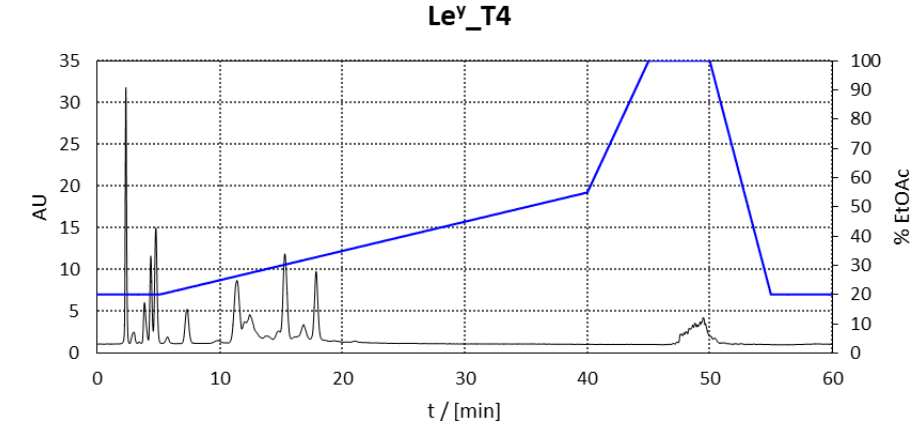
Glycosylation Conditions: GlcNAc: -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x, Fuc: -40°C (5 min) to -20°C (20 min), 10 eq, 1x, Gal: -30°C (5 min) to -10°C (20 min), 6.5 eq, 2x

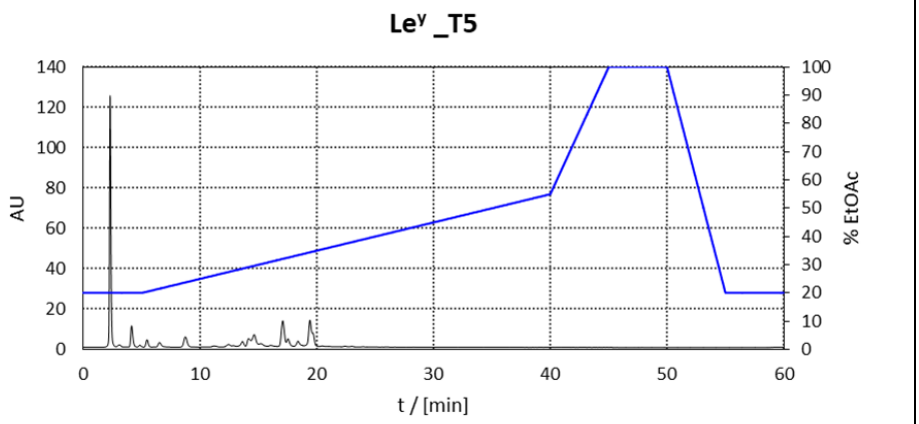
As indicated by the HPLC trace (Table 4f), the AGA of the trisaccharide was successful, as confirmed by the formation of product, and the absence of any deletion sequence. Therefore, with optimized glycosylation conditions for all the BBs required to assemble the Le^y epitope structure in hand, the target tetrasaccharide was synthesized again.

4.6.2 Synthesis of Le^{y} epitope structure using optimized conditions

With optimized glycosylation conditions for all the BBs (BB1, BB3 and BB5) in hand, the Le^{y} epitope was assembled using the Glyconeer.

Table 4g: Automated glycan assembly of the Le^{y} tetrasaccharide using optimized glycosylation conditions on the Glyconeer on the 0.0125 mmol scale

Final Analysis (HPLC Trace)	% Yield (Isolated)
	No Product
	No Product

	No Product

Glycosylation Conditions: GlcNAc: -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x, Fuc: -40°C (5 min) to -20°C (20 min), 10 eq,1x, Gal: -30°C (5 min) to -10°C (20 min), 6.5 eq, 2x

The HPLC traces (Table 4g), confirm that the automated glycan assembly of the Lewis^y epitope using the Glyconeer failed. On the other hand, the successful synthesis of the Lewis^x trisachharide using BB6 proved that the glycosylation conditions established for BB1, BB3 and BB6 were optimal and afforded the target trimer in satisfactory yield. These results led to the conclusion that the attachment of the second Fucose moiety was the most difficult synthetic step leading to repeated failed synthesis of the Lewis^y epitope structure.

In an effort to address this problem, the lactose spacer was included in the following synthetic trials, thus affording the complete Lewis^y antigen, in a bid to establish the effect of the lactose spacer in the automated glycan assembly of Lewis^y using the Glyconeer synthesizer.

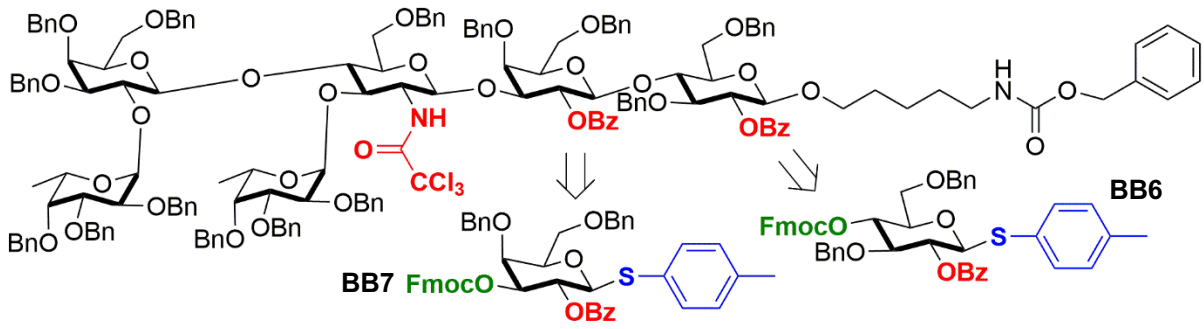
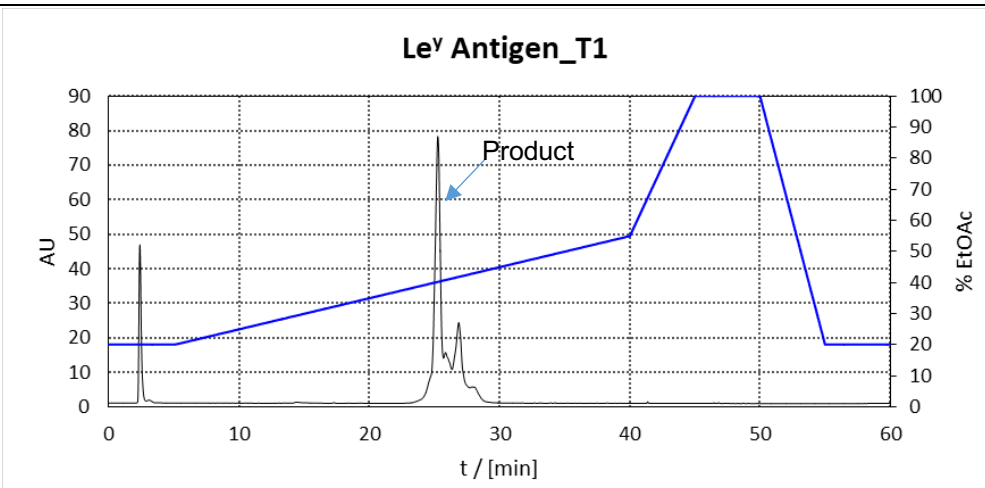
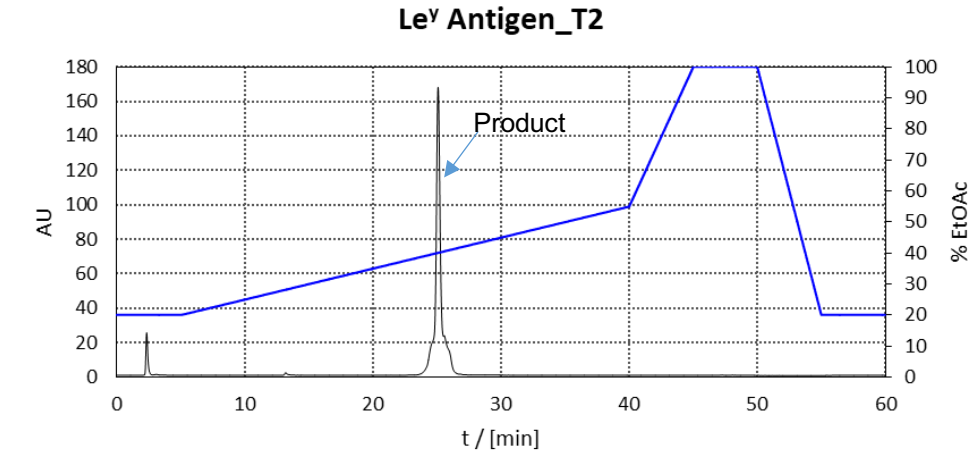


Figure 4: Retrosynthetic analysis of Le^y antigen structure and identification of building blocks

In order to assemble the Lewis^{y} antigen structure, the standard conditions used for all the building blocks, i.e. 6.5 equivalents, two glycosylation cycles were implemented for BB7 and BB8. For the remaining BBs (BB1, BB3 and BB6), the optimized glycosylation conditions mentioned above were used.

Table 4h: Automated glycan assembly of the Le^{y} antigen using the Glyconeer on the 0.0125 mmol scale

Final Analysis (HPLC trace)	**%Yield (Isolated)**
	30%
	32%

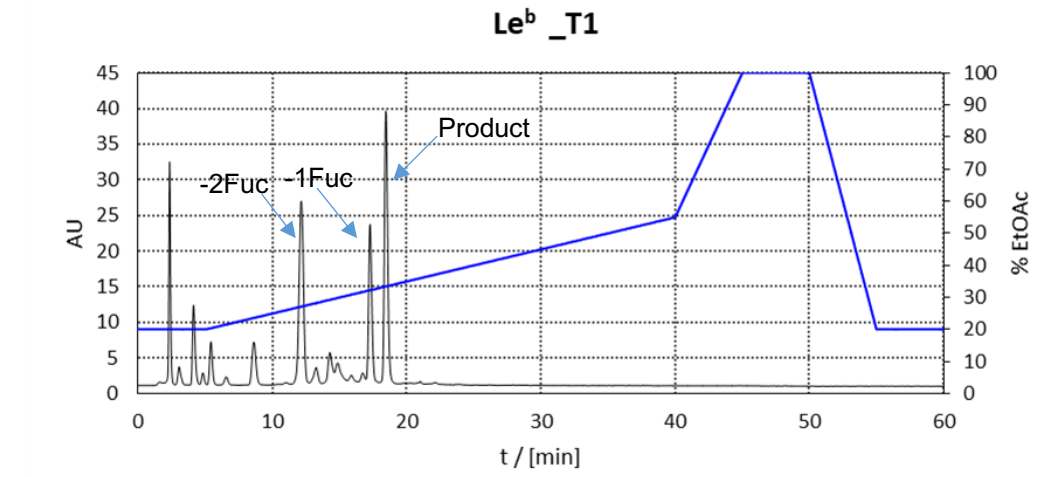
Glycosylation Conditions: Glc : -20^{o}C (5 min) to 0^{o}C (20 min), 6.5 eq, 2x, Gal: -20^{o}C (5 min) to 0^{o}C (20 min), 6.5 eq, 2x, GlcNAc: -20^{o}C (5 min) to 0^{o}C (20 min), 6.5 eq, 2x, Fuc: -40^{o}C (5 min) to -20^{o}C (20 min), 10 eq,1x, Gal: -30^{o}C (5 min) to -10^{o}C (20 min), 6.5 eq, 2x

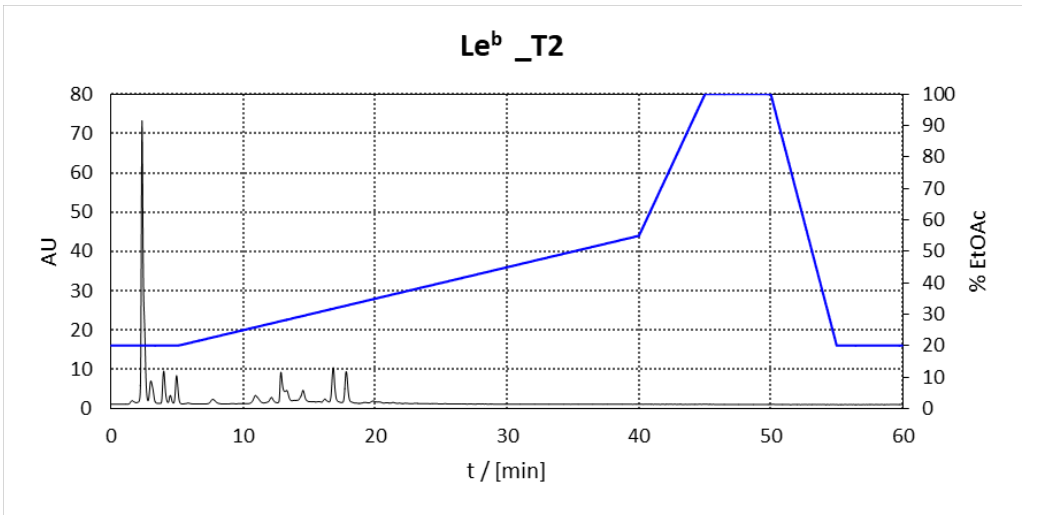
The successful synthetic trials of Le^{y} antigen via AGA (Table 4h) led to the speculation that the introduction of the lactose spacer relieved the spatial restriction imposed on the introduction of the second Fuc BB, thereby leading to successful synthesis.

In order to gain better insights into the complexity of difucosylation, the Lewis^{b} epitope structure bearing two fucose moieties was assembled, in a bid to observe whether the problem of fucosylation was specific only to the Lewis^{y} epitope, or persisted for all difcucosylated Lewis epitope structures.

Table 4h: Automated glycan assembly of the Le^{b} epitope structure using the Glyconeer on the 0.0125 mmol scale

Final Analysis (HPLC Trace)





Glycosylation Conditions: GlcNAc: -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x, Fuc: -40°C (5 min) to -20°C (20 min), 10 eq, 1x, Gal: -30°C (5 min) to -10°C (20 min), 6.5 eq, 2x

Although the HPLC trials for the synthesis of Le^b epitope indicate the formation of product, they also show the presence of by-products, namely the mono-fucosylated and un-fucosylated derivatives of Le^b. These results led to the conclusion that the complexity of achieving successful synthesis of difucosylated moieties persisted in this case as well, albeit to a lesser extent compared to $Lewis^y$ epitope structure, which could further be attributed to the fact that in case of the Le^y epitope, both the Fucose moieties appear in close spatial proximity to one another. On the other hand, in Le^b, the two Fucose units are farther away from one another, thereby reducing the rigidity in the molecule, ultimately resulting in formation of product compared to Le^y, wherein no product formation could be seen after several synthetic trials on the Glyconeer.

4.7 Conclusion and Outlook

The optimized synthetic protocols established for the synthesis of BB1 and BB3 led to the identification of a "common intermediate" **1e**, **2a** which was helpful in getting access to the final building block in fewer synthetic steps, thereby significantly reducing the time needed to synthesize the differentially protected building blocks for automation. The advantage provided by the intermediates **1e**, **2a** was evident in the synthesis of BB1 as well as BB4 wherein the final building block could be procured in merely four synthetic steps.

Next, the reproducibility of the glycosylation conditions established for BB1 and BB3 which are shared by $Lewis^x$ and $Lewis^a$ was proved by the successful synthesis of the later via automated glycan assembly using the Glyconeer. Additionally, the effect of increased argon mixing led to efficient fucoslyation in the scale up synthesis (0.025 mmol) of $Lewis^a$ epitope as well.

Finally, the automated glycan assembly of the $Lewis^y$ and $Lewis^b$ epitopes proved challenging, with no product formation seen in case of the $Lewis^y$ epitope. This problem could be solved by introducing the Lactose spacer to afford the complete antigen structure. This observation gave rise to the speculation that there is a spatial restriction in the introduction of the second fucose moiety thereby leading to repeated failed synthesis on the Glyconeer. However, it could be imagined that the lactose spacer reduces the rigidity in the structure, thereby leading to successful synthesis of

the Lewis^y antigen. This observation paves the way for possible investigations into the spatial arrangement of the Lewis^y epitope structure on the solid phase which could provide crucial details and also plausible solutions in order to overcome the challenge of synthesizing this structure via automated solid phase glycan assembly.

Chapter 5

Automated glycan assembly of poly-N-acetyl-glucosamines: Understanding their role in biofilm formation

5.1 Introduction

Poly-N-acetyl-β-(1,6)-glucosamine is an important constituent of the extracellular biofilm matrix of *Staphylococci* and *Bacillus* species.[2] The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear β-1,6-linked glucosaminoglycan:[8] Biofilm formation protects microorganisms from challenging environmental conditions, allow them to build communities, share resources and allows them to resist potential toxic compounds for example antibiotics.[3] However biofilms formed by pathogenic bacteria and fungi can pose a serious threat due to the enhanced resistance against common antibiotics and pharmaceuticals in the biofilm.[3] Therefore, a treatment, which would target and destroy the biofilm matrix may render the bacteria again susceptible towards classic antibiotics. Several glycoside hydrolases have been identified to be able to degrade the extracellular matrix though often it isn't entirely clear what the enzyme recognizes as substrate. Among these is family GH153, containing poly-N-acetyl-β-(1,6) hydrolases. Though, crystal structures are available, there are still no structural data available showing the interaction with the substrate, as well as the precise conformational itinerary of the catalytic cycle.

In order to synthesize the PNAG oligomers to support the structural analysis for biofilm degrading hydrolases, I decided to employ automated glycan assembly using the Glyconeer synthesizer to generate a variety of GlcNAc oligomers. I started the synthesis using the optimized synthetic protocols developed for the assembly of the Lewis antigens (see Chapters 3 and 4). This should prove the general usability of the established protocols and help to validate the Glyconeer for the synthesis of complex long carbohydrates. The resulting oligomers were provided to the group of Dr. Roth, where they were used to characterize a glucosamine hydrolase.

This chapter is divided into four sections. The first section describes the retrosynthetic analysis of the poly-N-acetyl-glucosamine oligomers, and identification of the necessary building block, required to assemble the desired oligosaccharides using the Glyconeer synthesizer. The second section discusses the advantage of having optimized protocols for the synthesis of a variety of structures via automation in a bid to extend the library of compounds assembled using the Glyconeer and prove the robustness of the established synthetic protocols. The third section highlights the global deprotection of the synthesized PNAG library. Finally, the fourth section describes use of the PNAG library to characterise the substrate specificity of the PelA glucosamine hydrolase domain from *Pseudomonas aeruginosa.*

5.2 Retrosynthetic analysis of target structure and identification of building blocks

Firstly, the building blocks were identified upon retrosynthetic analysis of the target structure (Figure 5). Since the oligosaccharide chain required to be extended along the C6 position, a temporary protecting group, namely, 9-fluorenylmethoxycarbonyl (FMOC) was installed in this position which could be cleaved during automation to elongate the oligosaccharide structure. Secondly, in order to ensure β-selectivity of the newly formed glycosidic linkage, the trichloroacetamide group (TCA) was installed in C2 position.

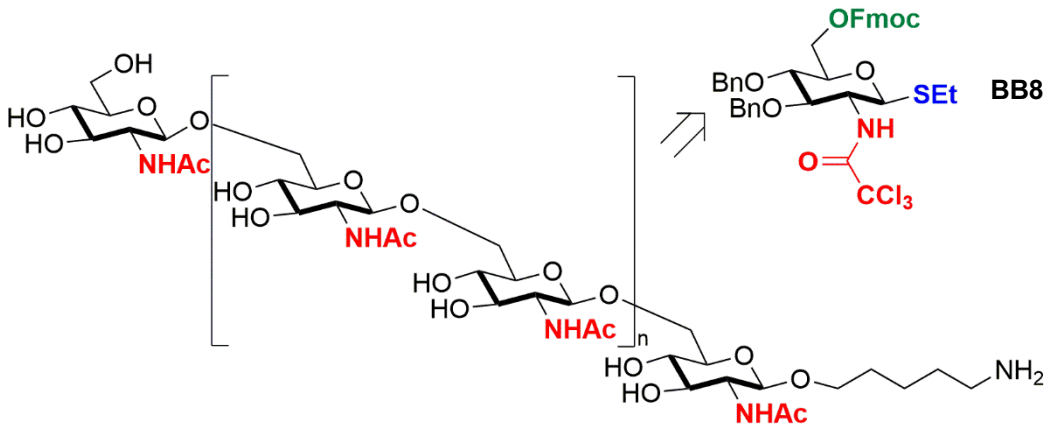
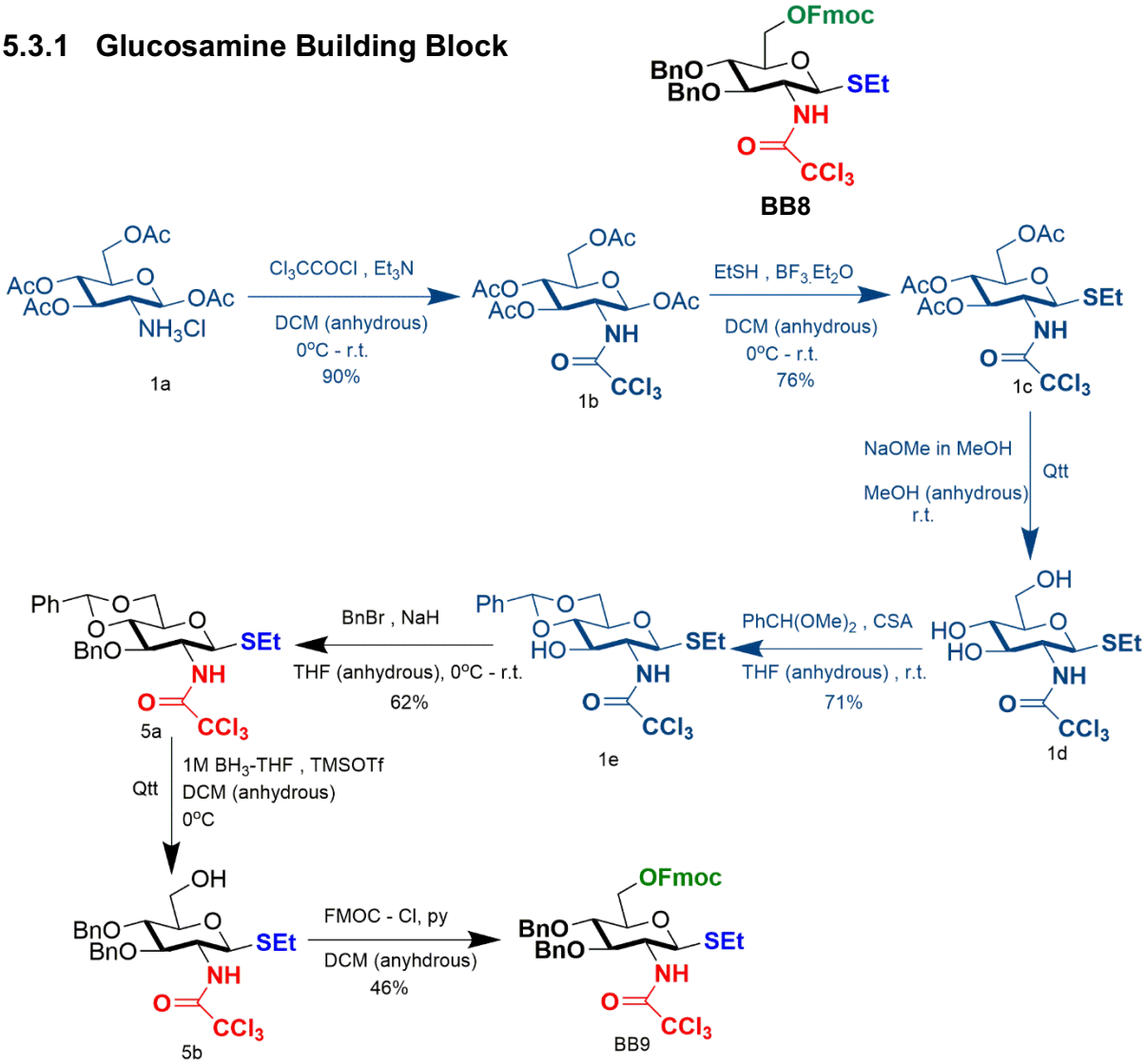


Figure 5: Retrosynthetic analysis of Poly-N-Acetyl-Glucosamine oligomers and identification of building block

5.3 Optimization and bulk synthesis of Building Blocks

The first four steps of the synthetic scheme leading to the formation of the benzylidene intermediate **1e** were optimized (Chapter 2) to obtain high yields. As a result, this intermediate was identified as a “common intermediate” which could be used to access a plethora of diverse, differentially protected building blocks for automated glycan assembly (Chapter 2). Therefore, the synthesis of this derivative was scaled up to a 40g batch size and stocked up in multi-gram quantities.

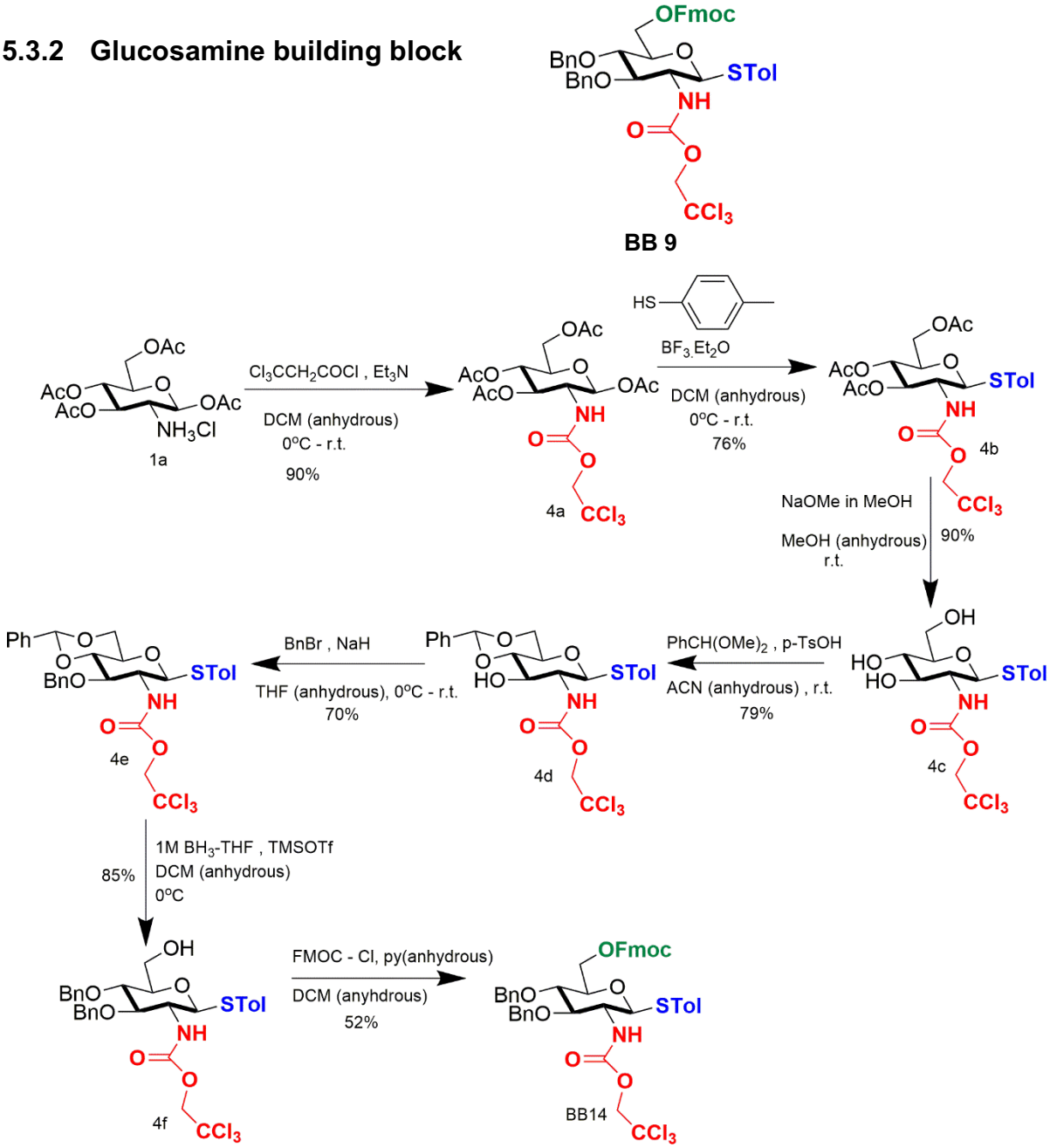
5.3.1 Glucosamine Building Block



The synthetic scheme in blue represents the optimized route to BB8 (Chapter 2). Therefore, these route was applied for the large scale synthesis of BB8 in order to

procure bulk quantities of the building block. As discussed in Chapters 2 and 3, all the derivatives in the above reaction scheme could be either crystallized/precipitated, or used for further steps without purification, thereby diminishing the need for column chromatography, and ultimately saving resources, which proved advantageous for the multigram synthesis of BB8.

5.3.2 Glucosamine building block



The synthesis of BB14 commenced from the commercially available starting material **1a**. The optimized synthetic protocols developed for the synthesis of BB9 were then applied to procure the intermediates **4a**, **4b**, **4c** and **4d**. As explained in Chapter 2, the

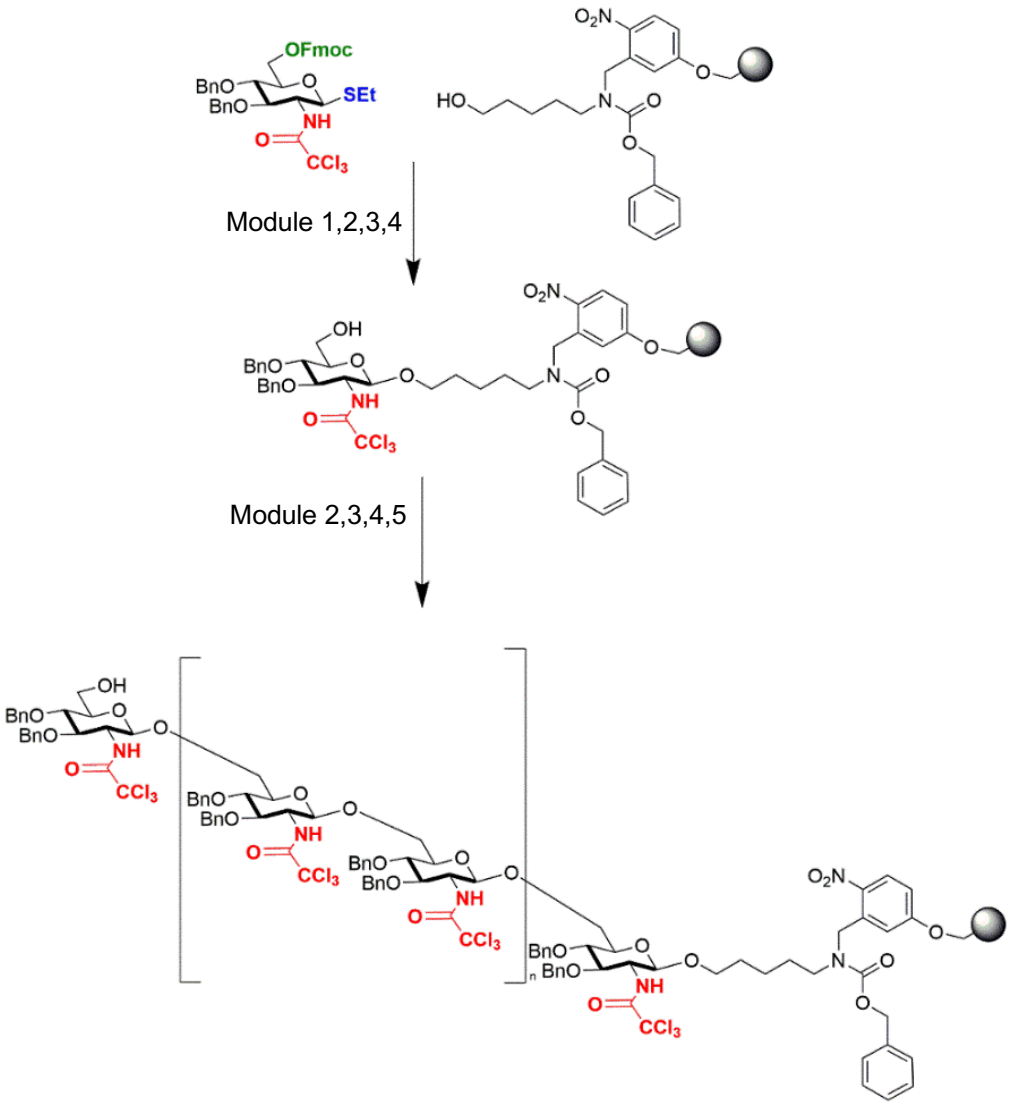
synthesis of intermediate **4e** was performed in acetonitrile since there was no product formation using THF.

5.4 Assembly of building block using the Glyconeer to obtain target oligomers

With optimized synthetic protocols and multi-gram quantities of BB9 in hand, the next step was to establish a workflow in order to assemble the target structures (Figure 5) via automation. The synthetic modules and the workflow used to assemble the target oligomers using the Glyconeer synthesizer are described in Table 5a.

Table 5a: Synthetic modules required for automated glycan assembly of PNAG oligomers using Glyconeer synthesizer

Module no.	Module name	Conditions
1.	Resin swelling	DCM, 25^{o}C, 30 min
2.	Acid wash	TMSOTf in DCM(anhydrous), -20^{o}C
3.	Thioglycoside Glycosylation	BB in DCM (anhydrous), T_1(5 min) to T_2 (20 min)
4.	Ac_2O acidic capping	10% Ac_2O, 2% Methanesulfonic acid in DCM(anhydrous)(v/v)
5.	FMOC Deprotection	20% Piperidine in DMF(v/v), 25^{o}C



Since the target structures are β-1,6-GlcNAc homopolymers, the GlcNAc (BB6) was added sequentially to the growing chain to generate the target oligomers. The synthesis was commenced by swelling the resin with DCM, followed by the glycosylation of the BB6, capping the deletion sequences, and finally cleaving the FMOC carbonate to propagate the oligosaccharide chain.

5.4.1 Post automation steps

After assembling the oligomers via AGA using the Glyconeer synthesizer, the resin loaded with the target oligosaccharides was subjected to photocleavage (detailed procedure in SI), and analysed as well as purified by HPLC.

The solvent gradient used for the analysis and purification of the crude samples by HPLC is governed by the solubility of the oligosaccharide in the solvent mixture used for analysis. While the glycans previously synthesized using the Glyconeer (Chapters 3 and 4) could be analysed using the gradient mentioned in Chapter 3, the poly-N-acetyl-glucosamine tetramer could not be dissolved in the usual solvent mixture, such as Hex/EA, 1:1. Therefore, the solvent gradient used for analysis and purification of the tetramer was modified based on the solubility of the crude sample (Hex/EA, 3:2) as shown in Table 5b.

Table 5b: Description of solvent gradient used for HPLC analysis of PNAG tetramer

Time (min)	%Ethyl Acetate	% Hexane	Flow Rate (mL/min)
0.00	40	60	1.000
5.00	40	60	1.000
40.00	65	35	1.000
45.00	100	0	1.000
50.00	100	0	1.000

5.5 Automated glycan assembly of poly-N-acetyl-glucosamine

The automated glycan assembly of three different PNAG oligomers, a tetramer, pentamer and hexamer was performed (Figure 5).

5.5.1 Automated glycan assembly of Poly-N-acetyl-Glucosamine tetramer

To begin with, the standard conditions for glycosylation, such as 6.5 equivalents of BB and two glycosylation cycles were applied to glycosylate BB8, and further optimized depending on the results obtained.

