UNIVERSITÀ DEGLI STUDI DI MILANO

Synthesis of Group B Streptococcus type II (GBS II) oligosaccharide for vaccine development

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Dedicated to my beloved parents

Abstract

Carbohydrates are among the most abundant molecules found on the cell surfaces of bacteria, parasites, and viruses. Apart from the conventional roles of carbohydrates as energy sources and structural polymers, carbohydrates are also associated with cancer metastasis, protein stabilization, pathogen infection and the immune response. Cells of our body have sensors made out of carbohydrates on outer surface of plasma membrane and acts as sensors and can detect many kinds of stimuli, and can signal the immune system to respond. Carbohydrate-protein molecular recognition processes have pivotal roles in infections and in immune response to pathogens. To date, several vaccines based on isolated capsular polysaccharides (CPSs) are marketed against infectious diseases. However, the use of isolated capsular polysaccharide poses several limitations, as natural sources are generally limited and the isolation is very challenging. Additionally, the isolated polysaccharides are heterogeneous and often contains impurities. Furthermore, limited protection of certain CPS antigens impairs the efficiency of vaccines. To overcome limitations associated with isolated polysaccharides, synthetic oligosaccharides present an effective alternative with great potential to understand glycan immunology and rationally design effective antigens. Consequently, characterization and reconstruction of carbohydrate epitopes with authentic composition has become one of the major target in glycoscience. To this end, strategies are needed to facilitate the streamlined design and generation of these antigens.

This thesis concerns the development of an effective synthetic strategy to obtain Group B Streptococcus (GBS) type II oligosaccharide for vaccine development. GBS, a Gram-positive bacterium, inhabits the intestinal and genitourinary tract of 10-30% of humans. GBS is one of the primary causes of bacterial infections among neonates and pregnant women, resulting in many severe diseases such as sepsis, meningitis, abortion, and so on. Type II GBS is one of the predominant GBS serotypes and is associated with about 15% of the invasive infections in adults and infants; therefore, represents an important human pathogen. The development of effective preventive vaccine against GBS is much needed to help pregnant women protect their newborns. This thesis describes the effective synthetic strategy to synthesize GBS type II oligosaccharide to be applied for vaccine development.

Herein, we present a new and convenient synthesis of the repeating unit of GBS type II capsular polysaccharide. The structure of GBS type II was elucidated in 1983 and the repeating unit of GBS type II is a heptasaccharide composed of **α-Neu5Ac (2-3)-ß-D-Gal-(1-4)- ß-D-GlcNAc-(1-3)-[-ß-D-Gal-(1-6)]-ß-D-Gal-(1-4)-ß-D-Gal-(1-3)-ß-D-Glc**. The presented synthetic strategy is based on the five subcomponents derived from the retro synthetic analysis. Suitably protected lactosamine and lactose derivatives are pivotal building blocks in our synthesis and both disaccharide fragments have been achieved from the cheap and readily available lactose. Having started from two disaccharides saves the efforts of glycosylation and reduces the number of synthetic steps. The building blocks have been obtained in good overall yield following the optimized synthetic approach. The synthesis of backbone linear chain trisaccharide [**ß-D-Gal-(1-4)-ß-D-Gal-(1-3)-ß-D-Glc**] and pentasaccharide [**ß-D-Gal-(1-4)-ß-D-GlcNAc-(1-3)-ß-D-Gal-(1-4)-ß-D-Gal-(1-3)-ß-D-Glc**] has been achieved in excellent yield (~80% yield). The final steps of the synthesis comprise- the incorporation of **ß-D-Gal** unit into the linear chain pentasaccharide (currently ongoing) followed by the enzymatic introduction of sialic acid (**NeuNAc** unit) and subsequent deprotection to yield the repeating unit of GBS type II capsular polysaccharide.

To conclude, in this thesis we present an efficient and easy handling synthetic approach to the heptasaccharide repeating unit of GBS type II. Readily available and cheap dairy side-product lactose has been used as a key structure in the presented scheme, allowing the efficient synthesis of the pentasaccharide backbone of the target compound. The synthetic GBS II fragments will be used for glycan array and structural studies and immunochemical characterization with specific monoclonal antibodies.

This thesis comprises of four main chapters and the experimental section containing the methods and synthetic procedures for the discussed schemes. Chapter one is a general introduction and deals with the necessity and the social importance of the described project. Chapter two of the thesis outlines the scientific background and pathogenesis of GBS, carbohydrates and their biological importance, and general introduction of vaccines and how the carbohydrates can be used as a suitable vaccine candidate. Chapter two establishes the importance of synthetic carbohydrates and how the synthetic carbohydrates can be used to develop suitable effective vaccines against GBS diseases.

Chapter three of the thesis contains the general introduction and structural features of GBS II CPS and the retrosynthetic analysis of GBS II CPS to identify the building blocks for the synthesis of GBS CPS II.

Chapter four of the thesis summarizes the synthetic strategies and results to achieve the building blocks described in chapter three and the recombination of fragments to achieve the final molecule GBS II CPS repeating unit.

The last part of the thesis will consists of the experimental methods and synthetic procedures to achieve the proposed molecule along with the characterization data.

Keywords: Group B Streptococcus (GBS), Carbohydrates, Oligosaccharide Synthesis, Vaccines, Enzymatic glycosylation, GBS infection, Capsular Polysaccharide (CPS)

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Abbreviations

Ac	Acetyl
AcOH	Acetic acid
All	Allyl
AllylBr	Allyl bromide
B-cells	Bone marrow- or bursa-derived cells
Bn	Benzyl
Bz	Benzoyl
CDC	Centers for Disease Control and Prevention
CDGs	Congenital disorders of glycosylation
CMP	Cytidine-5'-monophosphate
COSY	Correlation spectroscopy
CPS	Capsular polysaccharide
CRD	Carbohydrate recognition domain
CSA	Camphorsulfonic acid
DCM	Dichloromethane
DMAP	*N,N*-Dimethylaminopyridine
DMF	*N,N*-Dimethylformamide
EOD	Early onset disease
ESI	Electron spray ionization
Et	Ethyl
EtOAc	Ethyl acetate
EtOH	Ethanol
ETN	European Training Network
Fuc	Fucose
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GAVI	Global Alliance for Vaccines and Immunization
GBS	Group B Streptococcus
Glc	Glucose

GlcNAc	*N*-acetyl glucosamine
HIV	Human immunodeficiency virus
HPTLC	High performance thin layer chromatography
HRMS	High-resolution mass spectroscopy
HSQC	Heteronuclear single-quantum correlation spectroscopy
IAP	Intrapartum antibiotic prophylaxis
IgG	Immunoglobulin G
LOD	Late onset disease
Man	Mannose
Me	Methyl
MeCN	Acetonitrile
MeOH	Methanol
MHC	Major histocompatibility complex
NET	Neutrophil extracellular traps
NeuNAc	*N*-Acetylneuraminic acid/Sialic acid
NIS	*N*-Iodosuccinimide
NMR	Nuclear magnetic resonance
OTf	Triflate
PAMP	Pathogen-associated molecular pattern
PCV	Polysaccharide conjugate vaccine
PDVAC	Product Development for Vaccines Advisory Committee
Ph	Phenyl
Phth	Phthalimide
PS	Polysaccharide
PTSA	para-Toluenesulfonic acid
Py	Pyridine
Rha	Rhamnose
RT	Room temperature
SA	Sialic acid
Sym-collidina	2,4,6-Trimethylpyridine
TBAB	tetra-n-Butylammonium bromide
TBAF	Tetrabutylammonium fluoride

TBDMS	*t*-Butyldimethylsilyl
TBS	*tert*-butyldimethylsilyl
TBSCl	*tert*-butyldimethylsilyl chloride
T-cells	Thymus cells
TESOTf	Trimethylsilyl trifluoromethanesulfonate
TFA	Trifluoroacetic acid
Th cells	T helper cells
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
TOCSY	Total correlation spectroscopy
TOF	Time-of-flight
Tol	Toluene
Troc	Trichloroethoxycarbonyl
UN IGME	United Nations Inter-agency Group for Child Mortality Estimation
UTI	Urinary tract infection
VLPs	Viral-like particles
WHO	World Health Organization

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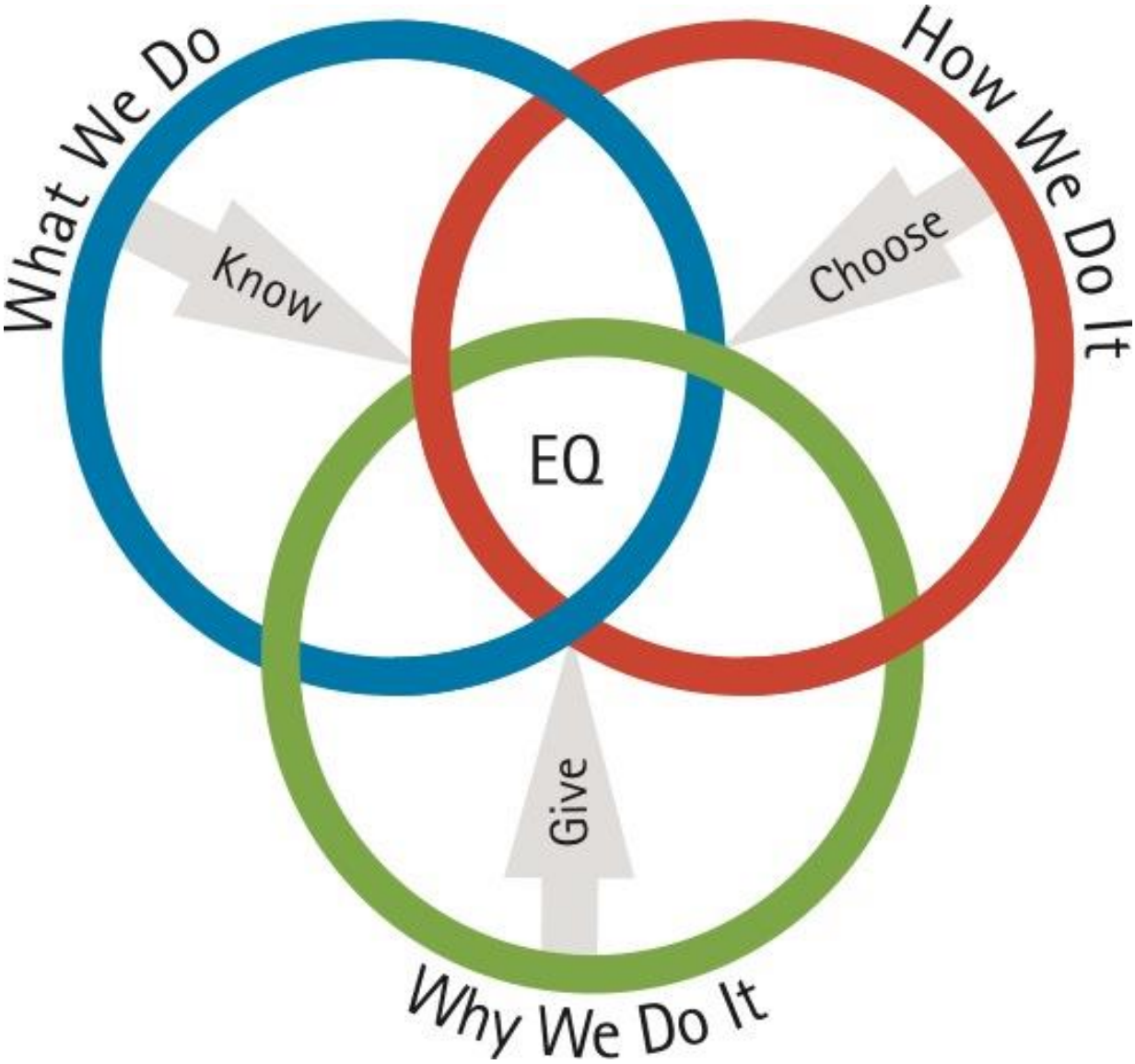
Chapter 1

General Introduction



1.1 Context

The first 28 days of life, also referred to as the neonatal period, are the most vulnerable time for a child's survival. Infants face the highest risk of dying in their first month of life at an average global rate of 18 deaths per 1,000 live births in 2017 according to UNICEF report.[1] Globally, 2.5 million children died in the first month of life in 2017 alone with approximately 7,000 neonatal deaths every day. Most of the neonatal deaths occurred in the first day and week, with about 1 million dying on the first day and about one million dying within the next six days.[1] Although under-5 mortality has declined up to 50% since 1990, 4.9 million children under 5 years of age died in 2015 (nearly 16,000 every day), with neonatal mortality accounting for approximately 45% of all deaths and with neonatal infection accounting for over a third of all deaths.[2,3,4] Reducing neonatal mortality is increasingly important not only because the proportions of under-five deaths that occur in the first 28 days is increasing as overall under-five mortality declines but also because the health interventions needed to address the major causes of neonatal deaths generally differ from those needed to address other under-five deaths. Despite progresses over the past quarter-century, millions of newborns, children and young adolescents die every year, mostly of preventable or treatable causes such as infectious diseases and injuries. In 2017, an estimated 6.3 million children and young adolescents died, mostly due to preventable causes. Children under age 5 accounted for 5.4 million of these deaths, with 2.5 million deaths occurring in the first month of life, 1.6 million at age 1-11 months, and 1.3 million at age 1−4 years. An additional 0.9 million deaths occurred among children aged 5−14.[5] These deaths reflect the limited access of children and communities to basic health interventions such as vaccination, medical treatment of infectious diseases, adequate nutrition and clean water and sanitation. Therefore, mortality rates among children and young adolescents are not only key indicators for child and young adolescent well-being, but more broadly, crucial for sustainable social and economic world. Two thirds of newborn deaths could be prevented if effective health interventions are provided during pregnancy, at birth and during the first week of life. Prevention of serious infections in pregnant mothers, newborns, and young infants through immunization during pregnancy and in early life has the potential to significantly reduce maternal and neonatal morbidity and mortality worldwide. Due to the recent scientific developments in the past decade, research in this field has advanced substantially, from the understanding of the

biology and immunology of pregnancy and early life, to the active development of several vaccine candidates. Vaccination is considered one of the most powerful means to save lives and to increase the level of health of humans.

One of the leading cause of global neonatal mortality and morbidity is Group B Streptococcus (GBS).[6] GBS was reported as a fatal human pathogen in 1938.[7] GBS is a Gram-positive bacterium, which is generally colonized in the intestine and genitourinary tract of 10-30 % of humans.[8] GBS emerged as a leading cause of neonatal infections in the 1960s and is known to cause a wide variety of infections.[9, 10] High clinical interest in GBS arises due to their ability to cause serious neonatal illnesses and is one of the primary causes of bacterial infections among neonates and pregnant women, resulting in many severe diseases such as sepsis, meningitis, abortion, and so on.[11, 12] If pregnant women carries the GBS bacterium in her vagina or rectum at the time of labour and delivery, there is a 1 in 100 chance that her baby will become sick from GBS infection.[13] The risk rises to 4% if a pregnant woman carries the bacterium and has certain risk factors such as- pre-term delivery before 37 weeks gestation, prolonged rupture of membranes (longer than 18 hrs without delivering the baby), or fever (38 °C or higher) during labour.[14] Other risk factors include having a previous pregnancy resulting in a GBS infected baby or having a urinary tract infection caused by GBS. Two distinct clinical syndromes of invasive GBS disease in the newborns exist are early onset disease (EOD) and late onset disease (LOD) depending on the age of the infant at the time of disease manifestation. Early onset neonatal disease refers to when the infant is infected by GBS in the first 6 days after birth and occurs secondary to vertical transmission. EOD is characterized primarily by sepsis, pneumonia, or less frequently meningitis and is most likely to manifest within the first 12-48 hours after birth.[15] About 60-70% of neonatal infections are early onset. Late onset neonatal disease occurs at 7-90 days after birth and are characterized by bacteremia, meningitis, or less commonly, organ or soft tissue infection.[15, 16] Late-onset disease is primarily acquired by horizontal transmission from the mother, but also can be acquired from hospital sources or from individuals in the community.[17] Late onset disease is less common then early onset disease. Maternal colonisation of GBS is a prerequisite for early onset and a risk factor for late onset disease.[18] Currently there is no effective strategy to help pregnant woman protect their newborns from GBS, therefore, the development of effective preventive strategies against GBS is urgent and much needed. The health and social care cost

for neonates with GBS disease is high, approximately two fold higher than neonates without GBS disease in the first 2 years and neonatal GBS disease poses a significant burden on society.[19] In view of this, finding prevention and treatments of morbidity for GBS-related diseases as well as early response and prevention is indeed an important issue that needs to be addressed. Finding cost-effective prevention therapies and treatments of GBS disease will benefit both patients and society globally.

Currently the only prevention strategy is a carriage or risk factor-based screening followed by intrapartum antibiotic prophylaxis (IAP). Antibiotics can reduce the risk of GBS disease in the first week of life, but this approach has only been partially successful.[20] Unfortunately, giving women antibiotics during labour is ineffective in preventing late-onset disease.[21] This successful public health intervention remains controversial. The UK National Health Service does not endorse routine screening-based GBS IAP, arguing that this approach does not adequately meet screening appraisal criteria.[22] The key issues driving the ongoing concerns about GBS IAP policies is due to the incomplete prevention of neonatal GBS disease.[23] The remarkable reduction of EOD contrasts with the unchanged incidence of neonatal GBS late-onset disease, for which IAP is not effective and for which, to our knowledge, there is no current prevention strategy. Vaccination presents the first step towards eliminating neonatal GBS disease and may be the only available approach for addressing the substantial international burden of GBS-associated stillbirth, preterm birth, and neonatal disease morbidity and mortality, therefore developing the effective vaccine against GBS is critically important.

Vaccination is one of the most powerful means to save lives and to increase the level of health globally.[24] The term vaccine elucidated by Edward Jenner's 1796 use of cowpox to immunize humans and providing them protection against smallpox.[25] A vaccine typically contains an agent that resembles a disease-causing microorganism and is often made of weakened or killed forms of the microbe, its toxins or one of its surface proteins, and cell surface carbohydrates.[26] This agent stimulates the body immune system to recognize the agent as foreign, destroy it, and keep a record of it, so that the immune system can more easily recognize and destroy any of such microorganisms that it encounters later. Vaccines are of two types- therapeutic such as vaccines against cancer, or prophylactic, which means to prevent or amend the effects of a future infection by any natural or wild pathogen.[26] Vaccines

will not only reduce neonatal GBS disease, vaccination could also lead to decline in maternal GBS urinary tract infection and chorio amnionitis and could potentially prevent preterm birth. In addition, if vaccination were a reality, the complications associated with IAP such as maternal anaphylaxis, antibiotic resistance and changes in neonatal sepsis rates would no longer cause problems.

In the last decade, the development of carbohydrate-based vaccines have successfully prevented many contagious diseases.[27] Carbohydrates are suitable and effective candidate for developing vaccines as they are among the most abundant molecules found on the cell surfaces of bacteria, parasites, and viruses.[28] Apart from their conventional roles as energy sources and structural polymers, carbohydrates are also associated with cancer metastasis, protein stabilization, pathogen infection and the immune response.[29] Cells of our body have sensors made out of carbohydrates on outer surface of plasma membrane. These cell surface carbohydrates act as sensors and can detect many kinds of stimuli, and can signal the immune system to respond. Invading microorganisms use surface-exposed carbohydrate and protein molecules to adhere to target cells in order to withstand natural fluxes and perturbations. This initial adhesion step is considered crucial for colonization and infection by pathogenic bacteria, which makes this carbohydrate-protein molecular recognition process crucial in infections and in immune response to pathogens.[30] Principally various types of cell-surface epitopes, characteristic of the invading organism or related to aberrant growth of cells, can be applied to develop vaccines.[31] Consequently, characterization and reconstruction of carbohydrate epitopes with authentic composition has become one of the major target in glycoscience. Isolation of carbohydrates from natural sources represents a viable method to provide samples for the biological testing of these molecules.[32] To date, several vaccines based on isolated capsular polysaccharides (CPSs) are marketed against pathogens.[33, 34] However, the use of isolated capsular polysaccharide poses several limitations, as natural sources are generally limited and the isolation is very challenging. Additionally, the isolated polysaccharides are heterogeneous and often contains impurities.[33] The manufacturer of these vaccines still suffers from shortfalls associated with the isolation of CPSs from natural sources. Furthermore, limited protectiveness of certain CPS antigens attenuate the efficiency of vaccines. To address the shortcomings associated with isolated polysaccharides, alternative strategies have been developed to produce immunogenic glycan antigens.

Oligosaccharide synthesis has become the most promising alternative method with great potential to understand glycan immunology and rationally engineer efficacious antigens.[35, 36] Synthetic oligosaccharides can be produced as homogeneous compounds with well-defined composition with high reproducibility, very precise construct, and the variables of chain length and chain density can be controlled because of the product purity. Additionally, the vaccines based on synthetic glycans have better safety profile and optimizing the vaccine formulation helps to fine-tune the immune responses elicited by oligosaccharide antigens. The progress in establishing the structure of carbohydrate immuno-determinants in conjunction with advancements in carbohydrate synthesis has rendered it possible to develop new generations of carbohydrate based vaccines. Recent advances in chemical synthesis, conjugation chemistry, engineered biosynthesis, and formulation design have spawned a new generation of vaccines that incorporate carbohydrate antigens. To this end, strategies are needed to facilitate the streamlined design and generation of these antigens. Novel synthetic methods fuel oligosaccharide assembly. Strategies to reverse engineer antibodies enable the rational antigen design. Concomitantly, optimizing the vaccine formulation helps to fine-tune the immune responses triggered by oligosaccharide antigens. Thanks to technological advances in glycobiology and glycochemistry, we entered in a new scientific era in which the rational design of carbohydrate vaccines has become an achievable goal.

To summarize, GBS is a dominant cause of serious neonatal illnesses especially meningitis and sepsis globally, and a prevention of GBS diseases is the need of the hour. Despite considerable advances in the diagnosis, prevention, and treatment of neonatal GBS infections, it remains an important public concern globally. Additionally, an increasing number of GBS infections in pregnant women and non-pregnant adults typically with underlying medical conditions, has been reported. In the first meeting of the Product Development for Vaccines Advisory Committee (PDVAC) by the World Health Organization (WHO) in 2014 for consultation regarding the development of GBS vaccines, GBS was identified as an important pathogen leading to a large burden of disease worldwide and a high priority for the development of a vaccine.[37] The meeting concluded that the native CPS vaccine is ineffective due to its poor immunogenicity, but the immunogenicity of the GBS polysaccharide conjugate vaccine may be able to induce a stronger and higher functional CPS-specific response. Although

vaccination is the most promising strategy for the prevention of GBS infection, currently no licensed GBS vaccine is available on the market.

The serious fatal threat posed by GBS on pregnant woman and neonates demands urgent prevention methods and carbohydrate-based vaccines present an effective solution to GBS diseases. This makes the development of an efficient synthetic method to obtain library of GBS related glycans critically demanding.

1.2 Aim of the thesis

Group B Streptococcus (GBS) is a leading etiologic agent of neonatal sepsis and meningitis and well known as one of the primary causes of bacterial infections among neonates and pregnant women.[1, 5] GBS, according to their type specific capsular polysaccharide (CPS), has been classified in ten structurally and antigenically unique types (Ia, Ib ,II, III, IV, V, VI, VII, VIII, X). [38, 39] Among 10 distinct serotypes of GBS, 90% of EOD are caused by serotypes Ia, Ib, II, III, and V while LODs are caused predominantly by serotype III.[40] Although all GBS serotypes are capable of causing invasive infection, six serotypes (Ia, Ib ,II, III, IV, V) account for the majority of disease both in neonates and adults. Type II GBS is associated with about 15% of the invasive infections in adults and infants; therefore, it represents a crucial human pathogen.[41]

Carbohydrate based vaccines present a solution to prevent the diseases due to GBS. The growing interest of the scientific and medical community in the vaccines based on synthetic carbohydrate is due to their advantages with respect to vaccines prepared from natural isolated carbohydrates, their better efficiency, and better safety profile. Advancement in the field of synthetic glycochemistry makes it possible to rationally design oligosaccharide assembly and prepare the optimized vaccine formulation. This thesis concerns the development of an effective synthetic strategy to obtain Group B Streptococcus (GBS) type II oligosaccharides for vaccine development. The project is a part of GLYCOVAX, a European Training Network (ETN) funded in the framework of H2020 Marie Skłodowska- Curie ITN programme with the aim to rationally design well-defined and innovative glycoconjugate

vaccines to improve current preventive therapies for neonatal GBS infections involving 8 academic groups and 2 industrial partners.[42]

The aim of the thesis is to develop an effective synthetic strategy to obtain the repeating unit of GBS type II capsular polysaccharide. The structure of GBS type II was elucidated in 1983 by the team of professor Kasper.[43] The repeating unit of GBS type II is a heptasaccharide consisting of α-Neu5Ac (2-3)-ß-D-Gal-(1-4)-ß-D-GlcNAc-(1-3)-[-ß-D-Gal-(1-6)]-ß-D-Gal-(1-4)-ß-D-Gal-(1-3)-ß-D-Glc. The development of an easy handling and convenient synthetic strategy will include the careful retrosynthetic analysis of the GBS type II repeating unit after an intensive literature survey. The synthetic approach to obtain the building blocks has been optimized to develop a high yielding and convenient method with suitable protecting groups to facilitate the recombining strategy. The synthesis of backbone linear chain pentasaccharide [ß-D-Gal-(1-4)-ß-D-GlcNAc-(1-3)-ß-D-Gal-(1-4)-ß-D-Gal-(1-3)-ß-D-Glc] unit has been optimized first, and it will be followed by the incorporation of ß-D-Gal unit into the linear chain pentasaccharide. The enzymatic introduction of sialic acid (NeuNAc unit) into the backbone hexasaccharide will be carried out at University of Manchester in the research group of Professor Sabine Flitsch, a GLYCOVAX network partner. After achieving the fully deprotected GBS type II repeating unit, synthesis of a library of glycans from GBS polysaccharide type II will be prepared. The synthetic GBS II fragments will be used for glycan array and structural studies and immunochemical characterization with specific monoclonal antibodies.