PNAG tetramer (Protected):

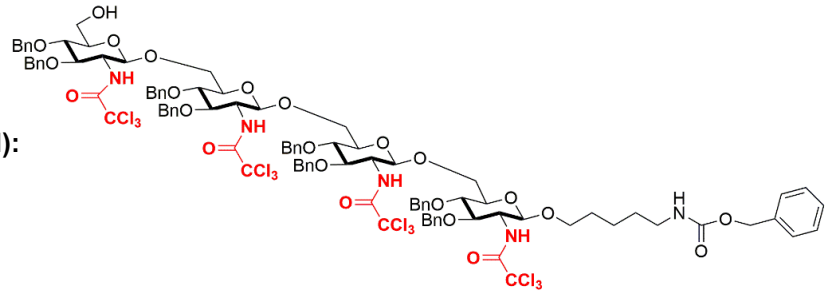
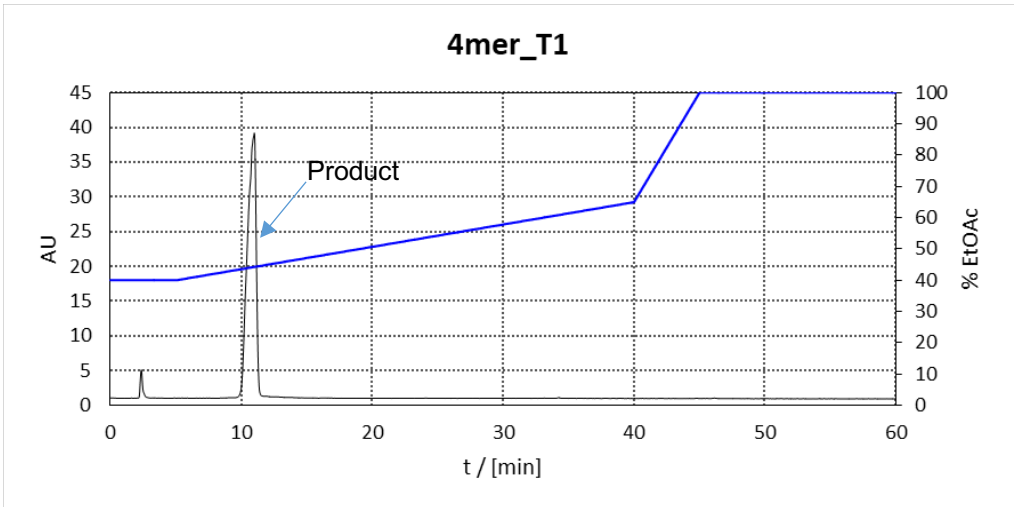
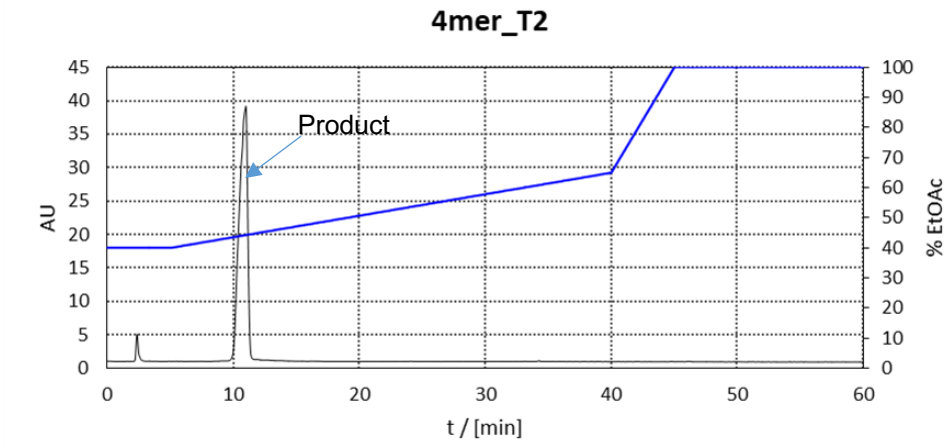


Table 5c: Automated glycan assembly of the PNAG tetramer using the Glyconeer synthesizer

HPLC Trace (Final Analysis)	% Yield (Isolated)
	30%

	35%

Glycosylation Conditions: GlcNAc(I): -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x, GlcNAc(II-IV): -20°C (5 min) to 0°C (20 min), 6.5 eq,1x

The HPLC trials in Table 5c show that the synthesis of the Poly-N-Acetyl glucosamine tetramer was successful in high purity. The HPLC traces also confirmed that the glycosylation conditions established for BB8 were optimal and reproducible, as shown by the consistent yields obtained for the consecutive trials depicted in Table 5c.

5.5.2 Automated glycan assembly of Poly-N-Acetyl-Glucosamine pentamer and hexamer

After the successful synthesis of the PNAG tetramer, efforts were then turned towards synthesis of the PNAG pentamer and hexamer structures by extending the number of glycosylation cycles accordingly. The established glycosylation conditions were applied once or two times more to obtain the desired structures. The crude material obtained upon photocleavage was found to be insoluble in Hex/EA used for HPLC analysis. Therefore, the coupling efficiencies were analyzed based on the obtained FMOC traces.

PNAG hexamer (protected):

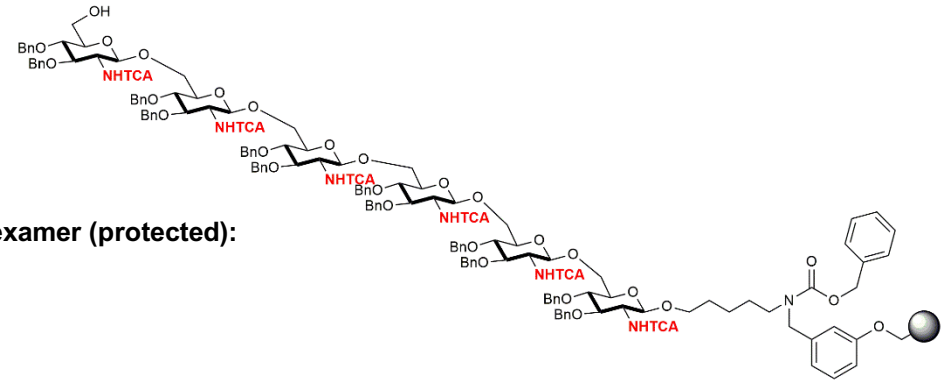
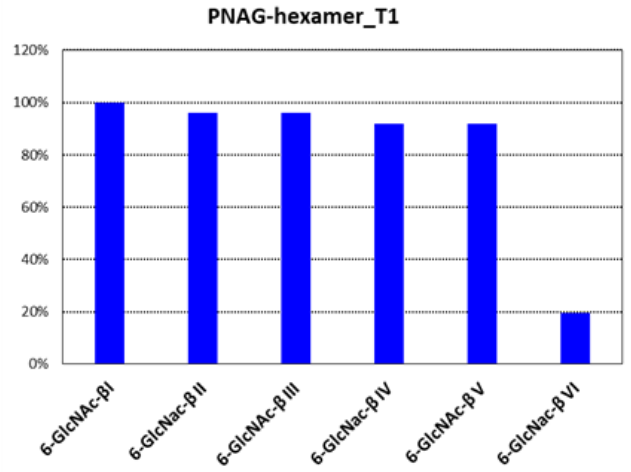
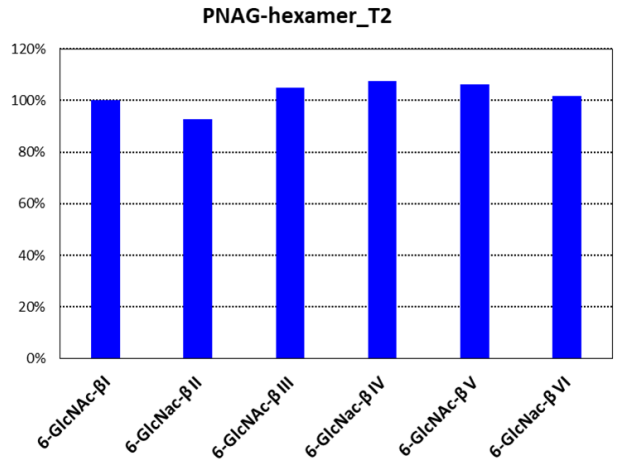


Table 5d: FMOC traces for automated glycan assembly of PNAG hexamer using the Glyconeer synthesizer

Preliminary Analysis
FMOC Trace





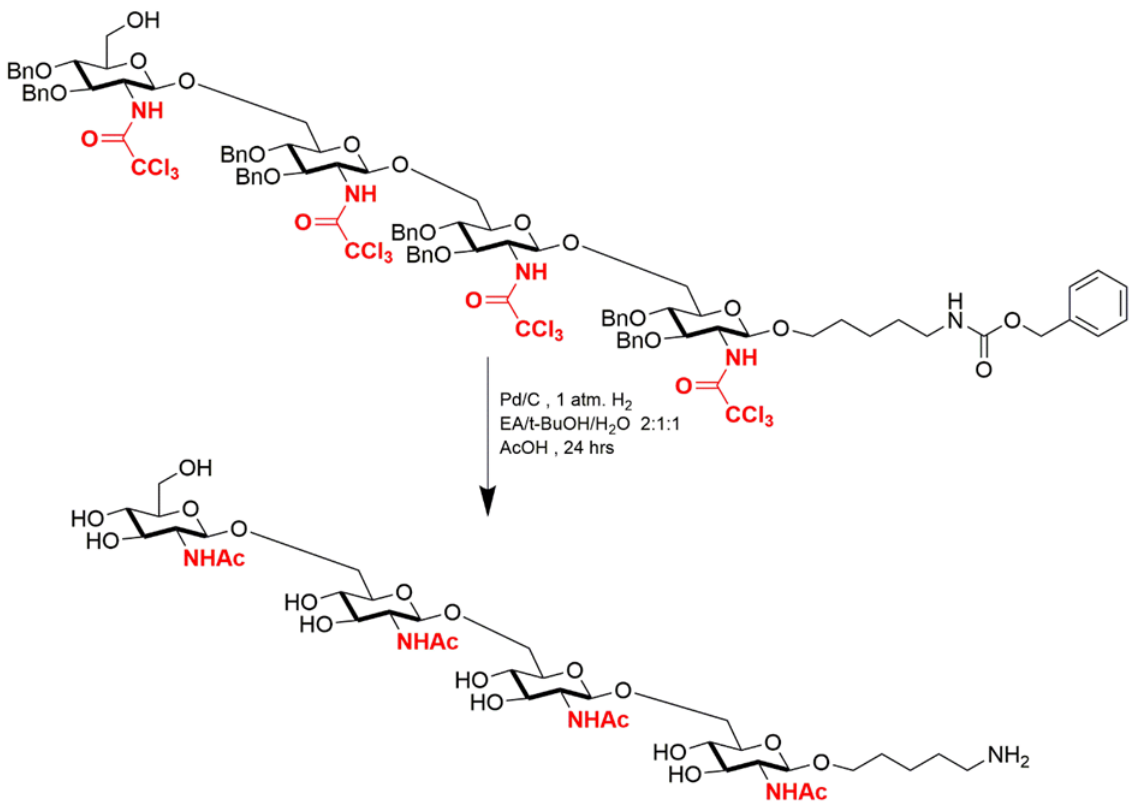
Glycosylation Conditions: GlcNAc (I): -20°C (5 min) to 0°C (20 min), 6.5eq, 2x, GlcNAc (II-VI): -20°C (5 min) to 0°C (20 min), 6.5eq, 1x

The FMOC traces in Table 5d show that each glycosylation step proceeds with nearly quantitative yield. Therefore, owing to the positive results obtained from the FMOC traces, the PNAG pentamer and hexamer obtained upon photocleavage was directly subjected to global deprotection.

5.6 Global Deprotection of poly-N-acetyl-glucosamine oligomers

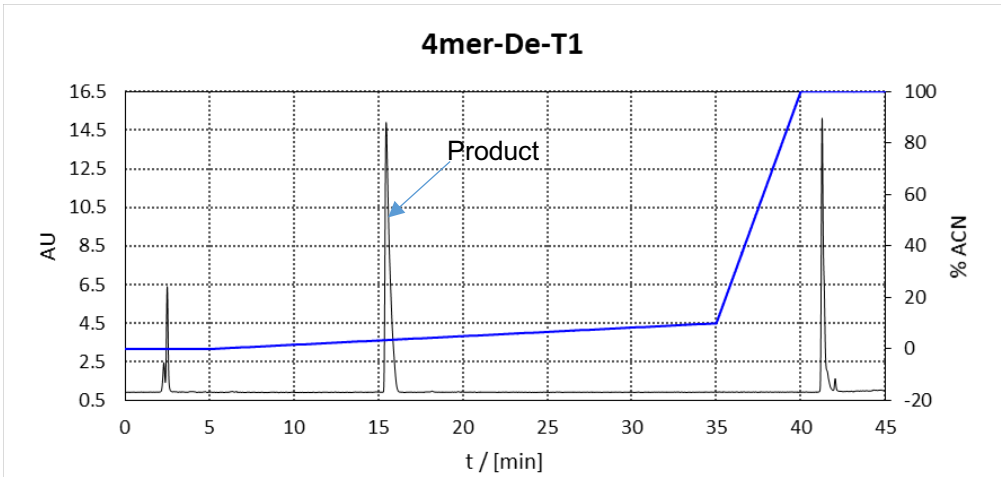
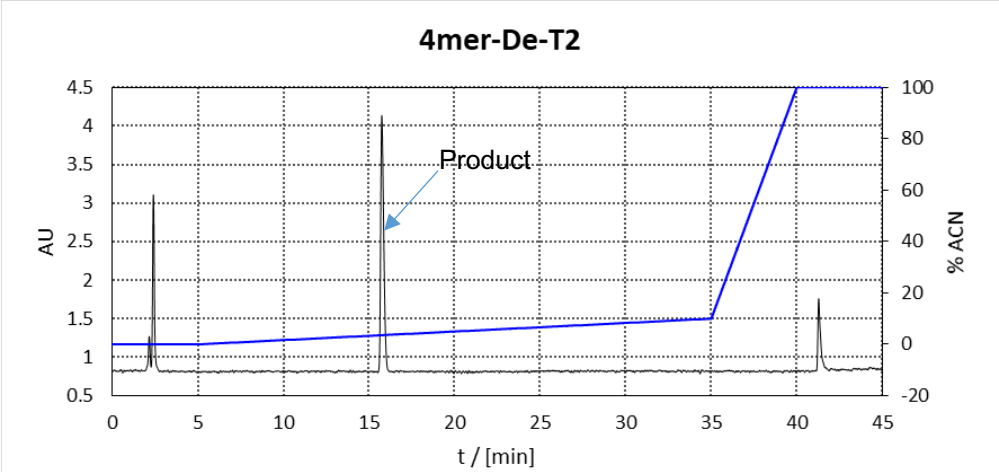
After purification and analysis of the protected PNAG oligomers, the permanent protecting group, namely, benzyl ether (OBn) was cleaved by hydrogenation using a Palladium catalyst, thereby affording the fully deprotected PNAG oligomers with a pentanyl amine spacer at the reducing end.

5.6.1 Global deprotection of PNAG tetramer



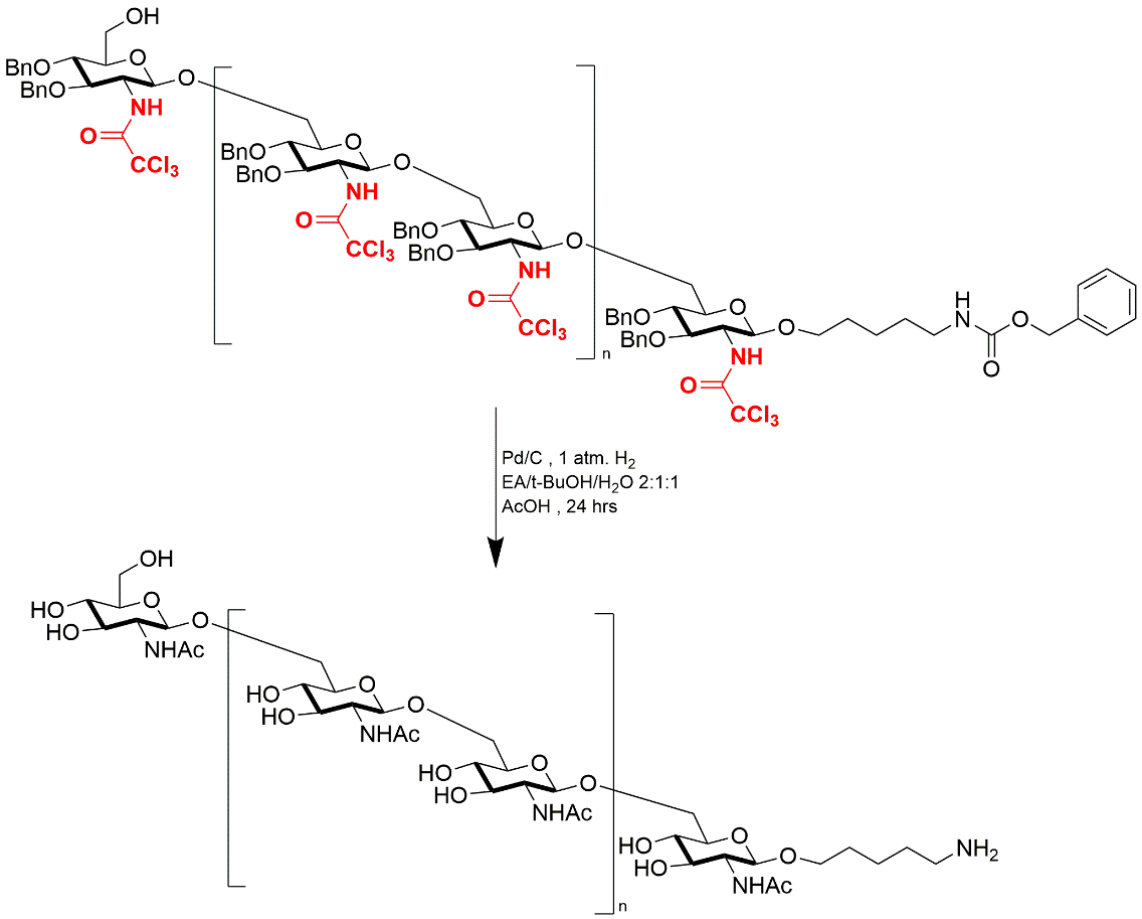
The global deprotection of the PNAG tetramer was performed according to the procedure described in section 5.6, and the results are shown in Table 5e.

Table 5e: Global deprotection of PNAG tetramer by applying the standard protocols developed for hydrogenation

Final Analysis (HPLC Trace)	% Yield
	40%
	42%

The HPLC trials in Table 5e indicate that the global deprotection of PNAG tetramer was successful, and proceeded with a satisfactory yield. The reproducibility of the reaction conditions was established by the consistent yields obtained for the successive trials.

5.6.2 Global deprotection of PNAG pentamer and hexamer



where n= 1,2

PNAG pentamer (deprotected):

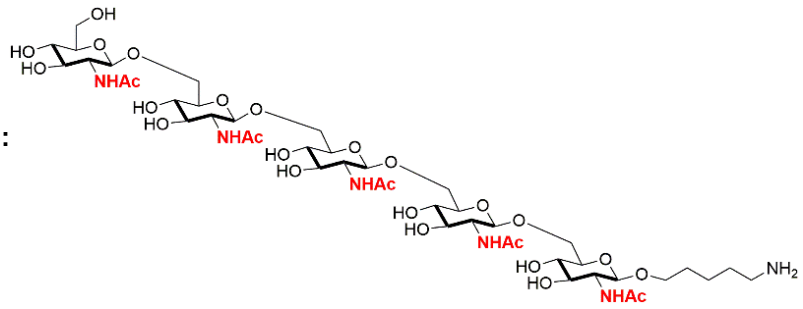
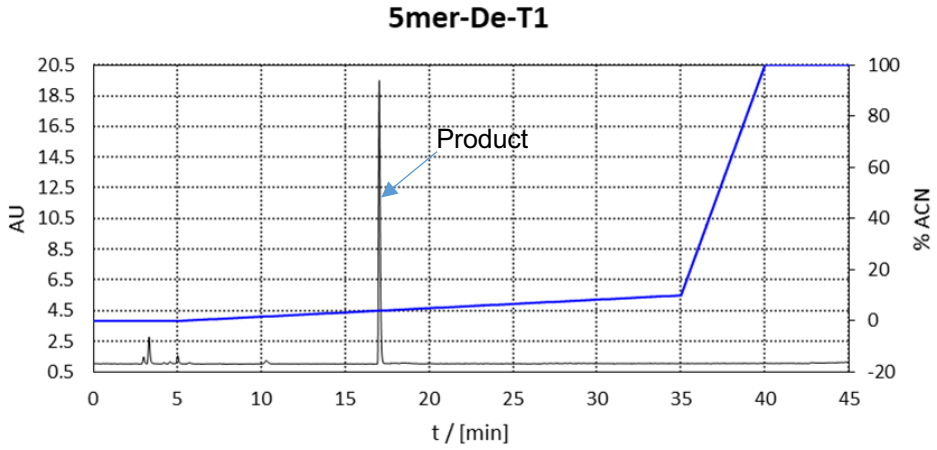
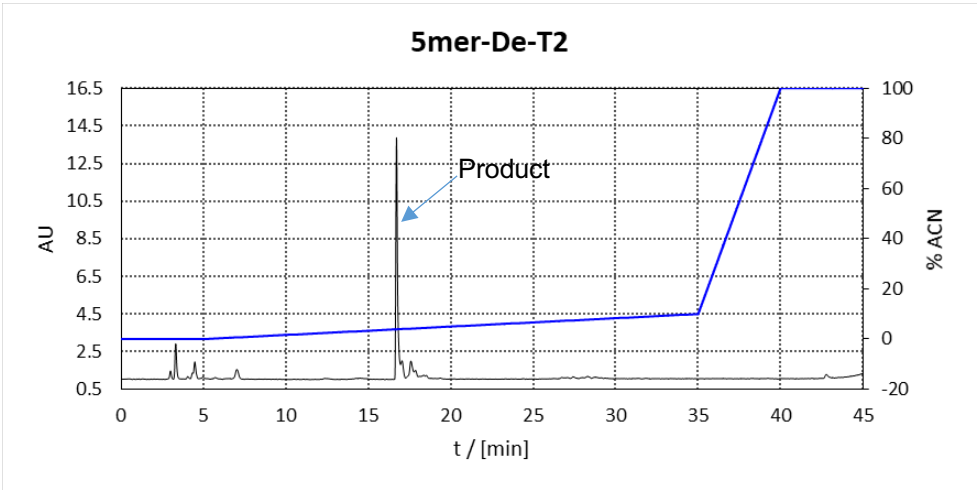


Table 5f: Global deprotection of PNAG pentamer

Final Analysis (HPLC Trace)	**% Yield**
	40%
	40%

PNAG hexamer (deprotected):

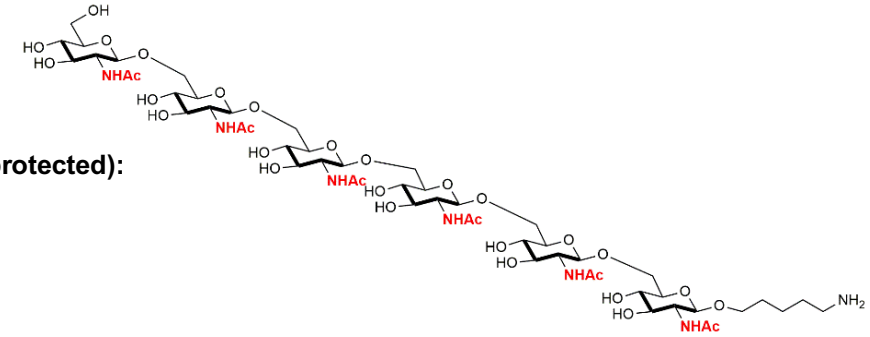
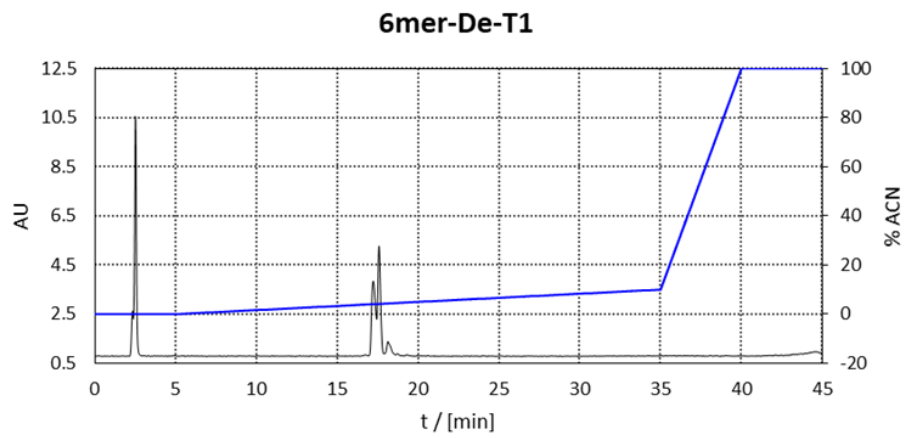
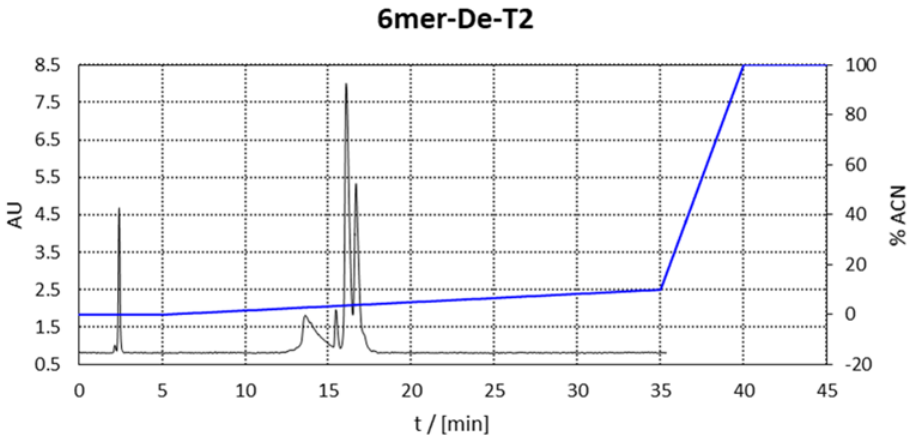


Table 5g: Global deprotection of PNAG hexamer

Final Analysis (HPLC Trace)	% Yield
	40%
	42%

With the fully deprotected PNAG oligomers in hand, the oligomers were used to evaluate the binding specificity of *P. aeruginosa* PaO1 PelA glycoside hydrolase domain.

5.7 Characterisation of the binding affinity of PaO1 hydrolase towards different oligomers

This ITC measurement was performed by Dr. Christian Roth and co-workers at Max Planck institute for Colloids and Interfaces. The affinity of the enzyme towards the synthesized PNAG oligomers was evaluated using Isothermal titration calorimetry (ITC). Initially only the tetramer was evaluated for binding (Fig.6). ITC confirmed binding of the tetramer to the enzyme albeit with only moderate affinity of 1.81±0.40 μM. Furthermore, the evaluation of the thermodynamic profile showed that the reaction is nearly exclusively driven by entropy, whereas the enthalpic contribution is negligible. This points towards a binding mechanism, which involves the binding via hydrogen bonds, combined with the desolvation of the ligand. Unfortunately, the low enthalpic contributions made the measurements challenging, due to the low measurable heat. Subsequently, with optimized experimental conditions the hexamer was evaluated for binding. Surprisingly, the affinity with a K_D of 2.85±0.97 μM is slightly worse compared to the tetramer. This indicates that the population of further subsites on the enzyme is not favourable. Recently, it was shown that glycoside hydrolase PgaB from *Bortadella bronchiseptica* favourably recognises partially deacetylated PNAG and is not active on homo-GlcNAc oligomers. It might be possible that the population of further subsites and a gain of affinity might require deacetylated glucosamine moieties within the oligomer in a particular spacing. Indeed, recently it was shown that most likely subsite +1 most likely needs to be populated by a deacetylated glucosamine residue for efficient catalysis.

A comprehensive study on fungal hydrolases revealed that the PAO1 hydrolase, studied here, is rather active on Pel type polymers or Galactosaminogalactans with an α-1,4 linkage. Thus, the hydrolase had to be classified into family 166 of the carbohydrate active enzyme database (Lombard 2014). Nevertheless the moderate to low affinity of the ligand might indicate some plasticity in the substrate binding cleft or a broader substrate spectrum than thought.

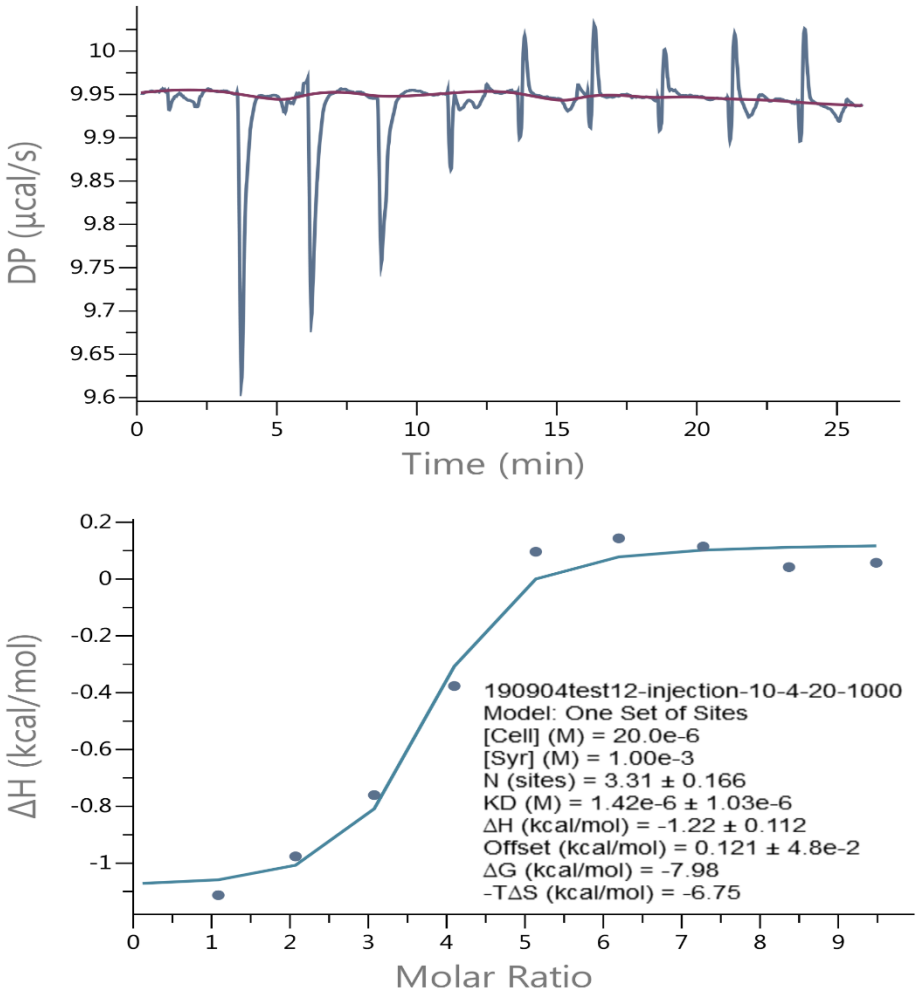


Figure 6: Evaluation of binding affinity of PNAG tetramer for PAO1 enzyme from *Pseudomonas aeruginosa* using ITC

5.8 Conclusion and Outlook

The optimized synthetic protocols developed for the synthesis of BB1 (see Chapters 2 and 3 for more details) were successfully applied for the synthesis of BB8 and BB9 in excellent yields and multi-gram quantities. The ready availability of optimized protocols enabled rapid access to the diverse, differentially protected building blocks (BB8 and BB9) in multi-gram quantities. With the building blocks in hand, the automated glycan assembly of the homo-GlcNAc oligomers were successfully attempted, affording the target structures in excellent yields. Unfortunately, the protected PNAG pentamer and hexamer could not be purified, therefore, the crude samples were subjected to global deprotection. The subsequent optimized global deprotection protocols led to fully deprotected ready to use oligomers, which were then used to characterise the substrate specificity of the glycosyl hydrolase PaO1 from *Pseudomonas aeruginosa* by Isothermal Titration Calorimetry (ITC). The experiments revealed that the PNAG tetramer and hexamer exhibited similar affinity towards the PaO1 enzyme. This observation indicated a saturation in the subsites of the enzyme towards homo-GlcNAc oligomers. Therefore, it could be speculated that deacetylated glucosamine moieties within the oligomer might lead to increased binding, which further opens up the possibility of synthesizing co-polymers containing different patterns of acetylated and deacetylated glucosamine (ABAB, AABB) oligomers in order to further expand the library of PNAG oligomers for evaluating binding affinities towards PaO1 enzyme.

Chapter 6

Automated Glycan Assembly of the N-glycan Core Structure: Exploring the Scope of Automated Glycan Assembly Using the Glyconeer

6.1 Introduction

Protein glycosylation is one of the most complex post-translational modifications.[39] Glycans are found on diverse protein classes and are often located on the extracellular side of the cell-surface. Toxins, lectins, antibodies, hormones, viruses and bacteria interact with membrane bound glycoproteins and thereby participate in various biological processes. Glycoproteins are also involved in intracellular interactions, and play a major role in signal transduction.[39] There are two major types of protein glycosylation, namely, N-glycosylation and O-glycosylation.[39]

The biosynthesis of N-glycans involves the transfer of a pre-synthesized tetradecasaccharide ($Glc_3Man_9GlcNAc_2$) from dolichol phosphate to an asparagine residue using the enzyme oligosaccharyl transferase (OTS). The terminal glucose residues are then removed by a complex pathway to form the common pentasaccharide core ($Man_3GlcNAc_2$). This core pentasaccharide is found to be highly conserved among eukaryotic N-glycans. It is further extended to obtain high mannose, hybrid- or complex type N-glycans. The recognition sites for N-glycans consist of a common peptide sequence of Asn-Xaa-Ser/Thr, where Xaa can be any of the 20 natural amino acids except proline.[39]

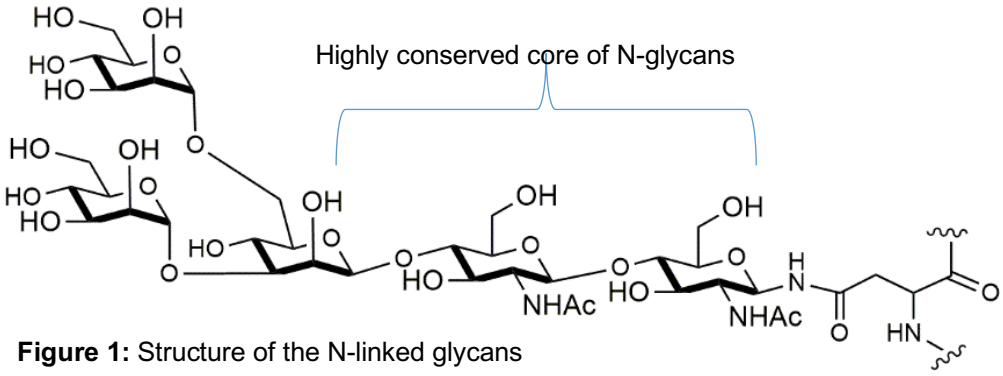


Figure 1: Structure of the N-linked glycans

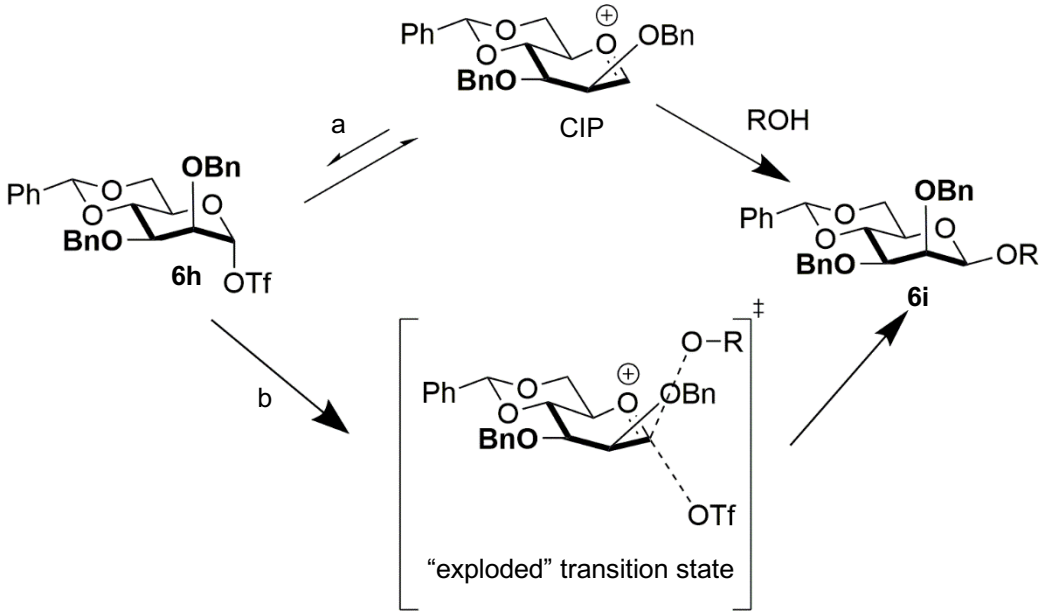
This chapter is divided into three sections. The first section highlights the retrosynthetic analysis of the target structure and identification of building blocks, followed by the strategy adopted to establish the beta mannose linkage. The second section describes the solution phase synthesis of the beta mannose containing dimer, followed by establishing optimized reaction conditions in order to achieve the optimal stereoselectivity. Finally, the third section discusses the synthesis of the N-glycan core structure via automation using the Glyconeer synthesizer.

6.2 Establishing the beta mannosidic linkage

The stereoselective formation of beta mannosidic linkages is one of the most challenging glycosidic linkages to establish via synthetic carbohydrate chemistry. A number of methods have been described in the literature for forming beta mannosidic linkages such as the synthesis of a beta glucoside, followed by inversion of C-2 configuration either by a nucleophilic displacement or an oxidation–reduction sequence.[6] Intramolecular aglycon delivery has also been developed by several research groups as a method for the synthesis of 1,2-cis glycosides and has been applied to the synthesis of beta mannosides. Crich et.al.[30] have reported the stereoselective formation of beta mannosides using 4,6-O-benzylidene intermediates in the presence of triflic anhydride and 2,6-di-tert-butyl-4-methylpyridine.[30] In this method, the 4,6-O-benzylidene mannosyl sulfoxide is first activated with triflic anhydride to give α-mannosyl triflate. The acceptor then displaces the triflate intermediate to afford the β-mannoside.

The method of choice adopted for establishing the beta mannosidic linkage is the Crich beta mannosylation using a mannose sulfoxide donor (BB11). As a start, a “proof of concept” study was performed to establish the beta mannosidic linkage via solution phase glycosylations. The beta mannose containing dimer was then used as a building block in order to assemble the N-glycan core structure using the Glyconeer synthesizer. Owing to the complexity of establishing the beta mannose linkage, this strategy provided the opportunity to procure the dimer in multi-gram quantities, and thereby minimize the time required to obtain the target structure. Finally, in order to extend the N-glycan chain using the Glyconeer, a differentially protected mannose

building block (BB12) was designed, with the possibility of selectively elongating the chain along O3 and O6 positions of BB12.



Scheme 1: Proposed glycosylation mechanism for formation of beta mannose linkage[30]

The mechanism proposed by Crich et.al.[30] involves the displacement of the triflate from **6h** by the carbohydrate acceptor (ROH) to give **6i** that proceeds with the development of substantial oxacarbenium ion character. This observation may be interpreted either by a fully dissociative mechanism involving the formation of a transient contact ion pair (CIP) (Scheme1, path a), or by an equivalent mechanism involving an “exploded” transition state (Scheme1, path b). In the CIP mechanism, the triflate anion is closely associated with the face of the oxacarbenium ion from which it departs and shields that face from the incoming alcohol. In the alternative mechanism there is a loose association of the nucleophile with the anomeric center as the leaving group departs. The minor amount of α-anomer formed in these reactions most likely arises through the formation of a looser, perhaps solvent-separated, ion pair (SSIP) intermediate, which is in equilibrium with an initial CIP. The function of the torsionally disarming benzylidene group is to oppose rehybridization at the anomeric carbon, thereby shifting the complete set of equilibria toward the covalent triflate and away from the SSIP, thereby minimizing α-glycoside formation.

6.3 Retrosynthetic analysis of target structure and identification of building blocks

The building blocks required for the synthesis of the target structures were identified upon retrosynthetic analysis as shown in Figure 2. In order to elongate the chain along the O4 position, BB10 was modified with the 9-fluorenylmethoxycarbonyl (FMOC) carbonate at this position. Secondly, to establish β-glycosidic linkage, participating protecting group such as trichloroacetamide (TCA) was installed in C2 position of BB10. The mannose building block (BB11) was modified as the benzylidene sulfoxide donor in order to establish the β-mannosidic linkage according to the Crich beta mannosylation method.

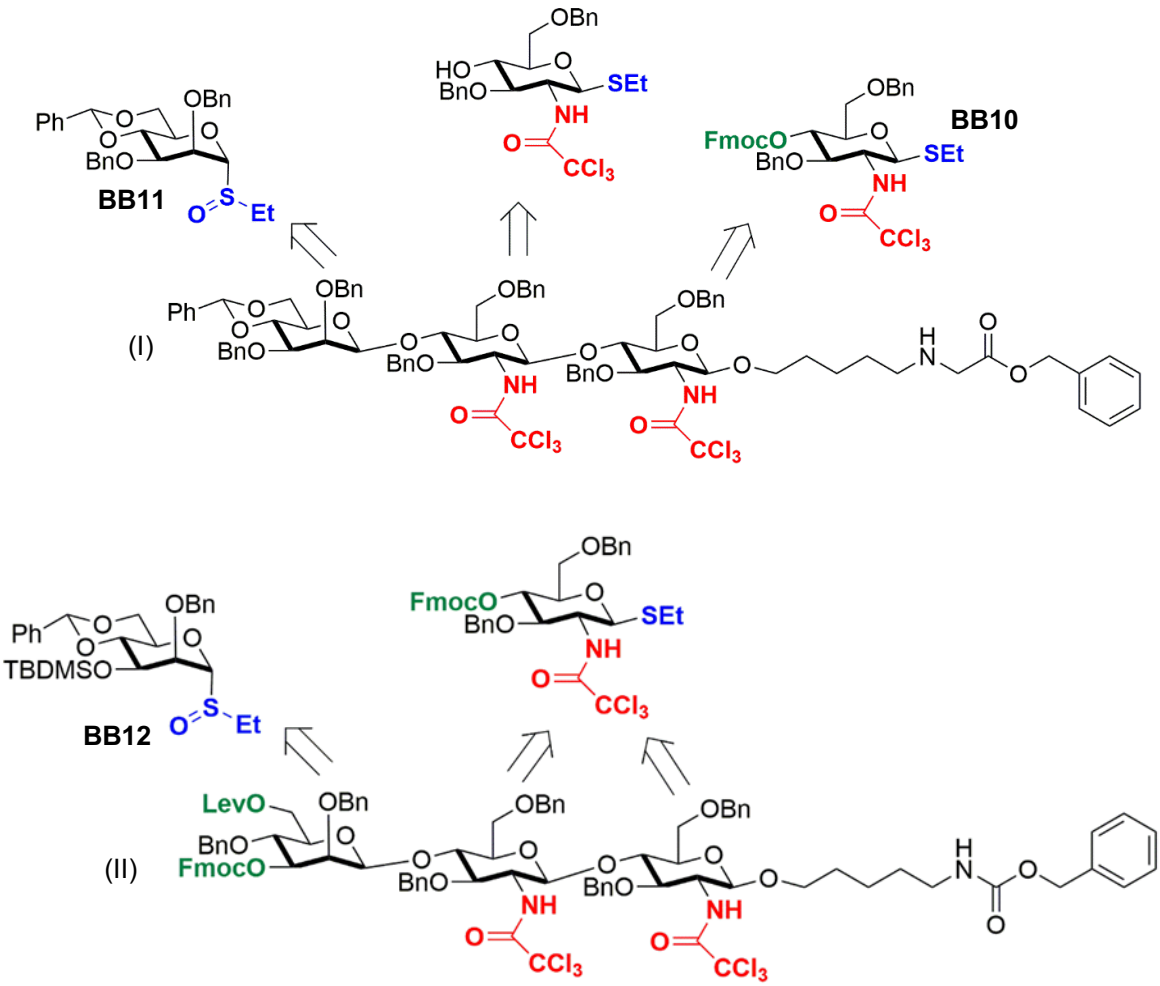
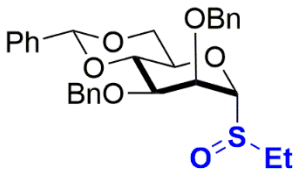


Figure 2: Retrosynthetic analysis of target structure and identification of building blocks (I) retrosynthetic analysis for "proof of concept" study (II) retrosynthetic analysis for extending the N-glycan chain using the Glyconeer

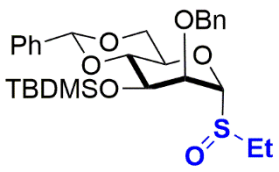
6.4 Synthesis of building blocks

The reaction conditions optimized for the synthesis of building blocks (see Chapter 2 for more details) were applied to procure BB11. The first four steps of the synthetic scheme, namely, peracetylation, thioglycosylation, removal of acetyl groups, and introduction of the benzylidene acetal protecting group could be performed faster due to the ready availability of optimized synthetic protocols (Chapter 2). Similarly, the synthesis of BB10 could be expedited by starting the synthesis from the intermediate **5a**.

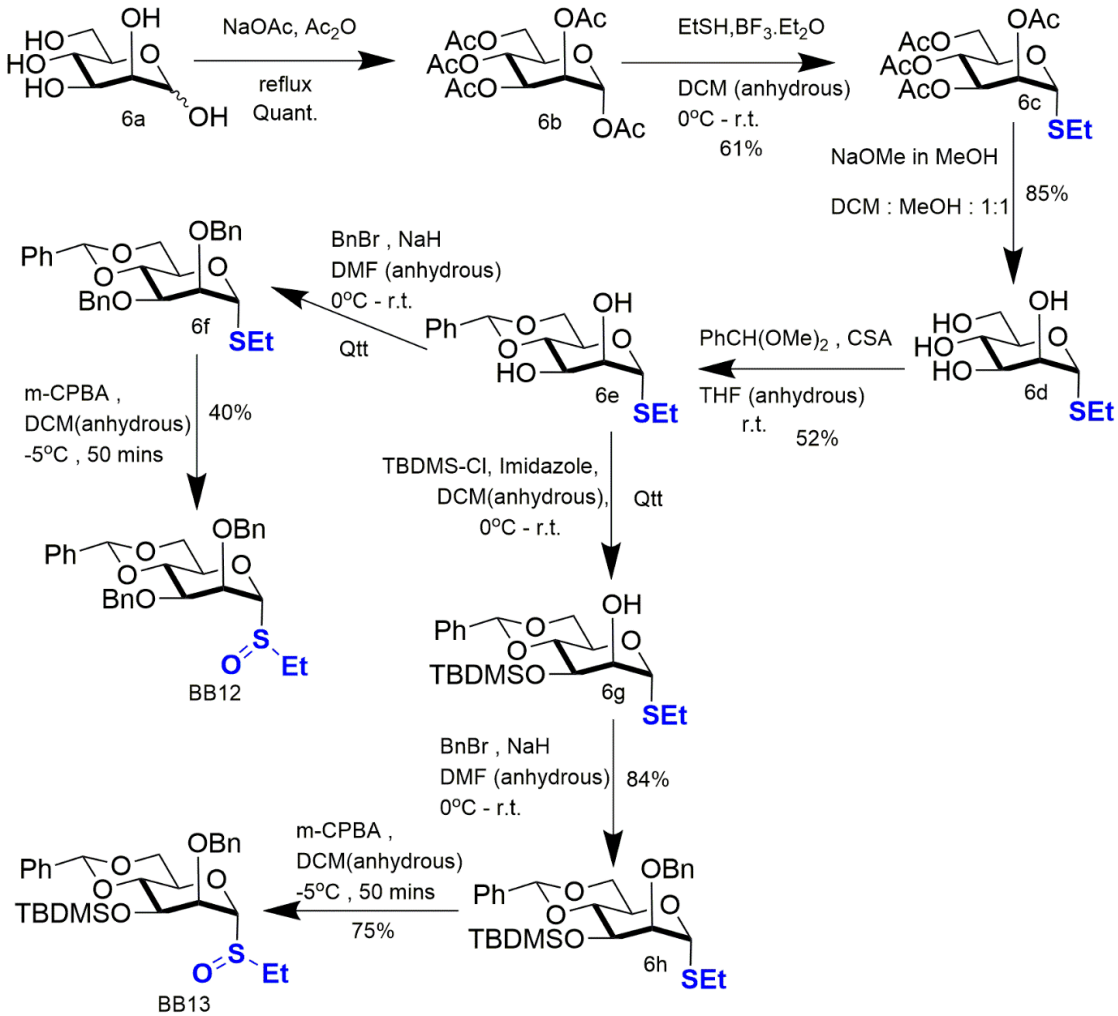
6.4.1 Mannose building block



BB11

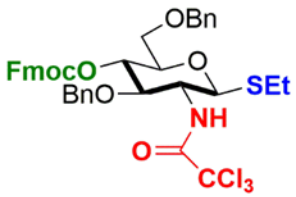


BB12

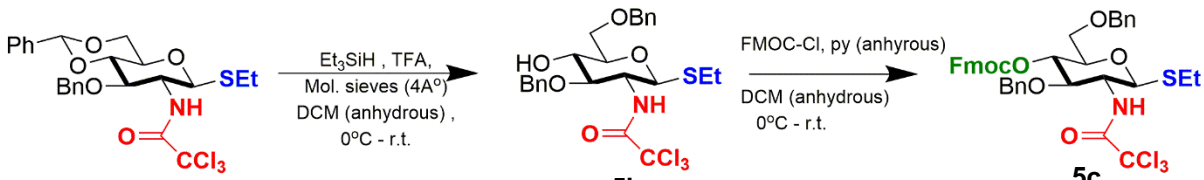


The synthesis of building blocks commenced from the free sugar **6a**, followed by synthesis of the peracetylated intermediate **6b**, that in turn was converted into the thioglycoside **6c**. Then, the acetate groups were removed to afford intermediate **6d**. The C4 and C6 positions were then modified as a benzylidene intermediate **6e**. Subsequently, the C2 and C3 positions were benzylated to obtain intermediate **6f**. Finally, the thioglycoside intermediate was then converted into a sulfoxide donor **BB11**. Secondly, the C3 position of intermediate **6e** was modified as a silyl ether to afford intermediate **6g**, which was the modified as a benzyl ether (OBn) at C2 position to obtain intermediate **6h**. It was then converted into the sulfoxide building block **BB12**. Additionally, in this synthetic scheme, the intermediates could either be crystallized, or used for the next synthetic step without any purification, thereby significantly reducing the time required to procure the final building blocks (**BB11**, **BB12**).

6.4.2 Glucosamine building block



BB10



5a **5b** **5c**

As depicted by the synthetic scheme, the BB10 was synthesized in two steps, thereby enabling faster access to the final building block. This synthetic scheme further highlights the importance of procuring intermediate **5a** in multi-gram quantities, which expedites the synthesis of diverse, differentially protected building blocks.

6.5 Performing solution phase glycosylation reaction to establish beta mannosidic linkage

Initially, the beta mannosylation was attempted via solution phase synthesis as a "proof of concept" to successfully achieve the formation of the beta mannosidic linkage. Since the method of choice was the Crich mannosylation, the mannosyl donor BB11 was glycosylated to the glucosamine acceptor **5b** in the presence of Tf_2O and TTBMP at -78°C. The various parameters associated with the reaction, namely, concentration of activators (Tf_2O, TTBMP), donor and acceptor, as well as the order of adding reagents were varied and their effect on the formation of the beta mannosidic linkage was studied.

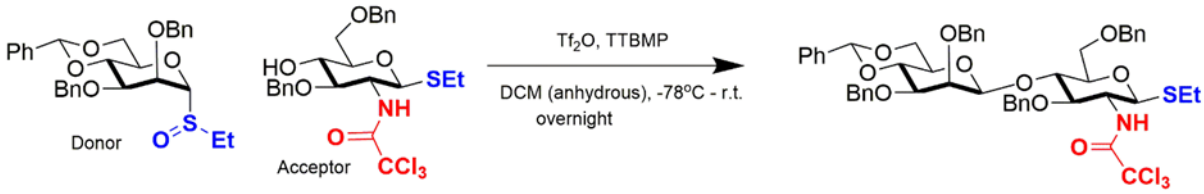


Table 6a: Effect of varying the reaction parameters on the stereoselectivity

Reaction Conditions	α/β Ratio*
Donor (1eq), Acceptor(1 eq), DTBMP(2.eq), Tf_2O (0.65 eq)	No product formed
Donor (1eq), Acceptor(1.6 eq), DTBMP(1.5 eq), Tf_2O (0.65 eq)	2:1
Donor (1eq), Acceptor(2.5eq), DTBMP(1eq), Tf_2O (0.5 eq)	3:1
Donor (1eq), Acceptor(2eq), DTBMP(2.5eq),Tf_2O (0.5 eq)	1:1

Note - * by NMR

The data shown in Table 6a indicate that the variations in reaction conditions were unsuccessful in driving the reaction to afford exclusively beta mannose product. On the contrary, the stereoisomeric ratios obtained mostly yielded the alpha anomer, except in one case, where both the anomers were obtained in 1:1 ratio. Therefore, owing to the ambiguous results obtained upon solution phase glycosylation, the strategy to obtain the N-glycan core structure was modified. Efforts were then

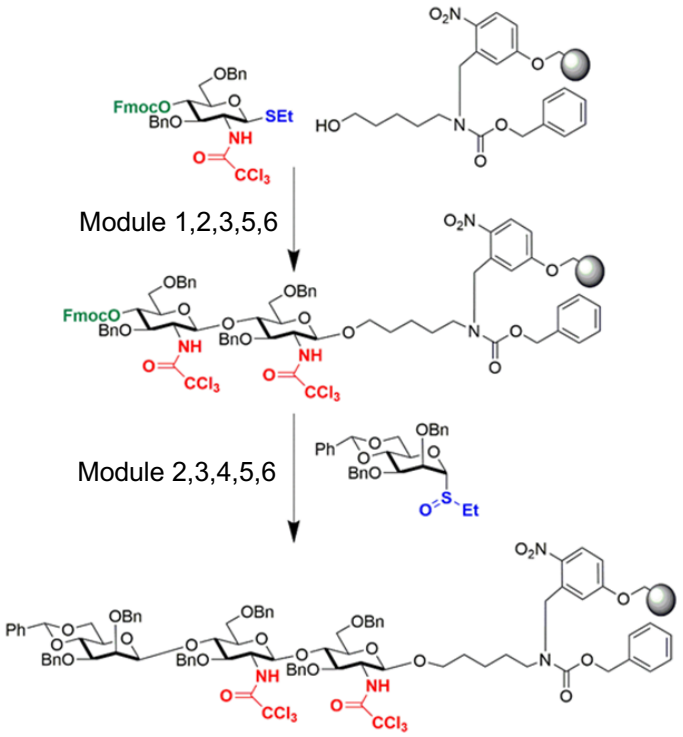
turned towards establishing the beta mannose linkage via automation using the Glyconeer synthesizer in a bid to obtain streoselective formation of the beta mannose linkage.

6.6 Automated glycan assembly of an N-glycan core structure

In order to assemble the N-glycan core structure via automation using the Glyconeer synthesizer, the optimized synthetic modules developed for synthesizing the Lewis antigens and PNAG oligomers (Chapters 3, 4 and 5) were used as described in Table 6b.

Table 6b: Synthetic modules used for automated glycan assembly of N-glycan core structure using the Glyconeer synthesizer

Module no.	Module name	Conditions
1.	Resin swelling	DCM, 25°C, 30 min
2.	Acid wash	TMSOTf in DCM(anhydrous), -20°C
3.	Thioglycoside Glycosylation	BB in DCM (anhydrous), T_1(5 min) to T_2 (20 min)
4.	Sulfoxide Glycosylation	Tf_2O/DTBMP, 1:3 in DCM(anhydrous)
5.	Ac_2O acidic capping	10% Ac_2O, 2% Methanesulfonic acid in DCM(anhydrous)(v/v)
6.	FMOC Deprotection	20% Piperidine in DMF(v/v), 25°C

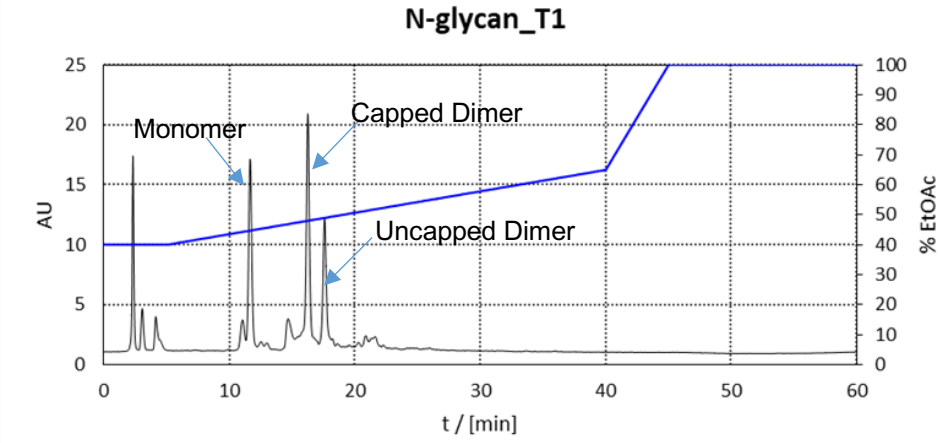


The synthesis was initiated by glycosylating **BB10** to the solid support, followed by capping the deletion sequences. The temporary protecting group, namely, FMOC was cleaved, and BB10 was glycosylated to the C4 position. Thereafter, **BB11** was glycosylated to the C4 position to afford the target structure.

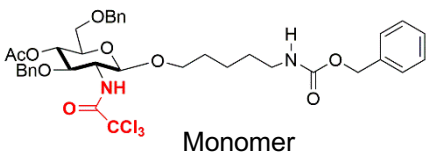
6.6.1 Establishing optimal glycosylation conditions for BB 10 and BB 11

Initially, 6.5 equivalents and one glycosylation cycle was used, and the coupling conditions were modified depending on the results obtained.

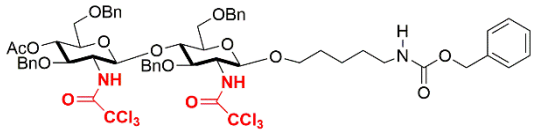
Table 6c: Automated glycan assembly of N-glycan core structure using the Glyconeer synthesizer

Final Analysis (HPLC Trace)	**% Yield**
	No product

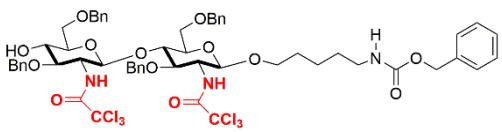
Glycosylation Conditions: GlcNAc (I) : -20°C (5 min) to 0°C (20 min), 6.5 eq, 1x, GlcNAc (II) : -20°C (5 min) to 0°C (20 min), 6.5 eq, 1x, Man: -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x



Monomer



Capped Dimer



Uncapped Dimer

The HPLC trace in Table 6c indicates that the glycosylation conditions used, such as 6.5 equivalents of BB10 with one glycosylation cycle led to inefficient coupling, thereby leading to the formation of a monomer and uncapped dimer side products. It also confirmed that the coupling of BB11 was unsuccessful, thereby affording no product. My efforts were turned towards improving the coupling efficiency of BB10 by increasing the number of glycosylation cycles to two for both rounds of glycosylation for BB10.

Chitin Dimer:

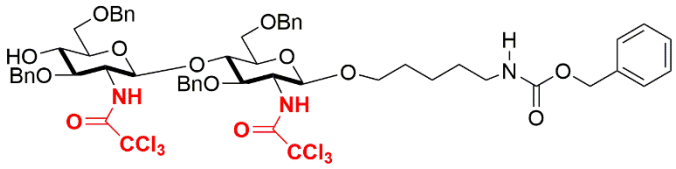
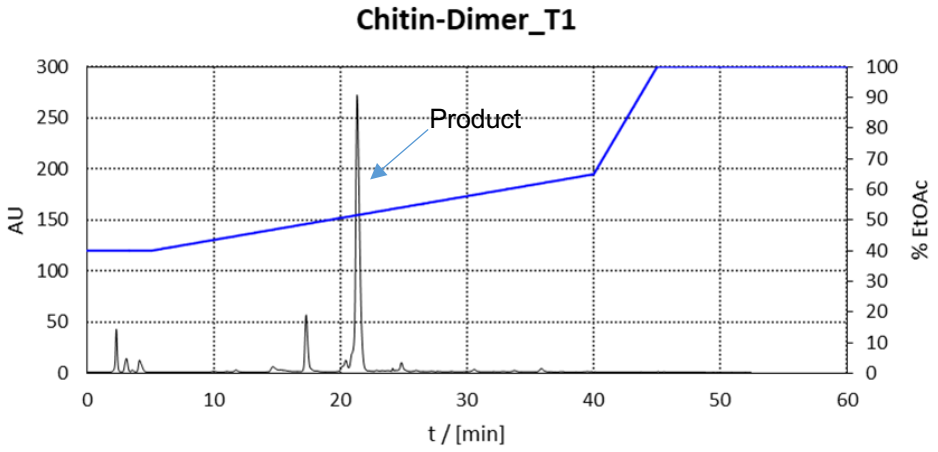


Table 6d: Automated glycan assembly of Chitin dimer

Final Analysis (HPLC Trace)	**%Yield**
	35%

Glycosylation Conditions: GlcNAc(I) : -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x, GlcNAc(II) : -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x

The results obtained in Table 6d indicate that the increased number of glycosylation cycles improved the coupling efficiency of BB10 significantly, which was proved by the absence of any deletion sequence. With optimized glycosylation conditions for BB10 in hand, efforts were then turned towards glycosylating the Mannose sulfoxide BB11. Similar to BB10, the BB11 was glycosylated using the standard conditions, such as 6.5 equivalents and two glycosylation cycles. Another important parameter, namely temperature was expected to play a major role in establishing the beta mannosidic linkage, as proven by the Crich method of beta mannosylation. Therefore, the

activation temperature was set to -60°C, and then adjusted according to the results obtained.

Man-β-1,4-GlcNAc:

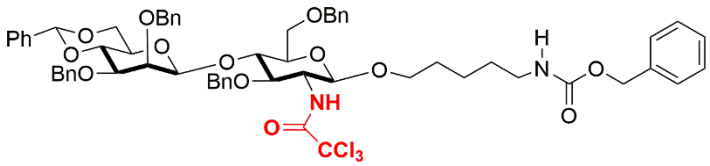
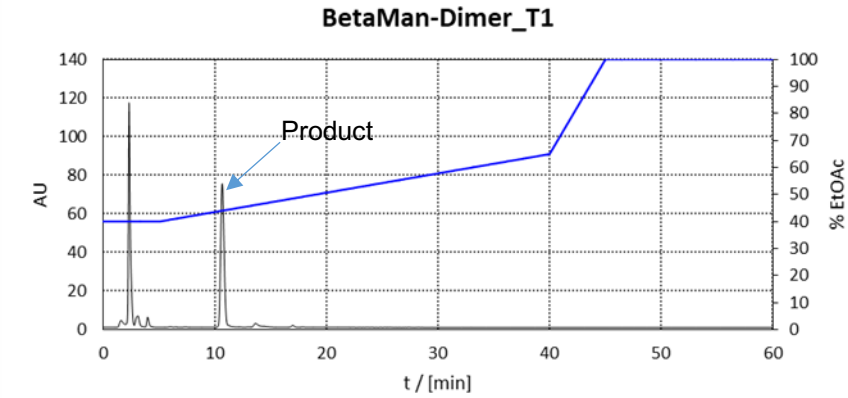


Table 6e: Automated glycan assembly of beta mannose containing dimer via automation using the Glyconeer synthesizer

Final Analysis (HPLC Trace)	**% Yield**
	30%

Glycosylation Conditions: GlcNAc(I):-20°C (5 min) to 0°C (20 min), 6.5 eq, 2x, GlcNAc(II) : -20°C (5 min) to 0°C (20 min), 6.5 eq, 2x, Man: -60°C(5 min to 20 min), 6.5 eq, 2x

The HPLC traces in Table 6e indicate that the synthesis of the beta mannose containing dimer was successful. The ^{1}H NMR of the reaction mixture confirmed the formation of beta mannoside. This finding proved beyond doubt that the glycosylation conditions used for the coupling of the BB11 lead to the successful and stereoselective installation of the beta mannose linkage via automation using the Glyconeer synthesizer.

6.7 Conclusion

The optimized synthetic protocols developed for procuring the building blocks (see Chapter 2 for more details) were successfully applied to obtain BB10 and BB11 in satisfactory yields. The ready availability of optimized protocols ensured faster access to diverse, differentially protected BBs **10-12**. Thereafter, with the building blocks in hand, the beta mannosylation was attempted via solution phase glycosylation, which proved to be unsuccessful in affording a stereoisomeric excess, instead leading to the formation of anomeric mixtures. However, the automated glycan assembly of the beta mannose containing dimer showed promising results. A stereoselective beta mannose anomer could be obtained, without any anomeric mixtures. Thus, with these promising results in hand, the reproducibility of the glycosylation conditions can be established. Secondly, the diverse, differentially protected mannose BB12 can be utilized to extend the N-glycan chain via automation using the Glyconeer synthesizer. Lastly, the results obtained in this chapter open up the possibility of synthesizing the N-glycans, including the beta mannose linkage using the Glyconeer.

Chapter 7

Conclusion and Outlook

Automated glycan assembly has enabled rapid access to a plethora of complex oligosaccharides. The aim of this study was to streamline the entire process of automation in order to access a repertoire of biologically relevant glycans. The first step towards achieving this goal was to get faster access to diverse, differentially protected building blocks. The synthesis of these monomers was streamlined, and optimized protocols were developed, which enabled procuring many building blocks in multigram quantities (as explained in Chapter 2). Additionally, in order to further reduce the time required to obtain the final building blocks, the benzylidene derivatives **1e, 2a** (Chapter 2) were identified as common intermediates that were stocked up in multi-gram quantities, thereby significantly reducing the number of synthetic steps required to access the building blocks, and hence saving time.

With the building blocks in hand, the target glycans were then assembled via automation using the Glyconeer synthesizer. Firstly, optimized synthetic protocols were developed for the automated glycan assembly of the Lewis^x epitope (see Chapter 3 for more details) to obtain the target glycan in excellent yields. Following the successful synthesis of the Lewis^x epitope structure on a 0.0125 mmol scale, the synthesis was then scaled up to a 0.025 mmol scale, wherein it was observed that a successful synthesis could only be achieved by increasing the argon mixing, which further established the importance of effective mixing in scale-up synthesis. These optimized protocols were then employed to obtain the Lewis^a, Lewis^y and Lewis^b antigens via automation (see Chapter 4 for more details). The automated glycan assembly of Lewis^y and Lewis^b epitopes proved to be challenging. However, this problem could be addressed by adding a lactose spacer to the Lewis^y epitope structure, thereby affording the target structure in satisfactory yields.

In order to further expand the repertoire of immunologically relevant glycans using the Glyconeer synthesizer, the N-acetyl-glucosamine oligomers, namely, tetramer, pentamer and hexamer were synthesized via automation with satisfactory yields (see Chapter 5 for more details). The successful synthesis of these oligomers not only

proved the reproducibility of the synthetic protocols developed for the AGA of complex glycans, but also validated the Glyconeer for synthesizing long structures.

Since the PNAGs are known to be important constituents in the biofilm matrix of *Staphylococci* and *Pseudomonas* species, the PNAG oligomers accessed via AGA were then used to characterise the substrate specificity of the glycosyl hydrolase PaO1 from *Pseudomonas aeruginosa* by Isothermal Titration Calorimetry (ITC). The experiments revealed that the PNAG tetramer and hexamer exhibited similar affinity towards the PaO1 enzyme. This observation indicated a saturation in the subsites of the enzyme towards homo-GlcNAc oligomers. Therefore, it could be speculated that deacetylated glucosamine moieties within the oligomer might lead to increased binding, which further opens up the possibility of synthesizing co-polymers containing different patterns of acetylated and deacetylated glucosamine (ABAB, AABB) oligomers in order to further expand the library of PNAG oligomers for evaluating binding affinities towards PaO1 enzyme.

Finally, in a bid to push the boundaries of AGA, the beta mannosidic linkage, which is known to be one of the most difficult glycosidic linkages to establish via synthetic carbohydrate chemistry was attempted via automation using the Glyconeer synthesizer. The successful synthesis of the beta mannose via AGA leads to the possibility of synthesizing the highly branched N-linked glycans (see Chapter 6 for more details), thereby providing rapid access to these glycans.

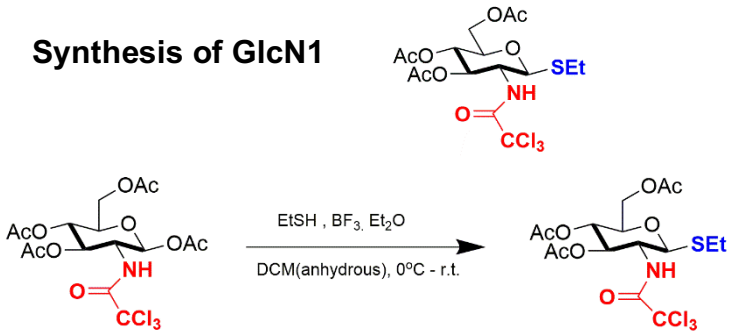
Experimental Section

8.1 General Methods and Materials

All reagents and solvents were acquired from commercial sources, unless stated otherwise. The resin equipped with a photocleavable linker (loading 0.40 mmol/g) was obtained from GlycoUniverse stock. 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) ,Varian 600 (Agilent) at 600 MHz (^{1}H) and 150 MHz (^{13}C), or an Ascend 700 (Bruker) at 700 MHz (^{1}H) and 176 MHz (^{13}C). $CDCl_3$ was used as solvent 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. ESI-HRMS were performed with a Xevo G2-XS Q-Tof (Waters). HPLCs were performed on Agilent 1200 Series systems, and analysed using YMC-Diol-300 column, 150 X 4.6 mm, ELSD Detector and DAAD, 280 nm.

8.2 Experimental procedures for synthesis of building blocks

I. Synthesis of GlcN1



Procedure: This procedure maintained an inert atmosphere throughout. To 45 mmol of compound **1b** (22.1 g) 50 mL anhydrous dichloromethane (final concentration ~ 0.9 M) were added under argon atmosphere and placed on an ice bath to cool down to 0 °C. Then, 50 mmol ethanethiol (3.7 mL, 1.1 eq.) were added via a syringe followed by dropwise addition of 33 mmol $BF_3 \cdot Et_2O$ (4.2 mL, 0.7 eq.) via a dropping funnel. The reaction was then allowed to stir at 0 °C for another 10 minutes, then removed from the ice bath and allowed to continue stirring for another 12 hours (overnight). The next day, the reaction mixture was poured into 250 mL water and kept to stir for some time. The organic phase was then separated, washed with 250 mL saturated aqueous $NaHCO_3$ (2x) and 250 mL brine (2x) and dried over $MgSO_4$ before it was concentrated and dried under high vacuum.

Purification: The crude material (22.0 g) was dissolved in ethyl acetate (~ 100 mL) at 50 °C. When all of it dissolved, it was poured into a 1 L Erlenmeyer flask and, while stirring, hexane (~ 800 mL) was slowly added dropwise via a dropping funnel until precipitation started. The remainder volume of hexane was then quickly added under vigorous stirring. The precipitate so obtained was kept in the fridge overnight. The next day, the precipitate was filtered and washed with cold hexane to give 16.5 g of pure compound **1c** after drying.

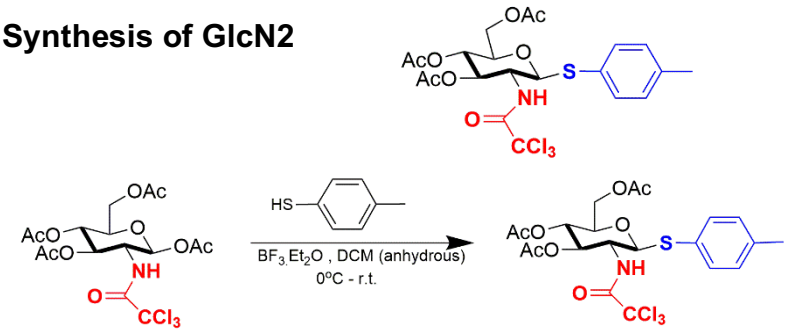
% Yield = 88

Analysis: TLC: R_f = 0.5 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ= 7.08 (d, *J* = 9.3 Hz, 1H, -NHCO), 5.39 – 5.32 (m, 1H, H-3), 5.10 (t, *J* = 9.7 Hz, 1H, H-4), 4.68 (d, *J* = 10.3 Hz, 1H, H-1), 4.23 (dd, *J* = 12.3, 5.3 Hz, 1H, H-6a), 4.17 – 4.09 (m, 1H, H-2, H-6b), 3.77 (ddd, *J* = 10.0, 5.2, 2.4 Hz, 1H, H-5), 2.77 – 2.65 (m, 2H, -SCH2), 2.06, 2.01 (d, *J* = 2.5 Hz, 6H, 3x-OAc), 1.25 (t, *J* = 7.4 Hz, 3H, $-CH_3$).

^{13}C NMR (176 MHz, $CDCl_3$) δ=171.21, 170.78, 169.31, 162.02 (4x C=O), 83.87 (C-1), 76.14 (C-5), 73.27 (C-3), 68.60 (C-4), 62.46 (C-6), 54.73 (C-2), 24.36 ($COCH_3$), 20.84 ($COCH_3$), 20.72 ($COCH_3$), 20.67(SCH_2), 14.98($-CH_3$).

II. Synthesis of GlcN2



Procedure: This procedure maintained an inert atmosphere throughout. In a 250 mL round bottom flask, 21 mmol of 1a (10g) were flushed with argon for several minutes before adding 23 mL anhydrous DCM (final concentration: ~ 0.9M). The reaction flask was then placed in an ice bath to cool down to 0°C. Then, p-toluylthiol (22 mmol, 3g) was added, followed by dropwise addition of $BF_3.Et_2O$ (16mmol, 2mL) via a dropping funnel. After the addition, the reaction was left to stir at room temperature for ~ 12 hours. Then, the reaction mixture was diluted with DCM (~ 100mL) and poured into a separating funnel. It was extracted with H_2O (~ 90 mL, 1x), Sat. aq. $NaHCO_3$ (~ 90 mL, 2x) and Brine (~90 mL, 1x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Purification: The crude material was dissolved in ethyl acetate (~ 25 mL) at 50°C on a heating mantle. Then, Hexane (~350 mL) was first added dropwise until precipitation starts, and then quickly under rigorous stirring. The precipitate so obtained was kept in the fridge overnight to induce more precipitation. Then, the precipitate was washed with cold Hexane to give 10g pure **GlcN2**.