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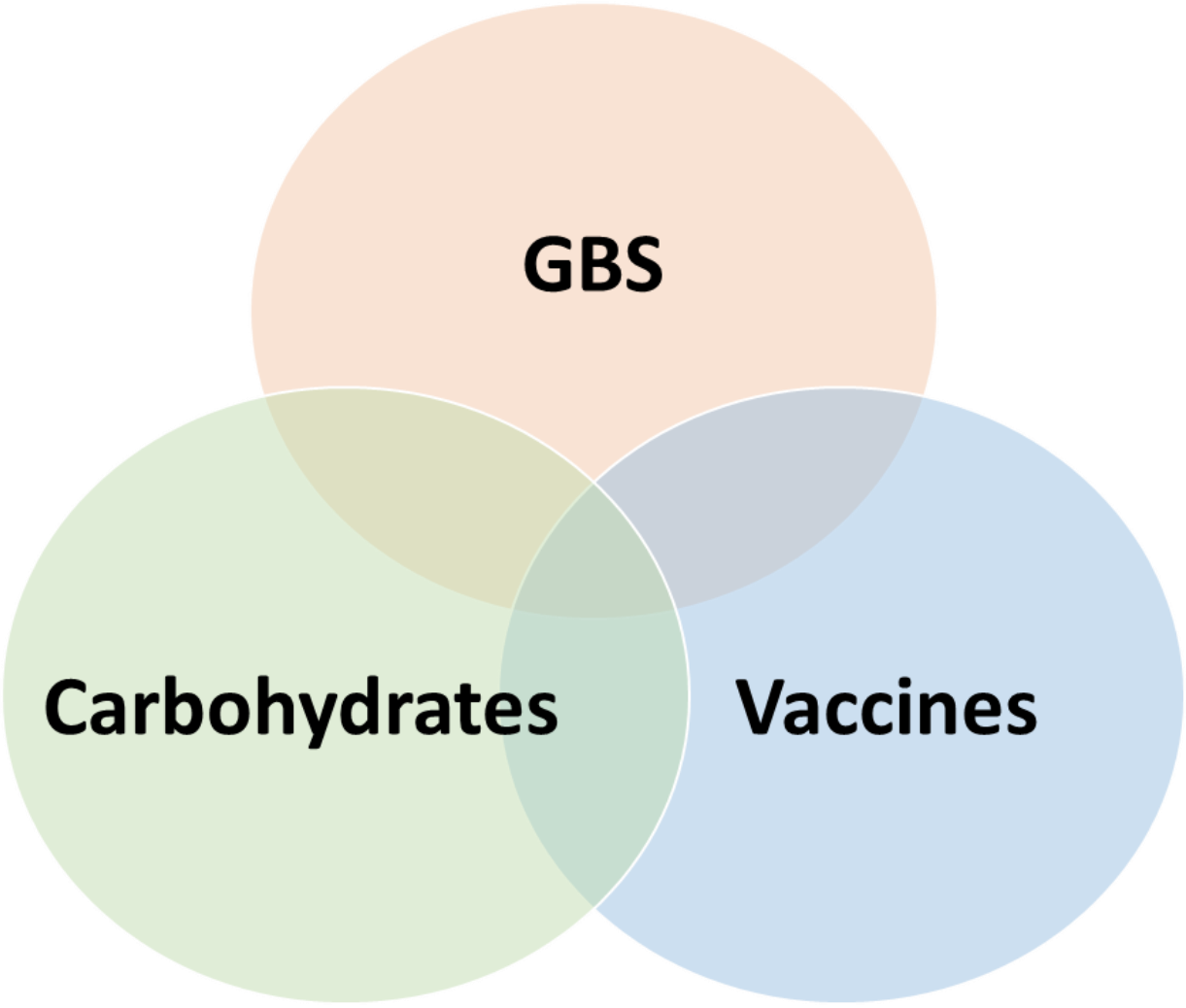
Chapter 2

GBS, Carbohydrates, & Vaccines: An Overview



Vaccination is an effective means to reduce death and morbidity caused by infectious diseases such as GBS infection. In recent years, the practice of vaccination with live attenuated bacteria, as originally conceived by Jenner to treat smallpox in 1796 and later developed by Pasteur, has most often left the stage to safer subunit vaccines. Subunit vaccines achieve protection by focusing the immune response on one or a few selected antigens. Among these, the structurally unique carbohydrates coating the surface of bacteria have become an optimal target for vaccine development.[1] Since the 1970s, it has been established that the purified capsular polysaccharide from *Streptococcus pneumoniae*, *Haemophilus influenzae* type b (*Hib*) and *Neisseria meningitidis* infections could be used for the prevention of infections in adults. However, it was only in the 1980s that revisiting the concept of sugar conjugation to a carrier protein, first introduced by Avery and Goebel in the early 1930s,[2] carbohydrate-based vaccines effective in infants could also be developed. Currently, glycoconjugate vaccines, as the product of chemical linkage of a carbohydrate antigen to a protein, are part of routine vaccinations in many countries.

This chapter will include the brief introduction of GBS, its classification and infection, GBS disease and preventive strategies in use, and the urgent need to develop effective preventive strategies against GBS. This will be followed by the brief introduction into carbohydrates and their role in inducing the immune system respond to pathogens and how these cell surface epitopes could be used to develop vaccines against a pathogen. The last section of this chapter will be about the origin of vaccines, their types and preparations, and general introduction of different type of vaccines with primary focus on carbohydrate based vaccines. The purpose of this chapter is to establish a correlation among carbohydrates, GBS, and vaccines.

2.1 Group B Streptococcus (GBS)

2.1.1 History of GBS

Streptococcus is a genus that comprises more than 50 recognized species of gram-positive and spherical bacteria that are able to colonize a variety of animal hosts.[3] While studying infections in cattle, Nocard and Mollereau identified a particular mastitis-causing Streptococcus in 1887, which they named Streptococcus nocardi.[4] Later, this species was renamed Streptococcus *agalactiae* (from the latin meaning of "no milk"), suggesting to its negative impact on bovine milk production. Based on their ability to cause the lysis of red blood cells, streptococci were divided into two groups: β-haemolytic and nonhaemolytic. Later, in 1933, Rebecca Lancefield further divided β-haemolytic streptococci into several categories, based on a carbohydrate found in the cell surface of the bacteria.[5] Hence, *S. agalactiae* is equally referred to as group B Streptococcus (GBS) as it is the only streptococcal species expressing the B antigen.

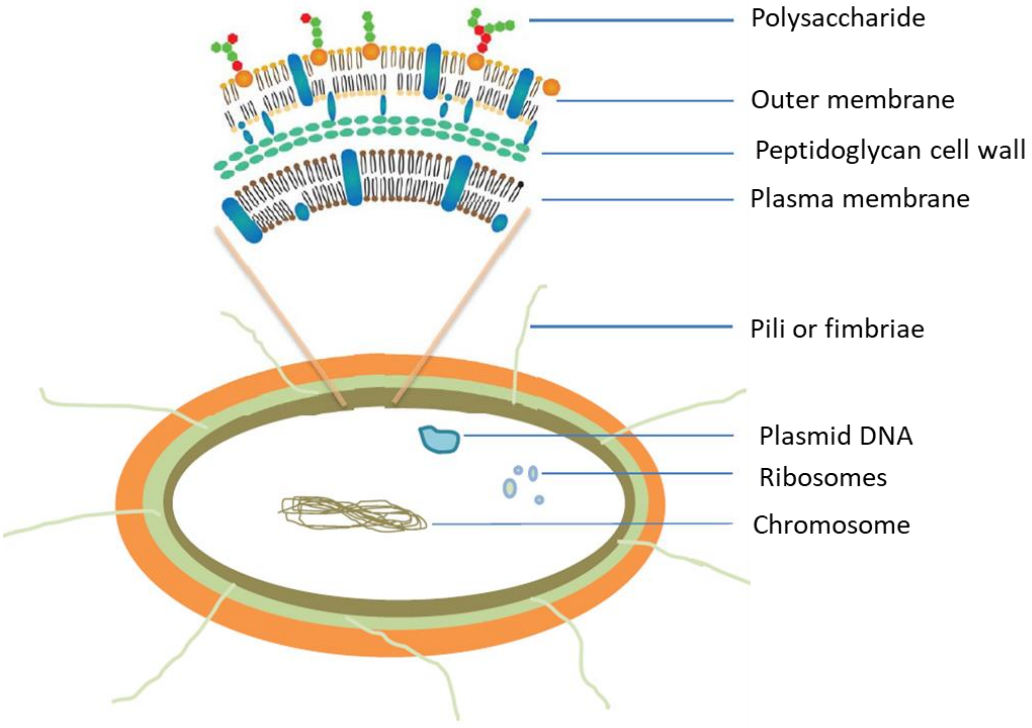


Figure 2.1: Molecular representation of Group B Streptococcus. (Adapted from Figure 4-35a, Brock Biology of Microorganisms, 11th Edition, Pearson Prentice Hall, 2006)

They are visualized as Gram-stain positive cocci on microscopy and grow non-fastidiously as 3-4 mm white colonies on blood agar. On a molecular level, the GBS cell surface is comprised of a capsule, peptidoglycan cell wall and a cell membrane (**Figure 2.1**).[6]

In nature, this bacterium has a broad range of hosts, but is most notably known for causing severe infections within humans, bovines and aquatic animals.[6-8] GBS inhabits the intestinal and genitourinary tract of 50% of humans.[9] In 1938, it was first identified as a human pathogen, causing human fatal puerperal sepsis,[10] but remained relatively unknown as sporadic asymptomatic cases were reported until the 1960s. By the 1970s, GBS had emerged as the predominant pathogen causing septicaemia and meningitis in neonates and infants, living in diverse regions.[11-13] In the adult population, colonization is mostly asymptomatic, although opportunistic infections can also occur among the elderly or in immunocompromised individuals.[14] In cattle, GBS adheres to the mammary epithelium causing bovine mastitis,[8] while it was also found to be responsible for outbreaks of invasive disease in several fish farms.[7] Each one of these reservoirs represents distinct ecological niches, reflecting the adaptive potential of GBS to very diverse host environments. Neonatal infections caused by GBS represent a significant cause of mortality and morbidity among infants. The primary clinical interest in the study of preventive strategies for GBS arises due to neonatal deaths caused by GBS.

2.1.2 GBS Classification

In the 1930s, Dr. Rebecca Lancefield described two polysaccharide antigens: the conserved Group B carbohydrate,[5] and the diverse S substance that generates type-specific antisera. Since then, 10 structurally and antigenically different types of capsular polysaccharides (CPS) with serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX), have been described.[15]

Biochemical capsular polysaccharide (CPS) structure of all ten GBS serotypes has been shown in **figure 2.2**. All identified CPSs of GBS are high-molecular weight polymers with the short side-chain capped terminally with a sialic acid (*N*-acetylneuraminic acid) residue. Each CPS consists of variously arranged monosaccharides such as glucose (Glc), galactose (Gal),

rhamnose (Rha), *N*-acetyl glucosamine (GlcNAc), and a sialic acid (SA) residue on the branching terminus of the repeating unit.[17]

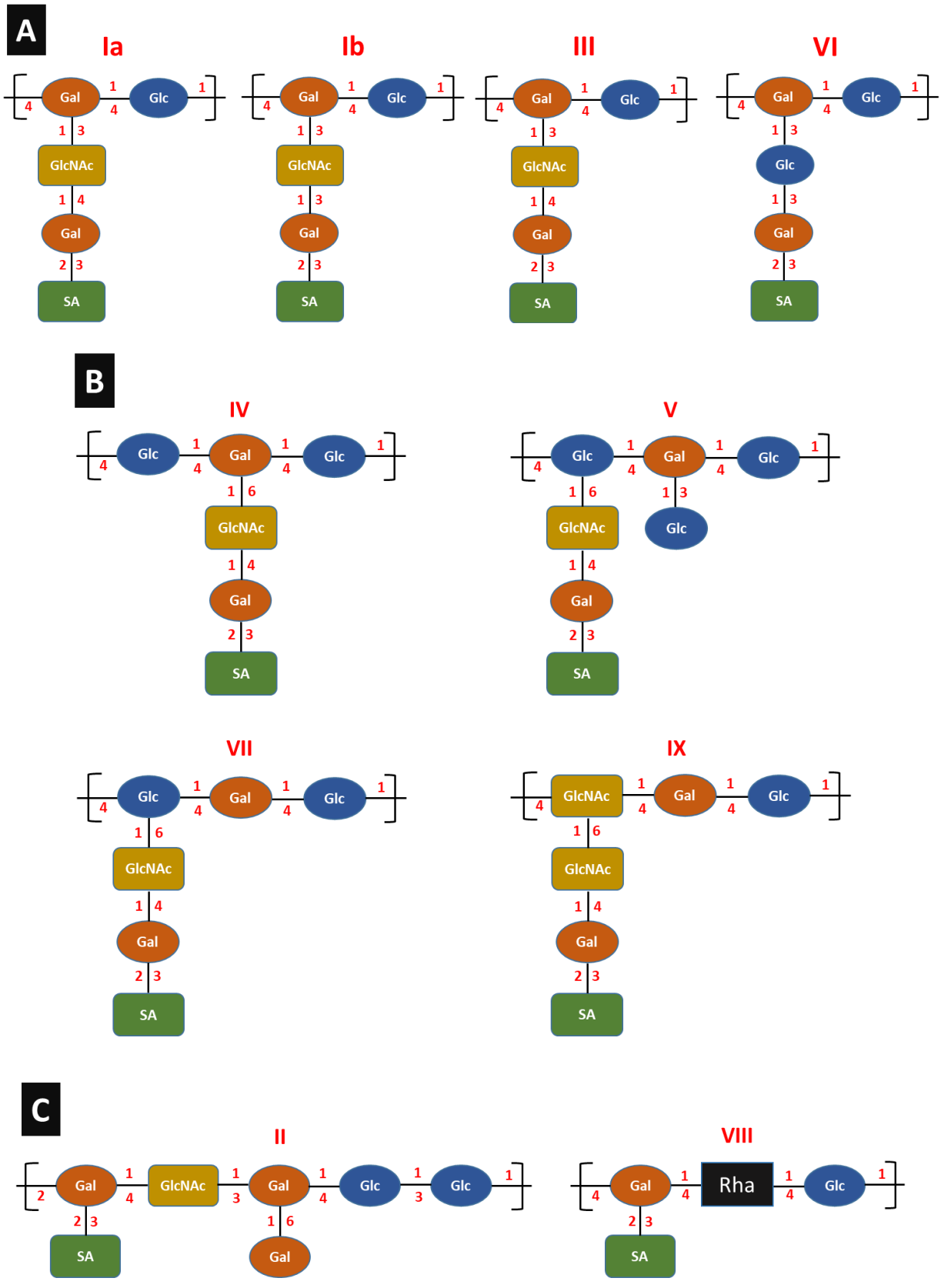


Figure 2.2. Chemical structure of the repeating unit of group B streptococcus (GBS) capsular polysaccharides. (A) Class A: GBS CPSs Ia, Ib, III, VI; (B) Class B: GBS CPSs IV, V, VII, IX; and (C) Class C: GBS CPSs II, VIII. Adapted from H. S. Sheo *at. al.*[16]

GBS CPSs are classified in three class depending on similarity of chemical structures and the involved enzymes in the assembly of the repeating units. GBS Serotypes belonging to class A (Ia, Ib, III, and VI) have the repeating unit which consists of two sugars containing a β (1→3) linked side chain and a terminal sialic acid unit (**Figure 2.2 A**). GBS CPS of class B (IV, V VII, and IX) have a repeating unit that consists of three sugars containing a β (1→6) linked side chain whose terminus is a sialic acid residue (**Figure 2.2 B**). GBS CPS type II and VIII belongs to class C and these CPSs have no similarity with GBS CPSs belonging to class A and class B and contain an α-(2→3) linked sialic acid residue (**Figure 2.2 C**).

2.1.3 Virulent factors and Pathogenesis

GBS expresses various virulence factors that are involved in its colonization, adherence, invasion, and immune evasion.[18-20] The GBS cell surface also comprises of multiple surface-proteins, some of which aid in the adherence of GBS to the epithelial surfaces. The virulence of GBS is attributed to the capsular and surface-proteins, and extracellular substances produced by the organism.[18] Surface antigens are major contributors to the pathogenic potential of GBS, and one of the most important ones is the capsule polysaccharide (CPS). The primary step in the pathogenesis of GBS is attachment to the host, followed by replication and evading host defences. These virulence factors may be used as potential vaccine candidates. Biochemical and molecular analyses of these factors can provide a better understanding of the infectious process, further assisting the development of new diagnostic techniques, specific antimicrobial compounds, and effective vaccines.

GBS expresses a unique CPS that is the most studied virulence factor contributing to the evasion of host immune defense mechanisms by protecting the bacteria from opsonophagocytosis by immune cells.[21-23] CPS can also increase the invasiveness of GBS by enhancing biofilm formation, inhibiting the binding of antimicrobial peptides and neutrophil extracellular traps (NET), and affecting bacterial adherence to the epithelium and mucus.[24-26]

Moreover, a correlation between the presence of CPS-specific antibodies in serum and the increased risk of GBS EOD and LOD development was reported.[27-28] Owing to its importance in GBS pathogenesis, CPS is considered the best target for the development of vaccine against GBS infection.

The development of GBS neonatal pneumonia, septicaemia and meningitis is a complex process. In the pathogenesis of these diseases, the bacterial need to adhere to and penetrate into different host cells including the vaginal epithelium, placental membranes, respiratory epithelium and blood-brain barrier endothelium (**Figure 2.3**). When GBS get into host blood and deeper tissues, it will trigger the host immune responses to eradicate the bacteria. GBS possess virulence factors, which can help the bacteria to avoid host immune clearance and activate host inflammatory responses. [9]

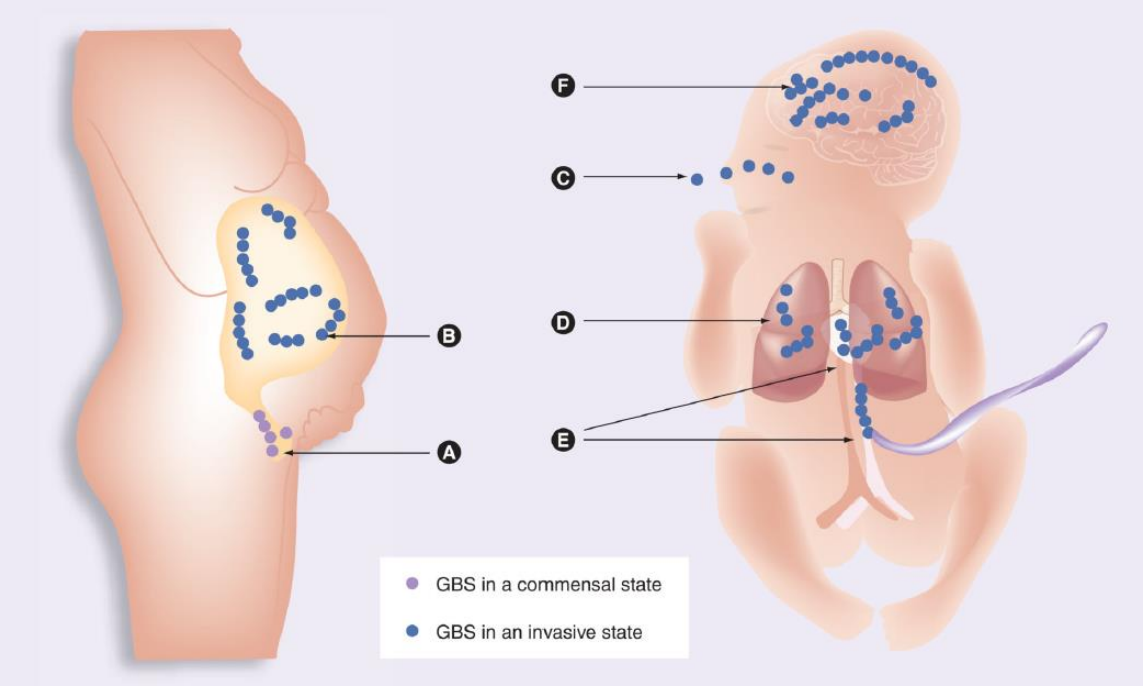


Figure 2.3: Lifecycle of Group B Streptococci as a neonatal pathogen. (A) GBS maternal colonization in vaginal and lower gastrointestinal tracts; (B) GBS penetration of the intrauterine compartment; (C) Neonatal aspiration of GBS during birth;(D) GBS invade the neonatal lung causing pneumonia; (E) GBS arrive in the neonatal bloodstream causing sepsis; (F) GBS penetration of the blood-brain barrier causing meningitis. Figure adapted from L. Rajagopal *et. al.* 2009. [18]

As per animal experiment, GBS was found in the lung, liver, spleen and brain in GBS infected neonatal primates.[29] The symptoms of sepsis and neonatal pneumonia include hypoxemia, hypercapnia and apnoea. The neonatal primates died with hypotension, metabolic acidosis and respiratory failure after a few hours without treatment.[29] There are inflammatory reactions and focal lesions at the early stage of GBS meningitis. This is accompanied with cerebral blood flow changes, cerebral hypoxia, ischaemia and oedema. This can result in permanent neuronal damage as necrosis in the cortex and neuronal loss and apoptosis were found in animal models. [30, 31]

GBS normally resides as a commensal organism in maternal genital and lower gastrointestinal tracts but can transits into an invasive pathogen that infiltrates a diverse array of host niches, such as the intrauterine compartment, neonatal lung and multiple neonatal organs, including the brain (**Figure 2.3**). This indicates that GBS is efficiently able to adapt to changing host environments.

Another specific mechanism by which GBS evades innate immunity is via molecular mimicry of a critical host glycan. All GBS capsular polysaccharide serotypes consists of a prominent α-(2,3)- linked terminal sialic acid in their repeat units, which is exactly same as a common host cell epitope present on glycolipids and glycoproteins present on the surface of all mammalian cells.[32]

2.1.4 Invasive GBS diseases

GBS has been identified as a major cause of invasive infections during the first three months of life since the 1970s. [33, 34] GBS is a cause of asymptotic bacteriuria, cystitis, and pyelonephritis during pregnancy. GBS bacteriuria in any concentration in a pregnant woman is a marker of heavy GBS colonization and bacteriuria and pyelonephritis are linked with preterm labour and preterm birth.[35] GBS causes bacteraemia, urinary tract infection (UTI), chorioamnionitis, postpartum bacteraemia, and endometritis in pregnant women.[36] When infection occurs during pregnancy, complications arise that may lead to abortion or preterm labour.[36]

In neonates, the most severe form of GBS infection is characterized by sepsis, pneumonia, and meningitis with a high fatality rate and residual damage.[9] The routes of GBS infection include intrauterine fetal infection, ascending infection from vagina of a GBS colonized woman, and contamination during passage through the birth canal.[35] GBS infection cases in the neonates and infants can be divided into two categories: early-onset disease (EOD) and late onset disease (LOD). Early-onset disease (EOD) is defined as presenting within the first 6 days of life, although more than 80% of EOD cases present within the first 12 hours of birth.[36] Neonatal bacteraemia, septicaemia, pneumonia, and meningitis are the most common representation of early onset GBS disease.[38] Risk factors for neonatal EOD include maternal GBS colonization, prolonged rupture of membranes, preterm delivery, GBS bacteriuria during pregnancy, birth of a previous infant with GBS disease, high fever, low levels of antibody to type specific CPS antigen, and young maternal age.[39] Late-onset disease (LOD) is defined as illness presenting from day 7 to 89 of life, with almost half of cases presenting with meningitis. Late-onset disease is primarily acquired by horizontal transmission from the mother, but also can be acquired from hospital sources or from individuals in the community.[40] Maternal colonization is also a strong risk factor for neonatal GBS infection in the late onset period.[41] Meningitis is the most common representation of LOD and there is risk of long-term neurological sequelae seems to be higher among survivors of late onset disease.

2.1.5 Current prevention strategies

Prevention of early-onset Group B Streptococcal infection received great clinical attention since GBS is the leading cause of neonatal sepsis, with a high fatality rate, especially in the very low birth weight infants.[38] The most effective strategy to prevent GBS infections during the perinatal period consists in intra partum antibiotic prophylaxis, which has been implemented since the 1990s in some high-income countries, including France and the US.[6] For this purpose, penicillin is the most frequent treatment choice, as GBS strains are universally sensitive to β-lactam antibiotics.[42] The pregnant women are tested for GBS bacteria when they are 35 to 37 weeks pregnant and giving antibiotics during labour to women at increased risk, including those who test positive for GBS bacteria. The revised 2010 guidelines for the prevention of perinatal group B streptococcal disease, issued by the Centers

for Disease Control and Prevention (CDC), state that if a patient has a history of penicillin allergy, they can also give other antibiotics to women. The antibiotics help during labour only because the bacteria can grow back quickly and the doctors cannot give antibiotics before labour begins. These preventive measures have only been shown to be effective at reducing the risk of EOD. Unfortunately, giving women antibiotics during labour does not prevent late-onset disease. Thus, even though there was a significant decrease in the incidence of early infections following the described treatment strategies, the level of LOD has remained largely the same.[43]

2.1.6 Progress towards GBS Vaccine

Currently, the only way to find women at risk of passing GBS onto their newborn during delivery is to screen for it during their third trimester of pregnancy and to monitor a course of antibiotics to the mother before the birth. However, this system is not foolproof and not practical in many overseas countries where health care systems are not as robust as in the western countries.

As per meeting of the PDVAC for consultation regarding the development of GBS vaccines in 2014,[44] native CPS vaccine is ineffective due to its poor immunogenicity, but the immunogenicity of the GBS polysaccharide conjugate vaccine (PCV) may be able to induce a stronger and higher functional CPS-specific immunoglobulin G (IgG) response.[45, 46] Several vaccine candidates are under clinical and preclinical investigations, but the low baseline incidence of the primary endpoint of GBS invasive disease requires phase III clinical efficacy trials to be very large.[47] Based on a good correlation between immune response and clinical protection, some experts suggested that GBS vaccine can be approved based on the immunogenicity assay.[48] Therefore, it is critical to develop a standardized immunogenicity assay and establish GBS serotype-specific protective cut-off values to succeed in the development of effective vaccines. Several efforts were made to modify the standard immunogenicity assay for pneumococcal PCV for the application in the GBS vaccine development.

GBS vaccine is the most promising strategy for the prevention of GBS infections in both newborns and adults with underlying diseases. However, numerous questions arise during the designing and evaluation of GBS vaccines. First, recent epidemiology studies have introduced the phenomenon of serotype switching and replacement occurring worldwide.[49, 50] Thus, a focused effort is required to update the global disease burden estimate and serotype distribution. Second, a standardized immunological assay is urgently needed. Hopefully, a vaccine will be soon available, to give to pregnant women the opportunity to prevent their newborn from GBS infection.

2.2 Carbohydrates

2.2.1 Background

The term "*carbohydrate*" was first used over 100 years ago to label molecules of the sum formula $(CH_2O)_n$. The carbohydrates are the compounds that provide energy to living cells. They are compounds of carbon, hydrogen and oxygen with a ratio of two hydrogens for every oxygen atom. The carbohydrates we use as foods have their origin in the photosynthesis of plants. They take the form of sugars, starches, and cellulose. Today this term also includes carbohydrate derivatives and refers to the whole class of molecules. Other names for carbohydrates are saccharides, sugars or glycans. Chemists referred to these compounds as "hydrates of carbon" or "carbohydrates". [51] The term "saccharide" was derived from the ancient Greek word sakhar meaning sugar or sweetness. In the 19th century, Emil Fischer elucidated the structure of the first monosaccharide, glucose, which was also referred as dextrose.[52] Glucose, for example, is a common monosaccharide that is oxidized to form carbon dioxide and water, providing energy for cellular processes such as protein synthesis, movement and transport. This was the development of chirality (from the greek "cheir" meaning "hand"), i.e. the differentiation between the D (dexter meaning "right") and L (laevus meaning "left") forms of carbohydrates. However, at that time science was still far from realizing the true biological impact of carbohydrates on living organisms.

The four major classes of organic molecules in living systems are proteins, lipids, nucleic acids and carbohydrates. Carbohydrates are by far the most abundant organic molecules found in nature, and nearly all organisms synthesize and metabolize carbohydrates.[51] The complex heterogeneity of carbohydrates in living systems (**Fig. 2.4**) is a direct result of several carbohydrate characteristics: the ability of different types and numbers of sugar residues to form glycosidic bonds with one another, the structural characteristics of these molecules, the type of anomeric linkage, the position and the absence or presence of branching.[53, 54] Carbohydrates play an essential part in a great deal of biological and biochemical processes. These molecules are commonly known as glycans in biochemical systems where they make up the carbohydrate chains of glycoproteins, proteoglycans and glycolipids. Glycosylation, substitution of these carbohydrate chains onto other molecules (most commonly proteins and glycolipids but also steroid skeletons), is a very common post-translational modification. At the most basic level, glycans are the union of saccharide chains to an underlying protein or lipid scaffold. Glycans are biologically important sugars, which may be single monosaccharides or oligosaccharides present as branched structures. About half of all known proteins in eukaryotes are glycosylated, indicating their importance as modifiers of different biological processes. Changes in oligo- or polysaccharide structures are associated with pathological and physiological events.[55] Most commonly, glycans are linked covalently via glycosidic linkages to either nitrogen provided by an asparagine residue (*N*-linked glycans) or by oxygen from serine or threonine residues (*O*-linked glycans).[54] Glycosylation of the human proteome is estimated to be at least 40% and glycans can comprise up to 90% of the total molecular weight of certain glycoproteins.[56, 57]

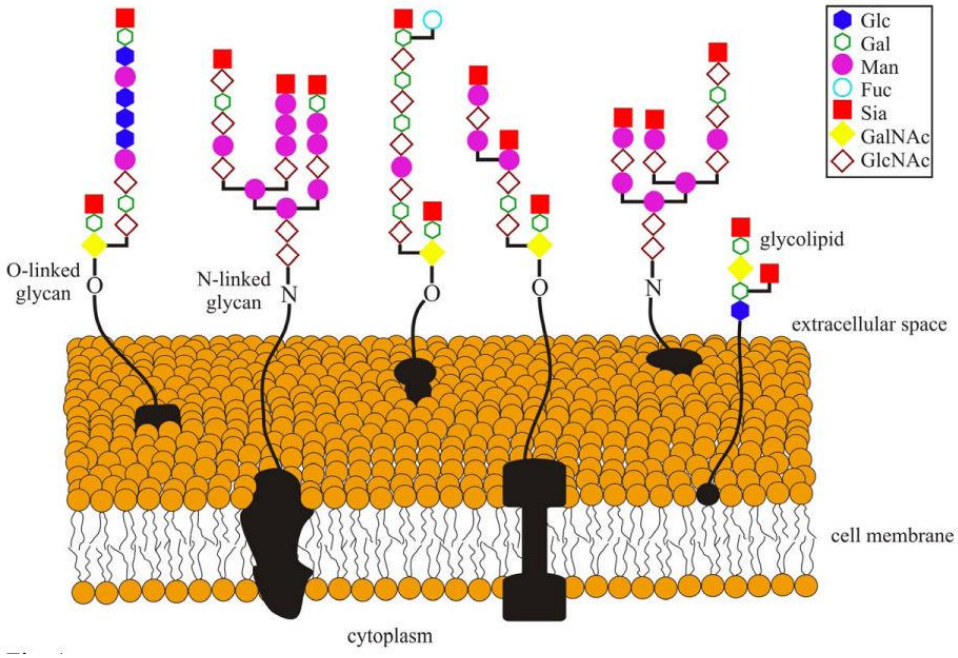


Figure 2.4: Pictorial illustration of carbohydrate heterogeneity found on cell surface glycoproteins and glycolipids. Sialic acids (Sia) are usually found at the terminal residue of *O*-linked and *N*-linked glycans of glycoproteins and glycolipids. [Glc (glucose); Gal (galactose); Man (mannose); Fuc (fucose); GalNAc (*N*-acetylgalactosamine); GlcNAc (*N*-acetylglucosamine)].

At least 2% of the genome is dedicated to creating, curating, maintaining, and recognizing glycans, and the loss of any component of these processes can result in drastic consequences or a threat to life.[58] To date, over 100 recessive Mendelian disorders resulting from hypomorphic mutations in glycosylation pathways have been identified, which result in the group of diseases known as congenital disorders of glycosylation (CDGs).[55]Although, approximately 2% of the genome encodes the required proteins needed for normal glycosylation, glycan structures themselves are not encoded. Therefore, glycans are an important form of post-translational modification and an epigenetic mechanism that can regulate gene expression.

Amazingly, glycans can be found in dense layers covering the surface of all natural cells. It is thought that the remarkable ubiquity of cell surface glycosylation that exists in nature is a consequence of its ability to shield a host cell from pathogens that would otherwise quickly adapt to recognize cell surface proteins.[59] Glycans are a common point of attachment for invading pathogens but can be rapidly remodeled without sacrificing underlying protein

function. Compared to the relatively limited diversity of linear structures achievable with any given quantity of amino acids, orders of magnitude more structures are possible with the same number of monosaccharides.[60]

Our understanding of the different glycoconjugates present on cells, proteins and entire organisms is lagging far behind compared to the advances in genomics and proteomics. [61, 62] Carbohydrate sequencing and advancement in the synthesis of defined oligosaccharides are two key approaches that have contributed to progress in glycomics research. Synthetic tools and high-throughput experiments such as carbohydrate arrays are beginning to affect biological research. These techniques are now being applied to the development of carbohydrate-based diagnostics, vaccines and therapeutics. [63]

2.2.2 Carbohydrates and Immunity

The immune system refers to a collection of cells and proteins that function to protect the skin, respiratory passages, intestinal tract and other areas from foreign antigens (any molecule recognized by the immune system as a foreign invader or as potentially dangerous for the host) such as microbes (organisms such as bacteria, fungi, and parasites), viruses, cancer cells, and toxins. The immune system responds to antigens by prompt an appropriate immune response. Protective immunity against pathogen exposure is attained by the amalgation of two distinct arms of the immune response (although these distinctions are not mutually exclusive): the innate and the adaptive (antigen-specific) responses.[64] Innate immunity refers to nonspecific defense mechanisms that come into play immediately or within hours of an antigen's appearance in the body.[65] These mechanisms include physical barriers such as skin, chemicals in the blood, and immune system cells that attack foreign cells in the body. The innate immune response is activated by chemical properties of the antigen. The innate response is rapid and unspecific and it is mediated by antigen-presenting cells (APCs) and establishes the first line of immune defense, detecting and responding to pathogen-associated molecular patterns (PAMPs).[65, 66] Adaptive immunity is an antigen-specific immune response and is more complex than the innate.[67] The antigen first must be processed and recognized. Once an antigen has been recognized, the adaptive immune system creates an army of immune cells specifically designed to attack that antigen. Thymus

cells (T cells) and bone marrow- or bursa-derived cells (B cells) mediate the adaptive response. The adaptive response recognizes pathogens with high affinity, providing the fine antigenic specificity required for complete elimination of the infective agent and the generation of the immunological memory. Adaptive immunity also includes a "memory" that makes future responses against a specific antigen more efficient.[67]

B and T cells are the major cellular components of the adaptive immune response. T cells are involved in cell-mediated immunity, whereas B cells are primarily responsible for humoral immunity (relating to antibodies). The function of T cells and B cells is to recognize specific "non-self" antigens, during a process known as antigen presentation. Once they have identified an invading pathogen, the cells produce specific responses that are optimized to eliminate specific pathogens or pathogen-infected cells. B cells respond to pathogens by producing large quantities of antibodies that further neutralize foreign objects like bacteria and viruses. In response to pathogens, some T cells, called T helper cells (Th cells), produce cytokines that direct the immune response while other T cells, called cytotoxic T cells, produce toxic granules that contain powerful enzymes that can induce the death of pathogen-infected cells. Following activation, B cells and T cells leave a lasting legacy of the encountered antigens in the form of memory cells. Throughout the lifetime of an animal, these memory cells will remember each specific pathogen encountered, and are able to mount a strong and rapid response if the same pathogen is detected again; this is known as acquired immunity.

Since carbohydrates are common surface molecules, it is reasonable to postulate that they are critical for immune recognition. Glycans influence immunity by regulating signal transduction and receptor activation, protein folding, and cell adhesion. Recent genomic studies have revealed the presence of carbohydrate recognition domains (CRDs) throughout evolution, suggesting an important role for glycans and their recognition in physiology. [68, 69] Pathogens are decorated with structurally distinct surface glycans and there are accumulating evidences have been demonstrating the importance of glycans and glycans binding proteins in the regulation of both innate and adaptive immune responses recognized by the immune system.[70] The chemically synthesized cell-surface glycans of clinically relevant bacterial and parasitic pathogens can be exploited as immunogens for vaccination. Chemically synthesized cell-surface glycans allow the generation of highly specific antibody responses to complex glycan structures. With the common presence of carbohydrate molecules on eukaryotic,

prokaryotic, and viral surfaces, the impact of carbohydrates on adaptive immunity is now indisputable.

2.2.3 Role of Carbohydrates

A dense layer of oligosaccharides, also referred to as glycocalyx, covers the surrounding membranes of mammalian cells and some bacteria.[71] Most human epithelia cells have a glycocalyx coating on the external surface of their plasma membranes.[72] This coating consists of several carbohydrates moieties, such as membrane glycolipids,[72] glycoproteins,[73] proteoglycans,[74] and other types of glyco-conjugates. These carbohydrates moieties display a remarkable degree of structural diversity and complexity. In general, these molecules contribute to cell-cell recognition, communication, and intercellular adhesion. Carbohydrate-specific receptors are called lectins,[75] that are found on the cell surface. They can interact with carbohydrates on correspondent cells. This process may control the adhesion of cells, such as carbohydrate-directed cell adhesion,[76] and it appears to be important in many intercellular activities including infection by bacteria[77] and viruses,[78] communication among cells of lower eukaryotes,[79] specific binding of sperm to eggs,[80] and recirculation of lymphocytes,[81] among others. An increasing number of studies demonstrate that carbohydrates play a very important role in both inter and intra cellular signal transmission.[82] Additionally, manipulation of relevant carbohydrate moieties may potentially impact or alter certain biological recognition events.[83]

Glycans are often large, highly charged structures. When positioned at the cell surface, they are uniquely poised to mediate the interaction of the cell with the outside environment. They can promote or obstruct interactions of receptors and their ligands, or alternatively of glycan-binding proteins known as lectins.[84] The structural variability and complexity of cell surface glycans allows them to function as signaling molecules, recognition molecules and adhesion molecules.[85-87] As such, cell surface glycans are involved in many physiologically important functions that include normal embryonic development, differentiation, growth, contact inhibition, cell-cell recognition, cell signaling, host-pathogen interaction during infection, host immune response, disease development, metastasis, intracellular trafficking and localization, rate of degradation and membrane rigidity.[85, 88-96] Although the physical and chemical

properties of simple carbohydrates are well known, it is unfortunate that we cannot say the same for complex carbohydrates in living systems. Glycobiology is a fertile area that we are just beginning to understand and appreciate. Studies in glycobiology have been advancing at ever-increasing rates in the past few years driven by advanced developments in new technologies and in genomics.[97, 98]

2.2.4 Carbohydrates as a vaccine candidate

The structural complexity of carbohydrate moieties on glycocalyx made them become possible bio-markers/antigen for vaccine or monoclonal antibodies development.[99, 100] There are some very successful examples of licensed vaccines that target carbohydrate antigens. For example, Pneumovax® 23 (PPV-23) is a 23-valent pneumococcal vaccine derived from capsular polysaccharides.[101, 102] Compared to proteins, carbohydrates are more conserved and more abundant on the cell surface. Consequently, development of carbohydrate-based vaccines against various diseases has been a hot area in recent decades, and many new and highly effective vaccines have been developed.[103-105]

The development of vaccines based on carbohydrates has a long history. As early as 1923, Heidelberger and Avery [106] described a 'soluble specific substance', of pneumococci to consist most likely of polysaccharides (PSs) and being typical for the serotype. Francis and Tillett [107] noted that intradermal injections of type-specific polysaccharides induced the development of circulating antibodies for heterologous types of Pneumococci. Later, Heidelberger et al. [108] established that pneumococcal capsular polysaccharides could be used as vaccines, providing a long lasting immunity. Despite the potential to apply such compounds as vaccines, the development of chemotherapeutics and antibiotics has led to a loss of interest in this application. Renewed interest for preventive vaccination was induced by the steady increase in resistance towards antibiotics. In 1983, Pneumovax was introduced, being a capsular polysaccharide vaccine derived from 14 pneumonia serotypes. Subsequently, Pneumovax 23 was presented containing isolated polysaccharides from 23 serotypes out of the about 90 known.[109, 110]

The cell surfaces of bacteria, parasites and viruses exhibit oligosaccharides that are often distinct from those of their hosts. Specific types of glycoconjugate are often more highly expressed on the surface of tumors than on normal cells. [111] Such cell-surface carbohydrate markers are the basis for carbohydrate-based detection systems and vaccines. An immune response against the carbohydrate antigens that results in the death of target cells is required for a carbohydrate-based vaccine. Such vaccines have been widely used against a host of diseases for several decades[112]. The carbohydrate antigens for antibacterial vaccines were isolated from biological sources. Recently, intense efforts focused on the use of defined carbohydrate antigens that are synthesized rather than isolated. A carbohydrate-based approach has also been pursued for anticancer vaccine candidates.[113-115]

Recently, intense efforts focused on the use of defined carbohydrate antigens that are synthesized rather than isolated from natural CPSs because of their limited availability and challenging isolation. The first commercial vaccine containing a synthetic carbohydrate antigen was developed in Cuba against Hib.[116] This vaccine, QuimiHib®, exhibits several advantages associated because of the use of the synthetic carbohydrate: potentially lower production costs compared to conventional vaccines using carbohydrates from natural sources, controlled production of a homogeneous single compound including the linker, minimal batch-to-batch inconsistency during manufacturing process, and higher quality control standards are permitted, compared with the use of naturally derived agents. In addition, synthetic carbohydrates can be modified to increase their immunogenicity.

Vaccine development could benefit greatly from the new glycomics technologies. The identification of specific oligosaccharide antigens has been aided substantially by sequencing and carbohydrate arrays. The procurement of defined oligosaccharides using improved solution- and solid-phase methods has become fast enough to be used reiteratively in drug-development efforts. A barrier to development of carbohydrate vaccines has been the lack of robust technologies that support the production of synthetic vaccines and analysis of the specificity of an immune response. Many scientific efforts have focused on advances in the synthesis, analysis of carbohydrates and analysis of carbohydrate immune responses that promise to accelerate the development of carbohydrate vaccines. This makes the development of new methods for oligosaccharide synthesis very lucrative and important.

2.3 Vaccines

2.3.1 History

Vaccines have been one of the biggest success stories of modern medicine. Vaccination is an effective means to reduce death and morbidity caused by infectious diseases. WHO estimates that at least 10 million deaths were prevented between 2010 and 2015, thanks to vaccinations delivered around the world. Many millions more lives were protected from the suffering and disability associated with diseases such as pneumonia, diarrhea, whooping cough, measles, and polio. It is not difficult to imagine the impact of vaccination if we just think that in the 20th century smallpox alone killed 300 million people, and that no one dies from it today because the virus has been eradicated thanks to vaccination in 1978.[117] Worldwide life expectancy also increased moving from 58.5 to 70 years from 1970 through 2010.[118] The highest decrease in mortality was measured in children and young adults up to 20 years of age. During this period, the Expanded Program on Immunization and more recently the establishment of the Global Alliance for Vaccines and Immunization (GAVI) increased complete infant immunization from less than 5% to more than 90%.[119] A study from the World Health Organization (WHO), reports that today vaccines save more than 2.5 million deaths annually.[120] The impact of communicable diseases worldwide decreased from 33% of the total deaths in 1990 to 25% in 2010 and non-communicable diseases became the first cause of global mortality and morbidity.[121]

The human immune system can detect a variety of pathogens and distinguish them from normal healthy tissues. The recognition of pathogenic patterns leads to the development of multiple highly specific defense mechanisms that eradicate or neutralize the pathogens in body. By harnessing the power of the immune system, vaccines have become one of the most significant achievements in the history of medical practice.[122] In 1796, Edward Jenner first inoculated healthy patients with a less lethal cowpox virus to help induce immunity against the smallpox virus.[123] Numerous successes post-Jenner have proven the great value of vaccines to society. Once the microbial origin of infectious diseases was discovered a century later, Pasteur pioneered vaccine development by exposing people to dead or attenuated microorganisms that mimicked the infectious agent, but did not cause disease.[124, 125] In the

1940s the discovery that viruses could be grown in in vitro cultures on animal cells allowed the development of many vaccines against poliomyelitis, measles, mumps, rubella, varicella, hepatitis A and, more recently, rotavirus and influenza. For more than a century vaccines have been developed following the principles of Pasteur by isolating, inactivating, and injecting the microorganism causing the disease or a portion of it. [124, 125] During the last 30 years, several new and unconventional technologies allowed for the development of vaccines that conquered several diseases.[44, 126] The advent of recombinant DNA in the late 1970s made it possible to safely make large quantities of hepatitis B vaccine by producing it in yeast viral-like particles (VLPs) identical to those released in the plasma by the hepatitis B virus.[127]

Currently there are several types of vaccines available in the medical practice, including attenuated or dead microorganisms, inactivated bacterial toxins, protein subunit, and capsular polysaccharides. Most of the successful vaccines in clinical applications are targeting epidemic diseases and have been developed empirically. For example, influenza vaccines are developed based on the specific influenza virus subunits, which are reconfigured regularly.

2.3.2 Type of Vaccines

The first human vaccines against viruses were based using weaker or attenuated viruses to generate immunity. The smallpox vaccine used cowpox, a poxvirus that was similar enough to smallpox to protect against it but usually did not cause serious illness.[123] Rabies was the first virus attenuated in a lab to create a vaccine for humans.[128] Vaccines are made using several different processes. They may contain live viruses that have been attenuated (weakened or altered so as not to cause illness); inactivated or killed organisms or viruses; inactivated toxins (for bacterial diseases where toxins generated by the bacteria, and not the bacteria themselves, cause illness); or merely segments of the pathogen (this includes both subunit and conjugate vaccines).[126]

There are several different types of vaccines. Each type is designed to teach to your immune system how to fight off certain kinds of pathogens and the serious diseases they cause. While creating vaccines, few factors must be considered such as- how your immune system responds to the pathogen, who needs to be vaccinated against the germ, the best technology

or approach to create the vaccine. Based on a number of these factors, scientists decide which type of vaccine needs to be developed. There are 4 main types of vaccines based on different preparation methods:

2.3.2.1 Live-attenuated vaccines

The idea of attenuation of virulent infections developed slowly over the course of centuries. Variolation was analogous to the use of small amounts of poison to render one immune to toxic effects. Jenner's use of an animal poxvirus (probably horsepox) to prevent smallpox was essentially based on the idea that an agent virulent for animals might be attenuated in humans. Live attenuated vaccines contain whole bacteria or viruses, which have been weakened so that they create a protective immune response but do not cause disease in healthy people. The immune response to a live attenuated vaccine is virtually identical to that produced by a natural infection. The immune system does not differentiate between an infection with a weakened vaccine virus and an infection with a wild virus. Live vaccines tend to create a strong and lasting immune response and are some of our best vaccines. Protection from a live, attenuated vaccine typically outlasts the protection provided by a killed or inactivated vaccine. Live attenuated vaccines are used against measles, mumps, rubella, yellow fever, rotavirus, smallpox, chickenpox, and influenza. [129, 130]

However, live vaccines are not suitable for people whose immune system does not work, due to either drug treatment or underlying illness. Live attenuated vaccines may cause severe or fatal reactions because of uncontrolled replication of the vaccine virus. This only occurs in persons with immunodeficiency (e.g., from leukemia, treatment with certain drugs, or human immunodeficiency virus [HIV] infection). This is because the weakened viruses or bacteria can multiply too much and might cause disease in these people.