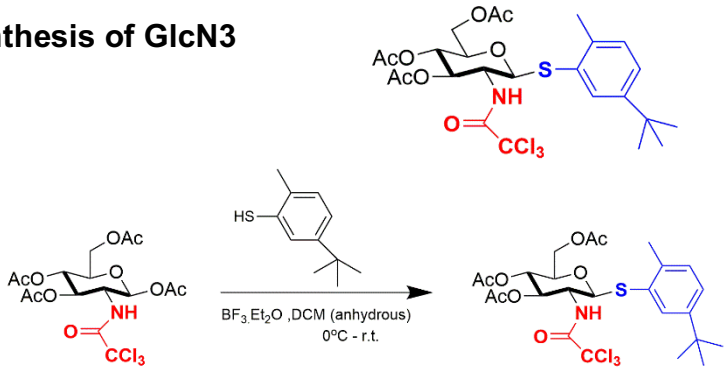
% Yield = 80

Analysis: TLC: R_f = 0.5 (Hex/EA 2:1)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.38 (m, 2H,-Ar), 7.11 (d, *J* = 3.1 Hz, 1H,-Ar), 7.10 – 7.08 (m, 1H), 5.35 (t, *J* = 9.9 Hz, 1H,H-3), 5.04 (t, *J* = 9.8 Hz, 1H,H-4), 4.76 (d, *J* = 10.4 Hz, 1H,H-1), 4.22 (dd, *J* = 12.3, 5.2 Hz, 1H,H-6a), 4.17 (dd, *J* = 12.3, 2.6 Hz, 1H,H-6b), 4.01 (q, *J* = 10.2 Hz, 1H,H-2), 3.74 (ddd, *J* = 10.1, 5.2, 2.6 Hz, 1H,H-5), 2.34 (s, 3H,-SCH_3), 2.08 (s, 3H,-$COCH_3$), 2.00 (s, 3H,-$COCH_3$), 1.88 (s, 3H,-$COCH_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 171.25 (OC=O), 170.68(OC=O), 169.27(OC=O), 161.74(OC=O), 139.19(-Ar), 134.18(-Ar), 129.87(-Ar), 127.66(-Ar), 92.38(CCl_3), 86.72(C-1), 76.04, 73.33, 68.43, 62.39, 54.41(C-2), 21.29(-CH_3), 20.67(-CH_3), 20.45(-CH_3).

III. Synthesis of GlcN3



Procedure: This procedure maintained an inert atmosphere throughout. In a 100 mL 3-necked round bottom flask, 22 mmol of **1a** (11g) were flushed with argon for several minutes before adding 26 mL anhydrous DCM (final concentration: ~ 0.9M). The reaction flask was then placed in an ice bath to cool down to 0°C. 5-tert-butyl-2-methyl-phenyl thiol (24 mmol, 4.4 mL) was added, followed by dropwise addition of $BF_3.Et_2O$ (16.4 mmol, 2 mL) via a dropping funnel. After the addition, the reaction was removed from the ice bath and left to stir at room temperature for ~ 12 hours. Then, the reaction mixture was diluted with EA (~100mL) and poured into a separating funnel. It was then extracted with H_2O (~ 75 mL, 1x) Sat. aq. $NaHCO_3$ (~ 75 mL, 2x) and Brine (~ 75 mL, 2x). The combined organic layer was dried with $MgSO_4$, concentrated and dried under high vacuum.

Purification: The crude material was dissolved in Ethyl acetate (~ 30 mL) at 50°C on a heating mantle. When all of it dissolved, Hexane (~ 150 mL) was first added dropwise until precipitation started, and then quickly under rigorous stirring. The precipitate so obtained was then kept in the fridge overnight, and then washed and flitered with cold Hexane to give 10g of pure **GlcN 3**.

% Yield = 58

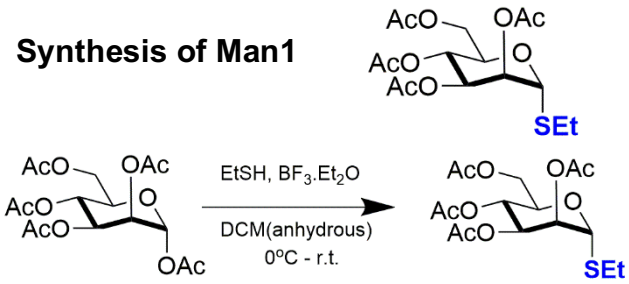
Analysis: TLC: R_f = 0.6 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.59 (d, *J* = 2.2 Hz, 1H), 7.25 (d, *J* = 9.5 Hz, 1H, -NHCO), 7.24 (d, *J* = 2.1 Hz, 1H), 7.13 (dd, *J* = 7.9, 0.9 Hz, 1H), 5.37 (dd, *J* = 10.4, 9.4 Hz, 1H,H-3), 5.10 (t, *J* = 10.5, 8.7 Hz, 1H,H-4), 4.73 (d, *J* = 10.5 Hz, 1H,H-1), 4.24 (dd, *J* = 12.3, 5.2 Hz, 1HH-6a), 4.18 (td, *J* = 10.4, 9.3 Hz, 2H,H-2), 4.11 (dd, *J* = 12.3, 2.2 Hz, 1H,H-6b), 3.71 (ddd, *J* = 10.1, 5.2, 2.2 Hz, 1H,H-5), 2.38 (s, 3H,-SCH_3), 2.03 (d, *J* = 20.2 Hz, 6H,-$COCH_3$), 1.75 (s, 3H,-$COCH_3$), 1.28 (s, 9H,-$C(CH_3)_3$).

^{13}C NMR (176 MHz, $CDCl_3$) δ 171.40(OC=O), 170.63(OC=O), 169.08(OC=O), 161.83(OC=O), 149.76(-Ar), 137.94(-Ar), 131.60(-Ar), 131.21(-Ar), 130.16(-Ar), 126.11(-Ar),

87.76, 77.24, 77.06, 76.88, 75.95, 73.43, 68.33, 62.43, 54.53(C-2), 31.33(-C(CH_3)$_3$, 20.73(-SCH_3), 20.49(-CH_3), 20.12(-CH_3).

IV. Synthesis of Man1



Procedure: This procedure maintained an inert atmosphere throughout. To 17mmol of peracetylated mannose (6.6g) were added 39 mL anhydrous DCM (final concentration: ~ 0.4M) under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, EtSH (3mmol, 2.5 mL) was added followed by dropwise addition of $BF_3.Et_2O$ (42mmol, 5.2mL). Then, the reaction was left to stir at room temperature for ~ 12 hours (overnight). Then, the reaction mixture was diluted with DCM (~ 85mL) and poured into a separating funnel. It was then extracted with Sat. aq. $NaHCO_3$ (~ 100 mL, 2x) and Brine (~ 100 mL, 2x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Purification: The crude material was dissolved in Ethyl Acetate (~ 20 mL) at 50°C on a heating mantle. When all of it dissolved, Hexane (~100mL) was first added dropwise at room temperature until precipitation starts, and then quickly under rigorous stirring. The precipitate so formed was kept in the fridge overnight to induce more precipitation. Then, it was washed and filtered with cold Hexane to give 4.2 g of pure **Man1**.

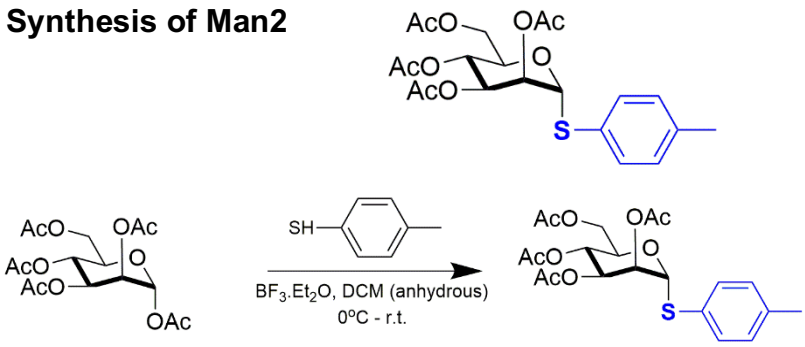
% Yield = 63

Analysis: TLC: R_f = 0.5 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 5.33 – 5.28 (m, 2H, H-3, H-4), 5.27 (d, *J* = 1.8 Hz, 1H,H-1), 5.24 (dd, *J* = 10.0, 3.3 Hz, 1H,H-5), 4.38 (dddd, *J* = 9.8, 5.4, 2.4, 0.7 Hz, 1H,H-2), 4.30 (dd, *J* = 12.3, 5.4 Hz, 1H,H-6a), 4.08 (dd, *J* = 12.2, 2.4 Hz, 1H,H-6b), 2.68 – 2.57 (m, 2H,-SCH_2), 2.15 (s, 3H,-$COCH_3$), 2.07 (s, 3H,-$COCH_3$), 2.03 (s, 3H,-$COCH_3$), 1.97 (s, 3H,-$COCH_3$), 1.29 (t, *J* = 7.4 Hz, 3H,-SCH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ 170.69(OC=O), 170.08(OC=O), 169.87(OC=O), 169.83(OC=O), 82.38(C-1), 71.28(C-3), 69.59(C-2), 69.02(C-4), 66.49(C-5), 62.54(C-6), 25.56(-SCH_2), 21.02(-$COCH_3$), 20.79(-$COCH_3$), 20.72(-$COCH_3$), 14.85(-SCH_3).

V. Synthesis of Man2



Procedure: This procedure maintained an inert atmosphere throughout. To 25 mmol of pentaacetate mannose (9.6g), were added 61 mL anhydrous DCM (final concentration: ~ 0.4 M) under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, p-toluylthiol (49 mmol, 6.1 g) were added followed by dropwise addition of $BF_3.Et_2O$ (61mmol, 7.6mL). Then, the reaction was allowed to stir at room temperature for ~ 12 hours. Then, the reaction was diluted with DCM (~ 100 mL) and poured into a separating funnel. It was then extracted with Sat. aq. $NaHCO_3$ (~ 100 mL, 2x) and Brine (~ 100mL, 2x). The combined organic layer was dried with $MgSO_4$, concentrated and dried under high vacuum.

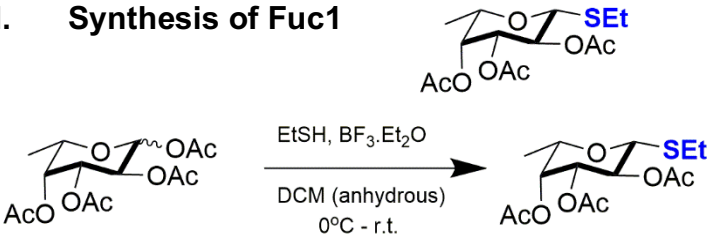
% Yield = 60

Analysis: TLC: R_f = 0.6 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.38 – 7.36 (m, 2H,-Ar), 7.13 – 7.11 (m, 2H,-Ar), 5.49 (dd, *J* = 2.8, 1.6 Hz, 1H,H-2), 5.41 (d, *J* = 1.6 Hz, 1H,H-1), 5.32 (dd, *J* = 3.8, 1.1 Hz, 2H,H-4,H-3), 4.60 – 4.52 (m, 1H,H-5), 4.29 (dd, *J* = 12.2, 5.9 Hz, 1H,H-6a), 4.10 (dd, *J* = 12.3, 2.4 Hz, 1H,H-6b), 2.32 (s, 3H,-$COCH_3$), 2.14 (s, 3H,-SCH_3), 2.07 (s, 3H,-$COCH_3$), 2.05 (s, 3H,-$COCH_3$), 2.01 (s, 3H,-$COCH_3$).

^{13}C NMR (176 MHz, $CDCl_3$) δ 170.59 (OC=O)), 169.97(OC=O), 169.86(OC=O), 169.80(OC=O), 132.71(-Ar), 130.04(-Ar), 129.93(-Ar), 129.88(-Ar), 86.10(C-1), 70.96, 69.52, 69.46, 66.51, 62.59, 21.21(-SCH_3), 20.99(-$COCH_3$), 20.95(-$COCH_3$), 20.79(-$COCH_3$), 20.72(-$COCH_3$).

VI. Synthesis of Fuc1



Procedure: This procedure maintained an inert atmosphere throughout. To 11.5mmol of peracetylated fucose (3.8g), 58mL anhydrous DCM (final concentration: ~ 0.2M) were added under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, EtSH (13mmol, 1mL) were added followed by dropwise addition of $BF_3.Et_2O$ (15mmol, 2mL). Then, the reaction was left to stir at room temperature for ~ 12 hours. Then, the reaction was quenched with Sat. aq. $NaHCO_3$ (~ 80mL) and stirred for a while. It was then poured into a separating funnel and the organic phase was extracted. The aqueous phase was then extracted with DCM (~80mL, 3x). The combined organic phase was then washed with Brine (~80mL, 3x). It was then dried with $MgSO_4$, concentrated and dried under high vacuum.

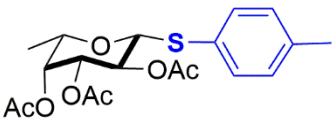
% Yield = 60

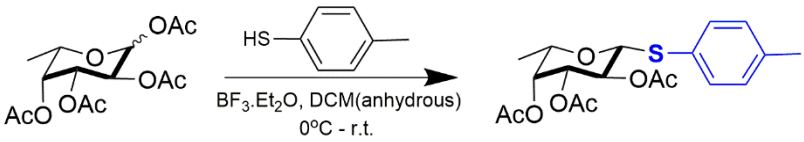
Analysis: TLC: R_f = 0.6 (Hex/EA 2:1)

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.25 (dd, *J* = 3.4, 1.1 Hz, 1H,H-4), 5.20 (t, *J* = 9.9 Hz, 3H), 5.03 (dd, *J* = 10.0, 3.5 Hz, 1H), 4.44 (d, *J* = 9.9 Hz, 1H), 3.80 (qd, *J* = 6.4, 1.1 Hz, 1H), 2.81 – 2.63 (m, 3H), 2.15 (s, 3H,-OCOCH$_3$), 2.04 (s, 3H,-OCOCH$_3$), 1.96 (s, 3H), 1.27 (t, 3H), 1.20 (d, *J* = 6.5 Hz, 3H,-CH$_3$).

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.76(OC=O), 170.25(OC=O), 169.78(OC=O), 83.63(C-1), 73.29, 72.47, 70.59, 70.57, 67.44, 24.23, 20.96, 20.81, 20.74, 16.53, 14.85.

VII. Synthesis of Fuc 2





Procedure: This procedure maintained an inert atmosphere throughout. To 11.5 mmol of peracetylated fucose (3.8g), 58 mL anhydrous DCM (final concentration: ~ 0.2 M) were added under argon atmosphere, and placed in an ice bath to cool down to 0°C. Then, p-toluylthiol (13mmol, 1.5g) were added followed by dropwise addition of $BF_3.Et_2O$ (15mmol, 1.85mL). Then, the reaction was allowed to stir at room temperature for ~ 12 hours. The reaction mixture was then quenched with Sat. aq. $NaHCO_3$ (~80 mL) and stirred for a while. Then, it was poured into a separating funnel, and the organic phase was extracted. The aqueous phase was extracted with DCM (~ 80mL, 2x). The combined organic phase was extracted with Brine (~80 mL, 2x). It was then dried with $MgSO_4$, concentrated and dried under high vacuum.

Purification: The crude material was dissolved in Ethyl acetate (~ 10 mL) at 50°C on a heating mantle. When all of it dissolved, Hexane (~ 80 mL) was added at room temperature under constant stirring. The precipitate so obtained was kept in the fridge. It was then washed and filtered with cold hexane to give 1.1 g of pure **Fuc2.**

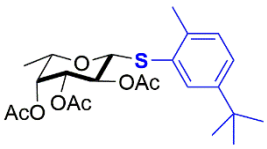
% Yield = 50

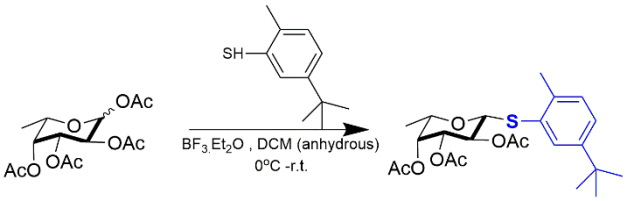
Analysis: TLC: R_f = 0.78 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ= 7.42 – 7.39 (m, 2H, -Ar), 7.13 – 7.11 (m, 2H, -Ar), 5.24 (ddd, *J* = 3.3, 1.1, 0.4 Hz, 1H, H-4), 5.22 – 5.16 (m, 1H, H-2), 5.03 (dd, *J* = 10.0, 3.4 Hz, 1H, H-3), 4.63 (d, *J* = 10.0 Hz, 1H, H-1), 3.80 (qd, *J* = 6.4, 1.1 Hz, 1H, H-5), 2.34 (s, 3H, $-SCH_3$), 2.13 , 2.08 , 1.96 (s, 3H, 3xOAc), 1.22 (d, *J* = 6.5 Hz, 3H, $-CH_3$).

^{13}C NMR (176 MHz, $CDCl_3$) δ = 170.90, 170.41, 169.77 (3x C=O), 138.50, 133.22, 129.91, 129.37 (4x -Ar), 87.14 (C-1), 73.42 (C-3), 72.77 (C-5), 70.67 (C-4), 67.73 (C-2), 21.46, 21.19, 20.97 (3x $–OCOCH_3$), 20.94 ($-SCH_3$), 16.78 ($-CH_3$).

VIII. Synthesis of Fuc3





Procedure: This procedure maintained an inert atmosphere throughout. To 11.5 mmol of peracetylated fucose (3.8g), 58 mL anhydrous DCM (final concentration: ~0.2 M) were added under argon atmosphere, and placed in an ice bath to cool down to 0°C. Then, 5-tert-butyl-2-methyl-phenyl thiol (13mmol, 2.3 mL) were added followed by dropwise addition of $BF_3.Et_2O$ (15mmol, 1.85mL). Then, the reaction was allowed to stir at room temperature for ~ 12 hours. The reaction mixture was then quenched with Sat. aq. $NaHCO_3$ (~80 mL) and stirred for a while. Then, it was poured into a separating funnel, and the organic phase was extracted. The aqueous phase was extracted with DCM (~ 80mL, 2x). The combined organic phase was extracted with Brine (~80 mL, 2x). It was then dried with $MgSO_4$, concentrated and dried under high vacuum.

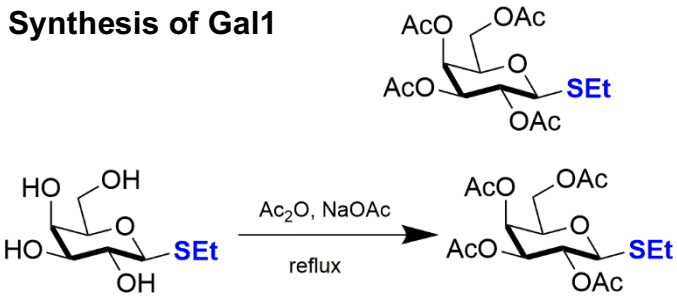
% Yield = 65

Analysis: TLC: R_f = 0.6 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.59 (d, *J* = 2.2 Hz, 2H,-Ar), 7.24 (d, *J* = 2.1 Hz, 2H,-), 7.13 (dd, *J* = 7.9, 0.9 Hz, 1H), 5.37 (dd, *J* = 10.4, 9.4 Hz, 1H,H-3), 5.10 (t, *J* = 10.5, 8.7 Hz, 1H,H-4), 4.73 (d, *J* = 10.5 Hz, 1H,H-1), 4.24 (dd, *J* = 12.3, 5.2 Hz, 1HH-6a), 4.18 (td, *J* = 10.4, 9.3 Hz, 2H,H-2), 2.38 (s, 3H,-SCH_3), 2.03 (d, *J* = 20.2 Hz, 6H,-$COCH_3$), 1.75 (s, 3H,-$COCH_3$), 1.28 (s, 9H,-$C(CH_3)_3$).

^{13}C NMR (176 MHz, $CDCl_3$) δ 171.40(OC=O), 170.63(OC=O), 169.08(OC=O), 161.83(OC=O), 149.76(-Ar), 137.94(-Ar), 131.60(-Ar), 131.21(-Ar), 130.16(-Ar), 126.11(-Ar), 87.76(C-1), 77.24, 77.06, 76.88, 75.95, 73.43, 68.33, 62.43, 54.53, 31.33, 20.73, 20.49, 20.12.

IX. Synthesis of Gal1



Procedure: This procedure maintained an inert atmosphere throughout. In a 100 mL 3-necked round bottom flask, NaOAc () and Ac_2O () were added and refluxed at 155^0C for ~ 5 minutes. Then, the temperature was reduced to 120^0C, and peracetylated mannose was added portion wise (23mmol, 5g). The reaction was allowed to stir at 120^0C for ~ 10 mins, and then allowed to cool down to room temperature and stir for ~ 30 minutes. Then, ice was added into the reaction mixture, and stirred at r.t. for a while. The yellow solid so obtained was diluted with DCM (~ 50 mL) poured into a separating funnel, and the organic phase was extracted with Sat. aq. $NaHCO_3$ (~ 50 mL,2x). The combined organic phase was then extracted with Brine (~80 mL,2x). It was then dried with $MgSO_4$, concentrated and dried under high vacuum.

Purification: The crude material was dissolved in EA (~ 50 mL) at 50°C. When all of it dissolved, Hex (~ 100 mL) was added first dropwise until precipitation starts, and then quickly under rigorous stirring. The precipitate so obtained was then washed and filtered with cold Hexane.

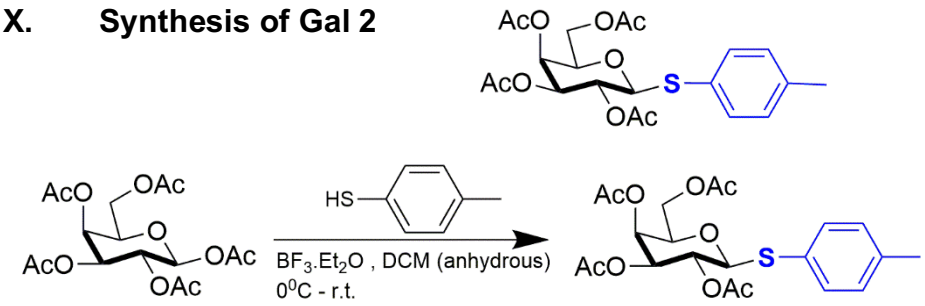
% Yield = 72

Analysis: TLC: R_f = 0.5 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 5.38 (dd, *J* = 3.4, 1.2 Hz, 1H,H-4), 5.19 (t, *J* = 10.0 Hz, 1H,H-2), 5.01 (dd, *J* = 10.0, 3.4 Hz, 1H,H-3), 4.46 (d, *J* = 10.0 Hz, 1H,H-1), 4.12 (dd, *J* = 11.3, 6.7 Hz, 1H,H-6a), 4.07 (dd, *J* = 12.1, 7.0 Hz, 1H,H-6b), 3.90 (td, *J* = 6.7, 1.2 Hz, 1H,H-5), 2.75 – 2.62 (m, 2H,-SCH_2), 2.11 (s, 3H,-$OCOCH_3$), 2.02 (s, 3H,-$OCOCH_3$), 2.00 (s, 3H,-$OCOCH_3$), 1.94 (s, 3H,-$OCOCH_3$), 1.24 (t, *J* = 7.5 Hz, 3H,-SCH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ 170.38(OC=O), 170.24(OC=O), 170.07(OC=O), 169.58(OC=O), 84.08(C-1), 74.43(C-5), 72.07, 71.96(C-3), 67.35(C-4), 67.27(C-2), 61.53(C-6), 24.39(-SCH_2), 20.85(-$COCH_3$), 20.70(-$COCH_3$), 20.62(-$COCH_3$), 14.92(-SCH_3).

X. Synthesis of Gal 2



Procedure: This procedure maintained an inert atmosphere throughout. In a 100 mL 3-necked round bottom flask, _ mmol _ (10.6g) was purged with argon for several minutes before adding 23 mL anhydrous DCM (final concentration: ~ 0.9M). Then, the reaction was placed in an ice bath to cool down to 0°C. Then, p-toluylthiol () was added into it, followed by dropwise addition of $BF_3.Et_2O$ (). After the addition, the flask was removed from the ice bath and allowed to stir at room temperature for ~ 12 hours. The reaction mixture was then diluted with DCM (~ 100 mL) and poured into a separating funnel. The organic phase was then extracted with Sat. aq. $NaHCO_3$ (~ 100 mL, 2x) and Brine (~ 100 mL, 2x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Purification: The crude material was dissolved in EA (~ 25 mL) at 40^0C on a heating mantle. When all of it dissolved, the flask was removed from the heating mantle, and hexane (~ 100 mL) was added dropwise until precipitation started and then quickly under rigorous stirring at room temperature. The precipitate so obtained was then kept in the fridge overnight. Then, it was washed and filtered with cold Hexane to give _g of pure **Gal2**.

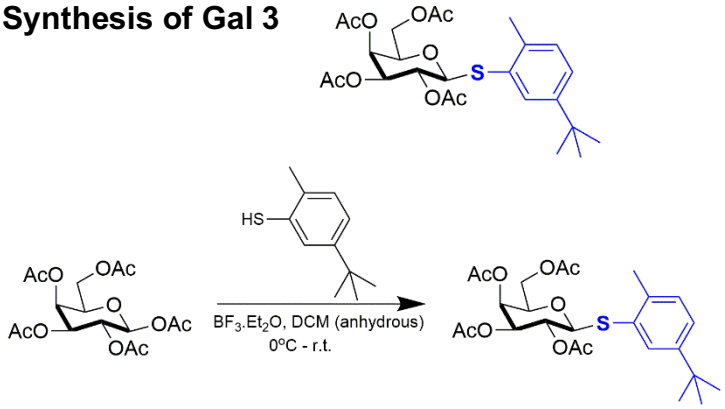
% Yield = 85

Analysis: TLC: R_f = 0.6 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.41 – 7.36 (m, 2H,-Ar), 7.13 – 7.08 (m, 2H,-Ar), 5.38 (dq, J = 2.7, 1.3 Hz, 1H,H-4), 5.19 (t, J = 10.0, 9.1 Hz, 1H,H-2), 5.02 (dd, J = 10.0, 4.9 Hz, 1H,H-3), 4.63 (d, J = 10.0 Hz, 1H,H-1), 4.16 (ddt, J = 11.4, 6.9, 1.4 Hz, 1H,H-6a), 4.12 – 4.03 (m, 3H), 3.89 (ddt, J = 6.6, 5.1, 1.2 Hz, 1H,H-5), 2.32 (d, J = 2.8 Hz, 3H,-SCH_3), 2.15 – 2.12 (m, 3H), 2.09 (d, J = 1.1 Hz, 3H), 2.09 – 2.06 (m, 5H), 2.02 (d, J = 1.1 Hz, 5H), 1.96 – 1.93 (m, 3H).

^{13}C NMR (176 MHz, $CDCl_3$) δ 138.51(-Ar), 133.21(-Ar), 129.70(-Ar), 128.69(-Ar), 89.78(C-1), 86.99, 74.43, 72.10, 68.83, 61.66, 61.31, 21.23, 20.93, 20.73, 20.70, 20.66.

XI. Synthesis of Gal 3



Procedure: This procedure maintained an inert atmosphere throughout. To 10 mmol of peracetylated galactose (4g), 51 mL anhydrous DCM (final concentration: ~ 0.2 M) were added under argon atmosphere, and placed in an ice bath to cool down to 0°C. Then, 5-tert-butyl-2-methyl-phenyl thiol (14mmol, 2.6mL) were added followed by dropwise addition of $BF_3.Et_2O$ (17mmol, 2.1mL). Then, the reaction was allowed to stir at room temperature for ~ 12 hours. The reaction mixture was then quenched with Sat. aq. $NaHCO_3$ (~80 mL) and stirred for a while. Then, it was poured into a separating funnel, and the organic phase was extracted. The aqueous phase was extracted with DCM (~ 80mL, 2x). The combined organic phase was extracted with Brine (~80 mL, 2x). It was then dried with $MgSO_4$, concentrated and dried under high vacuum.

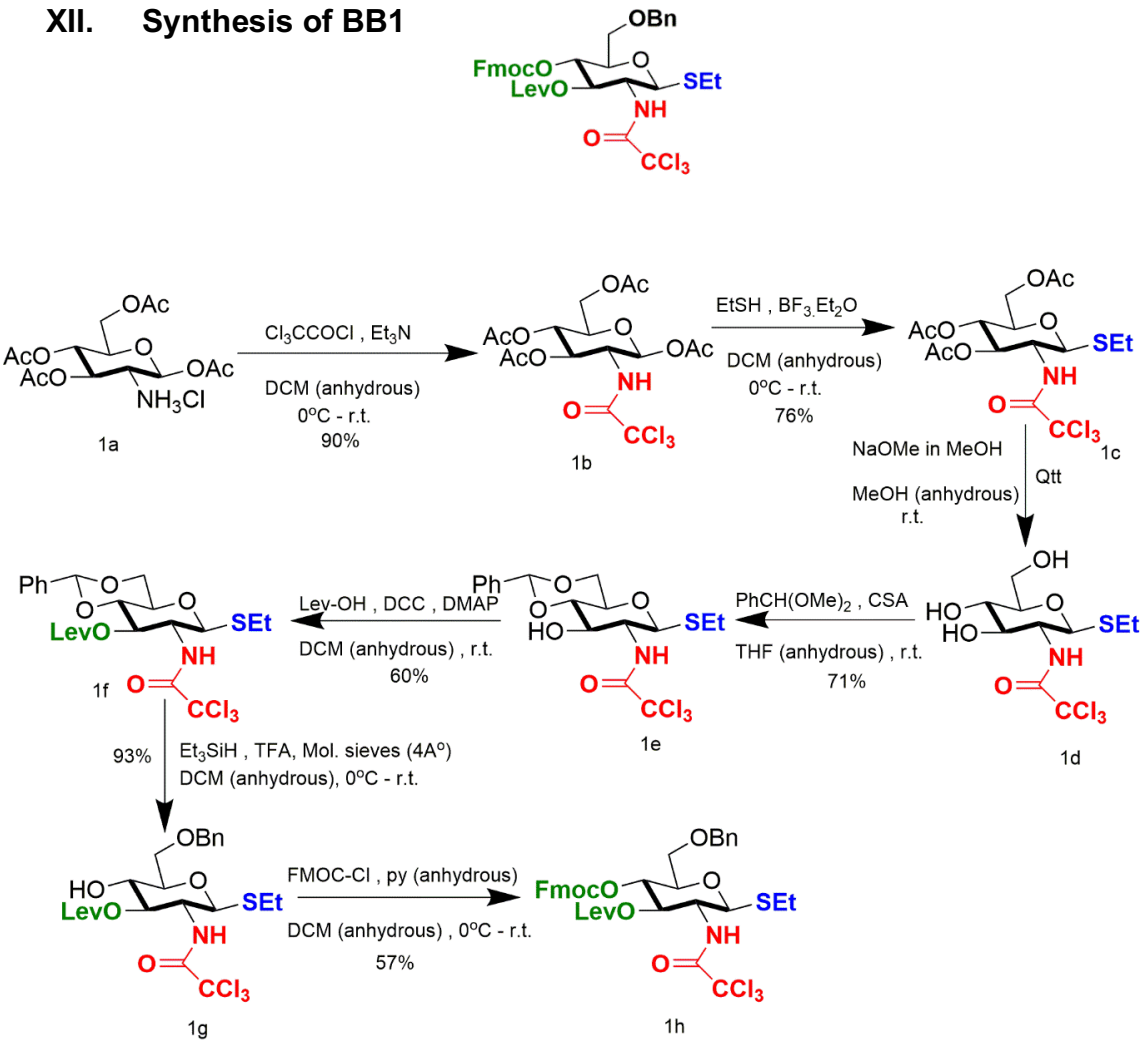
% Yield = 69

Analysis: TLC: R_f = 0.6 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.29 (d, *J* = 2.0 Hz, 2H,-Ar), 7.15 (dd, *J* = 7.8, 0.9 Hz, 1H,-Ar), 7.11 (d, *J* = 1.9 Hz, 1H,-Ar), 5.43 (dd, *J* = 3.4, 1.1 Hz, 1H), 5.06 (dd, *J* = 10.0, 3.4 Hz, 1H), 4.66 (d, *J* = 10.1 Hz, 1H,H-1), 4.15 (dd, *J* = 11.4, 7.0 Hz, 2H), 3.92 (td, *J* = 6.6, 1.2 Hz, 1H), 2.38 (d, *J* = 0.6 Hz, 3H), 2.29 (d, *J* = 0.6 Hz, 5H), 2.16 – 2.15 (m, 8H), 2.11 (d, *J* = 2.7 Hz, 4H), 2.04 (s, 2H), 2.02 (d, *J* = 2.5 Hz, 5H), 2.01 (s, 2H), 1.99 (s, 3H), 1.32 (s, 8H), 1.29 (s, 15H).

^{13}C NMR (176 MHz, $CDCl_3$) δ 170.40(OC=O), 170.32(OC=O), 170.18(OC=O), 170.11(OC=O), 169.92(OC=O), 169.50(OC=O), 168.96(OC=O), 149.78, 149.72, 149.65, 137.28, 133.36, 133.20, 131.86, 130.52, 130.38, 130.33, 130.16, 130.11, 127.14, 125.68, 123.17, 89.80(C-1), 87.71(C-1'), 77.34, 77.16, 76.98, 74.44, 72.08, 68.85, 67.56, 67.51, 67.45, 67.38, 66.54, 61.73, 61.32, 34.53, 34.42, 31.41, 31.36, 31.26, 31.07, 20.95, 20.91, 20.76, 20.72, 20.67, 20.61, 20.54, 20.42, 20.23.

XII. Synthesis of BB1



Step 1 Procedure: Installation of the trichloroacetyl group

This procedure maintained an inert atmosphere throughout. In a 500 mL 3-neck round bottom flask 105 mmol of per-*O*-acetylated glucosamine hydrochloride **1a** were flushed with argon for several minutes before adding 350 mL of anhydrous dichloromethane (final concentration: ~ 0.3 M). Gently stirring, the system was cooled to 0 °C on an ice bath followed by the addition of 210 mmol trimethylamine. Maintaining the temperature at 0 °C, 134 mmol trichloroacetyl chloride were added over a period of 10 minutes using a dropping funnel. The reaction was allowed to stir at 0 °C for another 2.5 hours. Then, the flask was removed from the ice bath and 350 mL water were added under vigorous stirring. After stirring for several more minutes, the reaction mixture was poured into a separating funnel, the organic phase was separated and subsequently washed with 350 mL saturated aqueous $NaHCO_3$ (1x), neutralised with 350 mL 1 N HCl (1x) and washed with 350 mL brine (2x). Finally, the organic phase was dried over $MgSO_4$, the solvent removed by evaporation and the resulting solid was dried under high vacuum to give 46.6 g of pure compound **1b** as a colourless foam.

Step 1 Analysis

TLC: R_f = 0.56 (Hex/EA 2:1)

^{1}H NMR (400 MHz, $CDCl_3$) δ= 7.23 (s, 1H, -NHCO), 5.80 (d, *J* = 8.8 Hz, 1H, H-1), 5.40 (dd, *J* = 10.8, 9.4 Hz, 1H, H-3), 5.16 (dd, *J* = 10.0, 9.5 Hz, 1H, H-4), 4.36 – 4.30 (m, 1H, H-2), 4.29 – 4.25 (m, 1H, H-6b), 4.15 (dd, *J* = 12.5, 2.2 Hz, 1H, H-6a), 3.90 (ddd, *J* = 10.0, 4.9, 2.2 Hz, 1H, H-5), 2.11, 2.10, 2.07, 2.05 (s, 3H, -3x OAc).

^{13}C NMR (101 MHz, $CDCl_3$) δ=171.66, 170.78, 169.47, 169.43, 162.43, (5x C=O), 92.32 (CCl_3), 92.10 (C-1), 73.27 (C-5), 72.01 (C-3), 68.02 (C-4), 61.83 (C-6), 54.56 (C-2), 20.87, 20.85, 20.69, 20.66 (4x $\underline{C}H_3C$=O).

Step 2 Procedure: Thioglycosylation

This procedure maintained an inert atmosphere throughout. To 45 mmol of compound **1b** (22.1 g) 50 mL anhydrous dichloromethane (final concentration ~ 0.9 M) were added under argon atmosphere and placed on an ice bath to cool down to 0 °C. Then, 50 mmol ethanethiol (3.7 mL, 1.1 eq.) were added via a syringe followed by dropwise addition of 33 mmol $BF_3 \cdot Et_2O$ (4.2 mL, 0.7 eq.) via a dropping funnel. The reaction was then allowed to stir at 0 °C for another 10 minutes, then removed from the ice bath and allowed to continue stirring for another 12 hours (overnight). The next day, the reaction mixture was poured into 250 mL water and kept to stir for some time. The organic phase was then separated, washed with 250 mL saturated aqueous $NaHCO_3$ (2x) and 250 mL brine (2x) and dried over $MgSO_4$ before it was concentrated and dried under high vacuum.

Step 2 Purification

The crude material (22.0 g) was dissolved in ethyl acetate (~ 100 mL) at 50 °C. When all of it dissolved, it was poured into a 1 L Erlenmeyer flask and, while stirring, hexane (~ 800 mL) was slowly added dropwise via a dropping funnel until precipitation started. The remainder volume of hexane was then quickly added under vigorous stirring. The precipitate so obtained was kept in the fridge overnight. The next day, the precipitate was filtered and washed with cold hexane to give 16.5 g of pure compound **1c** after drying.

Step 2 - Analysis

TLC: R_f = 0.5 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ= 7.08 (d, *J* = 9.3 Hz, 1H, -NHCO), 5.39 – 5.32 (m, 1H, H-3), 5.10 (t, *J* = 9.7 Hz, 1H, H-4), 4.68 (d, *J* = 10.3 Hz, 1H, H-1), 4.23 (dd, *J* = 12.3, 5.3 Hz, 1H, H-6a), 4.17 – 4.09 (m, 2H, H-2, H-6b), 3.77 (ddd, *J* = 10.0, 5.2, 2.4 Hz, 1H, H-5), 2.77 – 2.65 (m, 2H, -SCH2), 2.06, 2.01 (d, *J* = 2.5 Hz, 6H, 3X-OAc), 1.25 (t, *J* = 7.4 Hz, 3H, -CH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ=171.21, 170.78, 169.31, 162.02 (4x C=O), 83.87 (C-1), 76.14 (C-5), 73.27 (C-3), 68.60 (C-4), 62.46 (C-6), 54.73 (C-2), 24.36 ($COCH_3$), 20.84 ($COCH_3$), 20.72 ($COCH_3$), 20.67(SCH_2), 14.98(-CH3).