2.3.2.2 Inactivated vaccines

One alternative to attenuated vaccines is a vaccine from killed or inactivated pathogen. Vaccines of this type are created by inactivating a pathogen, typically using heat or chemicals such as formaldehyde or formalin. This destroys the pathogen's ability to replicate, but keeps

it intact so that the immune system can still recognize it. *Inactivated* is generally used rather than *killed* to refer to viral vaccines of this type, as viruses are generally not considered alive. Because killed or inactivated pathogens can not replicate at all, they cannot cause the diseases against which they protect, even in people with severely weakened immune systems. However, they tend to provide a shorter length of protection than live vaccines, and are more likely to require boosters to create long-term immunity. Therefore, you may need several doses over time (booster shots) in order to get ongoing immunity against diseases. Inactivated vaccines usually do not provide immunity (protection) that is as strong as live vaccines. In addition, inactivated vaccines usually require higher doses and multiple boosters, possibly causing inflammatory reactions at the site of injection. Adjuvants such as aluminium salts are often added to inactivated vaccines. These are substances, which help to strengthen and lengthen the immune response to the vaccine. As a result, common local reactions, such as a sore arm, may be more noticeable and frequent with inactivated vaccines. Inactivated vaccines are used to protect against Hepatitis A, Flu, Polio, and Rabies. [129, 130]

2.3.2.3 Toxoid vaccines

Some bacterial diseases are not directly caused by a bacterium itself, but by a toxin produced by the bacterium. The immune system recognises these toxins in the same way that it recognises polysaccharides or proteins on the surface of the bacteria. Some vaccines are made with inactivated versions of these toxins. They are called 'toxoids' because they look like toxins but are not poisonous. They trigger a strong immune response. One example is tetanus: its symptoms are not caused by the Clostridium tetani bacterium, but by a neurotoxin it produces (tetanospasmin).[131]

Immunizations for this type of pathogen can be made by inactivating the toxin that causes disease symptoms. As with organisms or viruses used in killed or inactivated vaccines, this can be done via treatment with a chemical such as formalin, or by using heat or other methods. Toxoids can actually be considered killed or inactivated vaccines, but are sometimes given their own category to highlight the fact that they contain an inactivated toxin, and not an inactivated form of bacteria. Toxoid vaccines are used to protect against diphtheria, and tetanus. [129, 130]

2.3.2.4 Subunit vaccines

Subunit vaccines do not contain any whole bacteria or viruses instead, these kind of vaccines contain polysaccharides (sugars) or proteins from the surface of bacteria or viruses.[132] These polysaccharides or proteins are recognised by our immune system recognises 'foreign', and they are referred to as antigens. Even though the vaccine might only contain a few out of the thousands of polysaccharide/proteins in a bacterium, they are enough in themselves to trigger an immune response, which can protect against the disease. Subunit vaccines can be produced either by chemically degrading a pathogen and isolating its key antigens or by producing the antigens through genetic engineering. Because these vaccines contain only the essential antigens of a pathogen, the risk of side effects is relatively low. Because of their immunity and low production costs, they are viable alternatives to the traditional vaccines.[103] They can also be used on almost everyone who needs them, including people with weakened immune systems and long-term health problems. Subunit vaccines can be further categorized into following three categories:

2.3.2.4.1 Protein based subunit vaccines

Protein based subunit vaccines present an antigen to the immune system without viral particles, using a specific, isolated protein of the pathogen. Commonly used protein-based subunit vaccines are against acellular pertussis and Hepatitis B. A weakness of this technique is that isolated proteins, if denatured, may bind to different antibodies than the protein of the pathogen.

2.3.2.4.2 Polysaccharide based subunit vaccines

Polysaccharide vaccines are a unique type of inactivated subunit vaccine composed of long chains of sugar molecules that make up the surface capsule of certain bacteria. Pure polysaccharide vaccines are available for three diseases: pneumococcal disease, meningococcal disease, and Salmonella Typhi.

In 1983, PneumoVax (Merck and Co.), the first polysaccharide vaccine, was commercially launched.[109] This vaccine was made up of unconjugated capsular polysaccharide isolated from 14 serotypes of the Streptococcus pneumoniae bacterium, whereas the current version includes 23 out of approximately 90 known serotypes.[110] In healthy adults, this vaccine induces good protection against most of the infections caused by these pathogens. Polysaccharide-based vaccines are poorly immunogenic, however, in infants, young children (under 2 years of age), the elderly, and immunocompromised patients, whereas in adults they induce only short-lasting antibody responses and fail to generate conventional B cell mediated immunological memory.

The immune response to a pure polysaccharide vaccine is typically T-cell independent, which means that these vaccines are able to stimulate B cells without the assistance of T-helper cells.[133] B cells directly recognize vaccine polysaccharides. No interaction occurs between Th and B cells (**Figure 2.5A**). T-cell independent antigens, including polysaccharide vaccines, are not consistently immunogenic in children younger than 2 years of age. Young children do not respond consistently to polysaccharide antigens, probably because of immaturity of the immune system.[103, 133]

2.3.2.4.3 Conjugate subunit vaccines

A conjugate vaccine is a type of subunit vaccine that consists of a protein conjugated to a capsule polysaccharide. Conjugate vaccines have been developed to enhance the efficacy of subunit vaccines against pathogens that have protective polysaccharide capsules that help them evade phagocytosis, causing invasive infections that can lead to meningitis and other serious conditions. Conjugate vaccines are made using a combination of two different components from the coats of bacteria. These coats are chemically linked to a carrier protein, and the combination is used as a vaccine.

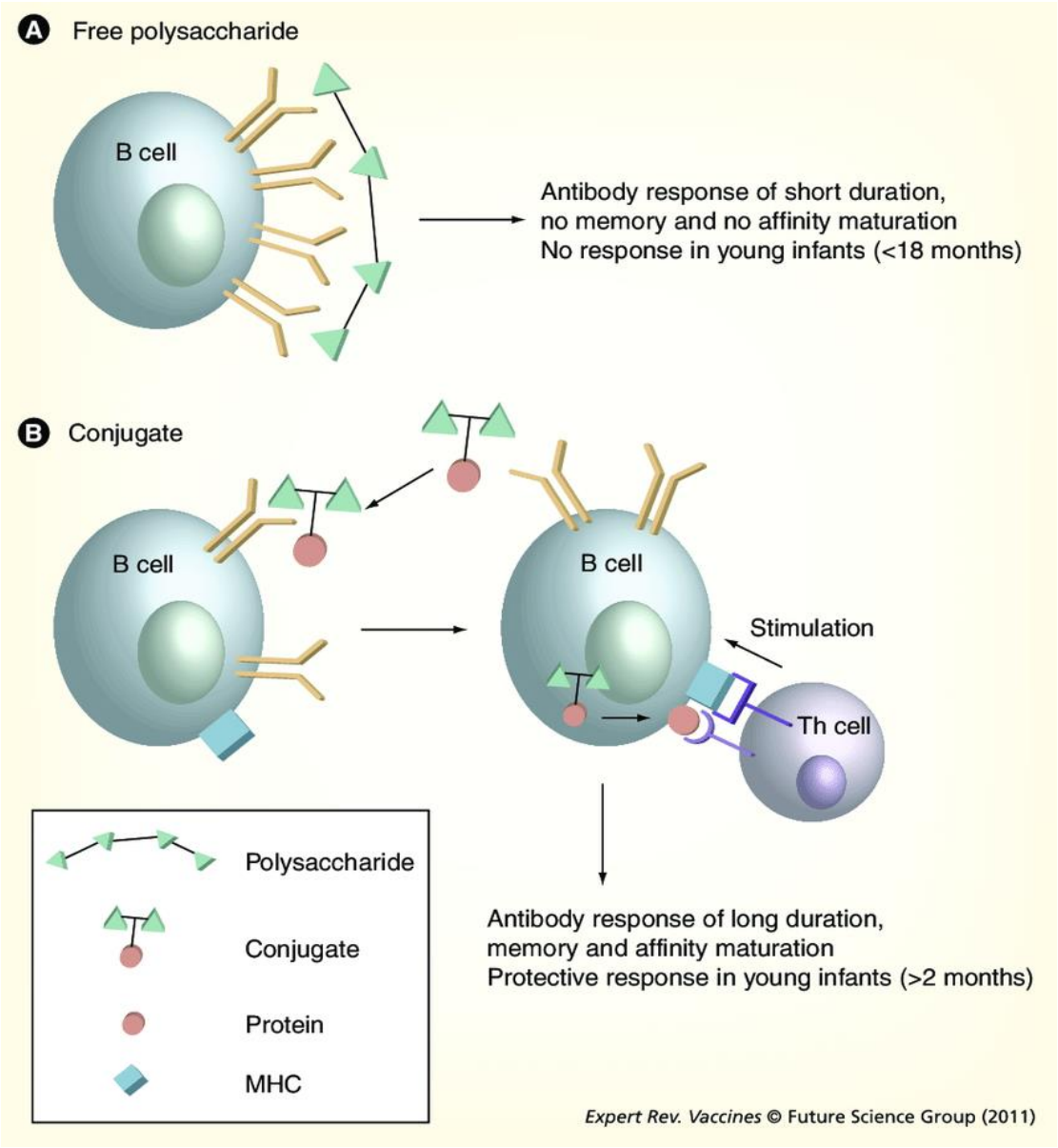


Figure 2.5: Depiction of T-cell-independent and T-cell-dependent responses to polysaccharide (**A**) and glycoconjugate vaccines (**B**). Adapted from J. Poolman *et. al.*[134]

In the late 1980s, it was discovered that the chemical conjugation of polysaccharide with a protein molecule changes the immune response from T-cell independent to T-cell dependent, leading to increased immunogenicity in infants and antibody booster response to multiple doses of vaccine (**Figure 2.5**).[135] Conjugate vaccines are used to create a more powerful, combined immune response as part of bacteria alone being presented would not generate a strong immune response on its own, while the carrier protein would. The piece of bacteria

can't cause illness, but combined with a carrier protein, it can generate immunity against future infection.[136] The vaccines currently in use for children against pneumococcal bacterial infections are made using this technique. In conjugate vaccines, B cells recognize the polysaccharide-protein conjugate of the vaccine. The protein carrier is processed and presented to T-helper (Th) cells in the context of the major histocompatibility complex (MHC), which induces T-cell activation. In turn, T cells stimulate the specific B cells to produce antibodies against the conjugate (**Figure 2.5 B**). The conjugate vaccines induce an antibody response of long duration. A key feature of conjugate vaccines is their ability to induce immunological prime in infants, with robust immune memory responses on subsequent booster vaccination with plain or conjugate polysaccharide.

In 1931, Avery and Goebel suggested that the polysaccharide immunogenicity can be significantly enhanced by conjugation to an immunogenic carrier protein, and more recently successfully adopted to target some bacterial infections associated with high mortality rates.[137] Glycoconjugate antigens are able to invoke T cell recruitment and immune B cell memory specifically directed toward the carbohydrate (**Figure 2.5B**).[138] Glycoconjugate vaccines exert useful immunological activities, even in persons in high-risk groups, and their generation has been one of the greatest success stories in the biomedical sciences.[139, 140] Several conjugate versions of polysaccharide vaccines are now either commercially available or in development.[141, 142]

The first conjugated polysaccharide vaccine was Hib. A conjugate vaccine for pneumococcal disease was licensed in 2000. A meningococcal conjugate vaccine was licensed in 2005.[141] The development of cost-effective, glycoconjugate vaccines based on fully synthetic saccharide antigens is gaining growing importance, as demonstrated by the outstanding success of the synthetic vaccine QuimiHib® in Cuba in 2004.[116]

2.4 Synthetic carbohydrates for Subunit vaccines

The carbohydrate-based antigens are necessary for inclusion in a vaccine, however, there are several limitations associated with the use of isolated antigens. Naturally isolated carbohydrates are not readily available from natural sources and are available in very limited

quantity. Apart from their scarce availability, the isolation and purification of glycans from the natural sources is very challenging. The isolated glycans are heterogeneous and often contains impurities, which hampers with the vaccine efficiency. In particular, the heterogeneity of naturally occurring glycans, obtained by means of challenging isolation, purification and identification techniques, and bacterial impurities are the main limitations to further expansion in this field.[143] Consequently, the development of future-generation vaccines is largely based on recourse to fully synthetic carbohydrate antigens with defined compositions, affording highly reproducible biological properties. Synthetic carbohydrate vaccines have important advantages over those isolated from natural sources because synthetic glycans can be produced as homogeneous compounds in a controlled manner with little or no batch-to-batch variability. The chain density and variables in chain length could be controlled and the optimized vaccine formulation can be obtained with fully synthetic glycans.[103]

Synthesis, taking advantage of the glycosylation methods developed in the last decades,[144, 145] progress in automated synthesis,[146-148] allows the construction of complex oligosaccharide fragments. The final product can be expected to have reliable structural integrity and appropriate purity for clinical application. Additionally, chemical modifications to the carbohydrate domain can be introduced during the optimization of the vaccine construct based on the requirement, an option often unavailable when the antigen has to be isolated from natural sources. Because of the danger of contaminating immunogens or disease-causing microbes that can occur in vaccines derived from live cultures, fully synthetic antigens also possess better safety profiles.

The synthetic approach which, starting from suitable monosaccharides, can provide pure and well-defined oligosaccharides for coupling to protein carriers, is increasingly attractive.[149] Improved synthetic protocols for oligosaccharide synthesis[150-152], as well as the use of automated carbohydrate assembly, have provided more straightforward access to usable quantities of pure oligo saccharides. Synthetic carbohydrates have allowed the development of chemical approaches to glycomics that provide a molecular picture of biological processes involving carbohydrates. Synthetic sugars are beginning to be used in the development of diagnostic tests, vaccines and carbohydrate therapeutics.

2.5 Conclusion

Over the last 50 years, GBS has remained a prominent concern for mother and neonatal health. Although universal screening and IAP have reduced the incidence of early-onset GBS sepsis, maternal and infant colonization rates remain unchanged, and IAP does not affect LOD and potential GBS-induced preterm birth. Despite the scientific advancements in the field of glycobiology, glycochemistry, and understanding of the immunology of pregnancy and early life, the neonatal mortality remains considerably. Although universal screening and IAP have reduced the incidence of early-onset GBS sepsis, maternal and infant colonization rates remain unchanged, and IAP does not affect LOD and potential GBS-induced preterm birth. Recent scientific discoveries in the molecular and microbial determinants of GBS vaginal colonization and placental disease have provided a better and clear understanding of host-microbe interactions within the female reproductive tract. Furthermore, advances in GBS vaccines and human trials, as well as the emergence of novel targeted strategies to control GBS vaginal colonization, point to a new era beyond broad-spectrum antibiotics, and its detrimental consequences, to prevent neonatal GBS pathogenesis.

Synthetic carbohydrate based conjugate vaccine present one of the most lucrative preventive strategy for neonatal GBS disease. In this regard, synthetic chemistry is a formidable tool to define the role of this crucial parameter, and each structural variable affecting the immunogenicity and efficacy of vaccine candidates. The rational design of carbohydrate antigens and the use of increasingly optimized synthetic strategies has made the synthesis of highly complex glycans possible. Nowadays, it is today possible to synthesize essentially any oligosaccharide or polysaccharide of interest. However, it may be very challenging, labourious and highly time consuming to attain the target molecule.

In this regard this, thesis describes the efficient synthetic strategy for the synthesis of GBS type II oligosaccharide repeating unit. The aim is to develop a synthetic route that is easy to handle and efficient (economically as well as less time consuming with higher yield).

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Chapter 3

Group B Streptococcus (GBS) type II Oligosaccharide: Retrosynthetic analysis

3.1 Introduction

Group B Streptococcus (GBS) is one of the leading causes of bacterial infections among neonates and pregnant women, resulting in many severe diseases such as sepsis, meningitis, abortion.[1,2] Among the ten different serotypes according to their structurally distinctive capsular polysaccharides (CPSs),[3, 4] type II GBS is associated with about 15% of the invasive infections in adults and infants.[5, 6] GBS type II is one of the main GBS serotype responsible for neonatal infection and hence represents an important human pathogen.

Rebecca Lancefield characterized the type-specific antigens of GBS type II in early immunological studies. [7-9] The type-specific antigens were polysaccharides and were originally extracted from whole organisms with hot hydrochloric acid. The extraction produced immunologically incomplete antigens, which form a lower molecular weight core to the complete native antigens.[10-17] In 1966, Lancefield and Friemer found that the extraction of type II organisms using milder acidic conditions (trichloroacetic acid) yielded a more immunologically complete antigen.[18] At that time, it was hypothesized that this was due to the presence of an acid-labile component in the original type II native polysaccharide, which was later identified as terminal sialic acid residue. [16, 18, 19]

Jennings *et al.*[19] characterized the structure of type II GBS CPS in 1983. The native polysaccharide antigen isolated from type II group B Streptococcus contains D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucosamine, D-sialic acid in the molar ratio of 3:2:1:1 (**Figure 3.1**). The type II GBS polysaccharide is also structurally unique among other native GBS serotypes due to an additional terminal D-galactopyranosyl unit beside a terminal sialic acid residue.

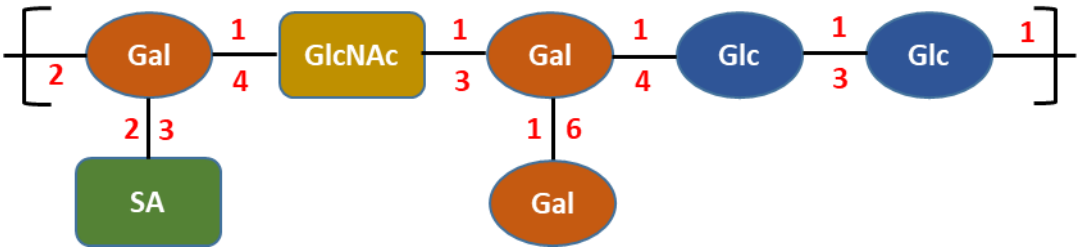


Figure 3.1: Structure of the heptasaccharide repeating unit of type II GBS capsular polysaccharide.

Although the antigenically critical terminal sialic acid residue unit present in all GBS serotypes (**Figure 2.2**), the terminal sialic acid residues of the native type II GBS antigen are also unique because they are not neuraminidase-sensitive and are connected directly to the backbone of the type II GBS antigen. While the terminal sialic acid residues of types Ia,[12] Ib,[14] and III[15] native GBS polysaccharides can be easily removed by treatment with bacterial neuraminidases, type II native GBS antigen are resistant to both viral and bacterial neuraminidases.[19] The resistance to neuraminidases associated with the type II native antigen could be attributed to steric bulk as the sialic acid residues is attached directly to its backbone and interferes the approach of the enzyme. Another factor that probably affects the resistance is that the sialic acid is attached to O-3 position of a backbone β-D-galactopyranosyl unit, which also has a vicinal β-D-glucopyranosyl substituent at O-2 (**Figure 2.2**). Consequently, the vicinal linkages of these two sugar units to the same β-D-galactopyranosyl residue put them in very close proximity to each other.

In this chapter, we discuss the structural features of GBS type II followed by the retrosynthetic analysis of GBS type II repeating unit to identify the suitable fragments used in our synthetic endeavour.

3.2 Retrosynthesis of GBS II

As characterized by Jennings *et. al.,*[19] the structure of type II GBS CPS consists of a repeating heptasaccharide unit of GBS type II is composed of α-Neu5Ac (2-3)-ß-D-Gal-(1-4)- ß-D-GlcNAc-(1-3)-[-ß-D-Gal-(1-6)]-ß-D-Gal-(1-4)-ß-D-Gal-(1-3)-ß-D-Glc. The chemical structure of GBS type II oligosaccharide repeating unit is shown in the **Figure 3.2** as the corresponding 3-aminopropyl glycoside. The glucose derived monosaccharide unit (Glc I and Glc II) has been presented in navy blue color; galactose based residues (Gal I, Gal II, and Gal III) in dark orange; N-acetylglucosamine unit (GlcNAc IV) in golden yellow; and terminal sialic acid unit (NeuNAc) in green in **figure 3.2**. It consists of a heptasaccharide repeating unit having pentasaccharide backbone ß-D-Gal-(1-4)-ß-D-GlcNAc-(1-3)-ß-D-Gal-(1-4)-ß-D-Gal-(1-3)-ß-D-Glc, and two decorating monosaccharide branches, an α-NeuNAc linked to the 3-O position of GalV and a ß-D-Gal linked to the 6-OH position of Gal III (**Figure 3.2**).

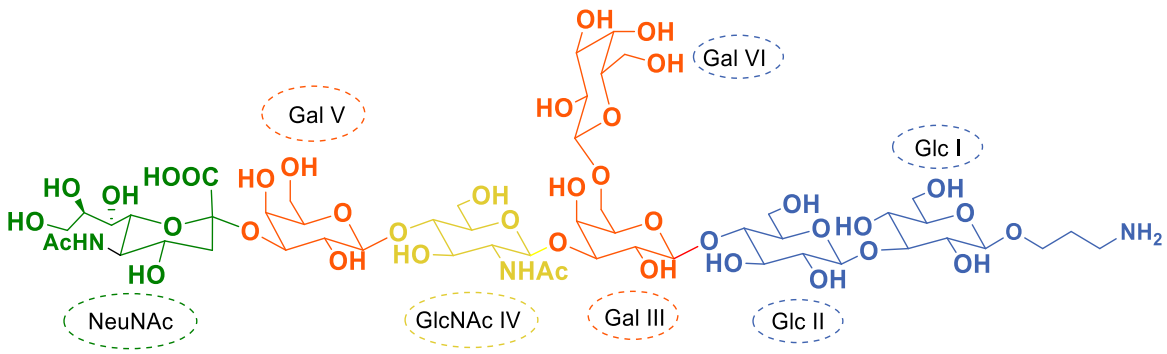
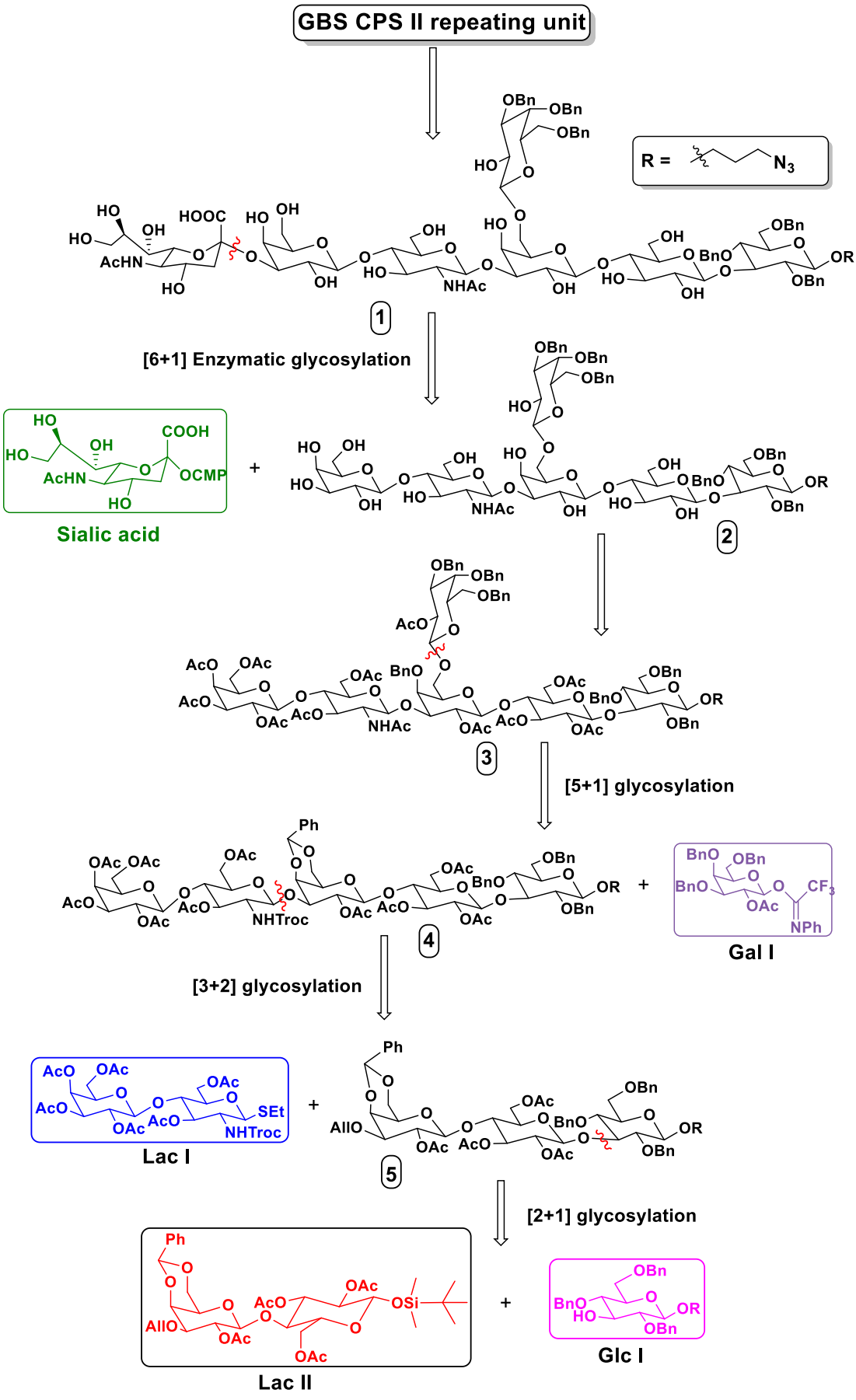


Figure 3.2: Chemical structure of the repeating unit of GBS type II capsular polysaccharide.

Although the structure of GBS was resolved in 1983, the first chemical synthesis of GBS type II repeating unit was not reported until recently in 2018.[20] The only reported synthesis of GBS type II has been achieved by following a conventional synthetic strategy where all the building blocks has been derived from a respective monosaccharide.

The first step to develop an efficient synthetic strategy to achieve the GBS type II heptasaccharide repeating unit relies on a proper disconnection schemes. To transform the target molecule GBS II CPS repeating unit into simpler precursor molecules, a retrosynthetic analysis of the GBS II CPS repeating unit was done based on an intensive literature survey.

The schematic representation of the retrosynthetic analysis of GBS type II is depicted **Scheme 3.1**- suitable and appropriate protecting groups have been selected to obtain molecule **1**, the partial protected form of GBS II CPS repeating unit.



Scheme 3.1: Retrosynthetic analysis of GBS type II repeating unit.

Partially protected heptasaccharide **1** could be achieved by enzymatic introduction of sialic acid residue to branched hexasaccharide **2**. The branched hexasaccharide **2,** obtained from fully protected branched hexasaccharide **3**, could be obtained from glycosylation of linear chain pentasaccharide **4** and Gal I unit. This could be achieved by first selectively opening the benzylidene ring in the N-acetyl protected derivative of **4**, followed by insertion of Gal I unit. The linear chain pentasaccharide **4** could be disconnected to yield protected lactosamine (Lac I) and trisaccharide **5**. This could be performed by first deprotections of allyl group in trisaccharide **5** followed by glycosylation with protected lactosamine (Lac I). The trisaccharide **5** could be obtained from the glycosylation of lactose based fragment (Lac II) and glucose based fragment (Glc I) based on the disconnection shown in **Scheme 3.1**. The retrosynthetic analysis of GBS II CPS repeating unit resulted in five building blocks (Lac I, Lac II, Glc I, Gal I, and Sialic acid) highlighted in the **Scheme 3.1**.

The presented synthetic strategy is based on the five subcomponents derived from the retro synthetic analysis. Suitably protected lactosamine (Lac I) and lactose derivative (Lac II) are pivotal building blocks in our synthesis.

3.3 State-of-the-art

Although, the structure elucidation of GBS II CPS was done in 1983,[19] the synthetic method to achieve the repeating unit of GBS II CPS was reported recently in April 2018 by Z. Guo and J. Gao *et. al*.[20] However, the reported synthesis of GBS type II has been achieved by following a conventional synthetic strategy where all the building blocks derived from a respective monosaccharide units and the assembly of the heptasaccharide requires a high number of steps (each monosaccharide building blocks requiring 5-8 synthetic steps from the respective sugars) and relatively more number of glycosylation reactions (for combining all the fragments and to attach the linkers), which make it an overall challenging process. On the contrary, we have designed a different approach to achieve the chemical synthesis of GBS CPS II. In our synthetic endeavour, we have used five subcomponents based on our retrosynthetic analysis. Suitably protected lactosamine and lactose derivatives are pivotal building blocks in our synthesis and both disaccharide fragments has been obtained from the

cheap and readily available lactose, produced in huge amounts as a side product in dairy industry.

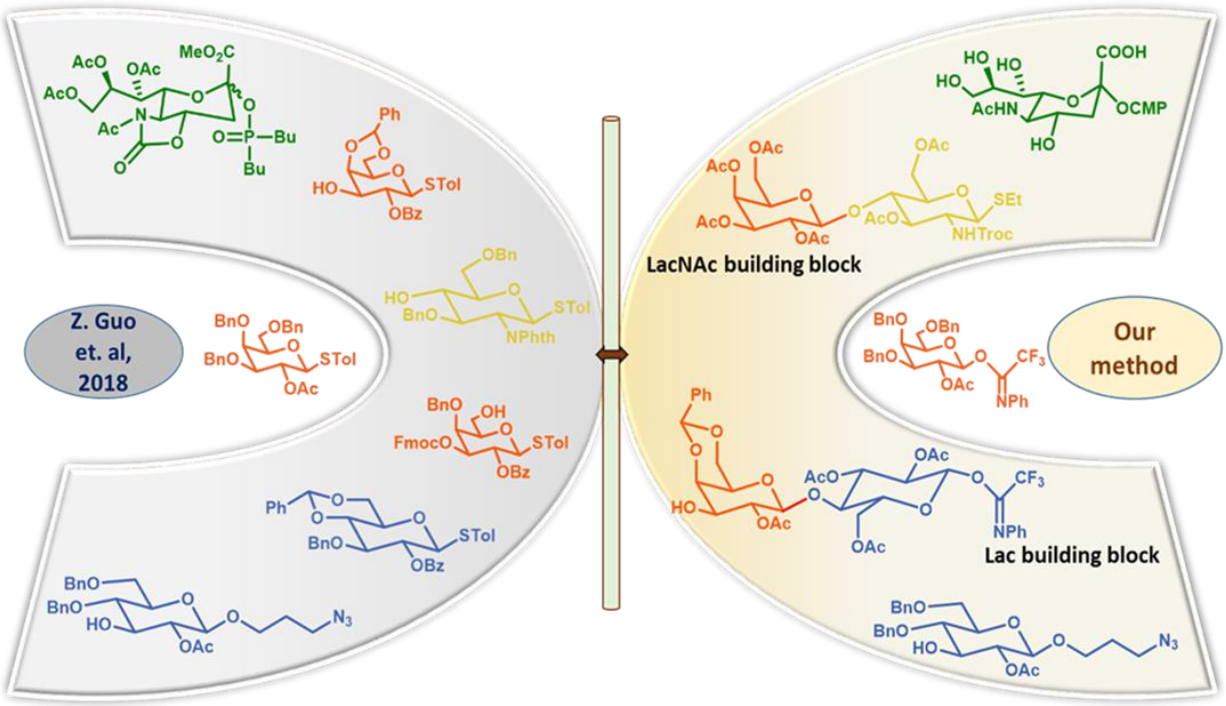


Figure 3.3: The building blocks for the synthesis of the repeating unit of GBS type II capsular polysaccharide reported by Z. Guo *et. al.* (to the left) and our synthetic approach (to the right).

The building blocks in the reported synthetic strategy and in our synthetic method have been presented in **Figure 3.3**. The key advantages of the presented synthetic strategy in the thesis are summarized here:

- **Number of synthetic steps:**

Since we are proposing the total synthesis of GBS II CPS repeating unit from five building blocks, comprising two disaccharide-derived building blocks, the total number of steps in the synthesis of proposed building blocks in our synthetic strategy are less than that in the strategy with all monosaccharide sub components. This will make the synthesis less time consuming.

- **Number of glycosylation reactions:**

The chemical glycosylation is a challenging process due to the necessity to control the desired stereoselectivity. Therefore, an inferior number of fragments (two disaccharide fragments) reduces the number of glycosylation reaction in the synthetic strategy and make the overall approach relatively less complex.

- **Enzymatic introduction of Sialic acid residue:**

The introduction of sialic acid in our approach is planned to be performed enzymatically, using specific sialyltransferase enzymes, to the partially protected branched hexasaccharide at University of Manchester with Glycovax partner Professor Sabine Flitsch. The use of sialyltransferase enzymes in our synthesis will provide advantage in selectivity and yield compared to the chemical glycosylation.

- **Economically efficient**

Two of our backbone building blocks are derived from readily available and very cheap starting material lactose.

To conclude, the presented strategy provides and efficient, easy-handling, economically efficient synthetic route towards GBS II CPS repeating unit.

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Chapter 4

Synthesis of GBS CPS II

This chapter consists of the synthetic strategy to achieve all the fragments derived from the retrosynthetic disconnections of GBS II CPS repeating unit, described in Chapter 3. Synthetic strategy to obtain the proposed fragments was optimized to produce the desired molecules in an efficient manner with the aim to reduce the complexity of the method as well as to get the sub-components in a decent yield. The strategy starts with the most challenging fragment so that the least difficult steps are carried out on more complex substrates at a later stage. Henceforth, the proposed fragment will be denoted as compounds **46, 24, 31, 38, and 45** as shown in **Figure 4.1** in different colours and separate boxes.

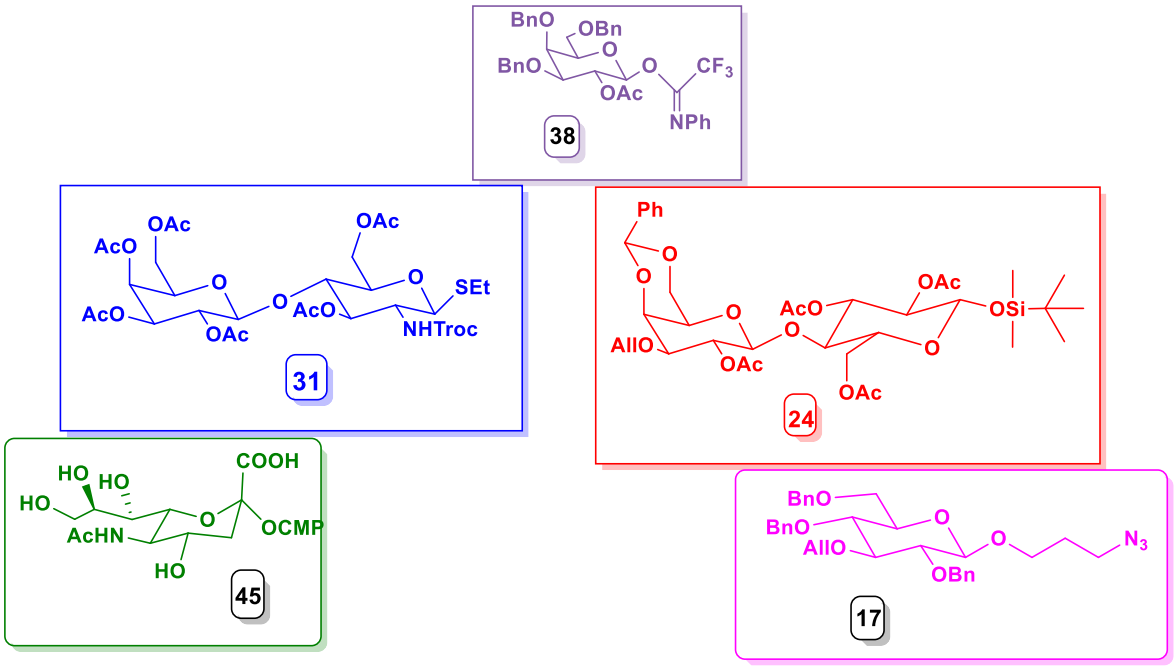


Figure 4.1: Building blocks for the synthesis of GBS II CPS oligosaccharide repeating unit.

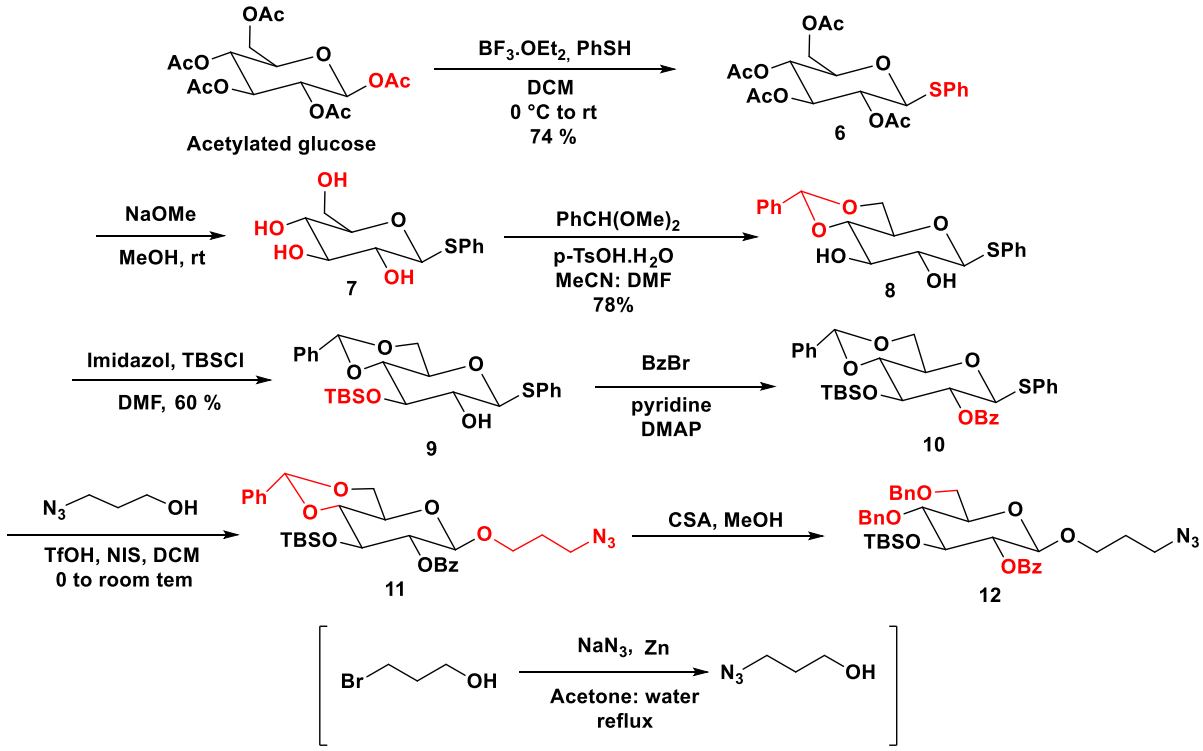
This chapter describes the synthetic strategy to achieve the building blocks **17, 24, 31, and 38** in good yields under optimized conditions.

4.1 Synthesis of Building block 17

Building block **17** is the glucose-derived fragment with the azide ending linker as shown in **Figure 4.1** in pink color. Compound **17** contains benzyl-protecting group at 2, 4, and 6-O position and orthogonal allyl protection at 3-OH. Two synthetic methods were explored to obtain compound **46**.

4.1.1 Synthesis of Building block 17: method I

Synthesis of compound **17** was first approached by following the method described in **Scheme 4.1**.[1, 2] Peracetylated glucose was used as a starting material.



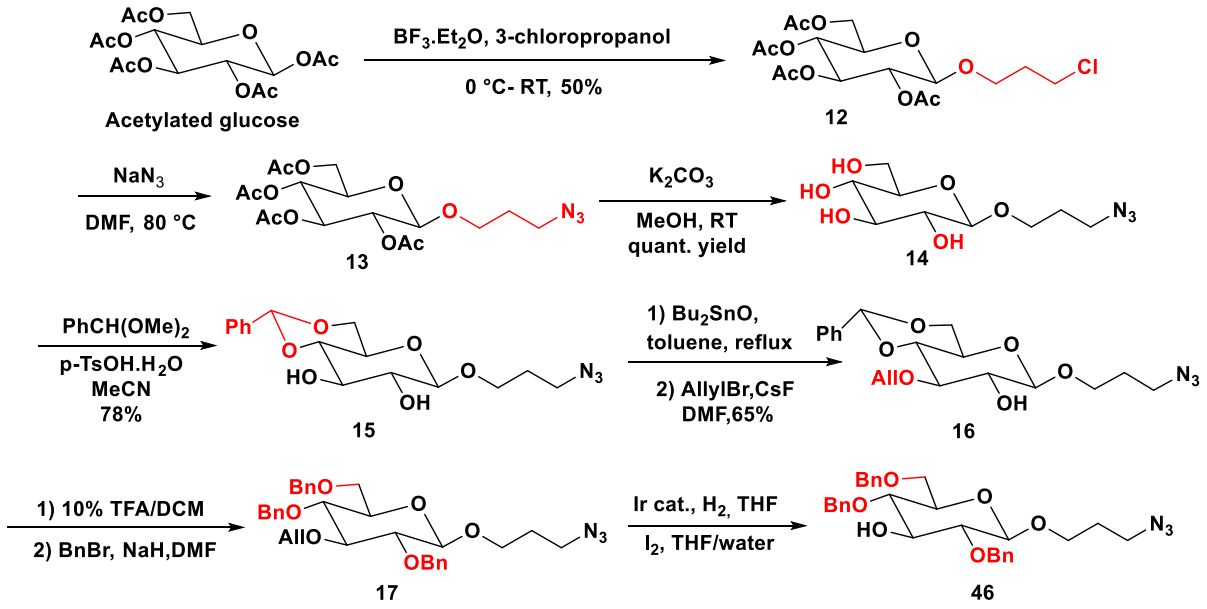
Scheme 4.1: Synthetic strategy to obtain compound **12** as a building block .[1,2]

First, the glycosylation of acetyl protected glucose using thiophenol as the acceptor and BF_3OEt_2 as the activator followed by deprotections of the remaining acetyl groups led to compound **7**. Then the benzylidene ring formation at 4- and 6-position using benzaldehyde dimethyl acetal and para-toluenesulfonic acid (p-TsOH) to result compound **8**, followed by

the regioselective *tert*-butyldimethylsilyl (TBS) protection at 3-OH with TBSCl in the presence of imidazole to yield molecule **9**. The benzoyl (Bz) protection of the free OH group at position 3- followed by the attachment of azidopropyl linker (prepared by reacting 3-bromopropanol with sodium azide and Zn)[2] using trifluoromethanesulfonic (TfOH) and *N*-iodosuccinimide afforded compound **11**. Up to compound **11**, the synthetic strategy worked well but when attempted the hydrolysis of the benzylidene ring with camphorsulfonic acid (CSA), the migration of TBS group was observed and the reaction provided the complex inseparable mixture of products. This complication affected the yield in a negative way and made the approach more challenging due to tough purification. Therefore, an alternative approach to obtain building block **17** was explored to overcome this limitation and obtain the desired molecule in a more efficient manner.

4.1.2 Synthesis of building block 17: method II

In our new approach to molecule **17,** peracetylated glucose was again used as a starting material. Building block **17** was prepared by glycosylation of per acetylated glycose using 3-chloro-1-propanol as the acceptor and BF_3OEt_2 as the activator (**Scheme 4.2**).[3, 4] Then, the alkyl chloride **12** was treated with sodium azide in DMF at 80 °C to introduce the azido group and afford compound **13**.



Scheme 4.2: Synthetic approach to obtain compound **17**. [3, 4]

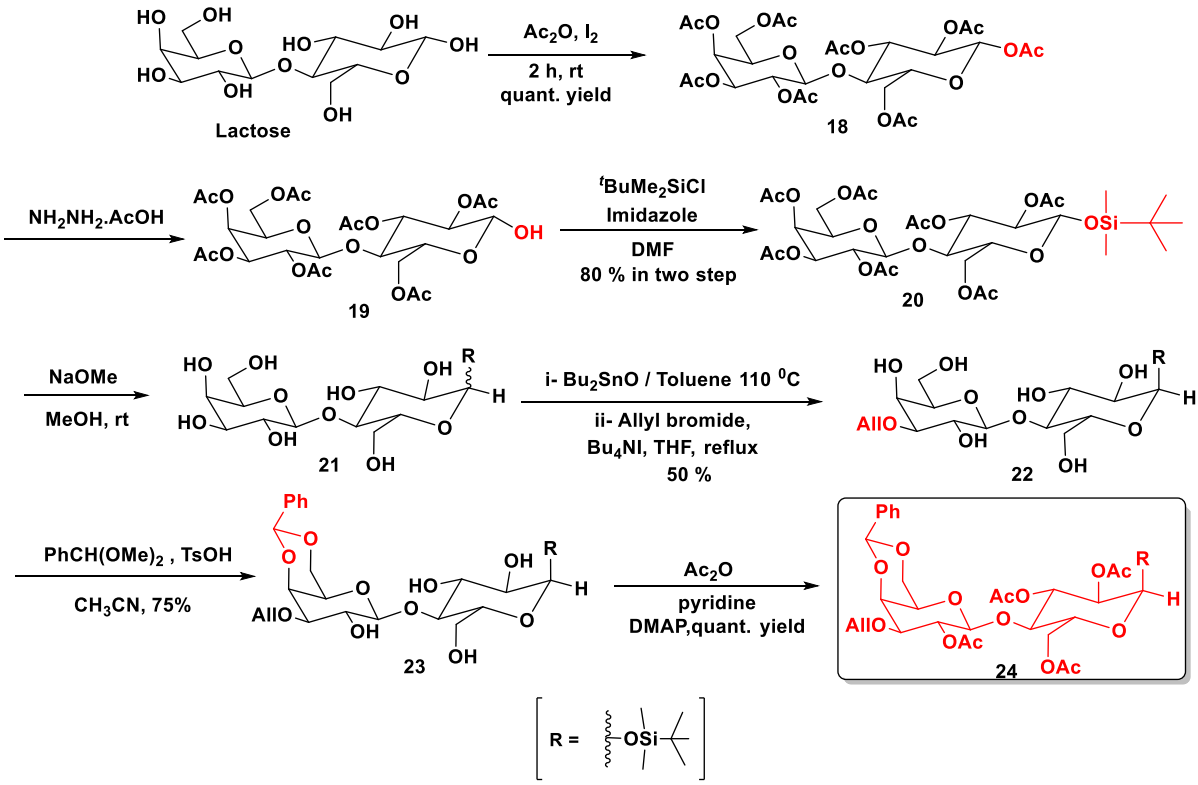
Deacetylation of glycosides **13** under with K_2CO_3 yielded the desired azidoglycoside **14** followed by the benzylidene ring formation on 4- and 6-position using benzaldehyde dimethyl acetal in the presence of para-toluenesulfonic acid (p-TsOH) to give compound **15**. Compound **15** was then treated with dibutyltin oxide (Bu_2SnO), cesium fluoride (CsF) and allyl bromide (AllylBr) to perform the selective allylation on position 3 to get alcohol **16**. Finally, the benzylidene acetal hydrolysis, followed by benzylation of the remaining free OH groups afforded building block **17** and the removal of the allyl ether afforded accepter **46** for glycosylation**.** (**Scheme 4.2**). Altogether, compound **17** was obtained in 35% overall yield over 9 steps.

4.2 Synthesis of Building block 24

Lactose, an abundant natural disaccharide, is the first and only carbohydrate every newborn mammal consumes in significant amounts. Lactose is composed of galactose and glucose monosaccharides linked with a β(-1→4) glycosidic linkage. Lactose comprises about 2-8 % of mammal's milk by weight where it is the principal carbohydrate and energy source.[5] Lactose production in nature is limited to the mammalian breast, which contains the enzyme system (lactose synthase) necessary to create β(-1→4) glycosidic linkage.[6] In addition, lactose is a cheap readily available side product in dairy industry, since several million tons of lactose are produced annually. Lactose, being water soluble, is associated with the *whey* portion of dairy foods.

On that basis lactose was used as a starting material to achieve building block **24** and building block **31** in our synthetic strategy. Synthesis of building block **24** was achieved using the method described in **Scheme 4.3.** [7] The acetyl protection of all free OH groups was carried out by using acetic anhydride in the presence of iodine, followed by the regioselective removal of 1-O-acetyl group with hydrazinium acetate to give compound **19**. Then 1-O-silylation with TBSCl in the presence of imidazole afforded silyl β lactoside followed by removal of all O-acetyl groups under Zemplen condition (sodium methoxide/methanol) to provide compound **21**. However, several complications arose in these steps. First, the 1-O-silylation occurred with poor stereoselectivity and afforded α/β mixture of the silyllactoside.