Step 3 Procedure: Removal of the acetyl groups

This procedure maintained an inert atmosphere throughout. In a 250 mL 3-necked round bottom flask 33 mmol of compound **1c** (16.5 g) were flushed with argon for several minutes before adding 77 mL of anhydrous methanol (final concentration: ~ 0.4 M). Gently stirring at room temperature, 13.33 mmol of 5.4 M sodium methanolate in MeOH (2.5 mL, 0.4 eq.) were added via a syringe. The reaction was allowed to stir at room temperature for another 2 hours. Then, the reaction mixture was passed through a column of 25 mL Amberlite-H^+ resin several times until pH of the filtrate stabilised at 6. The resin was the washed with MeOH (~ 3 bed volumes) and the combined filtrate and wash solution were then removed by evaporation. The resulting residue was dried under high vacuum to give 11.8 g of pure compound **1d** as white solid.

Step-3-Analysis

TLC: R_f = 0.6 (Hex/EA 1:1)

^{1}H NMR (700 MHz, D_2O) δ = 4.81 (d, *J* = 10.4 Hz, 1H, H-1), 3.96 – 3.93 (m, 1H), 3.84 (t, *J* = 10.2 Hz,1H, H-3), 3.77 (dd, *J* = 11.3, 4.0 Hz, 2H, H-6), 3.77 – 3.72 (m, 2H), 3.56 – 3.49 (m, 2H), 2.85 – 2.69 (m, 2H, -SCH_2), 1.28 (t, *J* = 7.4 Hz, 3H, -CH_3).

^{13}C NMR (176 MHz, D_2O) δ= 164.55 (NHC=O), 91.60 (CCl_3), 83.40 (C-1), 79.97 (C-4), 74.48 (C-3), 69.93 (C-6), 60.88 (C-5), 56.69 (C-2), 24.62 (-SCH_2), 14.48 (-CH_3).

Step 4 Procedure: Benzylidene formation

This procedure maintained an inert atmosphere throughout. To 74 mmol of compound **1d** (27.1 g) 150 mL anhydrous THF (final concentration: ~ 0.5 M) were added under argon atmosphere. Gently stirring at room temperature, 146 mmol of benzaldehyde dimethyl acetal (22 mL, 2.0 eq.) were added via a syringe followed by addition of 13 mmol camphor sulfonic acid (3.0 g, 0.2 eq.) via a powder funnel. The reaction was allowed to stir at room temperature for ~ 12 hours (overnight). The next day, the reaction mixture was diluted with 100 mL ethyl acetate and under vigorous stirring 200 mL of saturated aqueous $NaHCO_3$ was added. After stirring for several minutes, the reaction mixture was poured into a separating funnel, the organic phase was separated and subsequently washed with 150 mL brine (2x). Finally, the organic phase was dried with $MgSO_4$ before it was concentrated and dried under high vacuum.

Step 4 Purification

The crude material was dissolved in DCM (~220 mL) at on a heating mantle set to 55°C. When all of it dissolved, it was poured into a 1 L Erlenmeyer flask and then hexane (~ 800 mL) was added into it dropwise via a dropping funnel until precipitation started. The remainder volume of hexane was then quickly added under vigorous stirring. The precipitate so obtained was then kept in the fridge overnight. The next day, the precipitate was filtered and washed with cold hexane to give 24.6 g of pure compound **1e** after drying.

Step-4- Analysis

TLC: R_f = 0.47 (Hex.EA: 3:1)

^{1}H NMR (700 MHz,DMSO-d_6) δ = 8.88 (d, *J* = 9.3 Hz, 1H, -NHCO), 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).

^{13}C NMR (176 MHz, DMSO) δ = 161.26 (NHC=O), 137.66 (aryl-C), 128.87 (aryl-C), 128.03(aryl-C), 126.33 (aryl-C), 100.63(-PhCH), 93.08 (CCl_3), 83.78 (C-1), 81.12 (C-4), 70.93 (C-6), 70.22 (C-3), 67.68 (C-5), 56.98 (C-2), 23.51(-SCH_2), 15.09(-CH_3).

Step 5 Procedure: Installation of the levulinoyl group

This procedure maintained an inert atmosphere throughout. In a 1 L 3-neck round bottom flask 53 mmol of compound **1e** (24.3 g) were flushed with argon for several minutes before adding 380 mL anhydrous dichloromethane (final concentration: ~ 0.1 M). The mixture was stirred at room temperature. Meanwhile, in a separate 100 mL round bottom flask, 80 mmol *N*,*N*'-dicyclohexylcarbodiimide (16.5 g, 1.5 eq.) and 64 mmol levulinic acid (7.4 g, 1.2 eq.) were dissolved in 20 mL anhydrous dichloromethane and stirred at room temperature for 10 minutes. This mixture was then transferred to the reaction flask. The reaction was allowed to stir at room temperature for 3 hours. The precipitate that formed during the reaction was filtered off washed and with DCM. The filtrate so obtained was poured into a separating funnel and washed with 250 mL 1 N HCl (1x), 250 mL saturated aqueous $NaHCO_3$ (1x) and 250 mL H_2O (1x). The organic phase was dried over $MgSO_4$ before it was concentrated and dried under high vacuum.

Step 5 Purification

The crude material was dissolved in 150 mL ethanol (abs.) at 70 °C on a heating mantle. When all of it dissolved, it was kept in the freezer overnight. The next day, the crystals so obtained were washed and filtered with cold ethanol to give 17.8 g of pure compound **1f** after drying.

Step 5-Analysis

TLC: R_f = 0.6 (Hex/EA 4:1)

^{1}H NMR (700 MHz,$CDCl_3$) δ =7.61 (d, *J* = 9.8 Hz, 1H, -NHCO), 7.50 – 7.48 (m, 2H, -Ar), 7.37 – 7.32 (m, 3H, -Ar), 5.56 (t, *J* = 9.8 Hz, 1H, H-3), 5.49 (s, 1H, -PhCH), 4.52 (d, *J* = 10.4 Hz, 1H, H-1), 4.17 (q, *J* = 10.1 Hz, 1H,H-2), 3.93 (dd, *J* = 10.1, 4.8 Hz, 1H, H-6a), 3.67 (t, *J* = 9.5 Hz, 1H, H-4), 3.59 (t, *J* = 10.0 Hz, 1H, H-6b), 3.54 (td, *J* = 9.6, 4.8 Hz, 1H, H-5), 2.79 – 2.63 (m, 4H,-CH_2CH_2), 2.59 – 2.52 (m, 2H, -SCH_2), 2.13 (s, 3H, -$COCH_3$), 1.18 (t, *J* = 7.4 Hz, 3H, -CH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ =205.57 (ketone-C=O), 173.70 (O-C=O), 162.19 (NHC=O), 137.14 (aryl-C), 128.92 (aryl-C), 128.23 (aryl-C), 125.91 (aryl-C), 100.78 (-PhCH), 92.62 (CCl_3), 84.59 (C-1), 78.83 (C-4), 73.13 (C-3), 70.20 (C-5), 68.23 (C-6), 54.67 (C-2), 37.97 (-CH_2), 29.72 ($\underline{C}H_3$-C=O), 28.25 (-CH_2), 24.59(-SCH_2), 14.95(-SCH_3).

Step 6 Procedure: Opening of the benzylidene at C4

This procedure maintained an inert atmosphere throughout. In a 500 mL 3-neck round bottom flask 30 mmol of compound **1f** (16.6 g) were flushed with argon for several minutes before adding 150 mL of anhydrous dichloromethane (final concentration: ~ 0.2 M). Gently stirring at room temperature, 120 mmol Et_3SiH (19 mL, 4.0 eq.) were added via a syringe followed by addition of molecular sieves (4 Å, 5.0 g). The reaction was allowed to stir at room temperature for 30 minutes. Thereafter, the system was cooled to 0°C on an ice bath followed by addition of 120 mmol trifluoroacetic acid (9 mL, 4.0 eq.) over a period of 10 minutes via a dropping funnel. After the addition, the flask was removed from the ice bath and stirred at room temperature for 2.5 hours. Then, the reaction was quenched with ~ 20 mL triethylamine until no more gas evolved. It was then diluted with 100 mL DCM, poured into a separating funnel, and washed with 350 mL H_2O (2x), 350 mL saturated aqueous $NaHCO_3$ (2x) and 350 mL brine (2x). The organic phase was then dried over $MgSO_4$ before it was concentrated and dried under high vacuum.

Step 6 Purification

The crude material was dissolved in 50 mL absolute ethanol at 50 °C on a heating mantle and kept in the freezer overnight. The crystals so obtained were filtered and washed with cold ethanol. The filtrate obtained was concentrated and dried under high vacuum to 15.7 g of pure compound **1g**.

Step 6- Analysis

TLC: R_f = 0.78 (Hex/EA 1:1)

^{1}H NMR (700 MHz, DMSO-d_6) δ = 8.94 (d, *J* = 9.3 Hz, 1H, -NHCO), 7.39 – 7.27 (m, 5H, -Ar), 5.53 (dt, *J* = 5.9, 1.7 Hz, 1H, 4-OH), 5.11 (t, *J* = 10.4, 8.8 Hz, 1H, H-3), 4.83 (d, *J* = 10.4 Hz, 1H, H-1), 4.54 (d, *J* = 1.9 Hz, 2H, -CHBn), 3.79 – 3.71 (m, 2H, H-2, H-6b), 3.59 (dd, *J* = 11.3, 1.7 Hz, 1H, H-6a), 3.45 (ddd, *J* = 9.2, 5.6, 1.7 Hz, 1H, H-5), 3.41 (dddd, *J* = 10.0, 8.9, 5.9, 1.7 Hz, 1H, H-4), 2.69 – 2.55 (m, 4H, -CH_2CH_2), 2.48 (dd, *J* = 7.0, 1.7 Hz, 2H, -SCH_2), 2.39 (dtd, *J* = 17.2, 6.9, 1.7 Hz, 1H), 2.09 (t, *J* = 1.5 Hz, 3H, -$COCH_3$), 1.19 (d, *J* = 7.4 Hz, 3H, -SCH_3).

^{13}C NMR (176 MHz, DMSO) δ = 206.32 (ketone-C=O), 171.59 (O-C=O), 161.23 (NHC=O), 138.52 (aryl-C), 128.27 (aryl-C), 127.50 (aryl-C), 92.68 (CCl_3), 82.28 (C-1), 79.47 (C-5), 76.09 (C-3), 72.37 ($PhCH_2$-), 69.34 (C-6), 68.03 (C-4), 54.49 (C-2), 37.38 (-CH_2), 29.61 ($\underline{C}H_3$-C=O), 27.73 (-CH_2), 23.44 (-SCH_2), 15.11 (-SCH_3).

Step 7 Procedure: Installation of the Fmoc-group

This procedure maintained an inert atmosphere throughout. To 28 mmol of compound **1g** (15.7 g) 220 mL anhydrous dichloromethane (final concentration: ~0.1 M) were added under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, 85 mmol pyridine (7.0 mL, 3.0 eq.) were added via a syringe. After ~ 5 mins, 58 mmol Fmoc-chloride (15.0 g, 2.0 eq.) were dissolved in 15 mL DCM and added into the reaction flask via a syringe. The reaction was then allowed to stir on ice for another 30 minutes and for another ~ 12 hours (overnight) after removing the ice bath. The next day, 60 mL MeOH was added into the reaction mixture and kept to stir for some time. Then, it was poured into a separating funnel, and washed with 100 mL H_2O (2x). The aqueous layer was then extracted with 85 mL ethyl acetate (1x). The combined organic layers were then dried over $MgSO_4$ before it was concentrated and dried under high vacuum.

Step 7 Purification

The crude material was dissolved in 20 mL ethyl acetate at 45°C on a heating mantle. When all of it dissolved, it was poured into a 500 mL Erlenmeyer flask, and then 200 mL hexane were added into it dropwise via a dropping funnel until precipitation started. The remainder volume of hexane (~ 600 mL) was then quickly added under vigorous stirring. The precipitate so obtained was then kept in the fridge overnight. The next day, it was filtered and washed with cold hexane to give 12.5 g of pure compound **1h** after drying as an off-white powder.

Step 7 – Analysis

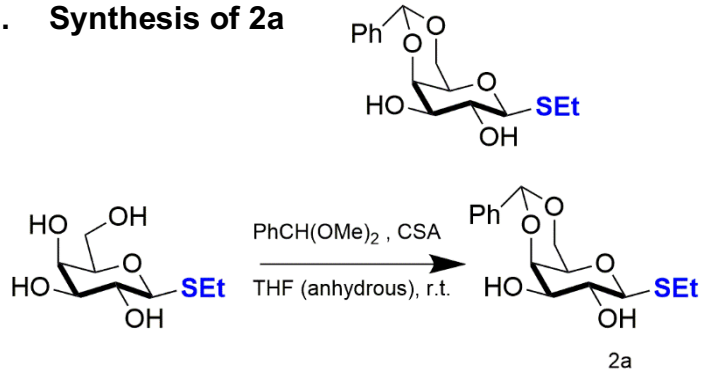
TLC: R_f = 0.6 (Hex/EA 4:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ = 7.80 – 7.76 (m, 2H), 7.55 (tq, *J* = 8.4, 0.9 Hz, 2H), 7.40 (ddt, *J* = 7.4, 6.6, 0.9 Hz, 2H), 7.34 (d, *J* = 9.4 Hz, 1H, -NHCO), 7.32 – 7.26 (m, 3H), 7.23 – 7.20 (m, 2H), 7.18 – 7.14 (m, 1H), 5.60 (dd, *J* = 10.4, 9.4 Hz, 1H, H-3), 5.11 (t, *J* = 9.7 Hz, 1H, H-4), 4.74 (d, *J* = 10.3 Hz, 1H, H-1), 4.52 – 4.45 (m, 3H), 4.30 (dd, *J* = 10.5, 7.9 Hz, 1H, H-6a), 4.23 (dd, *J* = 12.0, 7.4 Hz, 1H, H-6b), 4.19 (td, *J* = 10.4, 9.4 Hz, 1H, H-2), 3.95 (dt, *J* = 10.0, 3.9 Hz, 1H, H-5), 3.65 (dd, *J* = 11.2, 7.7 Hz, 2H, CH_2-Fmoc), 2.79 – 2.69 (m, 2H, -SCH_2), 2.69 – 2.43 (m, 4H, -CH_2CH_2), 2.07 (s, 3H, -$COCH_3$), 1.25 (t, *J* = 7.4 Hz, 3H, -CH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ = 205.88 (ketone-C=O), 173.20 (O-C=O), 162.09 (NHC=O), 154.13 (aryl-C), 143.67(aryl-C), 143.25 (aryl-C), 141.31 (aryl-C), 137.83 (aryl-C), 128.37(aryl-C), 128.02 (aryl-C), 128.00 (aryl-C), 127.31 (aryl-C), 127.29 (aryl-C), 125.26 (aryl-C), 125.18 (aryl-C), 120.16 (aryl-C), 92.47 (CCl_3), 83.63 (C-1), 77.02 (C-5), 73.76 (C-3), 73.68 (C-4), 73.30 ($PhCH_2$), 70.66 (C-6), 69.08 (Fmoc-CH_2), 54.78 (C-2), 46.70 (Fmoc-CH), 37.85 (-CH_2), 29.62 (CH3-C=O), 28.20 (-CH_2), 24.09 (SCH_2), 15.00 (SCH_3).

HRMS (ESI, positive mode): calculated m/z $[M+K]^+$ = 816.0965, found : 816.0947

XIII. Synthesis of 2a



2a

Procedure: This procedure maintained an inert atmosphere throughout. In a 1L 3-necked round bottom flask, 178mmol of Carbosynth intermediate_ (40g) was purged with argon for several minutes before adding 360mL anhydrous THF (final concentration: ~ 0.5M). This was followed by addition of CSA (31mmol, 7.2g) and $PhCH(OMe)_2$ (357mmol, 54mL). The reaction was then allowed to stir at room temperature. After ~ 2 hours, the reaction was diluted with EA (~100mL) and poured into a separating funnel. It was then extracted with Sat. aq. $NaHCO_3$ (~ 150mL, 2x) and Brine (~150 mL,2x). The combined organic phase was then dried with $MgSO_4$, concentrated and dried under high vacuum.

Purification: The crude material was dissolved in DCM (~220mL) at 50°C on a heating mantle. When all of it dissolved, the flask was removed from the heating mantle, and filtered hot into a 1L Erlenmeyer flask. Then Hexane (~800 mL) was first added dropwise until precipitation started, and then quickly under rigorous stirring. The precipitate so obtained was kept in the fridge overnight. It was then washed with cold DCM/Hexane 4:1 to get 4og of pure **2a** as a white solid.

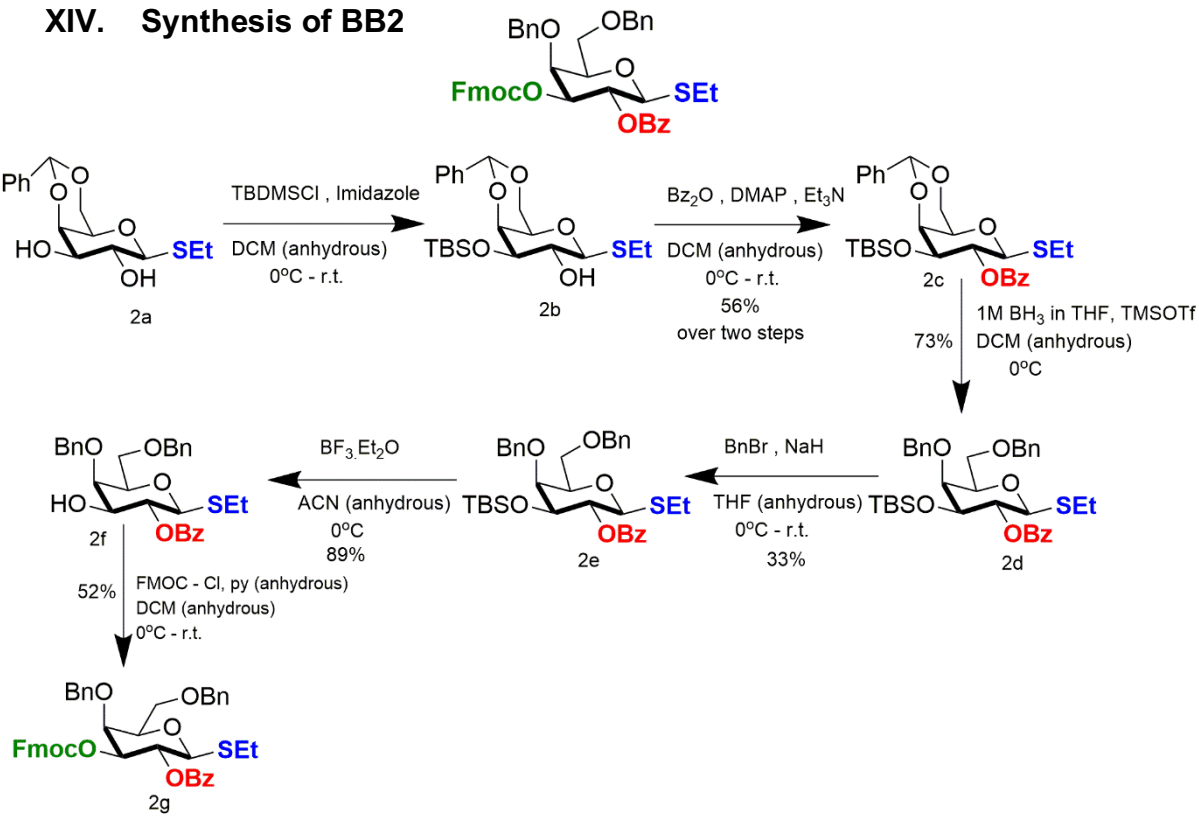
% Yield = 72

Analysis: TLC: R_f = 0.6 (Hex/EA 4:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.50 – 7.44 (m, 2H,-Ar), 7.34 (d, J = 6.5 Hz, 2H,-Ar), 5.48 (s, 1H,-PhCH), 4.31 (d, J = 9.5 Hz, 1H,H-1), 4.27 (d, J = 12.3 Hz, 1H,H-6a), 4.16 (d, J = 3.3 Hz, 1H,H-4), 3.96 (d, J = 12.3 Hz, 1H,H-6b), 3.78 (t, J = 9.4 Hz, 1H,H-2), 3.63 (dd, J = 9.2, 3.6 Hz, 2H,H-3,H-4), 3.41 (s, 4H), 2.84 – 2.66 (m, 3H,-SCH_2), 1.24 (t, J = 7.4 Hz, 1H,-SCH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ 137.78(-Ar), 136.40(-Ar), 134.53(-Ar), 129.80(-Ar), 129.27(-Ar), 129.04(-Ar), 128.31(-Ar), 126.53(-Ar), 101.42(-PhCH), 85.28(C-1), 75.76(C-4), 73.84(C-3), 70.04(C-5), 69.63(C-2), 69.33(C-6), 23.51(-SCH_2), 15.29(-SCH_3).

XIV. Synthesis of BB2



Step 1 Procedure: Installation of silyl group at C3

This procedure maintains an inert atmosphere throughout. In a 500 mL 3-necked round bottom flask, 64 mmol of **2a** (20g) were flushed with argon for several minutes before adding 160 mL anhydrous dichloromethane (final concentration: ~ 0.4M). The reaction flask was then placed on an ice bath, followed by addition of TBDMSCl (77 mmol, 12g) and Imidazole (90mmol, 6g). After the addition, the reaction was allowed to stir at room temperature for ~ 18 hours. After overnight stirring, the reaction was quenched with Sat. aq. $NaHCO_3$ (~200 mL). The reaction mixture was then poured into a separating funnel, and the organic ühase was extracted with DCM (~100 mL, 3x). The combined organic phase was then washed with Brine (~100mL, 2x), dried with $MgSO_4$, concentrated and dried under high vacuum to give 28g of **2b**.

Step 1- Analysis

^{1}H NMR (700 MHz, Chloroform-*d*) δ 7.50 (ddd, J = 8.0, 1.6, 0.6 Hz, 2H,-Ar), 7.38 – 7.31 (m, 3H,-Ar), 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(*C*H_3)$_3$), 23.24 (SCH_2), 18.34 (Si*C*(CH_3)$_3$), 15.35 (SCH_2CH_3), -4.24 ($SiCH_3$), -4.53 ($SiCH_3$).

Step 2 Procedure: Installation of benzoyl group at C2

This procedure maintained an inert atmosphere throughout. To 65 mmol of **2b** (28g), 405 mL anhydrous DCM (final concentration: ~ 0.2M) were added under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, benzoic anhydride (195 mmol, 44g) and DMAP (33mol, 4g) were added successively, followed by dropwise addition of Et_3N (390mmol, 54mL). The reaction was allowed to stir on ice bath for ~ 15 mins, and then stirred at room temperature for ~ 12 hours. After overnight stirring, the reaction mixture was poured into a separating funnel, and the organic phase was extracted with Sat. aq. $NaHCO_3$ (~ 300mL, 3x) and Brine (~300 mL, 2x). The combined organic phase was then dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 2 - Purification

The crude material was dissolved in EtOH (abs) (~ 60 mL) at 65°C on a heating mantle. When all of it dissolved, the crude material was kept in the freezer overnight to induce crystallization. The crystals so obtained were washed and filtered with cold EtOH to give 19g pure **2c.**

Step 2 – Analysis

^{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(*C*H_3)$_3$), 22.86 (SCH_2), 18.04 (Si*C*(CH_3)$_3$), 15.00 (SCH_2CH_3), -4.44 ($SiCH_3$), -4.59 ($SiCH_3$).

Step – 3 Procedure: Regioselective opening of benzylidene ring

This procedure maintained an inert atmosphere throughout. In a 500 mL, 3-necked round bottom flask, 89 mL anhydrous DCM (final concentration: ~ 0.4M) were added under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, 1M BH_3 in THF (4eq) was added dropwise via a dropping funnel followed by addition of TMSOTf (18mmol, 3mL) via a syringe. The reaction was stirred on the ice bath for ~ 1.5 hours. Then, the reaction was quenched with Et_3N/MeOH 1:10 till no more gas evolved. The reaction mixture was then concentrated, and the syrup so obtained was re-dissolved in DCM, and poured into a separating funnel, and the organic phase was extracted with Sat. aq. $NaHCO_3$ (~ 150 mL, 2x) and Brine (~ 150mL,2x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum to give 14g of **2d.**

Step 3 – Analysis

^{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-C*H*), 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 ($SiCH_3$).

Step 4 Procedure: Installation of benzyl group at C6

This procedure maintained an inert atmosphere throughout. To 26 mmol of **2d** (14g), 123 mL of anhydrous THF (final concentration: ~ 0.2M) were added under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, NaH (69mmol, 3g) was added, followed by dropwise addition of BnBr (39mmol, 5mL) via a dropping funnel. The reaction was left to stir on the ice bath for ~ 15 mins, and then left to stir at room temperature for ~ 18 hours. The reaction was then quenched with Sat. aq. NH_4Cl (~80 mL) till no more bubbling occurred. The reaction mixture was then transferred into a separating funnel, and the organic phase was extracted. The aqueous phase was then extracted with DCM (~60mL,1x) and the combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 4 – Purification

The crude material was dissolved in Ethyl Acetate (~ 20mL) at 50°C on a heating mantle. When all of it dissolved, Hexane (~50 mL) was then added dropwise via a dropping funnel. The crude material was then kept in the freezer overnight to induce crystallization. Then, the crystals so obtained were washed and filtered with cold Hexane to give pure 16g of pure **2e.**

Step 4 – Analysis

TLC: R_f = 0.78 (Hex/EA 4:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ =7.52 – 7.50 (m, 2H, -Ar), 7.43 – 7.28 (m, 15H,-Ar), 7.05 – 7.02 (m, 2H, -Ar), 5.02 (d, *J* = 11.6 Hz, 1H, -CH_2Bn), 4.82 (d, *J* = 10.2 Hz, 1H, -CH_2Bn), 4.78 – 4.73 (m, 3H, -CH_2Bn), 4.68 (d, *J* = 11.6 Hz, 1H, -CH_2Bn), 4.56 (d, *J* = 9.6 Hz, 1H, H-1), 3.91 (t, *J* = 9.4 Hz, 1H, H-2), 3.64 (dd, *J* = 2.9, 1.0 Hz, 1H, H-4), 3.60 (dd, *J* = 9.2, 2.8 Hz, 1H, H-3), 3.52 (qd, *J* = 6.4, 1.0 Hz, 1H, H-5), 2.31 (s, 3H, -SCH_3), 1.28 (d, *J* = 6.4 Hz, 3H, -CH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ= 138.80(-Ar), 138.51(-Ar),, 138.41(-Ar), 137.10(-Ar), 132.20(-Ar), 130.48(-Ar), 129.52(-Ar), 128.44(-Ar), 128.36(-Ar), 128.33(-Ar), 128.15(-Ar), 127.97(-Ar), 127.69(-Ar), 127.59(-Ar), 127.45(-Ar), 87.90(C-1), 84.61(C-4), 76.66(C-2), 75.55(-CH_2Bn), 74.58(-CH_2Bn, C-5), 72.87(-CH_2Bn, C-3), 21.13(-SCH_3), 17.34(-CH_3).

Step 5 Procedure: Removal of Silyl group from C3

This procedure maintained an inert atmosphere throughout. To 26 mmol of **2e** (16g), 172 mL anhydrous ACN (final concentration: ~ 0.15M) under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, $BF_3.Et_2O$ (28mmol, 3.5mL) was added dropwise via a syringe. The reaction was allowed to stir on ice bath for 5 mins, followed by quenching by dropwise addition of Sat. aq. $NaHCO_3$ (~ 80 mL). The reaction mixture was then poured into a separating funnel, and the organic phase was extracted. The combined organic phase was then washed with Brine (~ 100 mL, 1x). It was then dried with $MgSO_4$, concentrated and dried under high vacuum to give 7g of **2f.**

Step 5 – Analysis

^{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(OC=O), 138.98(-Ar), 138.20(-Ar), 133.23(-Ar), 130.05(-Ar), 129.35(-Ar), 128.61(-Ar), 128.24, 128.13, 127.63, 127.62, 127.49, 127.35, 82.77(C-1), 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(-SCH_2), 15.10(-CH_3).

Step 6 Procedure: Installation of FMOC group at C3

This procedure maintained an inert atmosphere throughout. To 13mmol of **2f** (7g) was added 100 mL (final concentration: ~ 0.13M) under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, pyridine (39mmol, 3.14mL) was added via a syringe, followed by addition of FMOC (pre-dissolved in DCM) (26mmol, 7g). The reaction was then allowed to stir on ice bath for ~ 10 mins, and then left to stir at room temperature overnight. The reaction was then quenched with 1N HCl (~ 20 mL), and added into a separating funnel. The organic phase was then extracted and the aqueous phase was extracted with DCM (~80 mL, 3x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 6 – Purification

The crude material was dissolved in EtOH(abs.)(300 mL) at 70°C on a heating mantle. Hen all of it dissolved, it was kept in the freezer to induce crystallization. The crystals so obtained were washed and filtered with cold EtOH to give 11g of pure **2g.**

Step 6 – Analysis

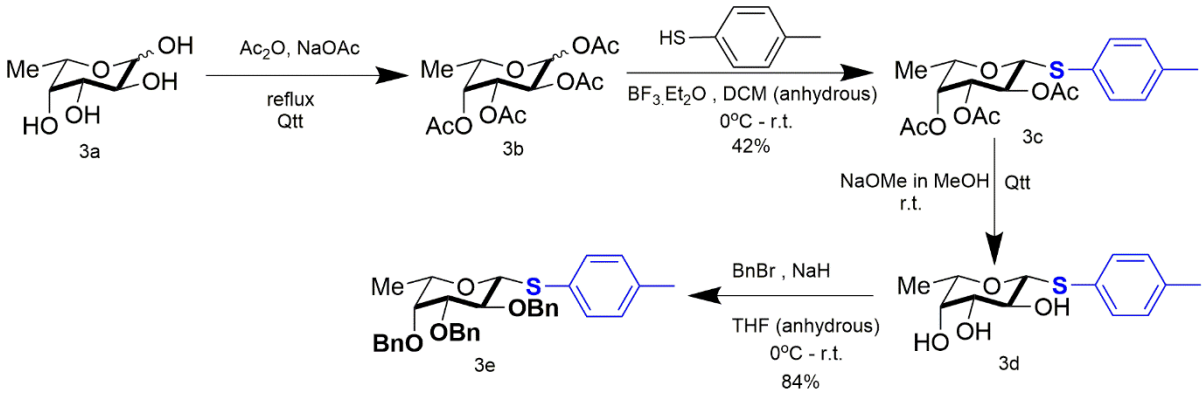
TLC: R_f = 0.5 (Hex/EA 5:1)

^{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, $PhCH_2$, PhC*H*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.

XV. Synthesis of BB3



Step1 Procedure: Peracetylation of L-Fucose

This procedure does not need an inert atmosphere. In a 250 mL 3-neck round bottom flask 61 mmol sodium acetate (5.0 g, 0.5 eq.) were mixed with 973 mmol acetic anhydride (92 mL, 8.0 eq.). A reflux condenser was fitted to one of the necks, and the flask was placed on a heating mantle. The reaction was then refluxed at 170 °C for 5 minutes. 122 mmol of L-fucose **3a** (20.0 g) were then added in portions over a period of 10 minutes and the temperature was decreased to 120°C. After the addition, the reaction was allowed to cool down to room temperature for 30 minutes. The reaction was then poured into ice in a 500 mL beaker and stirred for 1 hour. It was then poured into a separating funnel and extracted with 250 mL ethyl acetate (1X). Subsequently, the organic phase was washed with 150 mL saturated aqueous $NaHCO_3$ (2x) and 150 mL brine (2x). Finally, the organic phase was dried with $MgSO_4$, the solvent removed by evaporation and the resulting oil was dried under high vacuum to give 42.9 g of pure compound **3b**.

Step-1 Analysis

TLC: R_f = 0.57 (Hex/EA 2:1)

^{1}H NMR (700 MHz,$CDCl_3$) δ = 5.66 (d, *J* = 8.3 Hz, 1H, H-1), 5.29 – 5.26 (m, 1H, H-2), 5.24 (dd, *J* = 3.5, 1.1 Hz, 1H, H-4), 5.06 – 5.04 (m, 1H, H-3), 3.93 (qd, *J* = 6.4, 5.9, 1.4 Hz, 1H, H-5), 2.16, 2.08, 2.06, 2.00 (s, 3H, 4x-OAc), 1.19 (dd, *J* = 6.5, 0.7 Hz, 3H, -CH_3) .

^{13}C NMR (176 MHz, $CDCl_3$) δ= 170.55, 170.04, 169.49, 169.18 (4x C=O), 92.15 (C-1), 76.38 (C-3), 71.24 (C-2), 70.22 (C-5), 69.96 (C-4), 20.80, 20.69, 20.63, 20.60 (4x $\underline{C}H_3C$=O), 15.91(-CH_3).

Step 2 Procedure: Thioglycosylation

This procedure maintained an inert atmosphere throughout. To 130 mmol of compound **3b** (42.9 g) 250 mL anhydrous dichloromethane (final concentration: ~ 0.5 M) were added under argon atmosphere and placed on an ice bath to cool down to 0 °C. Then, 142 mmol p-toluenethiol (18.0 g, 1.0 eq.) were added via a powder funnel followed by dropwise addition of 168 mmol $BF_3 \cdot Et_2O$ (21 mL, 1.3 eq.) over 10 minutes via a dropping funnel. The reaction was allowed to stir on ice bath for another 5 minutes, then removed from the ice bath and allowed to stir at room temperature for 1.5 hours. Then, 250 mL Sat. aq. $NaHCO_3$ was added into the reaction flask and kept to stir for some time. It was then poured into a separating funnel, and the organic phase was separated. The aqueous phase was then extracted with 150 mL dichloromethane (2x). The combined organic phase was subsequently washed with 150 mL brine (2x). Finally, the organic phase was dried over $MgSO_4$ before it was concentrated and dried under high vacuum.

Step 2 Purification

The crude material was dissolved in ethyl acetate (~ 20 mL) at 50 °C on a heating mantle. When all of it dissolved, it was removed from the mantle, and hexane (~ 80 mL) was added into it dropwise via a dropping funnel until precipitation started. The remainder volume of hexane was then added quickly under vigorous stirring. The precipitate so obtained was then kept in the fridge overnight. The next day, the precipitate was filtered and washed with cold hexane to give 21.3 g of pure compound **3c** after drying.

Step 2 - Analysis

TLC: R_f = 0.78 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ= 7.42 – 7.39 (m, 2H, -Ar), 7.13 – 7.11 (m, 2H, -Ar), 5.24 (ddd, *J* = 3.3, 1.1, 0.4 Hz, 1H, H-4), 5.22 – 5.16 (m, 1H, H-2), 5.03 (dd, *J* = 10.0, 3.4 Hz, 1H, H-3), 4.63 (d, *J* = 10.0 Hz, 1H, H-1), 3.80 (qd, *J* = 6.4, 1.1 Hz, 1H, H-5), 2.34 (s, 3H, $-SCH_3$), 2.13 , 2.08 , 1.96 (s, 3H, 3xOAc), 1.22 (d, *J* = 6.5 Hz, 3H, $-CH_3$).

^{13}C NMR (176 MHz, $CDCl_3$) δ = 170.90, 170.41, 169.77 (3x C=O), 138.50, 133.22, 129.91, 129.37 (4x -Ar), 87.14 (C-1), 73.42 (C-3), 72.77 (C-5), 70.67 (C-4), 67.73 (C-2), 21.46, 21.19, 20.97 (3x -OAc), 20.94 ($-SCH_3$), 16.78 ($-CH_3$).

Step 3 Procedure: Removal of the acetyl groups

This procedure maintained an inert atmosphere throughout. In a 500 mL 3-neck round bottom flask 54 mmol of compound **3c** (21.3 g) were flushed with argon for several minutes before adding 125 mL methanol (final concentration: ~ 0.5 M). Gently stirring at room temperature, 22 mmol NaOMe in MeOH (5.4 M) (4.0 mL, 0.4 eq) were added into it via a syringe. The reaction was then allowed to stir at room temperature for ~ 12 hours (overnight). The next day, the reaction was passed through a column of Amberlite resin (~ 25 mL) in a sintered funnel till pH of the filtrate reaches 6. The solvent was then removed by evaporation and the resulting solid was dried under high vacuum to give 20.6 g of pure compound **3d** after drying.

Step 3-Analysis

TLC: R_f = 0.3 (Hex/EA 1:2)

^{1}H NMR (700 MHz, DMSO-d_6) δ = 7.35 – 7.30 (m, 2H, -Ar), 7.14 – 7.09 (m, 2H, -Ar), 4.49 (d, *J* = 9.2 Hz, 1H, H-1), 4.10 (s, 3H, -OH), 3.58 (qd, *J* = 6.5, 1.1 Hz, 1H, H-5), 3.48 – 3.46 (m, 1H,H-3), 3.34 (t, *J* = 10.5, 9.1 Hz, 2H, H-2, H-4), 2.27 (s, 3H, -SCH_3), 1.11 (d, *J* = 6.4 Hz, 3H, -CH_3)

^{13}C NMR (176 MHz, DMSO-d_6) δ =135.74, 130.21, 129.31 (-Ar), 87.67 (C-1), 74.74 (C-4), 73.87 (C-5), 71.05 (C-3), 68.82 (C-2), 20.47 (-SCH_3), 16.75 (-CH_3).