In addition, partial 1-OH and 2-OH silyl group migration was observed during the deprotection of the acetyl groups sodium methoxide, as depicted in the mechanism shown in **scheme 4.4**.[8]

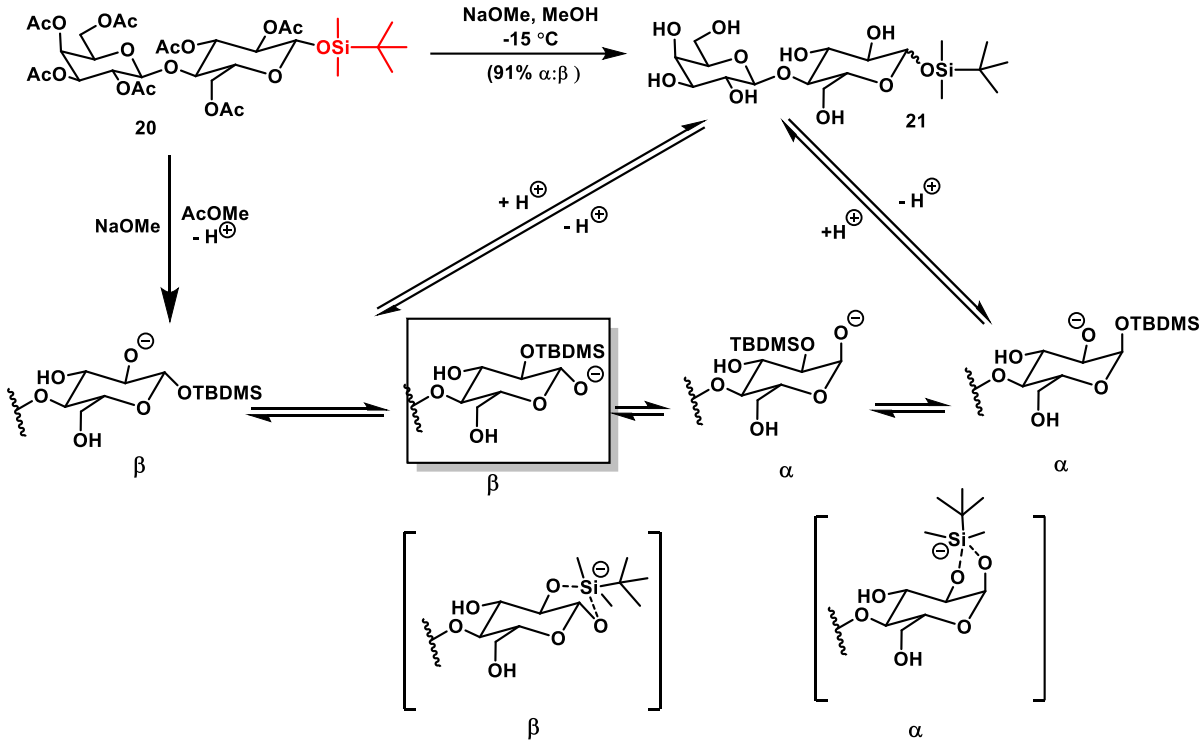


Scheme 4.3: Synthetic scheme to achieve building block **24**.[8]

Both anomerization and silyl migration caused problems when the subsequent steps were performed using the unseparated mixture of compounds. The issue of silyl migration could be addressed by lowering the reaction temperature to -15 °C, enabling to obtain the anomeric mixture of the silyllactoside in 91% yield (**Scheme 4.4**). The pure α-form of compound **21** was achieved by crystallization in ethylacetate/methanol (1:1) to reduce the complications in the next steps

Further, the regioselective allylation of pure α-form of compound **21** could be accomplished with Bu_2SnO/ toluene, and allyl bromide, Bu_4NI in THF to yield intermediate **22**. This compound was protected at C4, C6-OH position with a benzylidene acetal to yield **23**. The acetylation of the remaining hydroxyls of molecule **23** was performed by using anhydrous

pyridine and acetic anhydride with a catalytic amount of DMAP to give compound building block **24**.



Scheme 4.4: Mechanism of action for 1, 2 -O-silyl group migration.[8]

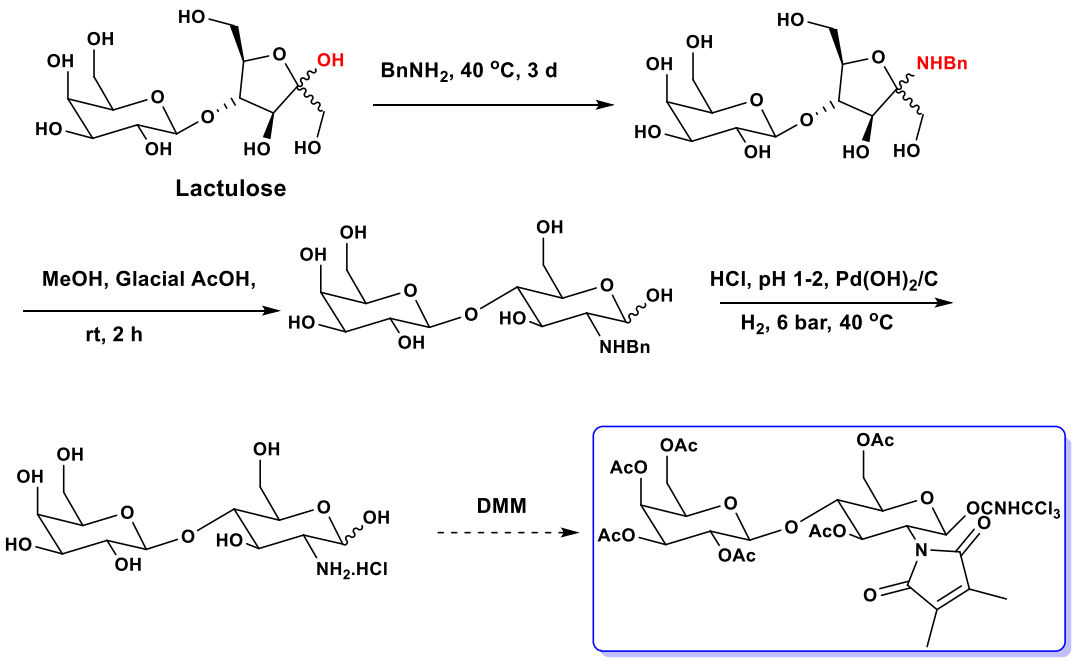
According to **scheme 4.3**, the lactose-based building block **24** was obtained in 33% overall yield over 7 steps.

4.3 Synthesis of Building block 31

Lactosamine is one of the most important building blocks of biologically relevant oligosaccharides and is a basic structural element of Lewis type, α-Gal type, and human milk oligosaccharides.[9] Lactosamine forms the backbone of several cell surface glycans such as sialylated glycans or keratin sulfates.[9] Oligo-*N*-acetyllactosamine and poly-*N*-acetyllactosamine are ubiquitous components of N- and O-glycans decorating the surface of mammalian cells. These structures are natural ligands for proteins of the galectin family with

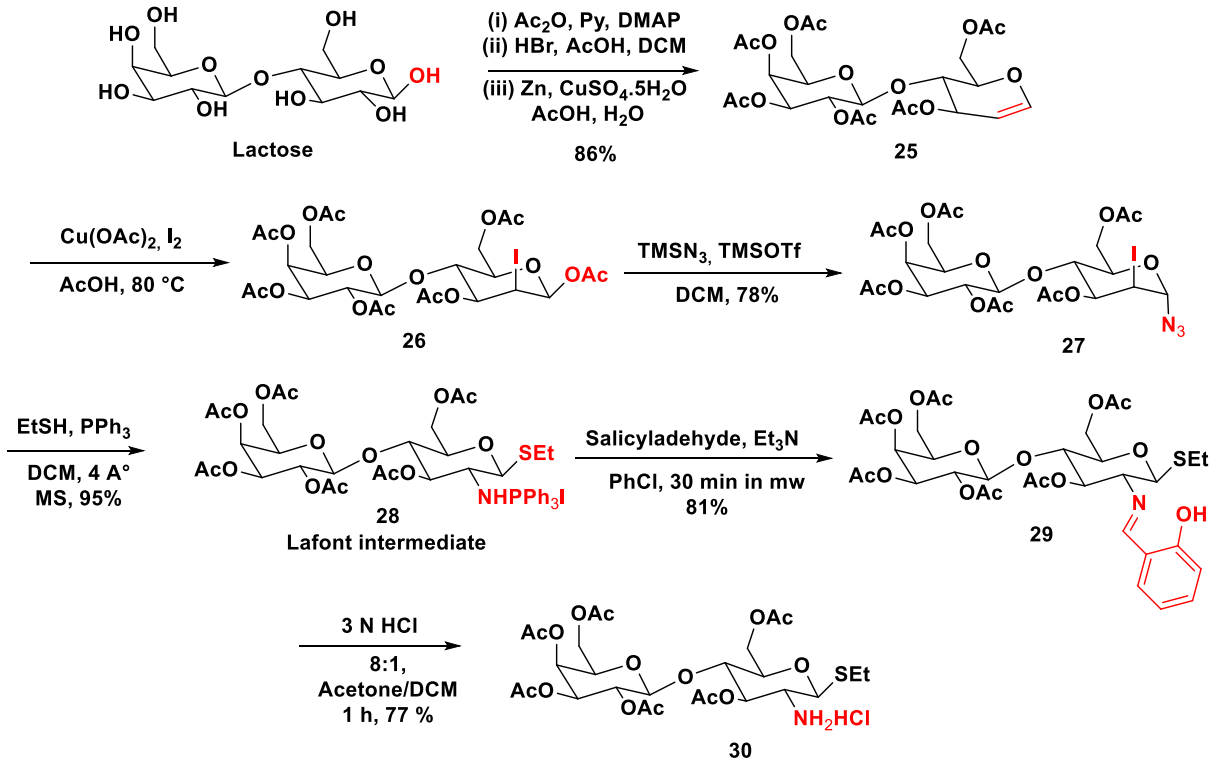
the binding interaction modulating cell-cell adhesion, inflammatory and immune responses, and a variety of disease-associated processes, e.g., tumour development and metastasis.[10] Therefore, a facile synthesis of the lactosamine building block serving as a universal building block would be of great importance in the assembly of the abovementioned glycans.

Due to its structural and biological importance, several enzymatic and chemical syntheses have been developed for the preparation of lactosamine and derivatives. The enzymatic synthesis is usually based on the transfer of a galactose unit onto glucosamine by glycosyl hydrolase or transferase enzyme.[11] The conventional strategy for synthesis of the lactosamine building block involves the glycosylation of a suitably protected glucosamine acceptor with a galactose donor; however, this is very tedious and time consuming.[12] Another strategy is to convert commonly available lactose into lactosamine via functionalizing the lactal via iodosulfonamidation, but this approach is similarly labourious as the previous one.[13] In this regard, a lengthy and labourious synthetic route is necessary to obtain the required specific linkage both in terms of regioselectivity and anomeric stereoselectivity, and the installation of orthogonal protecting groups (e.g., 5-6 steps for the synthesis of each building block). Stutz and co-workers have applied the Heyns rearrangement to convert lactulose into a lactosamine.[14]



Scheme 4.5: Synthetic strategy for building block **31** via Heyns rearrangement.[15]

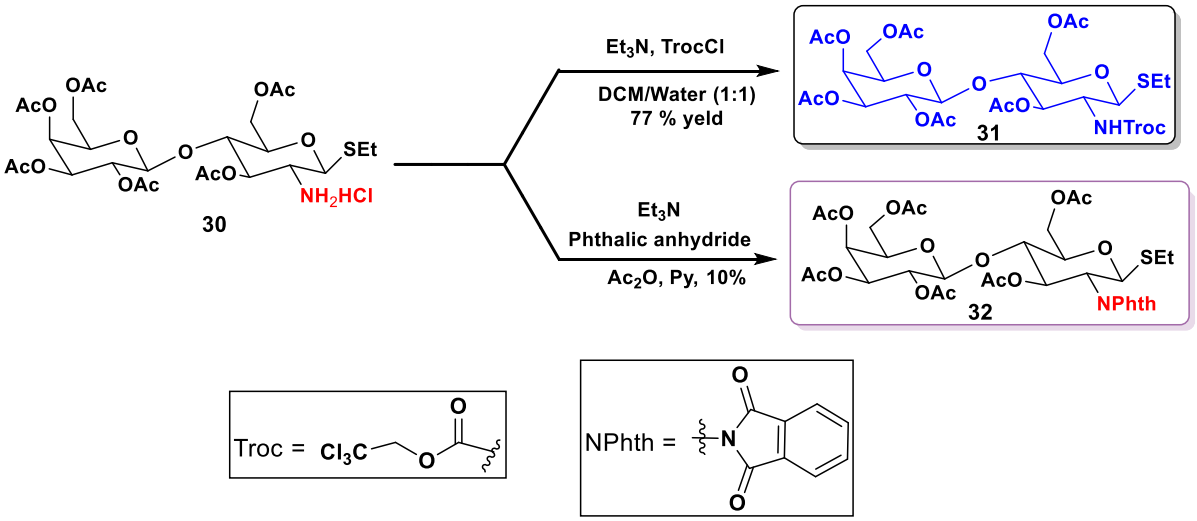
In our approach, we first tried to obtain building block **31** via Heyns rearrangement, but as we were working on a small scale, the one pot synthetic method (**Scheme 4.5**) provided a complex mixture of products that made the purification process very complicated. Therefore, we decided to adopt a different approach to obtain building block **31**.



Scheme 4.6: Synthetic strategy for compound **30** from lactose.[17]

Accordingly, a robust and easy-handling method to achieve suitably protected lactosamine building block from inexpensive lactose was investigated by following the work of Lafont and co-workers to obtain *N*-acetyl lactosamine from lactose.[16] The synthetic scheme is described in **Scheme 4.6**. First the complete acetyl protection of lactose was performed with acetic anhydride to give the octaacetate, which was then treated with HBr (25% in AcOH) to give the anomeric bromide. The reaction was quenched by the addition of sodium acetate, and the mixture was poured into a suspension of zinc (Zn) in AcOH in the presence of CuSO4 to afford the lactal **25** in high overall yield. Next, iodoacetoxylation of hexa-acetylated lactal **25** was achieved by addition of a slight excess of iodine in the presence of cupric acetate monohydrate in acetic acid at 80 °C. The reaction showed high stereoselectivity, affording **26**

in high yield. Then the glycosylation with trimethylsilyl azide afforded compound **27** with excellent stereoselectivity, followed by the Staudinger reaction with triphenylphosphine. The Staudinger reaction led to *situ* formation of iminophosphorane, followed by rearrangement with the elimination of iodine at C-2 position. The resulting aziridine intermediate reacted with ethane thiol and triphenyl phosphine to afford the corresponding 2-aminophosphonium iodide lactosamine ethylthio β-glycoside **28**, the Lafont intermediate. This compound appeared to be very stable and could be isolated and purified by silica gel column chromatography. To convert compound **28** into its free-amino group derivative, the Lafont intermediate was reacted with salicylaldehyde in presence of triethylamine under microwaves to form imine **29**, which could then be easily converted into the free-amino derivative **30** with aq. 3M HCl solution (**Scheme 4.6**) in 76% yield.



Scheme 4.7: Synthetic of lactosamine glycosyl donors **31**, and **32**.

A key point to note is the choice of *N*-protecting group on the glucosamine since it should be capable of providing neighbouring group participation to ensure high β-selectivity in the subsequent glycosylation reaction, and stable under both enzymatic and chemical reaction conditions.[18] A survey of different *N*-protecting groups led us to trichloroethoxycarbonyl (Troc) group. Troc is a suitable protecting group because it can be easily introduced and removed, and is able to promote β-selective glycosilations via anchimeric assistance. Additionally, the reactivity of donors with a C-2 *N*-Troc group is higher compared with the

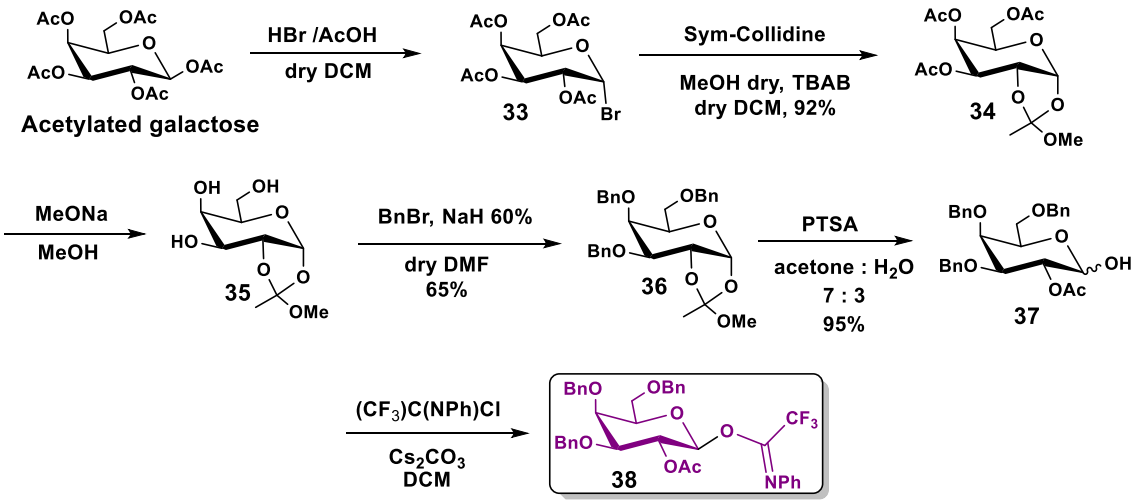
corresponding *N*-phthalimido-protected donors. Furthermore, the *N*-Troc group is stable under various standard conditions used for chemical and enzymatic oligosaccharide synthesis, although it is sensitive to alcoholysis under basic conditions.

To synthesize a suitable lactosamine glycosyl donor from free amino sugar **30**, and to further exemplify the efficiency of the method, compound **30** was protected with the commonly used protecting groups (**Scheme 4.7**); Phth (with Phthalic anhydride), and Troc (with 2,2,2-Trichloroethoxycarbonyl chloride). Troc protected lactosamine donor **31** was obtained in good yield (77%) by reaction with TrocCl in the presence of triethylamine, while the protection with phthalic anhydride provided the thioglycoside **32** in poor yield (10%).

The synthetic method paves the way to a rapid and robust synthesis of a suitably protected lactosamine building block from inexpensive starting material such as lactose via the Lafont intermediate. The stable 2-aminophosphonium iodide lactosamine glycoside has previously been shown not to be amenable to transformation into a useful glycosyl building block. However, as shown by X. Li *et. al.,* it could be converted to imine derivative on reaction with salicylaldehyde under microwave conditions which upon mild acid hydrolysis gave rise to the 2-amino lactosamine.[17] The described approach could also be implemented to convert other types of sugars to form the corresponding 2-aminosaccharides.

4.4 Synthesis of Fragment 38

Fragment **38**, required as a glycosyl donor, was synthesised in good yield from peracetylated galactose via ortho ester **34** in six steps (**Scheme 4.8**) following a commonly used synthetic approach.[19, 20] Acetylated galactose was first treated with HBr solution in acetic acid in dry DCM to provide glycosyl bromide **33**. The ortho ester **34** was prepared by heating **33** in anhydrous DCM in the presence of sym-collidine, anhydrous methanol and tetra-n-butylammonium bromide (TBAB). The acetyl groups were then removed with sodium methoxide and the resulting free hydroxy groups were benzylated to give ortho ester **36** in good yield.



Scheme 4.8: Synthetic approach to achieve building block **38**.

The ortho ester group was partially removed by treatment with p-toluenesulfonic acid (PTSA) in acetone/water mixture, and the resulting 2-O-acetylated compound **37** was then activated using 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride using cesium chloride in DCM to yield building block **38**. The compound **38** was obtained easily in good 30% overall yield in 6 steps.

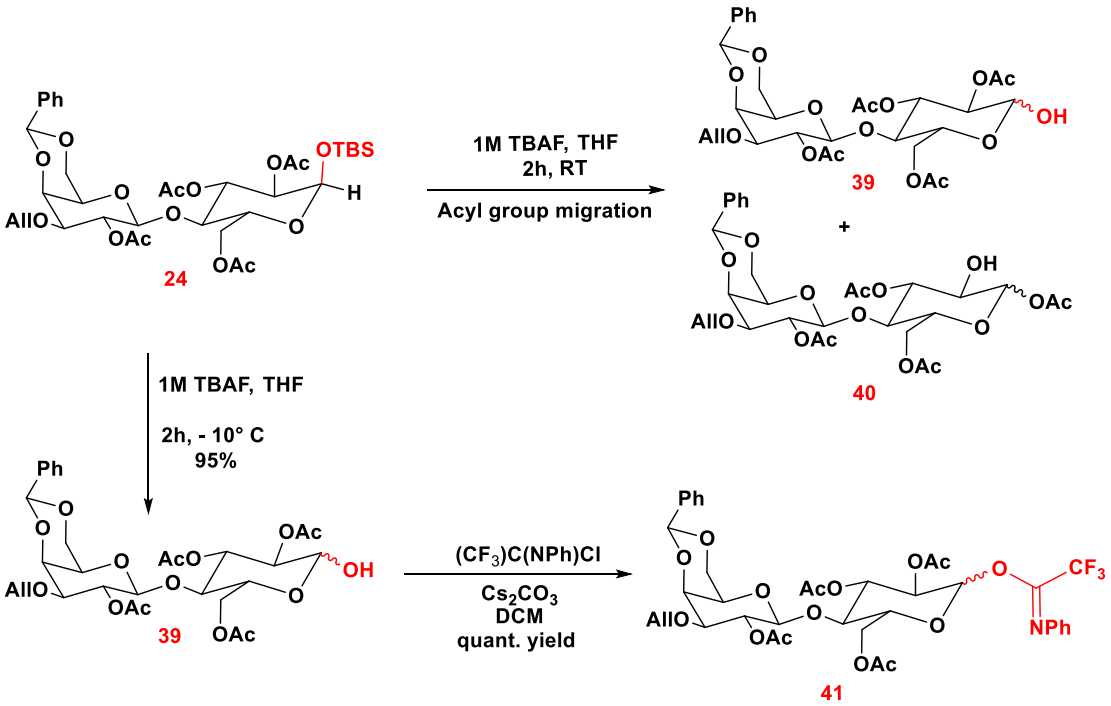
4.5 Combining of building blocks

After achieving the synthesis of core building blocks, the fragments were combined as described in the retrosynthetic analysis to obtain the target compound GBS II CPS.

4.5.1 Synthesis of trisaccharide 5- (2+1 glycosylation)

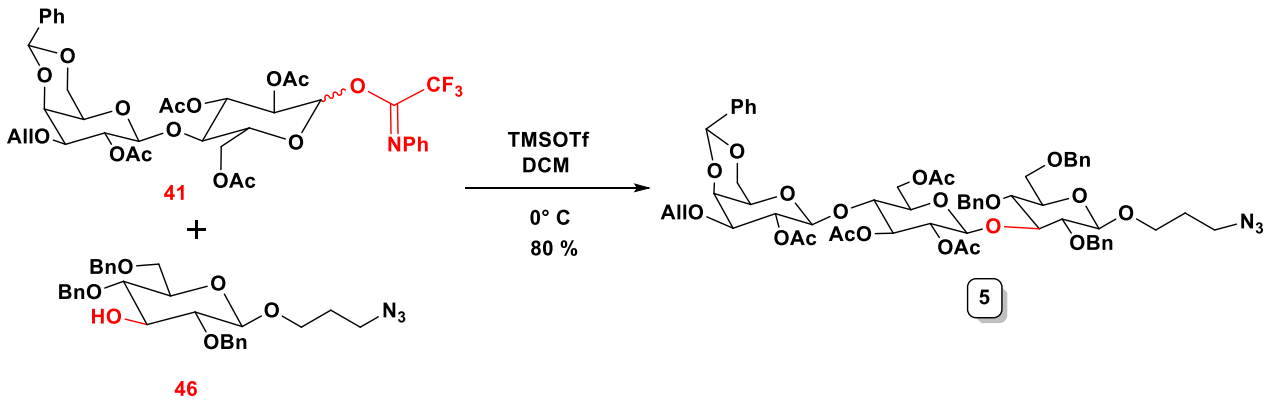
The first assembly step was a 2+1 glycosylation using lactose-based building block **24** as a donor and activated compound **46** as an accepter. First the activation of lactose donor **24** was performed by deprotection of the TBS group followed by activation of the anomeric hydroxyl with 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride and cesium chloride in DCM. Disappointingly, when the silyl deprotection was performed at room temperature using tetra-n-butylammonium fluoride (TBAF), the reaction provided both the TBS deprotected

disaccharide hemiacetal **39** and the acyl migrated sugar **40**. To avoid the acyl migration, the reaction was carried out at -10 °C and the compound **39** was achieved exclusively (**Scheme 4.9**).



Scheme 4.9: Activation of lactose glycosyl donor.

Lactose donor **41** was then reacted with glucose acceptor **46** at 0 °C in DCM and in the presence of TMSOTf as promoter to give trisaccharide **5** in 80% yield (**Scheme 4.10**). The stereochemistry of the new glycosidic bond of trisaccharide **5** was confirmed by ^{1}H NMR spectrometry by proton-proton coupling constant.

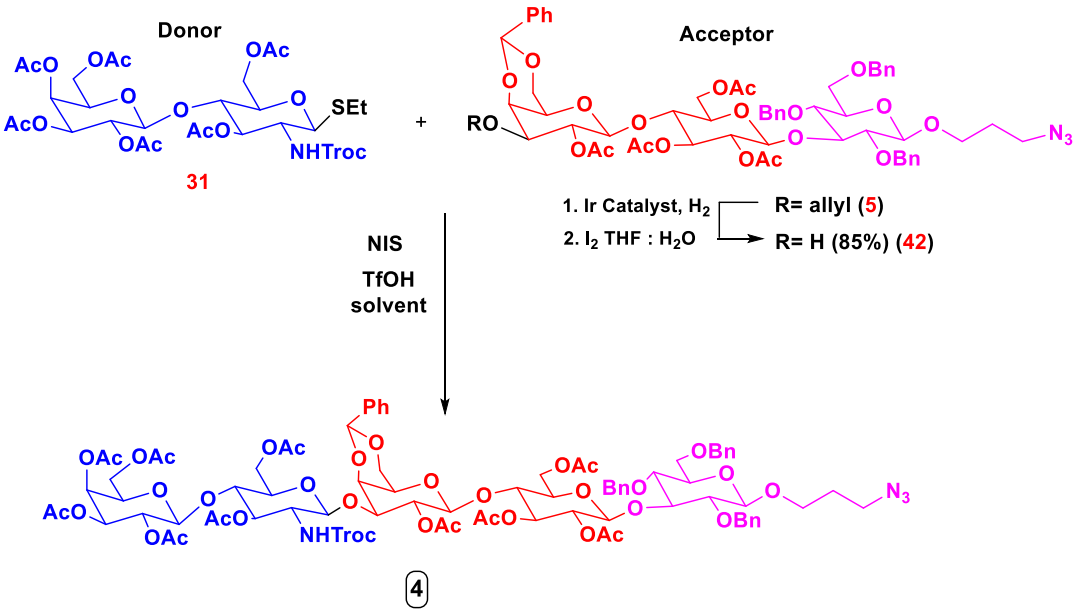


Scheme 4.10: (2+1) glycosylation to provide trisaccharide **5**.

The trisaccharide **5** was fully characterized by nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS). The full characterization was performed using 2-D ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{1}H-^{13}C heteronuclear single-quantum correlation spectroscopy (HSQC).

4.5.2 Synthesis of linear chain pentasaccharide 4 - (3+2 glycosylation)

Next, the synthesis of linear chain pentasaccharide **4** was performed. Troc-protected lactosamine **31** was used as a donor and allyl deprotected trisaccharide **42** as an acceptor with NIS and TfOH as promoters in the glycosylation reaction (**Scheme 4.11**). The allyl deprotection of compound **5** was carried out in two steps. First, allyl to propenyl ether isomerization catalysed by (1,5-Cyclooctadiene)bis(methyldiphenylphosphine)iridium(I) hexafluorophosphate (iridium complex) followed by the vinyl ether hydrolysis using I_2 in a THF/water mixture.[3] The structure of compound **42** was confirmed by 1-D NMR, COSY NMR, HSQC NMR, and mass spectroscopy.



Scheme 4.11: (3+2) Glycosylation to obtain linear chain pentasaccharide **4**.

Various promoters such as TESOTf (trimethylsilyl trifluoromethanesulfonate), TMSOTf (Trimethylsilyl trifluoromethanesulfonate), and TfOH (Triflic acid) were explored to optimize the reaction conditions and to obtain compound **4** in good yield (**Table 4.1**). The use of TfOH

as an acid promoter in DCM at -20 °C provided the linear chain pentasaccharide in high yield (80%).

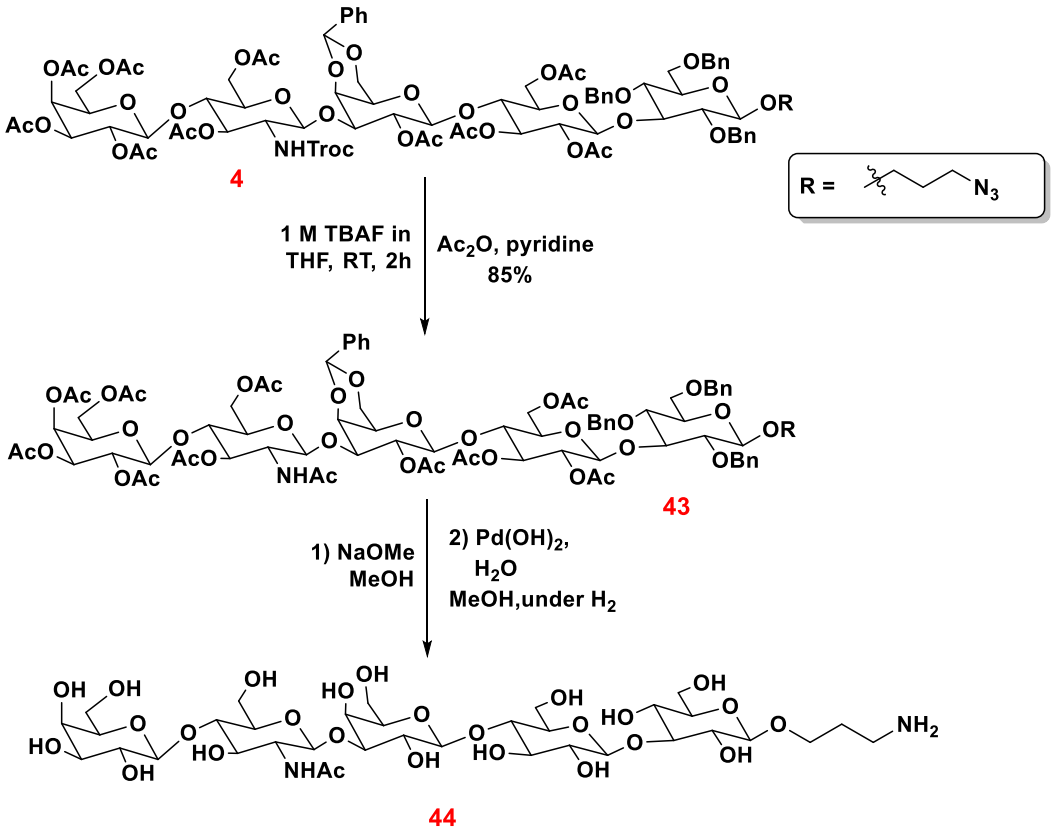
Entry	Donor (equiv)	Acceptor (equiv)	Promoter (equiv.)	NIS (equiv)	Solvent	Temp (^{0}C)	Yield
1	1.05	1.0	TESOTf (0.8)	2.1	Et_2O	0 - rt	20 -30 %
2	1.1	1.0	TMSOTf (0.8)	2.1	DCM	0 - rt	20-30 %
3	1.3	1.0	TfOH (0.7)	1.2	Et_2O	-20 - 0	20-30 %
4	**1.2**	**1.0**	**TfOH (0.4)**	**1.4**	**DCM**	**-20**	**80 %**

Table 4.1: Optimization of (3+2) glycosylation in scheme **4.11**.

The formation of pentasaccharide **4** was confirmed by mass spectroscopy and ^{1}H NMR spectroscopy. The mass spectroscopy and elemental composition report confirms the structure. The characterization was also performed by 2-D total correlation spectroscopy (TOCSY) and ^{1}H-^{13}C HSQC NMR correlation spectroscopy. The presence of five anomeric carbons in ^{13}C NMR spectrum confirms the formation of pentasaccharide **4**. The obtained pentasaccharide **4** was fully deprotected according to **scheme 4.12.** First, the Troc group was removed from the glucosamine unit using 1M TBAF solution in THF, followed by N-acetylation to yield compound **43**.[21] Partially deprotected pentasaccharide **43** was analysed with NMR and mass spectroscopy and is in good agreement with the structure. (See experimental section)

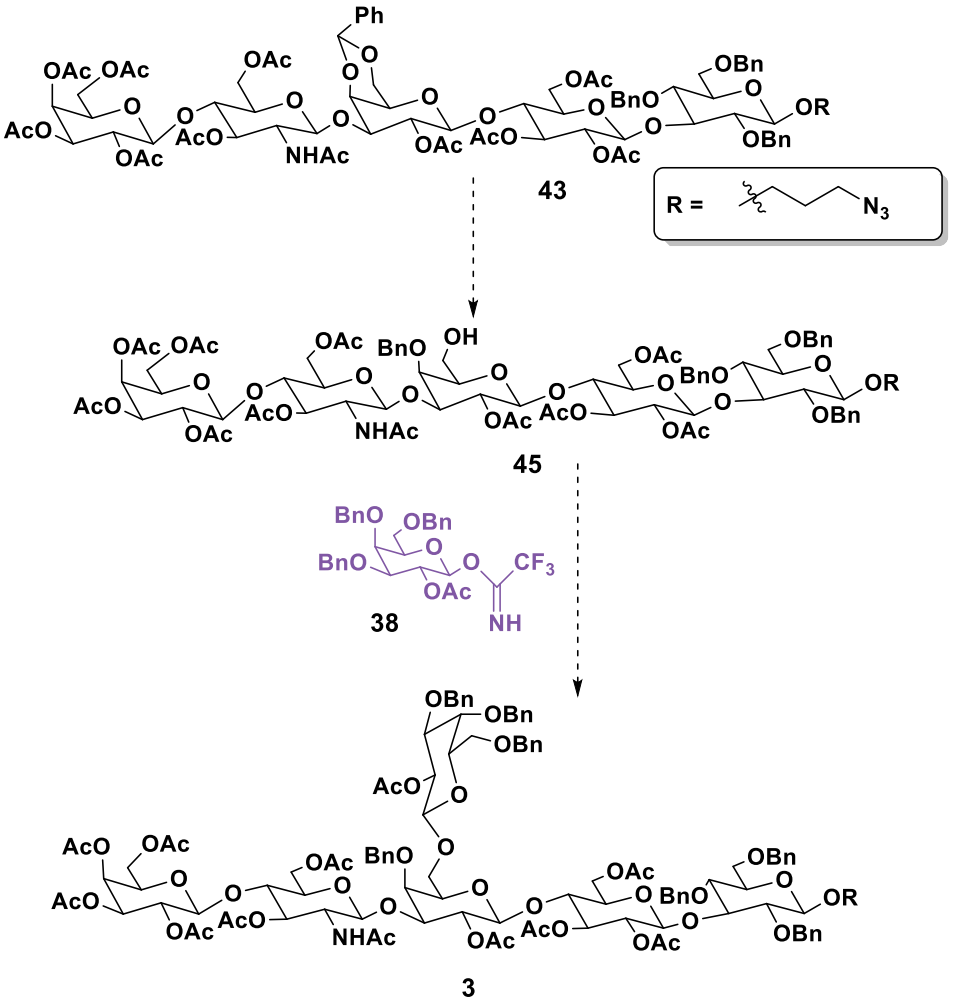
Compound **43** was then fully deprotected over two steps using sodium methoxide (for deacetylation) and final hydrogenation with palladium hydroxide as catalyst to afford pentasaccharide **44**. The fully deprotected pentasaccharide **44** was characterized by NMR spectroscopy. The low-resolution mass analysis confirms the formation of fully deprotected

pentasaccharide however; the full structural characterization including the purity and glycosidic linkages is still ongoing.



Scheme 4.12: Fully deprotected linear chain pentasaccharide **4**.

As far as the synthesis of the hexasaccharide, precursor of the acceptor for enzymatic sialylation, the final steps are currently ongoing. They includes the reductive opening of the benzylidene acetal of pentasaccharide **43** to deliver the 6-OH, followed by the glycosylation with donor **38** (**Scheme 4.13**).



Scheme 4.13: Scheme to obtain hexasaccharide **3**.

4.6 Results Summary

The results obtained so far are summarized in **table 4.2**.

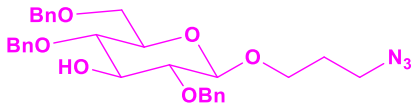
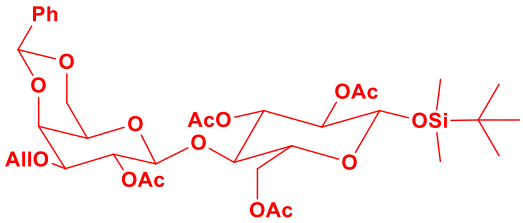
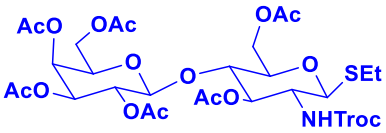
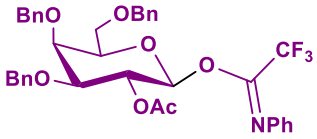
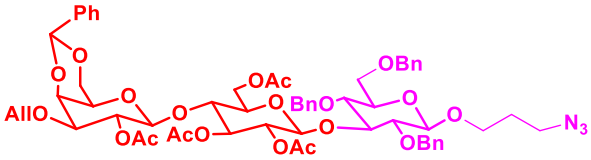
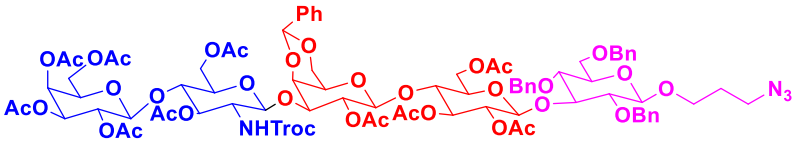
Molecule	Structure	Results
46		Completed in 35% overall yield over 9 steps
24		Completed in 33% overall yield over 7 steps from lactose
31		Completed in 33% overall yield over 7 steps from lactose
38		Obtained in overall 30% yield over 5 steps
5		Obtained in 80 % yield
4		Obtained in 80 % yield

Table 4.2: Summary of the results obtained in the synthesis of GBS type II CPS repeating unit.

4.7 Conclusions and perspectives

To conclude, this thesis work presents an efficient and easy handling synthetic approach to the heptasaccharide repeating unit of GBS type II. The presented strategy has been developed to make the synthetic procedure more expeditious in comparison to the one previously reported. The use of readily available and cheap dairy side-product lactose for the synthesis of two key building blocks **B** and **C** is an added value to our strategy, allowing the synthesis of the pentasaccharide backbone of the target compound. Since we employed lactose as a common precursor of two disaccharide intermediates, our methods allows to reduce the number of challenging glycosylation reactions. The results summarized in **Table 4.2** shows the efficiency of the developed synthetic approach.

The final steps of the synthesis comprise- the incorporation of building block 3**8** into the linear chain pentasaccharide (currently ongoing) followed by the enzymatic introduction of sialic acid. The sialic acid unit (NeuNAc fragment) will be incorporated enzymatically at the University of Manchester in the group of Professor Sabine Flitsch, a partner of the Glycovax network, during my upcoming secondment. Following the enzymatic introduction of the sialic acid unit, the obtained partially protected heptasaccharide will subsequently be fully deprotected to yield the repeating unit of GBS type II capsular polysaccharide.

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Chapter 5

Experimental Section

5.1 General experimental methods

5.1.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC) were performed on Merck precoated 60F254 plates (0.25 mm and 0.2 mm thickness, respectively). Reactions were always monitored by TLC analysis. Compounds were visualized, when appropriate by UV light (254 nm), and always by staining with the solutions given below and detected by heating at 196°C The solution used as stains are:

- molybdic solution (21 g of $(NH_4)_4Mo_4O_{24}$, 1 g of $Ce(SO_4)_2$, 31 mL of H_2SO_4 98%, 970 mL H_2O)
- sulphuric acid (50 mL of H_2SO_4 98%, 450 mL of MeOH, 450 mL H_2O)
- ninhydrin (2.7 g of 2,2-dihydroxyindane-1,3-dione, 27 mL of AcOH, 900 mL of EtOH)

5.1.2 Flash chromatography

According to Still procedure,[1] compounds were purified by flash chromatography, using Silica gel (SiO2, high-purity grade (Merck Grade 9385), pore size 60 Å, 230- 400 mesh particle size) from Sigma-Aldrich. In some cases it was also used a flash purification system, Biotage SP1. Normal- and reverse phase Biotage SNAP cartridges (sizes from 10 g to 340 g, standard 50 µm silica) were used to purify the compounds.

5.1.3 Nuclear Magnetic Resonance (NMR) analysis

NMR spectra were recorded on a Bruker AMX 400 instrument (400 and 100.6 MHz for ^1H and ^{13}C, respectively), and were all run at room temperature (298K), unless otherwise noted. The samples were prepared using deuterated solvents ($CDCl_3$, D_2O and CD_3OD from Sigma-Aldrich). Chemical shifts (δ) are reported in ppm and the coupling constants (*J*) in Hz. Chemical shifts were referenced to the residual proton in the solvent (e.g. the $CHCl_3$, 0.01 % in 99.99 % $CDCl_3$), according to Gottlieb and Nudelman.[2] Multiplicities are abbreviated as: br (broad), s (singlet), d (doublet), t (triplet), hept (heptet), m (multiplet) or combinations thereof. ^1H-NMR spectra were recorded for all the synthesized products. In the case of unknown structure, the

characterization is reported by ^{1}H-NMR and ^{13}C-NMR. 2-dimensional NMR experiments (COSY, TOCSY, HSQC and HMBC) were used to better assign peaks to the structure.

5.1.4 Mass analysis

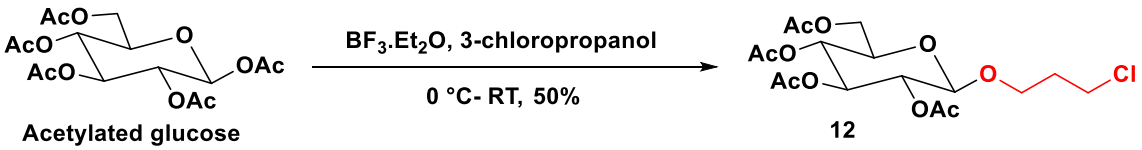
Low resolution mass analyses were recorded in negative or positive mode on a Thermo Finnigan LCQ Advantage equipped with an ESI source. High-resolution mass analyses were recorded on a Waters Micromass Q-Tof micro equipped with a LockSpray ESI source or on a Bruker Daltonics ICR-FTMS APEX II at C.I.G.A, University of Milan.

5.1.5 Anhydrous environment

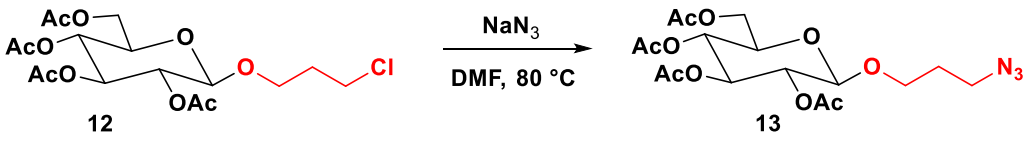
Unless otherwise stated, all the reaction were conducted under nitrogen atmosphere. When stirred overnight, the reaction were put under anhydrous atmosphere by an Argon-balloon. Solvents Unless otherwise stated, all the reactions were performed using dry solvents. DCM, Et_2O, MeOH, pyridine, toluene, DMF, CH_3CN and THF over molecular sieves were purchased from Sigma-Aldrich and used without further purifications.

5.2 Synthetic Procedures

5.2.1 Synthesis of Building block 17

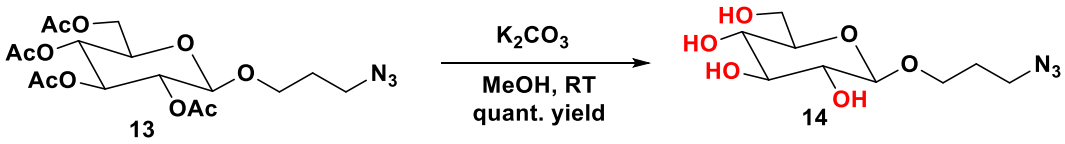


Synthesis of 3-chloropropyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (12)[3]: To a stirred solution of pentaacetylated glucose (8.0 g, 20.5 mmol) and 3-chloro-1- propanol (3.7 mL, 41.0 mmol) in anhydrous DCM (50 mL), $BF_3.Et_2O$ (5 mL, 41.0 mmol) was added dropwise at 0 °C. The reaction mixture was allowed to warm up to room temperature and then stirred for 18 hours. After the completion of the reaction, the reaction mixture was poured into ice water and stirring was continued until the ice had melted. After phase separation, the water layer was extracted with DCM (3x 100 mL) and the combined organic phase were washed with aq $NaHCO_3$ solution (100 mL), and 100mL brine solution. The organic phase was then dried over $MgSO_4$ and the solvent was evaporated in *vacuo* to yield the product as yellow viscous oil in 50 % yield (4.4 g). TLC- 6:4 (hexane:ethyl acetate). The product was used for further reaction without purification. Spectroscopic data were in agreement with those reported in the literature.[3]

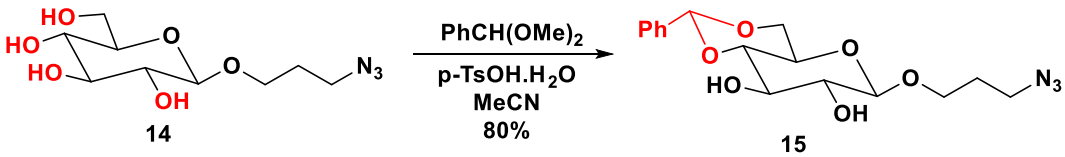


Synthesis of 3-azidopropyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (13)[3]: To the solution of **12** (8.0 g, 17.1 mmol) in dry DMF (50 mL), sodium azide (5.6 g, 85.4 mmol) was added. The resulting solution was stirred at 60 °C overnight and then the DMF was removed under reduced pressure. The reaction mixture was then diluted with DCM (120 mL) and the organic

phase was washed with water (100 mL) and aq. sodium bicarbonate solution. The organic phase was then dried over sodium sulphate and concentrated on rotary evaporator. The crude product was purified using column chromatography with hexane: ethyl acetate (2:1) as eluent and obtained compound **13** in quantitative yield (3.9 g). Spectroscopic data were in agreement with those reported in the literature.[3]

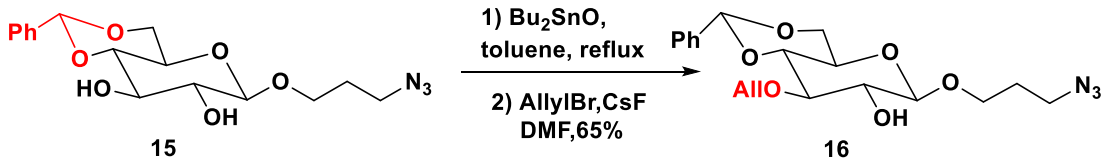


Synthesis of 3-azidopropyl β-D-glucopyranoside (14)[3]: Anhydrous potassium carbonate (0.13 g, 0.9 mmol) was added to solution of compound **13** (3.8 g, 9.0 mmol) in dry methanol (30 mL). The reaction mixture was then stirred overnight at room temperature. After completion of the reaction as indicated by TLC (15% MeOH in $CHCl_3$). The mixture was concentrated and compound was purified by silica gel column chromatography and eluted with 12% MeOH in $CHCl_3$ to give a viscous liquid: yield 95%, 2.2 g. Spectroscopic data were in agreement with those reported in the literature.[3]

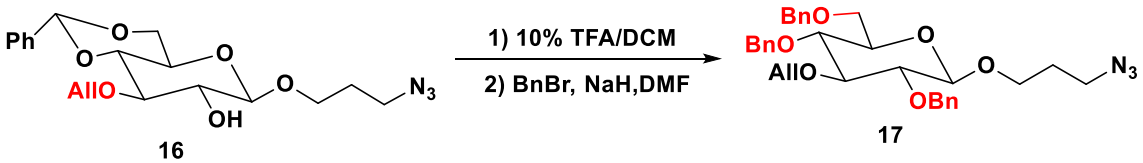


Synthesis of 3-azidopropyl 4,6-O-benzylidene-β-D-glucopyranoside (15)[3]: To a solution of **14** (1.9 g, 7.0 mmol) in MeCN (30 mL) was added benzaldehyde dimethylacetal (1.6 mL, 10.6 mmol) and p-TsOH (0.02 g, 0.1 mmol).The solution was stirred for 1 hour at room temperature and then the reaction was quenched with triethyl amine. The solution was then evaporated to dryness and purified with silica get column chromatography with hexane: ethyl acetate (1:2) and obtained in 80 % (2.0 g) yield as a white solid: Rf = 0.5. **^{1}H NMR (400 MHz, Chloroform-d)** δ 7.41-7.35 (2 H, m), 7.29-7.21 (3 H, m), 5.41 (1 H, s), 5.30 (1 H, s), 4.29 – 4.18 (2 H, m), 3.85 (1 H, dt, J 10.0, 6.1), 3.73 – 3.62 (1 H, m), 3.57 (1 H, dt, J 10.0, 6.1), 3.46 – 3.28

(3 H, m), 3.24 (3 H, s), 3.15 (1 H, s), 1.83 – 1.74 (2 H, m); **^{13}C NMR (101 MHz, Chloroform-d)** δ 137.1, 129.4, 128.5, 128.4, 128.3, 126.8, 126.4, 103.3, 102.0, 80.6, 77.5, 77.2, 76.8, 74.6, 73.3, 68.7, 67.2, 66.5, 52.8, 48.4, 29.2.