Step 4 Procedure: Installation of benzyl groups

This procedure maintained an inert atmosphere throughout. In a 500 mL 3-neck round bottom flask 19 mmol of compound **3d** (5.1 g) were flushed with argon for several minutes before adding 86 mL of anhydrous *N*,*N*-dimethylformamide (final concentration: ~ 0.22 M). Gently stirring, the system was cooled to 0 °C on an ice bath followed by the addition of 113 mmol sodium hydride (4.5 g, 6.0 eq.) via a powder funnel. Maintaining the temperature at 0 °C, 68 mmol benzyl bromide (8.06 mL) were added dropwise over 10 minutes via a dropping funnel. Then, the flask was removed from the ice bath, and allowed to stir at room temperature for 2 hours. The reaction was then quenched with 10 mL Sat.aq. NH_4Cl (until no more gas evolved). Then, it was diluted with 80 mL dichloromethane and poured into a separating funnel. The organic phase was separated, and the aqueous phase was extracted with 100 mL DCM (2x). The combined organic phase was washed with 150 mL brine (2x). Finally, it was dried over $MgSO_4$, before it was concentrated and dried under high vacuum.

Step 4-Purification

The crude material was dissolved in H_2O (~ 10 mL) at 60 °C on a heating mantle. When all of it dissolved, absolute ethanol (~ 60 mL) was added into it dropwise until precipitation started. The remainder volume of ethanol was the added quickly under vigorous stirring. The precipitate so obtained was kept in the fridge overnight. The next day, the precipitate was washed and filtered with cold ethanol to give 8.6 g of pure compound **3e** after drying.

Step 4- Analysis

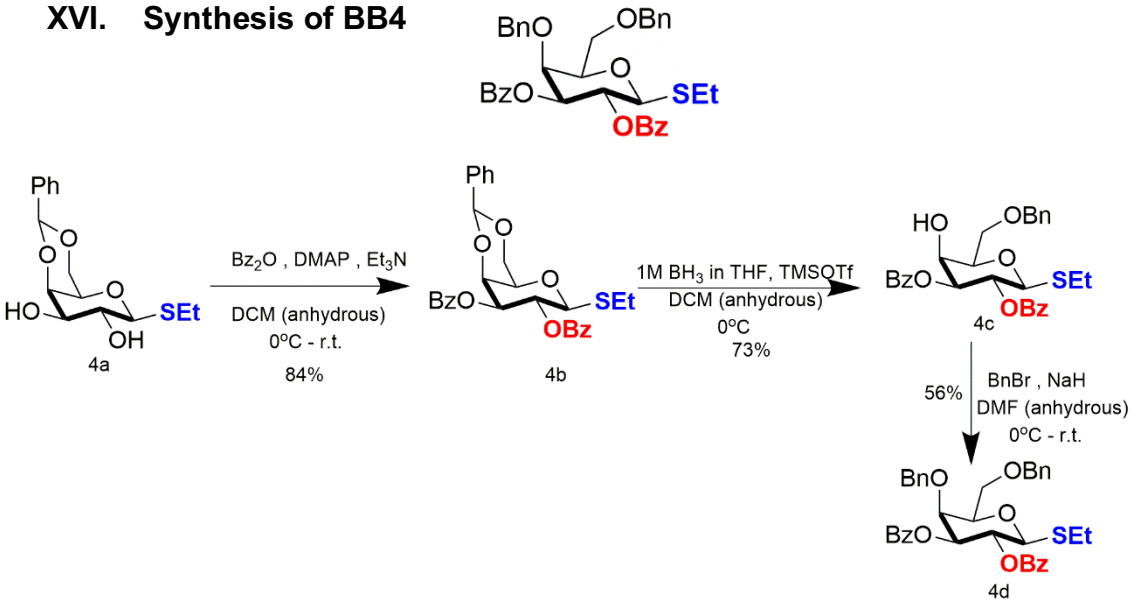
TLC: R_f = 0.76 (Hex/EA 4:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ =7.52 – 7.50 (m, 2H, -Ar), 7.43 – 7.28 (m, 15H,-Ar), 7.05 – 7.02 (m, 2H, -Ar), 5.02 (d, *J* = 11.6 Hz, 1H, -CH_2Bn), 4.82 (d, *J* = 10.2 Hz, 1H, -CH_2Bn), 4.78 – 4.73 (m, 3H, -CH_2Bn), 4.68 (d, *J* = 11.6 Hz, 1H, -CH_2Bn), 4.56 (d, *J* = 9.6 Hz, 1H, H-1), 3.91 (t, *J* = 9.4 Hz, 1H, H-2), 3.64 (dd, *J* = 2.9, 1.0 Hz, 1H, H-4), 3.60 (dd, *J* = 9.2, 2.8 Hz, 1H, H-3), 3.52 (qd, *J* = 6.4, 1.0 Hz, 1H, H-5), 2.31 (s, 3H, -SCH_3), 1.28 (d, *J* = 6.4 Hz, 3H, -CH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ= 138.80(-Ar), 138.51(-Ar),, 138.41(-Ar), 137.10(-Ar), 132.20(-Ar), 130.48(-Ar), 129.52(-Ar), 128.44(-Ar), 128.36(-Ar), 128.33(-Ar), 128.15(-Ar), 127.97(-Ar), 127.69(-Ar), 127.59(-Ar), 127.45(-Ar), 87.90(C-1), 84.61(C-4), 76.66(C-2), 75.55(-CH_2Bn), 74.58(-CH_2Bn, C-5), 72.87(-CH_2Bn, C-3), 21.13(-SCH_3), 17.34(-CH_3).

HRMS (ESI, positive mode): Calculated m/z $[M+Na]^+$ = 563.2227, Found: 563.2267

XVI. Synthesis of BB4



Step 1 Procedure: Installation of benzoyl groups at C2 and C3

This procedure maintained an inert atmosphere throughout. In a 500 mL 3-necked round bottom flask, 109 mmol of 2a (34g) were flushed with argon for several minutes before adding 340 mL anhydrous DCM (final concentration: ~ 0.32M). The reaction was then allowed to stir at room temperature, followed by addition of Bz_2O (327 mmol, 74g), Et_3N (436 mmol, 60 mL) and DMAP (3 mol% per OH group, 0. 740 g) successively. After 4 hours, the reaction mixture was washed with 1N HCl (300 mL, 2x), Sat. aq. $NaHCO_3$ (300 mL, 2x) and Brine (300 mL, 2x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 1 – Purification

The crude material was dissolved in Ethyl acetate (~ 100 mL) at 50°C on a heating mantle. When all of it dissolved, Hexane (~ 200 mL) was added at room temperature to induce precipitation. The precipitate so obtained was washed and filtered with cold Hexane to give 50 g of pure **4b**.

Step 1 – Analysis

^{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_2CH_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 (-PhCH), 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).

Step 2 Procedure: Regioselective opening of benzylidene ring at C6

This procedure maintained an inert atmosphere throughout. In a 500 mL, 3-necked round bottom flask, 36 mmol 4a (19g) were flushed with argon for several minutes before adding 89 mL anhydrous DCM (final concentration: ~ 0.4M) and placed in an ice bath to cool down to 0°C. Then, 1M BH_3 in THF (4eq) was added dropwise via a dropping funnel followed by addition of TMSOTf (18mmol, 3mL) via a syringe. The reaction was stirred on the ice bath for ~ 1.5 hours. Then, the reaction was quenched with Et_3N/MeOH 1:10 till no more gas evolved. The reaction mixture was then concentrated, and the syrup so obtained was re-dissolved in DCM, and poured into a separating funnel, and the organic phase was extracted with Sat. aq.

$NaHCO_3$ (~ 150 mL, 2x) and Brine (~ 150mL,2x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum to give 14g of **4c**.

Step 2 – Analysis

TLC: R_f = 0.3 (Hex/EA 3:1)

^{1}H NMR (700 MHz, DMSO-d6) δ 7.92 – 7.86 (m, 3H,-Ar), 7.83 – 7.80 (m, 2H,-Ar), 7.62 – 7.57 (m, 2H,-Ar), 7.56 – 7.52 (m, 1H,-Ar), 7.48 – 7.43 (m, 4H,-Ar), 7.33 – 7.23 (m, 4H,-Ar), 5.54 (dd, J = 9.8 Hz, 1H), 5.02 (d, J = 9.3 Hz, 1H), 4.99 (dd, J = 6.1, 4.9 Hz, 1H), 4.97 – 4.89 (m, 1H), 4.64 – 4.59 (m, 2H), 2.72 – 2.60 (m, 2H,-SCH_2), 1.17 (t, J = 7.4 Hz, 3H,-CH_3).

^{13}C NMR (176 MHz, DMSO) δ 164.95(OC=O), 138.97(-Ar), 138.23(-Ar), 133.69(-Ar), 133.55(-Ar), 129.84, 129.39, 129.25, 129.17, 129.13, 129.08, 129.04, 128.81, 128.67, 128.56, 128.52, 128.25, 128.12, 82.61(C-1),78.70, 75.87, 74.41, 68.63, 65.60, 56.02, 25.29(-SCH_2), 14.95(-SCH_3).

Step 3 Procedure: Installation of benzyl group at C6

This procedure maintained an inert atmosphere throughout. To 26 mmol of **4c** (14g), 123 mL of anhydrous THF (final concentration: ~ 0.2M) were added under argon atmosphere and placed in an ice bath to cool down to 0^{o}C. Then, NaH (69mmol, 3g) was added, followed by dropwise addition of BnBr (39mmol, 5mL) via a dropping funnel. The reaction was left to stir on the ice bath for ~ 15 mins, and then left to stir at room temperature for ~ 18 hours. The reaction was then quenched with Sat. aq. NH_4Cl (~80 mL) till no more bubbling occurred. The reaction mixture was then transferred into a separating funnel, and the organic phase was extracted. The aqueous phase was then extracted with DCM (~60mL,1x) and the combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 3– Purification

The crude material was dissolved in Ethyl Acetate (~ 20mL) at 50^{o}C on a heating mantle. When all of it dissolved, Hexane (~50 mL) was then added dropwise via a dropping funnel. The crude material was then kept in the freezer overnight to induce crystallization. Then, the crystals so obtained were washed and filtered with cold Hexane to give pure 16g of pure **4d**.

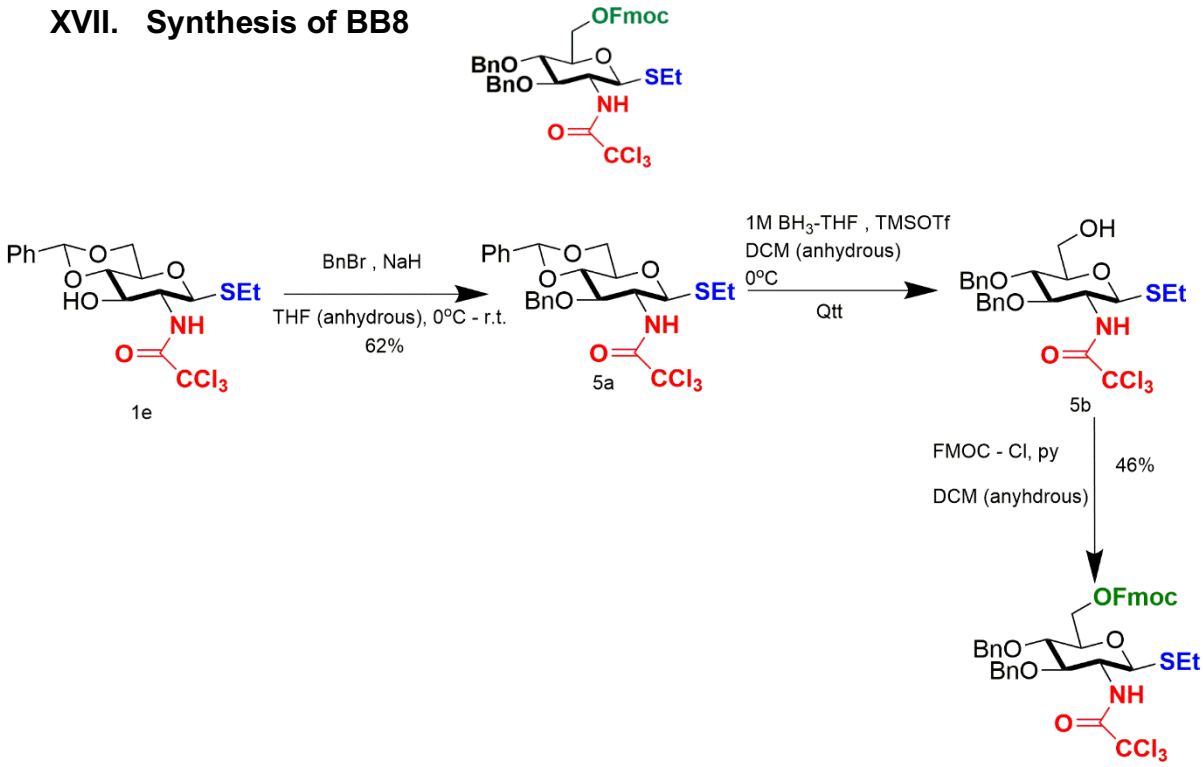
Step 3– Analysis

TLC: R_f = 0.5 (Hex/EA 4:1)

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 – 7.92 (m, 7H,-Ar), 7.65 – 7.54 (m, 3H,-Ar), 7.50 – 7.40 (m, 8H), 7.27 – 7.23 (m, 3H,-Ar), 5.32 (dd, J = 9.7, 3.1 Hz, 1H,H-3), 4.93 – 4.87 (m, 2H), 4.80 (dd, J = 8.9, 2.8 Hz, 1H), 4.63 (d, J = 9.7 Hz, 1H,H-1), 4.60 – 4.56 (m, 2H), 4.53 – 4.44 (m, 3H), 4.01 – 3.95 (m, 1H), 2.87 – 2.70 (m, 2H,-SCH_2), 1.36 – 1.32 (m, 4H,-SCH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.21(OC=O), 166.09(OC=O), 138.32(-Ar), 138.30(-Ar), 138.24(-Ar), 138.12, 137.86, 137.76, 137.66, 137.58, 137.49, 129.87, 129.84, 129.81, 129.80, 129.75, 85.64 (C-1), 76.02, 75.96, 75.78, 74.69, 73.57, 25.37 (-SCH_2), 15.24(-SCH_3)

XVII. Synthesis of BB8



Step1: Installation of benzyl group at C3

This procedure maintained an inert atmosphere throughout. **1e** (23.7 g , 51.887 mmol) was weighed into a 500 mL 3-necked R.B. flask fitted with a septum and Ar balloon and flushed with Ar for a while. THF (236 mL, 0.22 M) was added into it, and the reaction flask was then placed in an ice bath. NaH (60% dispersion in mineral oil) (5.39 g, 134.906 mmol) was added via a powder funnel, followed by dropwise addition of BnBr (9.24 mL, 77.83 mmol) via a dropping funnel. After adding all the reagents, the flask was removed from the ice bath and left to stir at room temperature overnight. After overnight stirring, the reaction was quenched with Sat. aq. NH_4Cl (~ 50 mL) till no more gas evolved and no more bubbling occurred. The

reaction mixture was diluted with DCM (~ 100 mL) and poured into a separating funnel. The organic phase was then extracted. The aqueous phase was then extracted with DCM (~ 100 mL, 2X). The organic phase was then subsequently washed with Brine (~ 150 mL, 2X). It was then dried with $MgSO_4$, concentrated and dried under high vacuum for ~ 1 hr.

Step 1-Puriifcation

Ethyl acetate (distilled) (~ 150 mL) was added to the crude material at 55^oC on a heating mantle. The precipitate so obtained was kept in the fridge overnight to induce more precipitation. It was then washed with cold ethyl acetate.

Step1-Analysis

TLC: R_f = 0.47 (Hex/EA 5:1)

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.16 (d, *J* = 9.0 Hz, 1H, -NHCO), 7.48 – 7.20 (m, 11H, -Ar), 5.73 (s, 1H, -PhCH), 4.82 (d, *J* = 10.1 Hz, 1H, H-1), 4.74 (d, *J* = 11.5 Hz, 1H, -CH_2Bn), 4.65 (d, *J* = 11.5 Hz, 1H, -CH_2Bn), 4.27 (dd, *J* = 10.1, 5.0 Hz, 1H, H-6a), 3.93 – 3.83 (m, 1H), 3.47 (td, *J* = 9.8, 5.0 Hz, 1H, H-5), 2.65 (qq, *J* = 12.8, 7.4 Hz, 2H, -SCH2), 1.19 (t, *J* = 7.4 Hz, 3H , -SCH_3).

^{13}C NMR (176 MHz, DMSO) δ 161.22 (NHC=O), 138.26(-Ar), 128.10(-Ar), 127.40(-Ar), 125.88(-Ar), 100.02(-PhCH), 92.93(-CCl_3), 83.74(C-1), 80.77, 78.91, 73.59, 69.97, 67.62, 55.61(C-2), 15.08(-SCH_3).

Step 2: Regioselective ring opening of benzylidene ring at C6

5a (8.6 g , 15.72 mmol) was weighed into a 3-necked 250 mL R.B. flask fitted with a septum and Ar balloon and flushed with Ar for a while. DCM (105 mL, 0.15 M) was added into it, and the reaction flask was placed in an ice bath. 1M BH_3 in THF (62.90 mmol) was added dropwise via a dropping funnel, followed by addition of TMSOTf (1.42 mL, 7.863 mmol) via a syringe. The reaction was allowed to stir on ice bath. After ~ 1.5 hrs, the reaction was quenched with Et_3N/MeOH 1:10 (~ 44mL) till no more gas evolved. It was then diluted with DCM (~ 75 mL) and poured into a separating funnel. It was subsequently washed with Sat. aq. $NaHCO_3$ (~ 150 mL, 2X) and Brine (~ 150 mL, 2X). The organic phase was collected, dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 2 – Analysis

TLC: R_f = 0.47 (Hex/EA 2:1)

^{1}H NMR (700 MHz, DMSO-d_6) δ 9.13 (d, *J* = 9.2 Hz, 1H, -NHCO), 7.36 – 7.22 (m, 10H, -Ar), 4.82 (t, *J* = 5.7 Hz, 1H, -OH), 4.72 (d, *J* = 11.1 Hz, 1H, -CH_2Bn), 4.64 (d, *J* = 11.1 Hz, 1H,-CH_2Bn), 4.31 (t, *J* = 5.2 Hz, 1H), 3.88 (t, *J* = 9.4 Hz, 1H), 3.81 (q, *J* = 9.7 Hz, 1H), 3.72 (ddd,

J = 12.1, 5.1, 1.9 Hz, 1H), 3.58 (dd, *J* = 11.4, 8.1 Hz, 1H), 3.52 (t, *J* = 9.4 Hz, 1H), 3.39 (td, *J* = 6.5, 5.0 Hz, 1H), 3.31 (ddd, *J* = 9.9, 4.9, 1.8 Hz, 1H), 2.66 (ddq, *J* = 45.3, 12.5, 7.4 Hz, 2H,-SCH_2), 1.20 (t, *J* = 7.4 Hz, 3H,-SCH_3).

^{13}C NMR (176 MHz, DMSO) δ 161.12(NHC=O), 138.34(-Ar), 138.21(-Ar), 128.22(-Ar), 128.11(-Ar), 127.70(-Ar), 127.5(-Ar), 127.44(-Ar), 93.04(-CCl_3), 82.91(C-1), 82.66, 80.06, 77.87, 74.25, 73.98, 66.99, 60.35, 56.02, 39.88, 39.76, 39.64, 25.11(-SCH_2), 14.88(-SCH_3).

Step 3 – Procedure: Installation of the FMOC group at C6

This procedure maintained an inert atmosphere throughout. **5b** (10.6 g, 19.311 mmol) was purged with Ar for a while. DCM (54 mL , 0.36 M) was added into it , and quickly transferred into a 3-necked 250 mL R.B: flask (already flushed with Ar). The reaction flask was then placed in an ice bath. Pyridine (4.67 mL , 57.93 mmol) was added into it via a syringe. FMOC-Cl (5.99 g , 23.17 mmol) was dissolved in DCM (~ 10 mL) and then added into the reaction flask. The reaction was allowed to stir on ice bath for ~ 10 mins and then left to stir at room temperature overnight. After overnight stirring, MeOH (~ 50 mL) was added into the reaction flask, and then poured into a separating funnel, and the organic phase was extracted. The aqueous phase was then extracted with EtOAc (~ 100 mL, 3X). The combined organic phase was then extracted with Brine (~ 100 mL, 2X). It was then dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 3- Purification

The crude material was dissolved in Ethyl acetate (~ 250 mL) at 55^{o}C on a heating mantle. When all of it dissolved, it was filtered hot into an Erlenmeyer flask, and EtOH (abs) (~ 250 mL) was added into it. It was then kept in the fridge overnight to induce crystallization. The crystals so obtained were washed and filtered with cold EtOH.

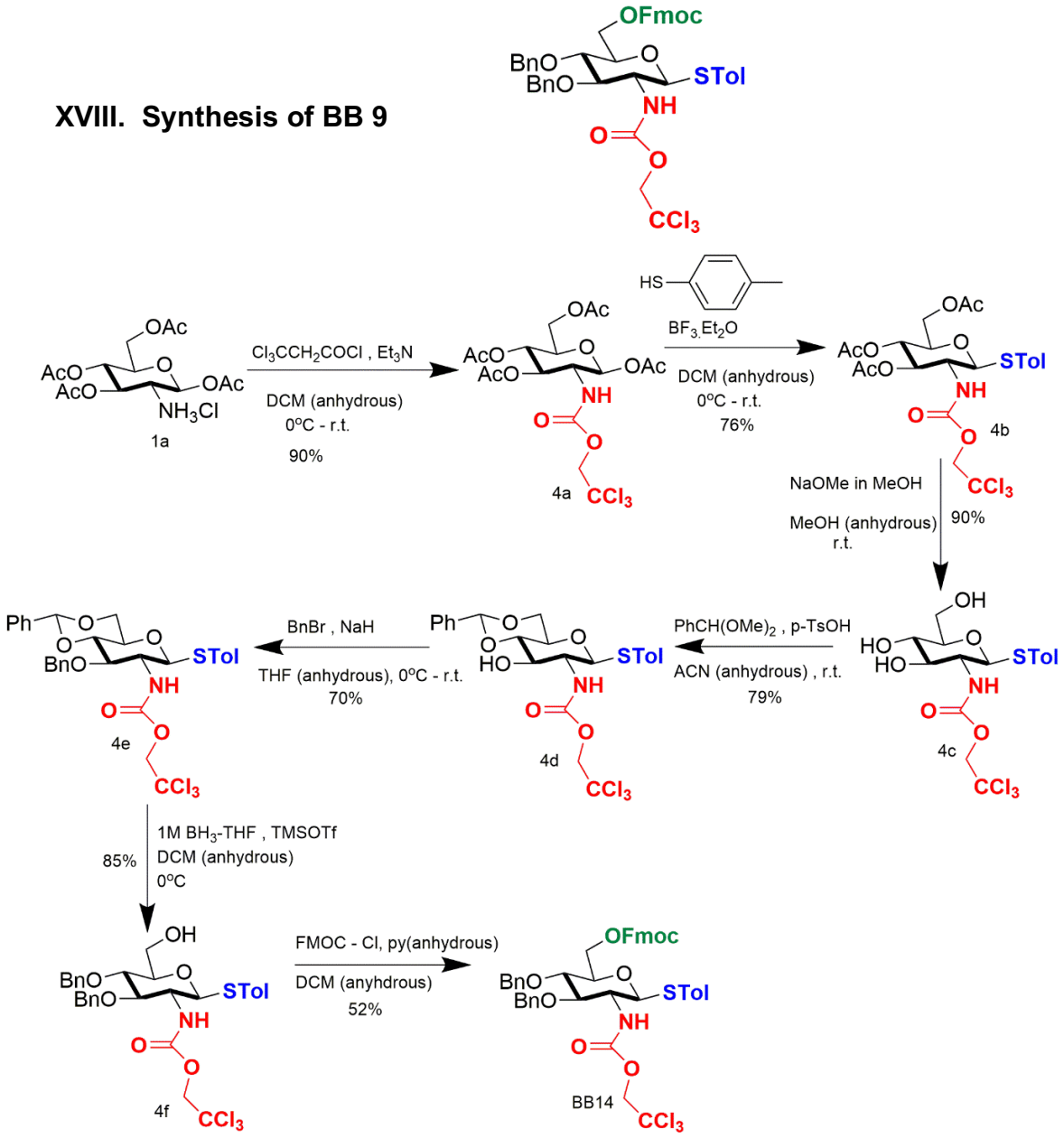
Step 3 – Analysis

TLC: R_f = 0.4 (Hex/EA 5:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.77 (ddt, *J* = 7.6, 2.0, 0.9 Hz, 2H), 7.62 (ddq, *J* = 9.2, 7.5, 0.9 Hz, 2H), 7.41 (tdt, *J* = 7.5, 1.9, 0.8 Hz, 2H), 7.36 – 7.27 (m, 13H), 6.91 (d, *J* = 8.3 Hz, 1H), 4.93 (d, *J* = 10.1 Hz, 1H), 4.85 (dd, *J* = 18.0, 10.9 Hz, 2H), 4.77 (d, *J* = 10.8 Hz, 1H), 4.63 (d, *J* = 11.0 Hz, 1H), 4.47 (dd, *J* = 11.7, 2.3 Hz, 1H), 4.40 (qd, *J* = 10.5, 7.5 Hz, 2H), 4.32 (dd, *J* = 11.6, 5.4 Hz, 1H), 4.26 (t, *J* = 7.5 Hz, 1H), 4.08 (dd, *J* = 9.7, 8.4 Hz, 1H), 3.77 (td, *J* = 9.9, 8.2 Hz, 1H), 3.70 (ddd, *J* = 9.6, 5.4, 2.3 Hz, 1H), 3.64 (dd, *J* = 9.5, 8.3 Hz, 1H), 2.78 – 2.66 (m, 2H), 1.27 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (176 MHz, $CDCl_3$) δ 161.76(NHC=O), 155.05(OC=O), 143.50(-Ar), 143.38(-Ar), 141.41(-Ar), 137.64(-Ar), 137.50(-Ar), 128.71(-Ar), 128.23(-Ar), 128.15(-Ar), 128.11(-Ar), 128.04(-Ar), 128.01(-Ar), 127.31(-Ar), 125.32(-Ar), 125.27(-Ar), 120.20(-Ar), 92.53(-CCl_3), 82.78(C-1), 81.85, 78.28, 70.15, 66.74, 57.49, 46.84(-FMOC (CH)), 24.81(-SCH_2), 15.27(-SCH_3).

XVIII. Synthesis of BB 9



Step 1: Procedure - Installation of the troc group

This procedure maintained an inert atmosphere throughout. In a 100 mL 3-necked round bottom flask, 8.3 mmol of **1a** (3.2 g) was purged with argon for several minutes before adding 21 mL of anhydrous DCM (final concentration: ~ 0.4M). The flask was the placed in an ice bath to cool down to 0°C. Then, Et_3N (16.6mmol, 2.3 mL) was added via a syringe, followed by dropwise addition of Cl_3CCH_2OCOCl (11mmol, 1.5 mL) via a dropping funnel. The reaction was then allowed to stir on ice bath. After ~ 2.5 hours, the reaction was quenched with H_2O (~ 40 mL) and allowed to stir for ~ 20 minutes. Then, the reaction mixture was poured into a separating funnel, and the organic phase was extracted. The organic phase was then extracted with 1N HCl (~ 50 mL, 1x), Sat. aq. $NaHCO_3$ (~ 50 mL, 1x) and Brine (~ 50 mL, 1x). The combined organic phase was then dried with $MgSO_4$, concentrated and dried under high vacuum to give 3.8 g of pure **4a**.

Analysis: TLC: R_f = 0.5 (Hex/EA 4:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 5.73 (d, J = 8.7 Hz, 1H,H-1), 5.40 (d, J = 9.5 Hz, 1H,H-3), 5.25 (d, J = 9.6 Hz, 1H,-NHCO), 5.10 (t, J = 9.7 Hz, 1H,H-4), 4.72 (d, J = 3.9 Hz, 2H,-Cl_3CCH_2), 4.28 (dd, J = 12.3, 4.6 Hz, 1H,H-6a), 4.11 (dd, J = 12.5, 4.6 Hz, 1H,H-6b), 3.96 (q, J = 9.6 Hz, 1H,H-2), 3.85 (ddd, J = 10.1, 4.6, 2.3 Hz, 1H,H-5), 2.10 (d, J = 3.2 Hz, 3H), 2.08 (s, 3H,-$OCOCH_3$), 2.04 (d, J = 2.3 Hz, 6H,-$OCOCH_3$).

^{13}C NMR (176 MHz, $CDCl_3$) δ 170.99(OC=O), 170.77(OC=O), 169.53(OC=O), 169.36(NHC=O), 95.53(-CCl_3), 92.42 (C-1), 72.91, 72.22, 68.16, 61.76, 55.20(C-2), 20.98($OCOCH_3$), 20.85(-$OCOCH_3$), 20.75($OCOCH_3$), 20.72(-$OCOCH_3$).

Step 2: Procedure – Thioglycosylation

This procedure maintained an inert atmosphere throughout. To 7.3 mmol of **4a** (3.8g), 10 mL anhydrous DCM (final concentration: ~ 0.7M) was added under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, p-toluylthiol (8 mmol, 1g) was added, followed by dropwise addition of $BF_3.Et_2O$ (5.4 mmol, 0.7 mL). Then, the flask was removed from the ice bath for ~ 5 minutes, and then left to stir at room temperature for ~ 12 hours. After 2.5 hours, the reaction mixture was diluted with DCM (~ 100 mL) and poured into a separating funnel. Then, it was extracted with Sat.aq. $NaHCO_3$ (~ 75 mL, 2x) and Brine (~ 75 mL, 2x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 2 – Purification

The crude material was dissolved in EA (~ 50 mL) at 55°C on a heating mantle. When all of it dissolved, the flask was removed from the heating mantle, and Hexane (~ 80 mL) was first added dropwise at room temperature, and then quickly under rigorous stirring. The precipitate so obtained was kept in the fridge overnight. Then, it was washed and filtered with cold Hexane to give 3.1 g pure **4b.**

Step 2 – Analysis

TLC: R_f = 0.6 (Hex/EA 2:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.42 – 7.39 (m, 2H,-Ar), 7.13 – 7.10 (m, 2H,-Ar), 5.29 – 5.25 (m, 2H,-NHCO,H-4), 5.01 (t, J = 9.7 Hz, 1H,H-3), 4.79 (d, J = 10.0 Hz, 1H,H-1), 4.72 (d, J = 12.1 Hz, 1H,-Cl_3CCH_2), 4.22 (dd, J = 12.2, 5.3 Hz, 1H,H-6a), 4.16 (dd, J = 12.2, 2.4 Hz, 1H), 3.70 (ddd, J = 10.0, 5.0, 2.3 Hz, 1H,H-5), 3.65 (q, J = 9.9 Hz, 1H,H-2), 2.34 (s, 3H,-SCH_3), 2.08 (s, 3H,-$OCOCH_3$), 2.00 (s, 3H,-$OCOCH_3$), 1.99 (s, 3H,-$OCOCH_3$).

^{13}C NMR (176 MHz, $CDCl_3$) δ 170.72(OC=O), 169.57(NHC=O), 133.79(-Ar), 129.87(-Ar), 95.53(-CCl_3), 86.81(C-1), 75.90, 74.67, 73.34, 68.66, 62.44, 55.15(C-2), 21.31(-SCH_3), 20.88($OCOCH_3$), 20.76($OCOCH_3$), 20.72($OCOCH_3$).

Step 3 – Procedure – Removal of the acetyl groups

This procedure maintained an inert atmosphere throughout. In a 100 mL 3-necked round bottom flask, 5.3 mmol of compound **4b** (3.1 g) was purged with argon for several minutes before adding 38 mL of anhydrous MeOH (final concentration: ~ 0.14M). Then, NaOMe in MeOH (2.11 mmol, 0.4 mL) was added via a syringe and the reaction was left to stir a room temperature for ~ 12 hours. Then, the reaction mixture was neutralized by passing the reaction mixture through a bed of amberlite resin till pH reaches 6. The filtrate so obtained was then concentrated and dried under high vacuum to give 2.1 g of pure **4c**.

Step – 3- Analysis

TLC: R_f = 0.6 (Hex/EA 1:1)

^{1}H NMR (700 MHz, DMSO-d6) δ 7.36 – 7.30 (m, 2H), 7.13 (d, J = 9.0 Hz, 1H), 7.11 (d, J = 8.0 Hz, 2H), 6.81 (t, J = 6.9 Hz, 1H), 5.10 (d, J = 5.3 Hz, 1H,-OH), 5.07 – 5.03 (m, 1H,-OH), 4.66 (d, J = 9.9 Hz, 1H,H-1), 4.56 (t, J = 6.3, 5.4 Hz, 1H), 4.04 (d, J = 6.8 Hz, 1H), 3.69 (ddd, J = 11.8, 5.3, 1.3 Hz, 1H), 3.45 (dd, J = 11.9, 5.9 Hz, 1H), 3.26 (d, J = 9.5 Hz, 1H), 3.12 (dt, J = 4.7, 2.6 Hz, 2H,H-3,H-4), 2.27 (s, 4H).

^{13}C NMR (176 MHz, DMSO) δ 135.89(-Ar), 131.53(-Ar), 129.80(-Ar), 129.47(-Ar), 86.83(-CCl_3), 81.14(C-1), 75.47, 75.30, 70.31, 61.00, 56.45, 51.28(C-2), 20.57(-SCH_3).

Step 4 – Procedure: Benzylidene formation

This procedure maintained an inert atmosphere throughout. To 7.8 mmol of compound **4c** (3.6 g) 31 mL anhydrous ACN (final concentration: ~ 0.25M) were added under argon atmosphere. Gently stirring at room temperature, benzaldehyde dimethyl acetal (16mmol, 2.3 mL) were added via a syringe followed by addition of p-toluenesulfonic acid acid (1.4 mmol, 0.3 g.) via a powder funnel. The reaction was allowed to stir at room temperature for ~ 12 hours (overnight). The next day, the reaction mixture was diluted with 75 mL ethyl acetate and under vigorous stirring 100 mL of saturated aqueous $NaHCO_3$ was added. After stirring for several minutes, the reaction mixture was poured into a separating funnel, the organic phase was separated and subsequently washed with 100 mL brine (2x). Finally, the organic phase was dried with $MgSO_4$ before it was concentrated and dried under high vacuum.

Step 4 Purification

The crude material was dissolved in DCM (~60 mL) at on a heating mantle set to 55°C. When all of it dissolved, it was poured into a 500 mL Erlenmeyer flask and then hexane (~ 200 mL) was added into it dropwise via a dropping funnel until precipitation started. The remainder volume of hexane was then quickly added under vigorous stirring. The precipitate so obtained was then kept in the fridge overnight. The next day, the precipitate was filtered and washed with cold hexane to give 3 g of pure compound **4d** after drying.

Step-4- Analysis

TLC: R_f = 0.47 (Hex.EA: 3:1)

^{1}H NMR (700 MHz, DMSO-d6) δ 7.47 – 7.30 (m, 6H), 7.29 (d, J = 9.5 Hz, 1H,-NHCO), 7.15 (d, J = 8.1 Hz, 2H), 5.60 (s, 1H,-PhCH), 5.46 (d, J = 5.9 Hz, 1H), 4.85 (d, J = 10.5 Hz, 1H,H-1), 4.19 (dt, J = 10.2, 4.7 Hz, 1H,H-6b), 3.69 (t, J = 10.1 Hz, 1H,H-6a,H-3), 3.60 (td, J = 9.2, 5.8 Hz, 1H,H-5), 3.45 (t, J = 9.3 Hz, 1H,H-4), 3.40 (t, J = 9.7 Hz, 1H,H-2), 2.50 (p, J = 1.8 Hz, 3H), 2.29 (s, 3H).

^{13}C NMR (176 MHz, DMSO) δ 130.64(-Ar), 129.61(-Ar), 128.00(-Ar), 126.32(-Ar), 100.63(-PhCH), 87.01, 80.79, 71.70, 69.99, 67.63, 56.97, 51.37, 20.60.

Step 5 – Procedure: Installation of benzyl group at C3

This procedure maintained an inert atmosphere throughout. **4e** (2.2g , 4mmol) was weighed into a 100 mL 3-necked R.B. flask fitted with a septum and Ar balloon and flushed with Ar for a while. THF (17mL, 0.2 M) was added into it, and the reaction flask was then placed in an ice bath. NaH (60% dispersion in mineral oil) (0.4g, 10.4mmol) was added via a powder funnel, followed by dropwise addition of BnBr (mL, mmol) via a dropping funnel. After adding all the reagents, the flask was removed from the ice bath and left to stir at room temperature overnight. After overnight stirring, the reaction was quenched with Sat. aq. NH_4Cl (~ 50 mL) till no more gas evolved and no more bubbling occurred. The reaction mixture was diluted with DCM (~ 100 mL) and poured into a separating funnel. The organic phase was then extracted. The aqueous phase was then extracted with DCM (~ 100 mL, 2X). The organic phase was then subsequently washed with Brine (~ 150 mL, 2X). It was then dried with $MgSO_4$, concentrated and dried under high vacuum for ~ 1 hr.

Step 5-Puriifcation

Ethyl acetate (distilled) (~ 150 mL) was added to the crude material at 55^oC on a heating mantle. The precipitate so obtained was kept in the fridge overnight to induce more precipitation. It was then washed with cold ethyl acetate.

Step 5-Analysis

TLC: R_f = 0.47 (Hex/EA 5:1)

Step 6 Procedure: Installation of FMOC group at C6

This procedure maintained an inert atmosphere throughout. **4f** (g, mmol) was purged with Ar for a while. DCM (mL , M) was added into it , and quickly transferred into a 3-necked 250 mL R.B: flask (already flushed with Ar). The reaction flask was then placed in an ice bath. Pyridine (mL , mmol) was added into it via a syringe. FMOC-Cl (g , mmol) was dissolved in DCM (~ 10 mL) and then added into the reaction flask. The reaction was allowed to stir on ice bath for ~ 10 mins and then left to stir at room temperature overnight. After overnight stirring, MeOH (~ 50 mL) was added into the reaction flask, and then poured into a separating funnel, and the organic phase was extracted. The aqueous phase was then extracted with EtOAc (~ 100 mL, 3X). The combined organic phase was then extracted with Brine (~ 100 mL, 2X). It was then dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 3- Purification

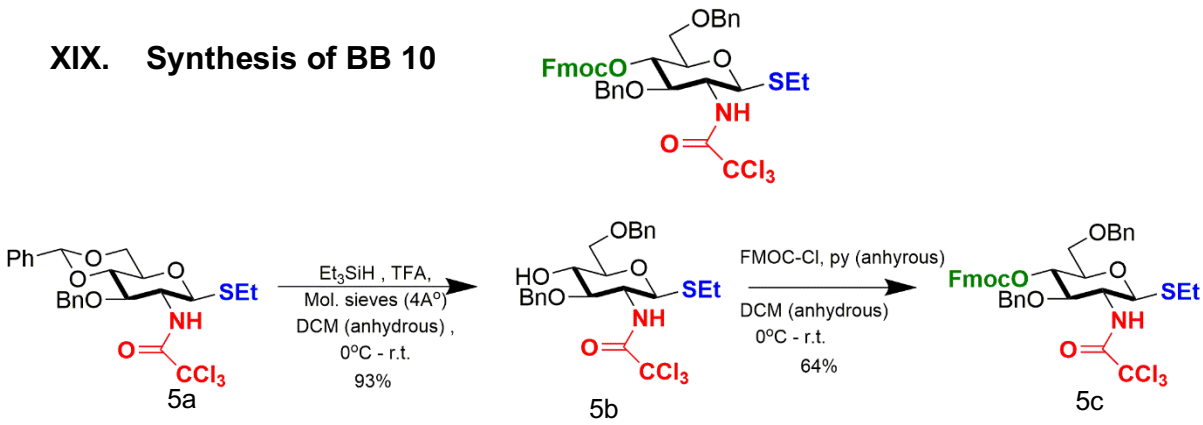
The crude material was dissolved in Ethyl acetate (~ 250 mL) at 55°C on a heating mantle. When all of it dissolved, it was filtered hot into an Erlenmeyer flask, and EtOH (abs) (~ 250 mL) was added into it. It was then kept in the fridge overnight to induce crystallization. The crystals so obtained were washed and filtered with cold EtOH.

Step 3 – Analysis

TLC: R_f = 0.4 (Hex/EA 5:1)

^{1}H NMR (700 MHz, Chloroform-*d*) δ 7.77 (ddt, *J* = 7.6, 2.0, 0.9 Hz, 2H,-Ar), 7.62 (ddq, *J* = 9.2, 7.5, 0.9 Hz, 2H,-Ar), 7.41 (tdt, *J* = 7.5, 1.9, 0.8 Hz, 2H,-Ar), 7.36 – 7.27 (m, 13H,-Ar), 6.91 (d, *J* = 8.3 Hz, 1H), 4.93 (d, *J* = 10.1 Hz, 1H,H-1), 4.85 (dd, *J* = 18.0, 10.9 Hz, 2H), 4.77 (d, *J* = 10.8 Hz, 1H), 4.63 (d, *J* = 11.0 Hz, 1H,-CH_2Bn), 4.47 (dd, *J* = 11.7, 2.3 Hz, 1H,-CH_2Bn), 4.40 (qd, *J* = 10.5, 7.5 Hz, 2H,-FMOC(CH_2)), 4.32 (dd, *J* = 11.6, 5.4 Hz, 1H), 4.26 (t, *J* = 7.5 Hz, 1H,-FMOC(CH)), 4.08 (dd, *J* = 9.7, 8.4 Hz, 1H), 3.77 (td, *J* = 9.9, 8.2 Hz, 1H), 3.70 (ddd, *J* = 9.6, 5.4, 2.3 Hz, 1H,H-5), 3.64 (dd, *J* = 9.5, 8.3 Hz, 1H,H-2), 2.78 – 2.66 (m, 2H,-SCH_2), 1.27 (t, *J* = 7.4 Hz, 3H,-SCH_3).

XIX. Synthesis of BB 10



Step1 Procedure: Regioselective opening of Benzylidene ring at C4

This procedure maintained an inert atmosphere throughout. In a 500 mL 3-necked round bottom flask, 22 mmol of **5a** (22 g) were purged with argon for several minutes before adding 170 mL of anhydrous DCM (final concentration: ~ M) and molecular sieves (4 Å). The suspension was stirred at room temperature for 10 minutes and then, Et_3SiH (88 mmol, 14 mL) was added, and placed in an ice bath to cool down to 0°C. After ~ 30 minutes, TFA (91 mmol, 7 mL) was added dropwise via a dropping funnel over 5 minutes. The reaction was allowed to stir at 0°C for ~ 15 minutes, and then left to stir at room temperature. After 3 hours,

the reaction mixture was quenched by dropwise addition of Et_3N (~ 40 mL) till no more gas evolved. The reaction mixture was then diluted with DCM (130 mL) and poured into a separating funnel. It was then extracted with Sat. aq. $NaHCO_3$ (300 mL, 3x) and Brine (300 mL, 2x). The combined organic phase was dried with $MgSO_4$, concentrated and subjected to purification.

Step 1 Purification

The crude material was subjected to column chromatography (Hex/EA 6:1 to 2:1) to give 9 g of pure **5b.**

Step 1 Analysis

^{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,-SCH_2), 1.22 – 1.18 (m, 3H,-SCH_3).

Step 2 Procedure: Installation of FMOC group at C4

This procedure maintained an inert atmosphere throughout. 10 mmol of **5b** (5.8 g) were purged with argon for several minutes before adding 75 mL of anhydrous DCM (final concentration: ~ 0.13M). The reaction was allowed to stir at room temperature and Pyridine (32 mmol, 2.6 mL) were added , followed by FMOC-Cl (11.6 mmol, 3.01 g). The colour of the reaction ixture turned from faint yellow to ornage to finally red. After 3 hours, the reaction was diluted with DCM (~ 75 mL) and extrcated with H_2O (150 mL, 2x) and Brine (150 mL, 2x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 2 Purification

The crude material was dissolved in EA (~ 15 mL) and Hexane (~15 mL) at 70^{o}C on a heating mantle. When all of it dissolved, it was kept in the freezer to induce crystallization. After ~ 4 days, crytsals could be incued by scratching. Then, the crystals so obatined were washed and filtered with cold Hex/EA 1:1, and dried in the desiccator to give 6 g of pure **BB 10**.

Step 2 Analysis

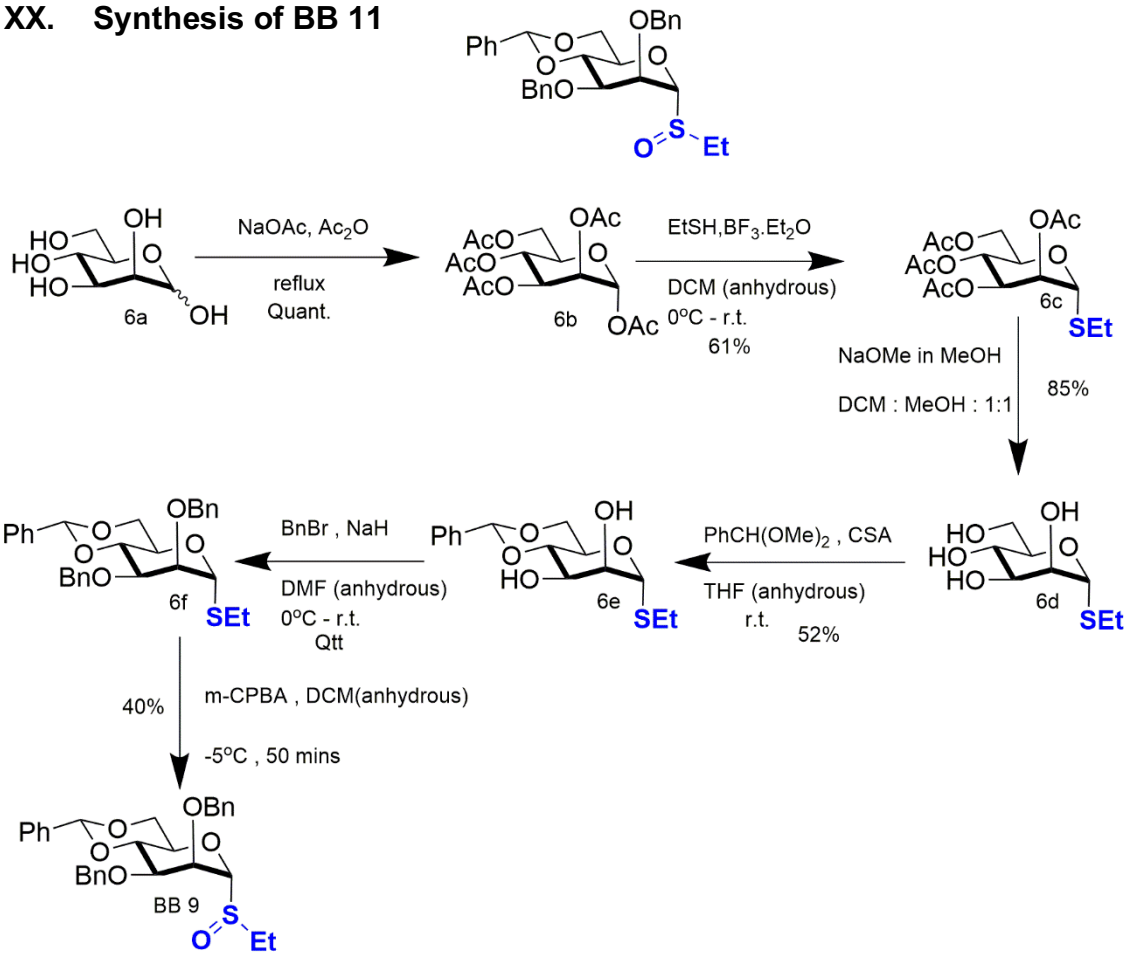
TLC: R_f = 0.5 (Hex/EA 4:1)

^{1}H NMR (500 MHz, Chloroform-d) δ 7.75 (ddt, J = 7.7, 3.0, 0.9 Hz, 2H,-Ar), 7.57 (dd, J = 7.5, 1.0 Hz, 1H,-Ar), 7.52 (dd, J = 7.5, 1.0 Hz, 1H,-Ar), 7.39 (td, J = 7.5, 1.0 Hz, 2H,-Ar), 7.36 –

7.17 (m, 12H,-Ar), 6.93 (d, J = 7.9 Hz, 1H, NHC=O), 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 (NHC=O), 154.39 (OC=O), 143.38(-Ar), 143.19(-Ar), 141.42(-Ar), 141.38(-Ar), 137.96(-Ar), 137.41(-Ar), 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 (C-1), 78.88 (C-3), 77.41 (C-2), 76.26 (C-4), 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).

XX. Synthesis of BB 11



Step 1- Procedure: Installation of acetyl groups

This procedure does not need an inert atmosphere. In a 250 mL 3-neck round bottom flask mmol sodium acetate (10 g, 0.5 eq.) were mixed with mmol acetic anhydride (101 mL, 10.0 eq.). A reflux condenser was fitted to one of the necks, and the flask was placed on a heating mantle. The reaction was then refluxed at 120 °C for 5 minutes. 122 mmol of D MANNOSE (20.0 g) were then added in portions over a period of 10 minutes and the temperature was maintained at 120°C. After the addition, the reaction was allowed to cool down to room temperature for 30 minutes. The reaction was then poured into ice in a 500 mL beaker and stirred for 1 hour. It was then poured into a separating funnel and extracted with 250 mL ethyl acetate (1X). Subsequently, the organic phase was washed with 150 mL saturated aqueous $NaHCO_3$ (2x) and 150 mL brine (2x). Finally, the organic phase was dried with $MgSO_4$, the solvent removed by evaporation and the resulting oil was dried under high vacuum to give 44 g of pure compound **6b** as a yellow oil.

Step 1 Analysis

TLC: R_f = 0.5 (Hex/EA 2:1)

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.08 (d, J = 1.5 Hz, 1H,H-1), 5.37 – 5.32 (m, 1H), 5.30 – 5.22 (m, 1H), 4.28 (ddd, J = 12.4, 10.0, 5.1 Hz, 1H), 4.09 (dd, J = 12.4, 2.4 Hz, 1H), 2.17 (d, J = 3.4 Hz, 2H), 2.10 – 2.08 (m, 6H,-OAc), 2.00 (d, J = 1.0 Hz, 3H,-OAc).

^{13}C NMR (101 MHz, $CDCl_3$) δ 177.25(OC=O), 170.86(OC=O), 170.39(OC=O), 169.93(OC=O), 169.72(OC=O), 90.70(C-1), 73.38, 70.77, 68.85, 65.63, 62.22(C-6), 20.98, 20.89, 20.88, 20.84, 20.78

Step 2 – Procedure: Thioglycosylation

This procedure maintained an inert atmosphere throughout. To 129 mmol of compound **6b** (50.2 g) 430 mL anhydrous dichloromethane (final concentration: ~ 0.3 M) were added under argon atmosphere and placed on an ice bath to cool down to 0 °C. Then, 257 mmol ethanethiol (19 mL, 2.0 eq.) were added via a powder funnel followed by dropwise addition of 321 mmol $BF_3{\cdot}Et_2O$ (40 mL, 2.5 eq.) over 10 minutes via a dropping funnel. The reaction was allowed to stir on ice bath for another 5 minutes, then removed from the ice bath and allowed to stir at room temperature for ~ 12 hours. Then, the reaction mixture was poured into an Erlenmeyer flask, and 500 mL Sat. aq. $NaHCO_3$ was added into it and kept to stir for some time. It was then poured into a separating funnel, and the organic phase was separated. The combined organic phase was subsequently washed with 400 mL brine (2x). Finally, the organic phase was dried over $MgSO_4$ before it was concentrated and dried under high vacuum.

Step 2 Purification

The crude material was dissolved in ethyl acetate (~ 125 mL) at 55 °C on a heating mantle. When all of it dissolved, it was removed from the mantle, and hexane (~ 750 mL) was added into it dropwise via a dropping funnel until precipitation started. The remainder volume of hexane was then added quickly under vigorous stirring. The precipitate so obtained was then kept in the fridge overnight. The next day, the precipitate was filtered and washed with cold hexane to give g of pure compound **6c** after drying.

Step 2 - Analysis

TLC: R_f = 0.5 (Hex/EA 3:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 5.33 – 5.28 (m, 2H, H-3, H-4), 5.27 (d, *J* = 1.8 Hz, 1H,H-1), 5.24 (dd, *J* = 10.0, 3.3 Hz, 1H,H-5), 4.38 (dddd, *J* = 9.8, 5.4, 2.4, 0.7 Hz, 1H,H-2), 4.30 (dd, *J* = 12.3, 5.4 Hz, 1H,H-6a), 4.08 (dd, *J* = 12.2, 2.4 Hz, 1H,H-6b), 2.68 – 2.57 (m, 2H,-SCH_2), 2.15 (s, 3H,-$COCH_3$), 2.07 (s, 3H,-$COCH_3$), 2.03 (s, 3H,-$COCH_3$), 1.97 (s, 3H,-$COCH_3$), 1.29 (t, *J* = 7.4 Hz, 3H,-SCH_3).

^{13}C NMR (176 MHz, $CDCl_3$) δ 170.69(OC=O), 170.08(OC=O), 169.87(OC=O), 169.83(OC=O), 82.38(C-1), 71.28(C-3), 69.59(C-2), 69.02(C-4), 66.49(C-5), 62.54(C-6), 25.56(-SCH_2), 21.02(-$COCH_3$), 20.79(-$COCH_3$), 20.72(-$COCH_3$), 14.85(-SCH_3).

Step 3 – Procedure: Removal of the acetyl groups

This procedure maintained an inert atmosphere throughout. In a 500 mL 3-neck round bottom flask mmol of compound **6c** (21.3 g) were flushed with argon for several minutes before adding mL DCM/MeOH 1:1 (final concentration: ~ 0.5 M). Gently stirring at room temperature, 22 mmol NaOMe in MeOH (5.4 M) (mL, 0.4 eq) were added into it via a syringe. The reaction was then allowed to stir at room temperature for ~ 12 hours (overnight). The next day, the reaction was passed through a column of Amberlite resin (~ 25 mL) in a sintered funnel till pH of the filtrate reaches 6. The solvent was then removed by evaporation and the resulting solid was dried under high vacuum to give g of pure compound **6d** after drying.

Step 3-Analysis

TLC: R_f = 0.5 (Hex/EA 1:1)

^{1}H NMR (400 MHz, MeOH-d4) δ 5.25 (d, J = 1.4 Hz, 1H,H-1), 3.90 (dt, J = 3.0, 1.9 Hz, 2H), 3.82 (dd, J = 11.9, 2.4 Hz, 1H), 3.73 (dd, J = 11.8, 5.8 Hz, 1H), 3.66 – 3.63 (m, 2H), 2.77 – 2.53 (m, 2H,-SCH_2), 1.28 (t, J = 7.4, 3H,-SCH_3).

^{13}C NMR (101 MHz, MeOH-D4) δ 84.64(C-1), 84.54, 73.51, 72.42, 71.89, 67.54, 61.45, 24.40(-SCH_2), 13.95(-SCH_3).

Step 4 –Procedure: Benzylidene formation

This procedure maintained an inert atmosphere throughout. To 31mmol of compound **6d** (7g), 62mL anhydrous THF (final concentration: ~ 0.5 M) were added under argon atmosphere. Gently stirring at room temperature, mmol of benzaldehyde dimethyl acetal (9.4mL, 2.0 eq.) were added via a syringe followed by addition of 5.5 mmol camphor sulfonic acid (1.3g, 0.2 eq.) via a powder funnel. The reaction was allowed to stir at room temperature for ~ 12 hours (overnight). The next day, the reaction mixture was diluted with 100 mL ethyl acetate and under vigorous stirring 200 mL of saturated aqueous $NaHCO_3$ was added. After stirring for several minutes, the reaction mixture was poured into a separating funnel, the organic phase was separated and subsequently washed with 150 mL brine (2x). Finally, the organic phase was dried with $MgSO_4$ before it was concentrated and dried under high vacuum.

Step 4 Purification

The crude material was dissolved in DCM (~ 80mL) at on a heating mantle set to 55°C. When all of it dissolved, it was poured into a 1 L Erlenmeyer flask and then hexane (~ 600 mL) was added into it dropwise via a dropping funnel until precipitation started. The remainder volume of hexane was then quickly added under vigorous stirring. The precipitate so obtained was then kept in the fridge overnight. The next day, the precipitate was filtered and washed with cold hexane to give g of pure compound **6e** after drying.

Step-4- Analysis

TLC: R_f = 0.5 (Hex/EA 3:1)

^{1}H NMR (700 MHz, DMSO-d6) δ 7.46 – 7.44 (m, 2H), 7.38 – 7.36 (m, 2H), 5.61 (s, 1H,-PhCH), 5.36 (d, J = 4.1 Hz, 1H,-OH), 5.23 (d, J = 1.2 Hz, 1H,H-1), 5.08 (d, J = 6.2 Hz, 1H,-OH), 4.10 (dd, J = 10.0, 4.8 Hz, 1H,H-6a), 3.95 (td, J = 9.8, 4.7 Hz, 1H,H-4), 3.89 (t, J = 9.5 Hz, 1H,H-3), 3.82 – 3.80 (m, 1H), 3.77 (t, J = 10.1 Hz, 1H), 3.67 (ddd, J = 9.6, 6.1, 3.4 Hz, 1H,H-5), 2.65 – 2.54 (m, 2H,-SCH_2), 1.22 (t, J = 7.4 Hz, 3H,-CH_3).

^{13}C NMR (176 MHz, DMSO) δ 137.89 (-Ar), 128.79(-Ar), 127.97(-Ar), 126.35(-Ar), 101.16(-PhCH), 85.65(C-1), 78.68, 72.46, 67.76, 64.46, 24.36(-SCH_2), 14.95 (-SCH_3).

Step 5 – Procedure: Installation of benzyl groups

This procedure maintained an inert atmosphere throughout. To 6 mmol of **6e** (2g), 28mL of anhydrous DMF (final concentration: ~ 0.2 M) were added under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, NaH (26mmol, 1g) was added, followed by dropwise addition of BnBr (15mmol, 2mL) via a dropping funnel. The reaction was left to stir on the ice bath for ~ 15 mins, and then left to stir at room temperature for ~ 18 hours. The reaction was then quenched with Sat. aq. NH_4Cl (~5mL) till no more bubbling occurred. The reaction mixture was then transferred into a separating funnel, and the organic phase was extracted. The aqueous phase was then extracted with DCM (~60mL,1x) and the combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 5 – Purification

The crude material was dissolved in Ethyl Acetate (~ 20mL) at 50°C on a heating mantle. When all of it dissolved, Hexane (~50 mL) was then added dropwise via a dropping funnel. The crude material was then kept in the freezer overnight to induce crystallization. Then, the crystals so obtained were washed and filtered with cold Hexane to give 4g of pure **6f**.

Step 5 – Analysis

TLC: R_f = 0.6 (Hex/EA 5:1)

Step 6 – Procedure: Formation of sulfoxide

This procedure maintained an inert atmosphere throughout. In a 250 mL 3-necked round bottom flask, 7.5 mmol of **6f** (3.7g) were purged with argon for several minutes before adding 50 mL of anhydrous DCM (final concentration: ~ 0.15M). The reaction flask was then placed in an ice bath to cool down to 0°C. Then, m-CPBA (7.5 mmol, 1.3g) was weighed into a 100 mL round bottom flask, and purged with argon for several minutes before adding ~ 20 mL DCM. This solution was then added dropwise to the reaction flask via a dropping funnel. The reaction was llowed to stir on ice bath. After ~ 1 hour, the reaction was quenched with Sat. aq. $NaHCO_3$ (~ 10 mL) till no more gas evolved. It was then poured into a separating funnel and the organic phase was extracted, and washed with Brine (~ 100 mL, 2x). The combined organic phase was then dried with $MgSO_4$, concentrated and dried under high vacuum.

Step 6 – Purification

The crude material was dissolved in EtOH (abs) (~50 mL) at 50°C on a heating mantle. When all of it dissolved, it was kept in the freezer overnight to induce crystallization. The crystals so obtained were washed and filtered with cold EtOH to give 1.4g of pure **6g**.

Step 6 - Analysis

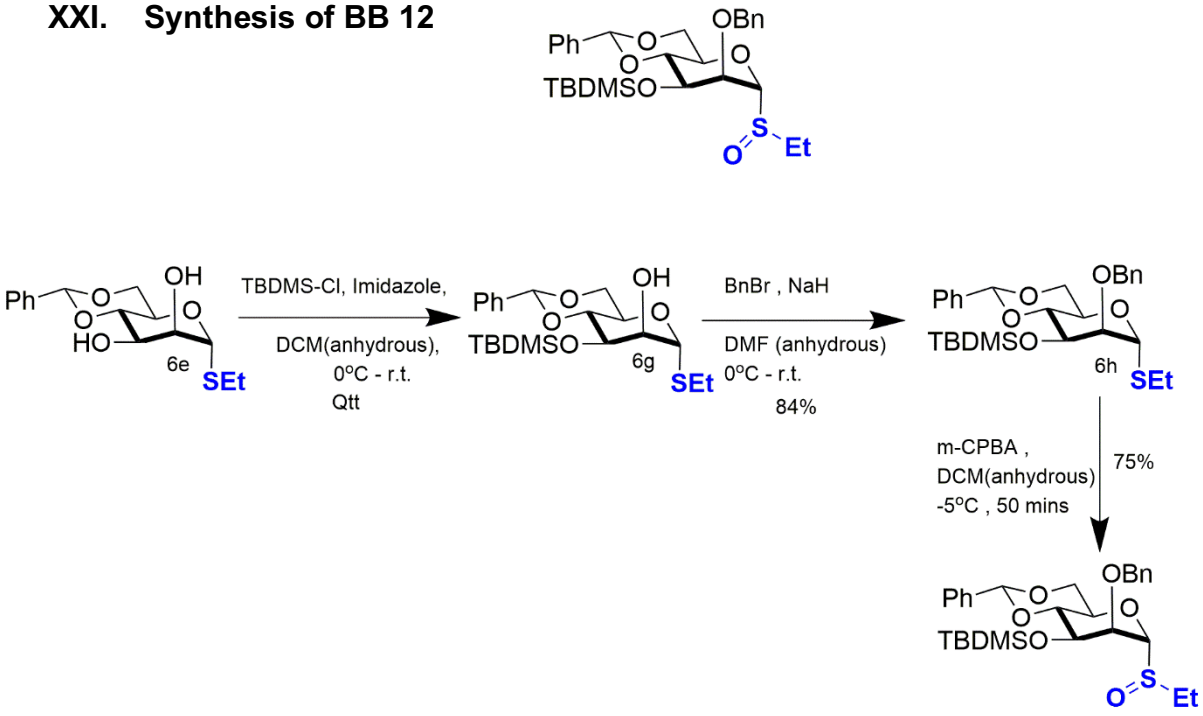
TLC: R_f = 0.5 (Hex/EA 4:1)

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.50 – 7.45 (m, 2H), 7.43 – 7.26 (m, 13H), 5.64 (s, 1H,-PhCH), 4.83 (dd, J = 17.8, 11.9 Hz, 2H,-CH_2Bn), 4.70 (dd, J = 16.7, 11.9 Hz, 2H,-CH_2Bn), 4.62 (d, J = 1.5 Hz, 1H,H-1), 4.52 (dd, J = 3.4, 1.5 Hz, 1H,H-2), 4.35 (dd, J = 10.1, 9.2 Hz, 1H,H-4), 4.21 (dd, J = 10.2, 4.6 Hz, 1H,H-6a), 4.13 (dd, J = 10.1, 3.4 Hz, 1H,H-6b), 3.72 (td, J = 9.5, 4.6 Hz, 1H,H-3), 2.78 (ddq, J = 134.0, 13.3, 7.4 Hz, 2H,-SCH_2), 1.35 (t, J = 7.5 Hz, 3H,-SCH_3).

^{13}C NMR (126 MHz, $CDCl_3$) δ 138.22(-Ar), 137.70(-Ar), 137.29(-Ar), 129.15(-Ar), 128.59(-Ar), 128.55(-Ar), 128.48(-Ar), 128.44(-Ar), 128.36(-Ar), 128.12(-Ar), 127.79(-Ar), 127.77(-Ar), 127.75(-Ar), 126.16(-Ar), 101.73(-PhCH), 92.87(C-1), 78.02, 76.27, 74.19, 73.29, 73.17, 70.19, 68.31, 44.16(-S(O)CH_2), 6.00(-S(O)CH_3).

HRMS (ESI, positive mode): Calculated m/z $[M+Na]^+$ = 531.1920, Found: 531.1772

XXI. Synthesis of BB 12



Step 1 – Procedure: Installation of silyl group at C3

This procedure maintained an inert atmosphere throughout. In a 100 mL3-necked round bottom flask, 3.2 mmol of **6e** (1g) were purged with argon for several minutes before adding 25 mL of anhydrous DCM (final concentration: ~ 0.13M). The reaction flask was then placed in an ice bath to cool down to 0°C. Then, TBDMS-Cl (3.8 mmol, 0.58g) were added, followed by Imidazole (4.48 mmol, 0.305g). The reaction was then allowed to stir at room temperature for ~ 12 hours. Then, the reaction was quenched with Sat. aq. $NaHCO_3$ (~ 50 mL) under constant stirring, and stirred for a while. Then, it was poured into a separating funnel, and the organic phase was extracted and washed with Brine (~ 100 mL, 2x). The combined organic phase was then dried with $MgSO_4$, concentrated and dried under high vacuum to give 1g of **6g**.

Step 1 – Analysis

TLC: R_f = 0.6 (Tol/EA 3:1)

^{1}H NMR (700 MHz, DMSO-d6) δ 7.46 – 7.42 (m, 3H,-Ar), 7.38 – 7.34 (m, 4H,-Ar), 5.64 (s, 1H,-PhCH), 5.30 (d, J = 4.2 Hz, 1H), 5.24 (d, J = 1.4 Hz, 1H,H-1), 4.10 (dd, J = 10.1, 4.3 Hz, 1H), 3.91 – 3.88 (m, 1H), 2.62 – 2.58 (m, 2H), 1.22 (t, J = 7.4 Hz, 4H), 0.84 (d, J = 3.1 Hz, 9H,-$C(CH_3)_3$, -0.02 (s, 3H,-$Si(CH_3)$), -0.04 (s, 3H,-$Si(CH_3)$).

^{13}C NMR (176 MHz, DMSO) δ 127.91(-Ar), 126.00(-Ar), 100.86(-PhCH), 85.79(C-1), 78.17, 72.78, 70.39, 67.65, 64.71, 25.70(-SCH_2), 14.94(-SCH_3), -3.21(-$C(CH_3)_3$), -4.53(-$Si(CH_3)$), -4.91(-$Si(CH_3)$)

Step 2 – Procedure: Installation of benzyl group at C2

This procedure maintained an inert atmosphere throughout. To 2.3 mmol of **6g** (1g), 11 mL of anhydrous DCM (final concentration: ~ 0.2M) were added and purged with argon for several minutes and placed in an ice bath to cool down to 0^{o}C. Then, NaH (4.7 mmol, 0.18g) were added, followed by dropwise addition of BnBr (2.8 mmol, 0.3 mL) via a dropping funnel. The reaction was then removed from the ice bath and allowed to stir at room temperature. After ~ 4.5 hours, the reaction was quenched with Sat. aq. NH_4Cl (~ 5mL) till no more bubbling occurred. The reaction mixture was then diluted with DCM (~ 75 mL) and poured into a separating funnel. The organic phase was extracted and washed with Brine (~ 100 mL, 2x). The combined organic phase was then dried with $MgSO_4$, concentrated and subjected to purification.

Step 2 - Purification

The crude material was purified with dry flash chromatography. (Tol/EA 6:1 to 2:1) to give 0.8 g of pure **6h**.

Step 2 – Analysis

TLC: R_f = 0.7 (Tol/EA 3:1)

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.53 – 7.49 (m, 3H,-Ar), 7.41 (t, J = 7.4 Hz, 3H,-Ar), 7.38 – 7.33 (m, 7H,-Ar), 5.58 (s, 1H,-PhCH), 5.29 (d, J = 1.4 Hz, 1H,H-1), 4.88 (d, J = 11.9 Hz, 1H,-CH2Bn), 4.82 – 4.74 (m, 1H), 4.71 (d, J = 12.0 Hz, 1H,-CH2Bn), 4.15 (dd, J = 9.8, 3.1 Hz, 1H,H-3), 4.08 (d, J = 9.2 Hz, 1H,H-4), 3.94 – 3.89 (m, 1H,H-6a), 3.89 – 3.85 (m, 1H), 3.80 (dd, J = 3.2, 1.4 Hz, 1H,H-2), 2.66 – 2.52 (m, 2H,-SCH_2), 1.27 – 1.25 (m, 3H,-CH_3), 0.90 (s, 9H,-$(C(CH_3)_3)$, 0.09 (s, 3H,-$Si(CH_3)$, 0.05 (s, 3H,-$Si(CH_3)$.

^{13}C NMR (176 MHz, $CDCl_3$) δ 128.96(-Ar), 128.93(-Ar), 128.55, 128.44, 128.40, 128.31, 128.22, 128.18, 128.14, 128.06, 127.96, 127.90, 127.75, 127.69, 126.36, 126.22, 102.03(-PhCH), 84.23(C-1), 81.23, 79.49, 74.07, 71.07, 68.76, 64.98, 26.02(-SCH_2), 15.09(-SCH_3), -4.33(-$Si(CH_3)$), -4.68(-$Si(CH_3)$).

Step 3 – Procedure: Formation of sulfoxide

This procedure maintained an inert atmosphere throughout. To 4 mmol of **6h** (2.1 g), 27 mL of anhydrous DCM (final concentration: ~ 0.1M) and placed in an ice bath to cool down to 0°C. Then, m-CPBA (4 mmol, 0.7 g) was weighed into a 100 mL round bottom flask and purged with argon for several minutes before adding DCM (~ 10 mL), and added into the reaction flask dropwise via a dropping funnel. The reaction was then allowed to stir on ice bath. After ~ 1 hour, the reaction was quenched with Sat. aq. $NaHCO_3$ (~ 10 mL) and poured into a separating funnel. The organic phase was then extracted, and washed with Brine (~ 100 mL, 2x). The combined organic phase was dried with $MgSO_4$, concentrated and subjected to purification.

Step 3 – Purification

The crude material was purified by flash chromatography with Tol/EA as eluents. (9:1 6:1 4:1). Product eluted at 4:1. The product fractions were collected, concentrated and dried under high vacuum to give 0.104g of pure **BB 10**.

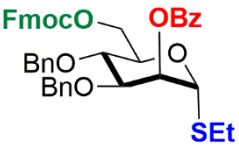
Step 3 – Analysis

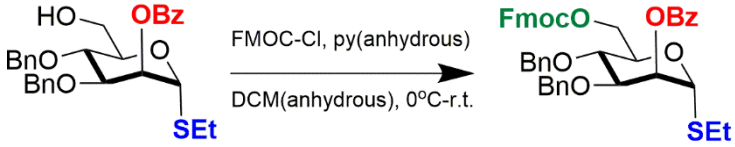
TLC: R_f = 0.6 (Tol/EA 3:1)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 – 7.34 (m, 11H,-Ar), 5.58 (s, 1H,-Ar), 4.97 (d, J = 11.4 Hz, 1H,-CH_2Bn), 4.69 (d, J = 11.4 Hz, 1H,-CH_2Bn), 4.62 (d, J = 1.2 Hz, 1H,H-1), 4.35 – 4.31 (m, 2H), 4.20 – 4.11 (m, 2H), 3.79 – 3.68 (m, 2H), 2.91 (dq, J = 13.5, 7.5 Hz, 1H), 2.64 (dq, J = 13.5, 7.4 Hz, 1H), 1.36 (t, J = 7.5 Hz, 3H), 0.90 (s, 9H), 0.11 (s, 3H), 0.07 (d, J = 3.8 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 137.97(-Ar), 137.24(-Ar), 128.60(-Ar), 128.29(-Ar), 128.27(-Ar), 128.09, 126.29, 102.16(-PhCH), 93.24(C-1), 78.15, 76.02, 75.06, 70.70, 68.28, 44.31, 25.98, 25.98, 18.48, 6.14, -4.38, -4.65.

XXII. Synthesis of BB 14





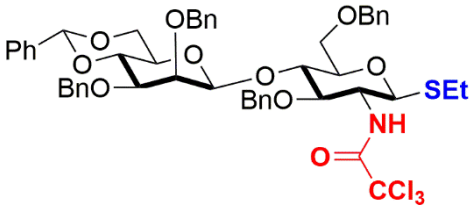
Procedure: This procedure maintained an inert atmosphere throughout. To 4mmol of Compound_ (2g), 15mL of anhydrous DCM (final concentration: ~ 0.3M) were added under argon atmosphere and placed in an ice bath to cool down to 0°C. Then, FMOC-Cl (8mmol, 2.1g) were added, followed by addition of pyridine (13mmol, 1mL). The colour of the reaction mixture changed from yellow to orange upon addition of pyridine. After the addition, the reaction flask was removed from the ice bath, and allowed to stir at room temperature for ~ 12 hours. Then, the reaction was quenched with 1N HCl, and poured into a separating funnel. The organic layer was then extracted, and washed with Sat. aq. $NaHCO_3$(~80mL,2x) and Brine (~80mL,2x). The combined organic phase was then dried with $MgSO_4$, concentrated and dried under high vacuum.

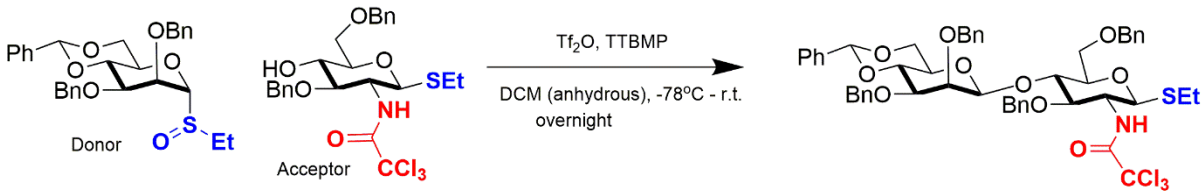
Purification: The crude material was subjected to purification by column chromatography with Hex/EA as solvents. (Hex/EA 6:1, 4:1, 2:1). The product fractions were collected, concentrated and dried under high vacuum.

Analysis:

TLC: R_f = 0.6 (Hex/EA 2:1)

8.3 Synthesis of β-mannose containing dimer by solution phase glycosylation





Procedure: Donor (0.4mmol, 0.2g) and acceptor (0.6mmol, 0.354g) were co-evaporated with Toluene (~5mL,3x) and dried under high vacuum overnight. Then, the donor was dissolved in (~ 7mL) and poured into a 100 mL 3-necked round botton flask (pre-dried in the oven and cooled under Ar), and the reaction flask was placed in a cooling bath and the temperature was set to -78°C using dry ice and acetone. Then, DTBMP (0.8mmol, 0.166g) was added, and the reaction mixture was stirred for ~ 10 minutes. Then, Tf_2O (0.4mmol, 0.061mL) were added dropwise. Then, the reaction was strirred for ~ minutes, and the acceptor (dissolved in 7mL DCM) was added dropwise into the reaction flask. Then, the reaction was allowed to stir on the cooling bath overnight. Then, the reaction was quenched with Sat. Aq. $NaHCO_3$(~30mL) and poured into a separting funnel. The organic phase was extracted and washed with Brine (~ 50mL,2x). The combined organic phase was dried with $MgSO_4$, concentrated and dried under high vacuum.

Purification: Product was purified by flash column chromatography (10:1 to 6:1 Toluene/EA

Analysis:

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 – 7.46 (m, 1H), 7.42 – 7.26 (m, 15H), 6.91 (d, J = 0.7 Hz, 2H,H-1), 5.64 (s, 1H,-PhCH), 4.92 – 4.89 (m, 1H), 4.89 – 4.82 (m, 1H), 4.81 – 4.74 (m, 2H,H-1(GlcNAc (H-1)), 4.70 (dd, J = 15.2, 11.8 Hz, 1H), 4.62 (d, J = 1.4 Hz, 1H,H-1 (alpha)), 4.60 – 4.55 (m, 1H), 4.52 (dd, J = 3.4, 1.5 Hz, 1H), 4.35 (dd, J = 10.1, 9.2 Hz, 1H), 4.20 (dd, J = 10.2, 4.6 Hz, 1H), 4.13 (dd, J = 10.0, 3.4 Hz, 1H), 3.92 – 3.87 (m, 1H), 3.85 – 3.77 (m, 2H), 3.72 (dd, J = 4.9, 0.7 Hz, 1H), 3.56 (dt, J = 9.7, 4.9 Hz, 0H), 2.94 – 2.86 (m, 1H), 2.76 – 2.60 (m, 2H), 0.98 (t, J = 7.9 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 167.54, 146.55, 138.24, 137.72, 137.30, 129.15, 128.74, 128.66, 128.65, 128.60, 128.48, 128.36, 128.25, 128.23, 128.15, 128.12, 128.07, 127.94,

127.80, 127.77, 126.17, 116.32, 101.75, 92.90, 82.91, 82.77, 81.48, 81.35, 78.05, 77.94, 76.31, 74.96, 74.22, 73.94, 73.68, 73.32, 73.22, 70.85, 70.22, 68.33, 57.10, 44.18, 37.54, 24.60, 24.37, 21.60, 15.24, 6.79, 6.02, 4.35.

8.4 General procedures for pre-automation steps

8.4.1 Attachment of Lenz linker to Merrifield resin

The resin (2g, loading: 0.5mmol/g) was weighed into a filter syringe, and swollen in DCM for 1 hour. Then, Lenz linker (441mg, 1.104mmol) was weighed into a 100 mL round bottom flask, and dissolved in EtOH (2mL) and deionized H_2O (0.55mL). The pH was then adjusted to 7 by adding 2M Cs_2CO_3 (~ 0.5mL). The solution was then evaporated to dryness. Then, it was co-evaporated with anhydrous 1,4-Dioxane (3x) and dried under high vacuum for ~ 30 minutes. DCM was then drained from the resin and allowed to sit on DMF for ~ 15-20 minutes. The Cs salt of the Lenz linker was then taken off from the high vacuum and dissolved in 10mL DMF. Then, NaI (0.092mmol, 0.014g) was added into it. DMF was then drained out of the filter syringe containing resin. Then, a needle was fiited to the syringe and the Cs salf was drwan up into the syringe. The needle was taken out, and the syringe was capped. It was then kept in a 1L conical flask, and covered with parafilm. The flask was kept at 50^oC in the oven overnight.

8.4.2 Attachment of FMOC carbonate to Lenz resin/photocleavable resin

~ 150mg Lenz resin was weighed into a filter syringe, and swollen in DCM for 1 hour on a shaker. Meanwhile, 550mg FMOC-Cl was weighed into a round bottom flask, and dissolved in 5mL DCM. Then, 0.5mL pyridine was added into it. Then, the filter syringe was removed from the shaker, and the FMOC solution was drawn up the syringe using a needle. Upon complete addition, the needle was removed, and the syringe was capped. Then, the syringe was the left on the shaker overnight. The syringe was then removed from the shaker, and the solution was drained out of the syringe. The resin was then washed with DCM and MeOH (~ 5mL, 3x each). It was then dried under high vacuum overnight.

8.4.3 Loading determination of Linker loaded resin(Lenz resin/photocleavable resin)

For the loading determination, three independent samples of Lenz/photocleavable resin with FMOC carbonate attached were prepared. Approximately, 50 mg of dried resin were weighed into a grdauated 100 mL flask and 20mL DMF (using the 25 mL graduated pipette) were added. The suspension was left to sit for 30 minutes under occassional gentle swirling and then 400 µL of DBU were added (using the 1 mL volumetric pipette) , affording a final concentration of 2% DBU (v/v). This mixture was left to sit under occassional swirling for another 30 minutes. After 30 min, ACN was added to make up the final volume to 100 mL. Then, 2 mL of this solution was transferred to a graduated 25mL flask and diluted with ACN to 25mL. The reference solution was prepared similarly without the resin. Then, the UV absorption maxima of the reference and the three independent samples were measured at 304 or 294 nm. Three measurements were prformed for each of the resin sample and reference.

The data were recorded and imported into Excel. The raw data was then “corrected” by subtracting the average of teh three refernce measurements from each of the individual samples. The loading was then calculated using the following formula:

Loading

$$\frac{\mathrm{Abs}\ (294\ \mathrm{nm}) \times 142.14}{\mathrm{m}\ (\mathrm{mg})}$$

Loading

$$\frac{\mathrm{Abs}\ (304\ \mathrm{nm}) \times 163.96}{\mathrm{m}\ (\mathrm{mg})}$$

8.5 Commonly used commands for Automated Glycan Assembly using the Glyconeer

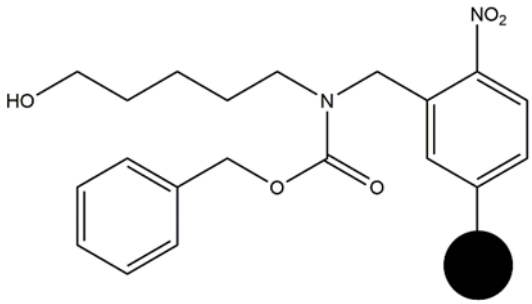
Commands	Function	Parameters				
		P1	P2	P3	P4	P5
Argon Mix	Mixes the contents of the reaction vessel via bubbling	Bubble on(s)	Bubble off (s)			
Deliver BB	Delivers the specified BB to the reaction vessel	Vial number	Delivery Volume (µL)	Number of deliveries	Bubble on (s)	Bubble off (s)
Deliver Duration	Delivers the specified solvent/reagent by opening the required valves for the specified amount of time	Reagent/ Solvent	Delivery Time (s)	Bubble on (s)	Bubble off (s)	
Discharge	Discharges the contents of the reaction vessel to the desired destination	Discharge Duration (s)	Destination (Waste, FC or UV Sensor)			
Incubation	Allows the previously set conditions to be maintained for the desired amount of time	Incubation Time (s)	Bubble on (s)	Bubble off (s)		
Pause	Pauses the process for the desired amount of time	Pause Duration (s)				
Prime	Primes the lines for the selected solvent or reagent directly into the waste. The central manifold id then washed with argon	Reagent/ Solvent	Prime Duration (s)			
Rinse BB Waste	Uses the desired solvent to wash the lines and delivery needle. Solvent is initially delivered	Vial Number (1-64)	Rinse Solvent	Solvent Duration (s)	Destination	Via

	through the cannula into the specific vial					
Rinse Manifold Waste	Flushes the specific manifold with the desired solvent or argon via the bypass or reaction vessel into the chosen destination	Manifold	Rinse solvent	Solvent Duration (s)		
Set Temp	Sets the cryostat to the desired target temperature	Temperature (° C)				
Start Loop	Starts a loop with the specified number of cycles	Number of cycles				
Wait for Temp	Pauses the run until the cryostat reaches the set temperature	Desired Temperature (° C)				

8.6 Automated Glycan Assembly using the Glyconeer

8.6.1 General methods and Materials

Anhydrous* solvents used to prepare building block as well as activator, TMSOTf and capping stock solutions were taken from a solvent drying system (jcmeyer-solvent systems). HPLC Grade DCM was used for washing. All other washing solvents (DMF, THF, Dioxane and MeOH) were reagent grade. Building blocks are dried by co-evaporation with Toluene (3x) and drying under high vacuum for approximately 1 to 2 hours. All synthesis were carried out on a scale of 0.0125 mmol using a Merrifield Resin modified with a photocleavable linker (Loading = 0.41 mmol/g).



The polymer matrix is Copoly(stryol-1% divinylbenzol) 100 - 200 mesh

Stock Solutions

Activator solution: 150 mM NIS/15mM TfOH in DCM*:Dioxane*

Acid wash solution: 62 mM TMSOTf in DCM*

Capping solution: 10% (v/v) Ac_2O/2% (v/v) $MeSO_3H$ in DCM*

Pyridine "pre-wash" solution: 10% (v/v) pyridine in DMF

FMOC deprotection solution: 20% (v/v) Piperidine in DMF

Automation Modules

Module A: Initial Resin Swelling/Beginning of Synthesis Wash

The resin is washed with DCM, DMF and THF (3x, 2 mL, 25 s each) before swelling in DCM (2 mL) for 30 mins with occasional mixing using pulsed Argon bubbling.

Module B: Acidic Wash with 62 mM TMSOTf

DCM (2 mL) is delivered into the reaction vessel, and the temperature is adjusted to -20 °C. The DCM is drained and replaced by another 2 mL of DCM before TMSOTf solution (1 mL) is added dropwise. The mixture is incubated for 1.5 mins under Ar bubbling before draining washing with 2 mL DCM for 25 s.

Action	Cycles	Reagent	Amount	T(°C)	Incubation Time
Cooling	-	-	-	-20	-
Deliver	2	DCM	2 mL	-20	-
Deliver		TMSOTf	1 mL	-20	1.5 min
Wash		DCM	2 mL	-20	25 s

Module C: Thioglycoside coupling

Building Block stock solutions and glycosylation parameters:

	Building Block	Excess	c (mM)	T1 (°C)	T2 (°C)
A	BB1, BB2, BB4, BB6, BB7, BB8	6.5 eq.	81	-20	0
B	BB3	10 eq.	125	-40	-20

Glycosylation cycle:

DCM (2 mL) is added to the resin and the temperature is set to the activation temperature T1-2 K. While cooling down, the building block solution is delivered to the reaction vessel. After the set temperature has stabilized at T1 - 2 K, the reaction is started by adding 1 mL of activator solution. The mixture is kept at T1 for 5 minutes before a second 20 minute incubation cycle is started during which the temperature is raised to a temperature T2. Upon completion

of the incubation cycle the reaction mixture is drained and the resin is washed once each with DCM/Dioxane 1:1 (2 mL) and DCM (2mL). The module finishes by raising the temperature to 25 °C while performing two additional DCM washes (2 mL).

Action	Cycles	Solution	Amount	T(°C)	Incubation Time
Cooling	-	-	-	T1 - 2	-
Deliver	2	BB Solution	1 mL		
Deliver		Activator Solution	1 mL		
Incubation				T1	5 min
Incubation				T2	20 min
Wash	1	DCM:Dioxane 1:1	2mL	T2	25 s
Wash	1	DCM	2mL	0	25 s
Heating	-	-	-	25	-
Wash	2	DCM	2mL	25	25 s

Module D: Capping

The resin is washed with DMF (2x, 25 s) and the temperature of the reaction vessel was set to 25 °C. 2 mL of 10 % Pyridine in DMF is delivered into the reaction vessel. After 1 min, the solution is drained and the resin is washed with DCM (3x, 2 mL, 25 s). Then, 4 mL of capping solution is delivered into the reaction vessel and incubated for 20 mins under Argon bubbling. The cycle concludes by draining the reaction mixture washing the resin with DCM (3x, 2mL, 25 s).

Action	Cycles	Solution	Amount	T(°C)	Incubation Time
Heating	-	-	-	25	
Wash	2	DMF	2 mL	25	25s
Deliver	1	10% Py in DMF	2 mL	25	1 min
Wash	3	DCM	2 mL	25	25s
Deliver	1	Capping Solution	4 mL	25	20 mins
Wash	3	DCM	2 mL	25	25s

Module E: FMOC Deprotection

The resin is washed with DMF (3x, 2 mL, 25s) and the temperature of the reaction vessel is adjusted to 25 °C. 2 mL of FMOC deprotection solution is delivered into the reaction vessel. After 5 mins, the solution was drained through the UV-sensor and the resin is washed with DMF (3x, 2 mL) and DCM (5x, 2mL, 60 s each). The temperature of the reaction vessel was decreased to -20 °C in preparation of the next cycle.

Action	Cycles	Solution	Amount	T(°C)	Incubation Time
Heating	-	-	-	25	
Wash	3	DMF	2 mL	25	25 s
Deliver	1	FMOC Deprotection Solution	2 mL	25	5 mins
Wash	1	DMF	2 mL		
Cooling	-	-	-	-20	-
Wash	3	DMF	2 mL		25 s
Wash	5	DCM	2 mL		25

8.7 General procedure for post automation steps

8.7.1 Photo-induced cleavage of the product from the solid support

The sample (resin loaded with target oligosaccharide) was taken up in 20 mL DCM (stabilized with amylene, HPLC grade) and injected into the reactor (Wavelength = 300 nm) at the rate of 2.0 mL/min. When all of the resin was inside the reactor, it was allowed to incubate inside the reactor for ~ 20 mins. After this, fresh DCM (20 mL) was injected to retrieve the photocleaved resin. The filtrate so obtained was concentrated in-vacuo and subjected to HPLC analysis and purification.

8.7.2 Quantification of 1st building block

The building block was co-evaporated with Toluene (~ 5 mL, 3x) and dried under high vacuum for ~ 2 hours. It was then dissolved in DCM (anhydrous) (2mL) and put in the BB vials. The photoresin was then added into the reaction vessel of the Glyconeer, and the synthetic modules were programmed as follows: Initial resin swelling ⟶ Thioglycoside Glycosylation Ac_2O capping ⟶ FMOC deprotection. The discharge solution upon FMOC cleavage was collected in the fraction collector, and pooled into 100 mL volumetric flask. The tubes were washed down with ACN several times, and added into the volumetric flask. Then, the volume was made upto 100 mL with ACN. The reference solution was prepared in the same way, without addition of the resin. Then, 2ml of this solution was taken in a 25 mL volumetric flask, and diluted with ACN to make up the volume to 25 mL. This solution was then subjected to UV measurements, and the loading was calculated as described in section 8.4.3.

8.8 General procedure for global deprotection of glycans

8.8.1 Global deprotection of Lewis^x

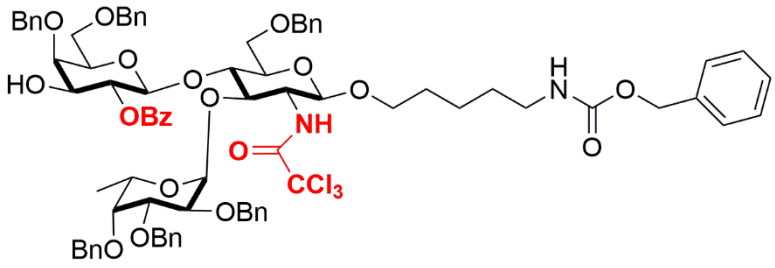
The starting material (protected Lewis^x epitope structure) was dissolved in DCM/MeOH 1:1 and 0.1 mL NaOMe in MeOH (0.5M) was added into it via a syringe. The reaction was then allowed to stir at room temperature overnight. Thereafter, the reaction mixture was analysed using MALDI to monitor the progress of reaction. Then, the crude mixture was neutralized using amberlite resin till PH reached 7. The filtrate thus obtained was concentrated, and subjected to hydrogenation. The debenzoylated crude sample was dissolved in EtOAc/t-BuOH/H_2O 2:1:1 (4 mL). Then $Pd(OH)_2$ on C (10%) was added into it, and H_2 (1 atm)was purged through the reaction mixture for ~ 10 mins. Then, two H_2 balloons were attached to the septum, and the reaction mixture was allowed to stir at r.t for 24 hours. Then, the reaction mixture was added to a filter syringe attached to a syringe filter. The catalyst was then washed with EA (2x), t-BuOH (2x) and finally with H_2O (6-8x). The filtrate so obtained was concentrated and analysed by MALDI and HPLC.

8.8.2 Global deprotection of PNAG oligomers

The crude samples (PNAG tetramer, pentamer and hexamer) were dissolved in EtOAc/t-BuOH/H_2O 2:1:1 (4 mL). Then Pd on C (10%) was added into it, and H_2 (1 atm) was purged through the reaction mixture for ~ 10 mins. Then, two H_2 balloons were attached to the septum, and the reaction mixture was allowed to stir at r.t for 24 hours. Then, the reaction mixture was added to a filter syringe attached to a syringe filter. The catalyst was then washed with EA (2x), t-BuOH (2x) and finally with H_2O (6-8x). The filtrate so obtained was concentrated and analysed by MALDI and HPLC.

8.9 Analytical Data for target glycans

8.9.1 Lewis^x epitope

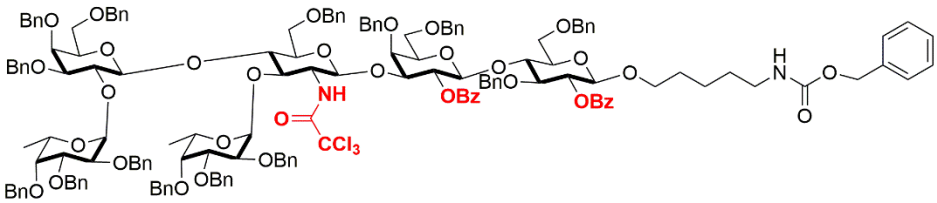


^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.96 – 7.93 (m, 1H), 7.60 – 7.56 (m, 1H), 7.44 (t, *J* = 7.8 Hz, 1H), 7.41 – 7.27 (m, 14H), 7.26 – 7.20 (m, 3H), 5.18 – 5.16 (m, 1H, H-1''), 4.75 (d, *J* = 2.6 Hz, 1H), 4.72 (dd, *J* = 4.9, 1.6 Hz, 2H), 4.69 (d, *J* = 7.7 Hz, 1H, H-1), 4.67 (d, *J* = 7.9 Hz, 1H, H-1'), 4.45 (d, *J* = 12.2 Hz, 1H), 4.40 (q, *J* = 11.8 Hz, 1H), 4.28 (d, *J* = 11.4 Hz, 1H), 4.07 (t, *J* = 8.2 Hz, 1H), 4.01 (dd, *J* = 10.2, 3.7 Hz, 1H), 3.90 (dd, *J* = 10.2, 2.8 Hz, 1H), 3.83 (dd, *J* = 10.9, 3.2 Hz, 1H), 3.74 – 3.68 (m, 2H), 3.68 – 3.64 (m, 1H), 3.60 (dd, *J* = 10.1, 3.5 Hz, 1H), 3.43 (d, *J* = 2.8 Hz, 1H), 3.12 (q, *J* = 6.9 Hz, 1H), 2.05 (s, 1H), 1.46 (dp, *J* = 30.2, 7.0, 6.6 Hz, 2H), 1.28 – 1.23 (m, 2H).

HRMS (ESI-QTOF): calcd for $C_{82}H_{89}Cl_3N_2O_{18}$ $[M+Na]^+$: 1517.5176 Found: 1517.5109

NMR in agreement with Literature[27]

Lewis^y antigen

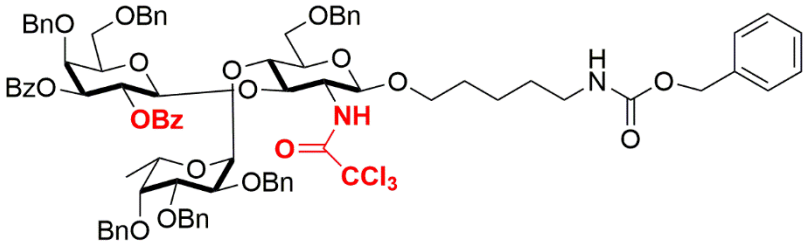


^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.94 (dd, *J* = 19.9, 7.7 Hz, 6H), 7.35 – 7.28 (m, 18H), 7.24 – 7.16 (m, 7H), 5.14 (d, *J* = 3.6 Hz, 1H), 5.05 (d, *J* = 8.4 Hz, 5H), 4.90 (d, *J* = 11.1 Hz, 1H), 4.78 – 4.72 (m, 3H), 4.35 – 4.25 (m, 7H), 4.21 – 4.16 (m, 2H), 4.09 – 3.98 (m, 3H), 3.92 – 3.86 (m, 2H), 3.79 (ddd, *J* = 10.2, 7.5, 2.5 Hz, 2H), 3.76 – 3.64 (m, 4H), 3.54 (ddd, *J* = 22.2, 10.9, 6.4 Hz, 4H), 3.44 (td, *J* = 13.3, 9.2 Hz, 2H), 3.34 (ddd, *J* = 21.7, 9.0, 5.0 Hz, 2H), 3.16 (d, *J* = 14.8 Hz, 2H), 2.86 (d, *J* = 12.0 Hz, 5H), 2.35 (t, *J* = 7.8 Hz, 1H), 2.23 (t, *J* = 7.8 Hz, 1H), 1.17 – 1.02 (m, 4H).

HRMS (ESI-QTOF): calcd for $C_{163}H_{171}Cl_3N_2O_{33}$ $[M+2Na]^{2+}$= 1417.5307 Found: 1417.5304

NMR in agreement with Literature[27]

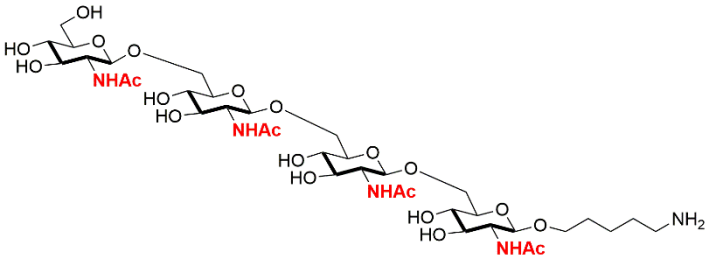
8.9.3 Lewis[a] epitope



^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.99 – 7.96 (m, 1H), 7.44 – 7.37 (m, 4H), 7.37 – 7.25 (m, 18H), 7.24 – 7.12 (m, 8H), 5.19 (d, *J* = 3.5 Hz, 1H), 5.10 (d, *J* = 3.9 Hz, 1H), 4.92 (dd, *J* = 11.5, 2.6 Hz, 1H), 4.75 – 4.68 (m, 2H), 4.66 – 4.62 (m, 1H), 4.61 – 4.57 (m, 3H), 4.53 – 4.49 (m, 2H), 4.37 (d, *J* = 7.1 Hz, 1H), 4.24 – 4.20 (m, 1H), 4.06 (t, *J* = 3.6 Hz, 1H), 4.01 – 3.95 (m, 2H), 3.89 (q, *J* = 6.5 Hz, 1H), 3.83 (dt, *J* = 10.0, 3.8 Hz, 2H), 3.77 (dd, *J* = 9.9, 6.1 Hz, 1H), 3.58 (d, *J* = 3.0 Hz, 1H), 3.30 (dtd, *J* = 9.6, 6.8, 3.8 Hz, 1H), 3.26 – 3.17 (m, 1H), 3.13 – 3.06 (m, 1H), 2.23 (td, *J* = 7.7, 2.6 Hz, 1H), 2.11 – 2.02 (m, 1H), 1.82 (dddd, *J* = 19.6, 16.2, 12.4, 7.6 Hz, 1H), 1.72 – 1.60 (m, 4H), 1.58 – 1.43 (m, 2H), 1.38 – 1.27 (m, 1H), 1.08 (d, *J* = 6.4 Hz, 2H).

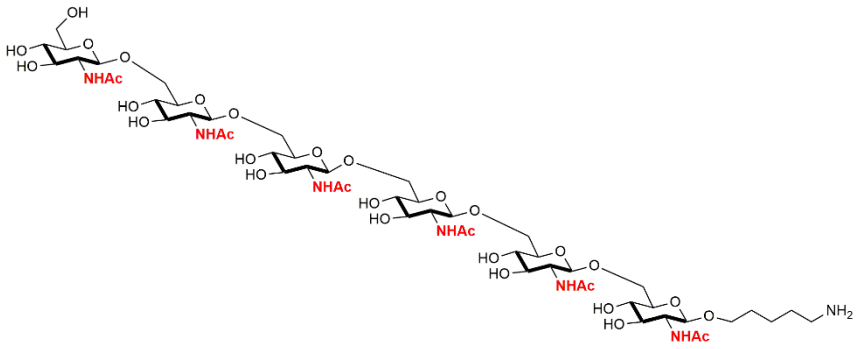
HRMS (ESI-QTOF): calcd for $C_{89}H_{93}Cl_3N_2O_{19}$ $[M+Na]^+$= 1621.5438 Found: 1621.5266

8.9.4 PNAG tetramer



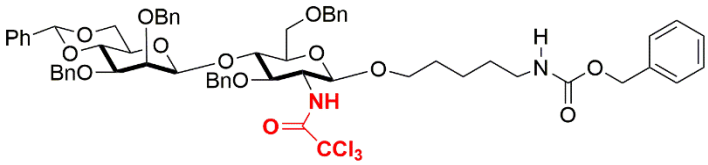
^{1}H NMR (600 MHz, Deuterium Oxide) δ 7.46 – 7.37 (m, 3H), 5.83 (d, *J* = 8.7 Hz, 1H), 4.59 – 4.48 (m, 4H), 4.24 – 4.10 (m, 5H), 3.94 (dd, *J* = 12.1, 8.0 Hz, 3H), 3.80 – 3.72 (m, 5H), 3.71 (q, *J* = 5.1, 4.1 Hz, 2H), 3.68 – 3.53 (m, 9H), 3.47 (d, *J* = 5.0 Hz, 3H), 3.48 – 3.37 (m, 3H), 3.01 (q, *J* = 7.7 Hz, 4H), 2.12 (s, 13H), 2.09 (d, *J* = 5.4 Hz, 2H), 2.06 (dd, *J* = 12.1, 4.8 Hz, 15H), 1.62 (dq, *J* = 20.6, 7.6 Hz, 4H), 1.43 – 1.35 (m, 3H), 1.35 – 1.27 (m, 17H).

8.9.5 PNAG Hexamer



^{1}H NMR (600 MHz, Deuterium Oxide) δ 4.60 – 4.50 (m, 6H), 4.27 – 4.17 (m, 8H), 3.97 (d, *J* = 12.1 Hz, 2H), 3.90 (tt, *J* = 12.6, 6.5 Hz, 2H), 3.84 – 3.72 (m, 12H), 3.03 (td, *J* = 8.3, 7.9, 2.8 Hz, 5H), 2.11 – 2.03 (m, 20H), 1.76 – 1.67 (m, 6H), 1.62 (q, *J* = 7.6 Hz, 5H), 1.45 (dt, *J* = 15.1, 7.7 Hz, 5H).

8.9.7 Man-β-1,4-GlcNAc:



^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.40 – 7.26 (m, 14H), 7.24 – 7.13 (m, 4H), 5.06 (s, 2H), 5.03 (dd, *J* = 9.8, 8.8 Hz, 1H), 4.98 (d, *J* = 8.1 Hz, 1H, H-1), 4.73 (d, *J* = 7.7 Hz, 1H, H-1'), 4.64 (d, *J* = 11.1 Hz, 1H), 4.55 (d, *J* = 11.1 Hz, 1H), 4.52 – 4.50 (m, 6H), 4.31 (dd, *J* = 10.3, 8.9 Hz, 1H), 3.88 (dt, *J* = 9.6, 6.1 Hz, 1H), 3.64 (ddd, *J* = 9.6, 5.1, 4.0 Hz, 1H), 3.58 – 3.54 (m, 2H), 3.48 (dt, *J* = 9.4, 6.4 Hz, 1H), 3.41 (dt, *J* = 10.4, 7.7 Hz, 1H), 3.15 (qq, *J* = 13.3, 6.4 Hz, 3H), 2.21 (td, *J* = 7.6, 2.6 Hz, 1H), 1.43 – 1.27 (m, 16H).

Bibliography

1. Feng, D.; Shaikh, A. S.; Wang, F., Recent advance in tumor-associated carbohydrate antigens (TACAs)-based antitumor vaccines. *ACS Chem. Biol.* **2016,** *11* (4), 850-863.

2. Venkatesan, N.; Perumal, G.; Doble, M., Bacterial resistance in biofilm-associated bacteria. *Future Microbiology* **2015,** *10* (11), 1743-1750.

3. Mack, D.; Fischer, W.; Krokotsch, A.; Leopold, K.; Hartmann, R.; Egge, H.; Laufs, R., The intercellular adhesin involved in biofilm accumulation of Staphylococcus epidermidis is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J. Bacteriol.* **1996,** *178* (1), 175-183.

4. Garegg, P. J.; Henrichson, C.; Norberg, T., A reinvestigation of glycosidation reactions using 1-thioglycosides as glycosyl donors and thiophilic cations as promoters. *Carbohydr. Res.* **1983,** *116* (1), 162-165.

5. Früchtel, J. S.; Jung, G., Organic Chemistry on Solid Supports. *Angew. Chem. Int. Ed. Engl.* **1996,** *35* (1), 17-42.

6. Fischer, E., Alkylderivate des Benzoïns. *Berichte der deutschen chemischen Gesellschaft* **1893,** *26* (3), 2412-2415.

7. Ferrier, R. J.; Hay, R. W.; Vethaviyasar, N.]A potentially versatile synthesis of glycosides. *Carbohydr. Res.* **1973,** *27* (1), 55-61.

8. Dube, D. H.; Bertozzi, C. R., Glycans in cancer and inflammation — potential for therapeutics and diagnostics. *Nat. Rev. Drug Discovery* **2005,** *4* (6), 477-488.

9. Chao, C.-S.; Lin, C.-Y.; Mulani, S.; Hung, W.-C.; Mong, K.-k. T., Neighboring-Group Participation by C-2 Ether Functions in Glycosylations Directed by Nitrile Solvents. *Chem. Eur. J.* **2011,** *17* (43), 12193-12202.

10. Bochkov, A. F.; Kochetkov, N. K., A new approach to the synthesis of oligosaccharides. *Carbohydr. Res.* **1975,** *39* (2), 355-357.

11. Zhang, Z.; Ollmann, I. R.; Ye, X.-S.; Wischnat, R.; Baasov, T.; Wong, C.-H., Programmable One-Pot Oligosaccharide Synthesis. *J. Am. Chem. Soc.* **1999,** *121* (4), 734-753.

12. Yu, Y.; Kononov, A.; Delbianco, M.; Seeberger, P. H., A capping step during automated glycan assembly enables access to complex glycans in high yield. *Chem. Eur. J.* **2018,** *24* (23), 6075-6078.

13. Goto, K.; Sawa, M.; Tamai, H.; Imamura, A.; Ando, H.; Ishida, H.; Kiso, M., The Total Synthesis of Starfish Ganglioside GP3 Bearing a Unique Sialyl Glycan Architecture. *Chem. Eur. J.* **2016,** *22* (24), 8323-8331.

14. Hanessian, S.; Bacquet, C.; Lehong, N., Chemistry of the glycosidic linkage. Exceptionally fast and efficient formation of glycosides by remote activation. *Carbohydr. Res.* **1980,** *80* (2), C17-C22.

15. Knoben, H.-P.; Schlüter, U.; Redlich, H., Synthesis of N-unsubstituted, mono- and disubstituted carbohydrate-1-O-carbamates and their behaviour in glycoside syntheses. *Carbohydr. Res.* **2004,** *339* (18), 2821-2833.

16. Kochetkov, N. K.; Backinowsky, L. V.; Tsvetkov, Y. E., Sugar thio orthoesters as glycosylating agents. *Tet. Lett.* **1977,** *18* (41), 3681-3684.

17. Ley, S. V., The Engineering of Chemical Synthesis: Humans and Machines Working in Harmony. *Angew. Chem. Int. Ed.* **2018,** *57* (19), 5182-5183.

18. Takahashi, T.; Adachi, M.; Matsuda, A.; Doi, T., Combinatorial synthesis of trisaccharides via solution-phase one-pot glycosylation. *Tet. Lett.* **2000,** *41* (15), 2599-2603.

19. Teruaki, M.; Takashi, N.; Shin-ichiro, S., AN EFFICIENT GLUCOSYLATION OF ALCOHOL USING 1-THIOGLUCOSIDE DERIVATIVE. *Chem. Lett.* **1979,** *8* (5), 487-490.

20. Trobe, M.; Burke, M. D., The Molecular Industrial Revolution: Automated Synthesis of Small Molecules. *Angew. Chem. Int. Ed.* **2018,** *57* (16), 4192-4214.

21. Seeberger, P. H., The logic of automated glycan assembly. *Acc. Chem. Res.* **2015,** *48* (5), 1450-1463.

22. Seeberger, P. H., Automated oligosaccharide synthesis. *Chem. Soc. Rev.* **2008,** *37* (1), 19-28.

23. Routenberg Love, K.; Seeberger, P. H., Automated solid-phase synthesis of protected tumor-associated antigen and blood group determinant oligosaccharides. *Angew. Chem. Int. Ed.* **2004,** *43* (5), 602-605.

24. Plante, O. J.; Palmacci, E. R.; Seeberger, P. H., Automated solid-phase synthesis of oligosaccharides. *Science* **2001,** *291* (5508), 1523-1527.

25. Hahm, H. S.; Schlegel, M. K.; Hurevich, M.; Eller, S.; Schuhmacher, F.; Hofmann, J.; Pagel, K.; Seeberger, P. H., Automated glycan assembly using the Glyconeer 2.1 synthesizer. *PNAS* **2017,** *114* (17), E3385-E3389.

26. Hahm, H. S.; Liang, C.-F.; Lai, C.-H.; Fair, R. J.; Schuhmacher, F.; Seeberger, P. H., Automated glycan assembly of complex oligosaccharides related to blood group determinants. *J. Org. Chem.* **2016,** *81* (14), 5866-5877.

27. Guberman, M.; Bräutigam, M.; Seeberger, P. H., Automated glycan assembly of Lewis type I and II oligosaccharide antigens. *Chem. Sci.* **2019,** *10* (21), 5634-5640.

28. Eller, S.; Collot, M.; Yin, J.; Hahm, H. S.; Seeberger, P. H., Automated solid-phase synthesis of chondroitin sulfate glycosaminoglycans. *Angew. Chem. Int. Ed.* **2013,** *52* (22), 5858-5861.

29. Nigudkar, S. S.; Demchenko, A. V., Stereocontrolled 1, 2-cis glycosylation as the driving force of progress in synthetic carbohydrate chemistry. *Chem. Sci.* **2015,** *6* (5), 2687-2704.

30. Crich, D.; Chandrasekera, N. S., Mechanism of 4,6-O-Benzylidene-Directed β-Mannosylation as Determined by α-Deuterium Kinetic Isotope Effects. *Angew. Chem. Int. Ed.* **2004,** *43* (40), 5386-5389.

31. Panza, M.; Pistorio, S. G.; Stine, K. J.; Demchenko, A. V., Automated Chemical Oligosaccharide Synthesis: Novel Approach to Traditional Challenges. *Chem. Rev.* **2018,** *118* (17), 8105-8150.

32. Tsai, B.-L.; Han, J.-L.; Ren, C.-T.; Wu, C.-Y.; Wong, C.-H., *Tetrahedron. Lett.,* **2011,** *52* (17), 2132-2135.

33. Toepfer, A.; Schmidt, R. R., *Tetrahedron Lett.,* **1992,** *33* (36), 5161-5164.

34. Munneke, S.; Painter, G. F.; Gainsford, G. J.; Stocker, B. L.; Timmer, M. S., *Carbohydr. Res.,* **2015,** *414*, 1-7.

35. Chen, C.; Zhang, Y.; Xue, M.; Liu, X.-w.; Li, Y.; Chen, X.; Wang, P. G.; Wang, F.; Cao, H., *Chem. Commun.,* **2015,** *51* (36), 7689-7692.

36. Moore, C. J.; Auzanneau, F.-I., *Beilstein J. Org. Chem.,* **2012,** *8* (1), 1134-1143.

37. R. Apweiler, H. Hermjakob, N. Sharon, Biochim. Biophys. Acta, Gen. Subj. 1999, 1473,4.

38. R. A. Dwek, Chem. Rev. 1996, 96, 683.

39. A. Varki, Glycobiology 1993, 3, 97