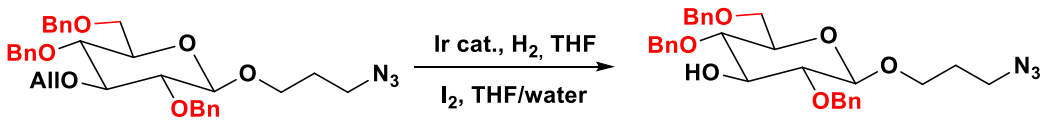
Synthesis of 3-azidopropyl 3-O-allyl-4,6-O-benzylidene-β-D-glucopyranoside (16)[4]: A mixture of compound **15** (2.0 g, 5.9 mmol) and Bu_2SnO (2.9 g, 11.8 mmol) in toluene (70 mL) was refluxed under N_2 overnight. The reaction was then cooled down to room temperature and toluene was removed under reduced pressure. Then the resulting residue was dissolved to dry DMF (30 mL) and mixed with CsF (2.6 g, 17.7 mmol) followed by the addition of allylbromide (765.3 µL, 8.8 mmol). The solution was stirred at room temperature for 24 hours and after completion of the reaction, the solution was diluted by ethylacetate (200 mL) and washed with saturated aq. NaCl solution (100 mL). The organic layer dried over Na_2SO_4 and concentrated under reduced pressure. The purification of the residue by flash column chromatography with ethyl acetate/hexane (1:3) as eluent yielded the desired compound in 52 % yield (1.2 g) as white foam. Spectroscopic data were in agreement with those reported in the literature.[4]



Synthesis of 3-azidopropyl 3-O-allyl-2,4,6-O-benzoyl-β-D-glucopyranoside (17)[4]: The compound **16** (1.0 g, 2.5 mmol) in 10% TFA in CH_2Cl_2 (20 mL) and H_2O (2 mL) was stirred at room temperature for 2 h and progress of the reaction was monitored using TLC. On

completion of the reaction, as indicated by TLC, the mixture was co-evaporated with toluene under vacuum to provide a residue.

To a solution of this obtained residue in 20 mL of anhydrous DMF was added NaH (0.5 g, 13.3 mmol) and BnBr (1.3 mL, 10.6 mmol) at 0 °C under a N_2 atmosphere. After an hour, MeOH was added slowly to quench sodium hydride the reaction followed by the addition of H_2O. The aqueous phase was extracted with EtOAc (3 × 100 mL), and the combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by a flash silica gel column chromatography with EtOAc/ hexanes (1:11) as the eluents to give colourless syrup in 70 % (1.0 g) yield over 2 steps. Spectroscopic data were in agreement with those reported in the literature.[4]

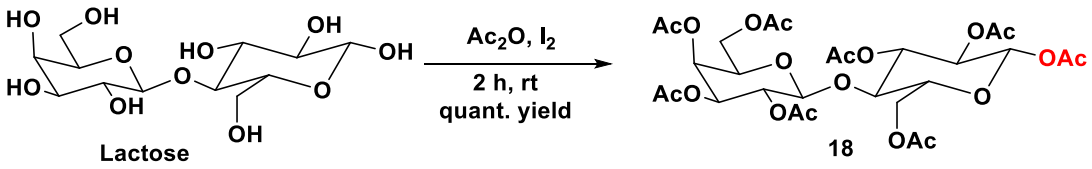


17 46

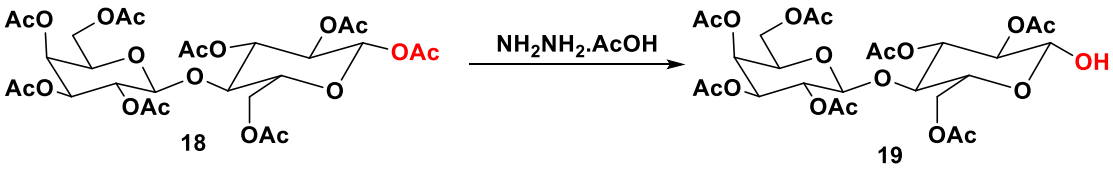
Synthesis of 3-azidopropyl 2,4,6-O-benzoyl-β-D-glucopyranoside (46): Building block **17** (0.60 g, 1.1 mmol) was dried and reaction flask was evacuated. Iridium catalyst was added to the flask following the evacuation of the flask. THF was then added to the flask and the reaction mixture was stirred at room temperature. A balloon of H2 gas was attached and this reaction was monitored until the red color of the solution turned to pale yellow. H2 balloon was then removed and the flask was flushed with Argon gas and stirred for another 6 hours. The resulting solution was concentrated and dissolved in 80% THF in water (5 mL) followed by the addition of I_2. The resulting solution was stirred at room temperature for an hour, quenched by addition of saturated sodium thiosulfate solution, and washed with sodium bicarbonate, brine and water. Concentrated and evaporated then separated by Flash Chromatography to afford **46** in 70% yield over 2 steps. **^{1}H NMR (400 MHz, Chloroform-d)** δ 7.39 – 7.23 (13 H, m), 4.93 (1 H, d, J 11.5), 4.84 (1 H, d, J 11.2), 4.68 (1 H, d, J 11.5), 4.63 (1 H, d, J 12.2), 4.60 – 4.53 (2 H, m), 4.38 (1 H, d, J 7.8), 4.04 – 3.97 (1 H, m), 3.78 – 3.69 (3 H, m), 3.69 – 3.61 (1 H, m), 3.56 – 3.49 (1 H, m), 3.47 (1 H, d, J 6.2), 3.42 (2 H, t, J 6.8), 3.28 (1 H, dd, J 9.1, 7.8), 2.44 (1 H, s), 2.01 – 1.82 (2 H, m), 1.59 (1 H, s); **^{13}C NMR (101 MHz, Chloroform-d)**

δ 138.3, 138.2, 128.6, 128.4, 128.4, 128.0, 127.8, 127.7, 103.2, 81.4, 77.4, 77.4, 77.0, 76.7, 74.8, 74.5, 73.5, 68.9, 66.5, 48.4, 29.3.

5.2.2 Synthesis of Building block 24



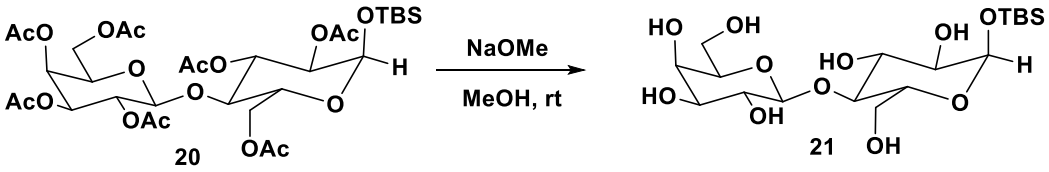
Synthesis of β-Lactose octaacetate (18)[5]**:** Solid I_2 (1.5 g, 5.8 mmol) was dissolved in 150 mL of Ac_2O. After cooling the solution using cold water bath, D lactose monohydrate (20.0 g, 58.4 mmol) was slowly added and to it. The reaction mixture was allowed to stir at room temperature for 1-2 hours and the reaction progress was monitored by TLC. After the completion of the reaction, reaction mixture was poured into a cold 5% sodium thiosulfate solution (300 mL) the mixture is allowed to warm up to room temperature for 20 min. The crude is diluted with DCM (500 mL) and the organic layer was washed with 5% $NaHCO_3$ solution. The organic layer was dried on *vacuo*. The product **18** was obtained in quantitative yield (39.0 g) and used for next step without purification. Spectroscopic data were in agreement with those reported in the literature.[5]



Synthesis of β-D-Lactose heptaacetate (19)[6]**:** Compound **18** (30.0 g, 44.2 mmol) was dissolved in dry DMF (150 mL) and hydrazinium acetate (4.5 g, 48.6 mmol) was added to the reaction flask under nitrogen atmosphere. The reaction mixture was heated at 40 °C for 1 to 2 hours and progress of the reaction was monitored by thin layer chromatography (TLC) using ethyl acetate: hexane (7:3) as solvent system. After completion of the reaction, as indicated by TLC, DMF was removed in *vacuo* and saturated NaCl solution was added to the flask and extracted

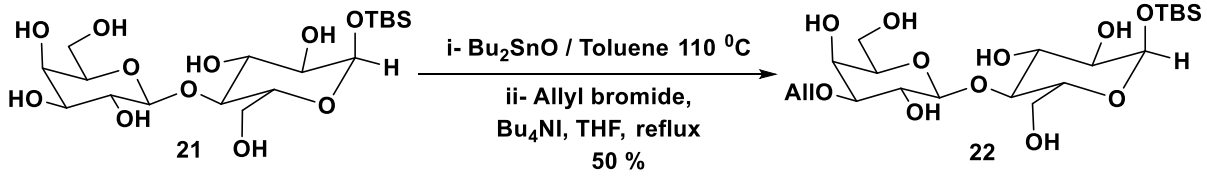
using ethyl acetate. The combined organic layer was dried over Na_2SO_4 and concentrated on rotary evaporator. Crude mixture was purified by column chromatography using ethyl acetate: hexane (7:3) as eluent. (Rf = 0.3). Compound **19** was obtained in quantitative yield. Spectroscopic data were in agreement with those reported in the literature.[6]

Synthesis of tertbutyl dimethylsilyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1-4)-2,3,6-tri-O-acetyl-β–D-glucopyranoside (20)[6]: To the stirred solution of crude compound **19** (30.0 g, 47.1 mmol) and imidazole (16.0 g, 235.6 mmol) in dry DMF (150 mL) was added the tert-butyl dimethylsilyl chloride (8.5 g, 56.6 mmol). Reaction mixture was stirred at room temperature for 24 hours and then the volume of the reaction flask was reduced to half. The remaining reaction mixture was dissolved in 1.5 L of Et_20 and washed with a saturated aq NH_4Cl (3 × 200 mL) solution. The organic layer was dried over Na_2SO_4 and concentrated in *vacuo*. The crude product (28.0 g, 80% yield) was used for further steps without purification. TLC - 1:1 EtOAc-hexane; Rf 0.62. Spectroscopic data were in agreement with those reported in the literature.[6]

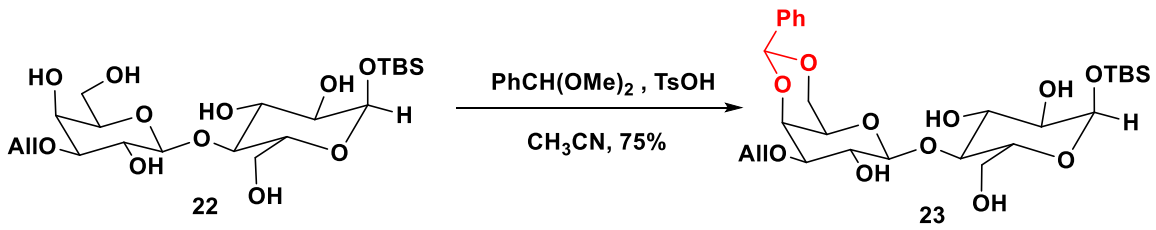


Synthesis of tert-butyldimethylsilyl β-D-galactopyranosyl-(1-4)-α/β-D-glucopyranoside (21): Compound **20** (20.0 g, 26.6 mmol) was dissolved in dry MeOH (200 mL) and the reaction flask was cooled to -10 °C. Then 1 M NaOMe solution in methanol was added slowly to the reaction at -10 °C until the pH of the solution reached ~9 and then the reaction mixture was stirred at -10 °C overnight. Then the reaction mixture was neutralized using Amberlite IR –

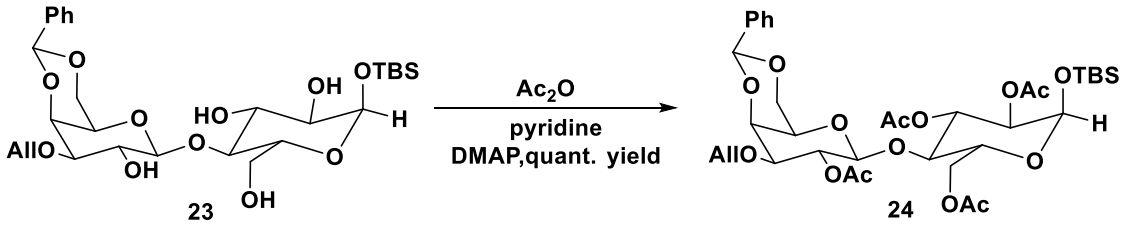
120 acidic resins, filtered and concentrated under reduced pressure gave 12.0 g of compound **21** which on crystallization in methanol/ethyl acetate (2:1) afforded pure α-form of compound **21.** TLC (65:30:5 $CHCl_3$: MeOH: H_2O); Rf 0.42. **^{1}H NMR (400 MHz, Deuterium Oxide)** δ 5.24 (1 H, d, J 3.7), 4.70 (1 H, dd, J 11.2, 7.7), 4.48 (2 H, dd, J 8.0, 4.1), 4.04 – 3.51 (23 H, m), 3.40 – 3.22 (1 H, m), 0.95-0.91 (5 H, s), 0.28 – 0.06 (3 H, m).**^{13}C NMR (101 MHz, Deuterium Oxide)** δ 102.9, 97.1, 95.8, 92.9, 91.8, 78.8, 78.5, 78.4, 75.4, 74.8, 74.6, 74.4, 74.2, 73.8, 72.6, 72.0, 71.4, 71.3, 71.2, 71.0, 70.1, 68.6, 61.0, 60.1, 60.0, 25.1.



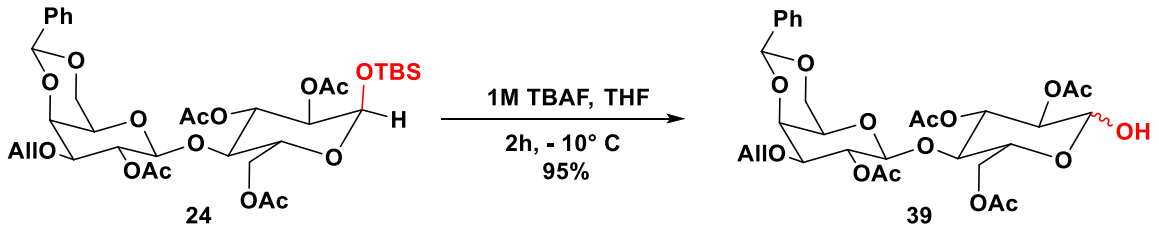
Synthesis of tert-butyldimethylsilyl 3-O-allyl-β-D-galactopyranosyl-(1-4)-α/β-D-glucopyranoside (22): The mixture of compound **21** (α- form) (2.0 g, 4.0 mmol) and dibutyltin oxide (1.1 g, 4.6 mmol) in dry Toluene (80 mL) was heated at reflux for 4 hours while using dean stark trap to remove water from the reaction. Then toluene was removed completely on rotary evaporator and dry THF (50 mL) was added to the reaction flask. Then allyl bromide (3.9 mL, 46.3 mmol) followed by the addition of tetrabutylammonium iodide (0.8 g, 2.2 mmol).The reaction mixture was then stirred at 60 °C for 14 hours and then cooled down to room temperature. THF was removed under reduced pressure and purification by column chromatography using ethyl acetate/methanol (15:1) as eluent yielded **22** (1.1 g) in 50 % yield. RF= 0.2. **^{1}H NMR (400 MHz, Chloroform-d)** δ 5.95 (1 H, ddt, J 17.2, 10.3, 5.8), 5.33 (1 H, dq, J 17.2, 1.5), 5.22 (1 H, dt, J 10.3, 1.4), 5.15 (1 H, d, J 3.4), 4.78 (1 H, s), 4.50 (1 H, d, J 7.9), 4.27 – 4.15 (2 H, m), 4.06 (1 H, d, J 3.2), 4.04 – 3.89 (3 H, m), 3.89 – 3.67 (6 H, m), 3.63 (2 H, t, J 5.3), 3.54 (1 H, dd, J 9.7, 3.5), 3.40 (1 H, dd, J 9.5, 3.1), 3.25 (1 H, s), 2.96 (1 H, s), 2.33 (1 H, d, J 11.2), 0.91 (9 H, s), 0.12 (6 H, d, J 4.9); **^{13}C NMR (101 MHz, Chloroform-d)** δ 134.3, 118.2, 103.1, 93.3, 80.4, 78.0, 77.3, 77.0, 76.7, 74.7, 72.8, 72.6, 71.2, 70.4, 69.5, 67.2, 62.4, 61.4, 25.7, 18.0. **HRMS (ESI):** m/z calcd for $C_{21}H_{40}O_{11}Si$ $[M+Na]^+$– 519.2236, found 519.2238.



Synthesis of tert-butyldimethylsilyl 3-O-allyl-4,6-O-benzylidene-β-D-galactopyranosyl-(1-4)-α/β-D-glucopyranoside (23): Compound **22** (1.0 g, 1.8 mmol), benzaldehyde dimethyl acetal (0.4 g, 2.9 mmol) and p-toluenesulfonic acid monohydrate (0.03 g, 0.2 mmol) was taken in a round bottom flask and CH_3CN was (10 mL) was added to it. The reaction mixture was stirred overnight at room temperature and after completion of the reaction; the reaction was neutralize with triethyl amine. The resulting mixture was concentrated on rotary evaporator. After that mixture was dissolved in ethyl acetate and washed with saturated NaCl solution. The organic phase was dried over sodium sulphate and concentrated under reduced pressure to give the crude product **23**. The purification using column chromatography in ethylacetate/MeOH (15:1) as an eluent afforded the product in 75% yield (0.8 g). Rf=0.5. **^{1}H NMR (400 MHz, Chloroform-d)** δ 7.51 – 7.46 (2 H, m), 7.40 – 7.29 (3 H, m), 5.95 (1 H, ddt, J 17.2, 10.3, 5.8), 5.51 (1 H, s), 5.33 (1 H, dq, J 17.2, 1.6), 5.22 (1 H, dq, J 10.3, 1.3), 5.18 (1 H, d, J 3.6), 4.56 (1 H, d, J 8.1), 4.34 – 4.15 (4 H, m), 4.09 – 4.01 (3 H, m), 3.99 – 3.92 (1 H, m), 3.88 – 3.66 (5 H, m), 3.55 – 3.42 (3 H, m), 2.88 (1 H, t, J 7.1), 1.95 (1 H, d, J 9.9), 0.91 (9 H, s), 0.13 (6 H, d, J 3.2); **^{13}C NMR (101 MHz, Chloroform-d)** δ 137.3, 134.6, 129.1, 128.3, 126.3, 118.0, 103.3, 101.5, 93.1, 78.8, 77.3, 77.0, 73.1, 73.0, 72.8, 70.8, 70.6, 69.2, 68.5, 67.2, 61.4, 25.7.

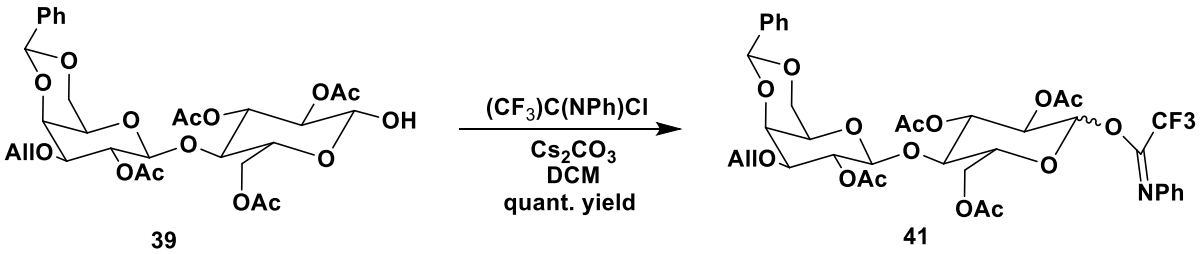


Synthesis of tert-butyldimethylsilyl 2-O-acetyl-3-O-allyl-4,6-O-benzylidene-β-D-galactopyranosyl-(1-4)-2,3,6-O-acetyl-α/β-D-glucopyranoside (24): Molecule **23** (0.7 g, 1.3 mmol) was dissolved in 10 mL of pyridine/acetic anhydride (1:1). The catalytic amount of DMAP (0.008 g, 0.06 mmol) was added to the reaction flask and the reaction mixture was stirred at room temperature overnight. Then the reaction mixture was then concentrated in *vaccuo* and co-evaporated with toluene azeotropically. The compound was dried over vacuum and obtained in quantitative yield (0.96 g). The compound was used for next steps without further purification. Toluene/acetone (8:2); Rf =0.42. **HRMS (ESI):** m/z calcd for $C_{36}H_{52}O_{15}Si$ $[M+Na]^+$– 775.2968, found 775.2964.



Synthesis of 2-O-acetyl-3-O-allyl-4,6-O-benzylidene-β-D-galactopyranosyl-(1-4)-2,3,6-O-acetyl-α/β-D-glucopyranose (39): Compound **24** (0.8 g, 1.1 mmol) was dissolved in 20 mL of anhydrous THF under argon atmosphere at -10 ◦C followed by the dropwise addition of 1M TBAF solution in THF (1.2 mL) was added dropwise to the flask. The reaction mixture turns yellow immediately after addition of TBAF. The reaction mixture was stirred at -10 ◦C for 2 hour and after completion of reaction (monitored by TLC), the reaction mixture was neutralized with sat. aq. NH_4HCO_3 solution. During the neutralization process a colourless precipitate was formed which was taken up in DCM. The organic phase was subsequently washed with brine, dried over Na_2SO_4 and the solvent was removed in *vacuo.* The purification

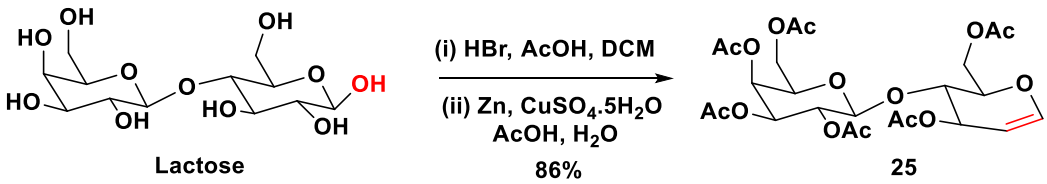
of crude compound by column chromatography yielded the compound **39** as white foam in 95% yield (0.65 g) using toluene/acetone (9:1). Rf = 0.7. **^{1}H NMR (400 MHz, Chloroform-d)** δ7.51 – 7.42 (2 H, m), 7.36 (3 H, d, J 7.0), 5.93 – 5.75 (1 H, m), 5.60 – 5.44 (2 H, m), 5.36 (1 H, t, J 3.5), 5.30 – 5.11 (3 H, m), 4.95 – 4.66 (1 H, m), 4.49 (1 H, d, J 10.1), 4.40 (1 H, t, J 8.1), 4.25 – 3.99 (5 H, m), 3.81 – 3.63 (2 H, m), 3.51 (1 H, dd, J 10.1, 3.3), 3.36 (1 H, s), 3.31 (1 H, d, J 3.4), 2.11 (3 H, s), 2.06 (9 H, d, J 2.0), 1.71 (1 H, s); **^{13}C NMR (101 MHz, Chloroform-d)** δ 170.65, 170.41, 170.12, 168.99, 137.55, 134.59, 129.12, 128.21, 126.57, 117.24, 101.54, 101.33, 101.25, 95.37, 90.24, 77.45, 77.33, 77.02, 76.70, 75.96, 75.90, 73.46, 73.21, 71.74, 71.22, 70.48, 70.40, 69.29, 68.95, 68.60, 66.72, 62.33, 62.19, 20.82, 20.75, 20.64.



Synthesis of 2-O-acetyl-3-O-allyl-4,6-O-benzylidene-β-D-galactopyranosyl-(1-4)-2,3,6-O-acetyl-α/β-D-glucopyranosyl triflouro-*N*-phenylacetimidate (41): To a solution of hemiacetal compound **39** (0.44 g, 0.7 mmol) in dry DCM (10 mL) was added 2,2,2-trifluoro-N-phenylacet-imidoyl chloride (223.5 µL, 1.4 mmol). The reaction mixture was cooled to 0 ^{0}C and to the cool solution Cs_2CO_3 (0.45 g, 1.4 mmol) was added. The reaction mixure was allowed to warm up to room temperature and stirred for 2 hours. Reaction progress monitor by TLC and on completion of the reaction, the reaction mixture was filtered over celite and the filtrate was concentrated in *vacuo*. The product was purified by column chromatography using toluene/acetone (8:2) as eluent and obtained **41** in quantitative yield (0.54 g) as colorless oil. **^{1}H NMR (400 MHz, Chloroform-d)** δ 7.48 (2 H, dd, J 7.7, 1.7), 7.36 (3 H, t, J 6.1), 7.30 (2 H, t, J 7.8), 7.21 – 7.08 (3 H, m), 6.80 (2 H, d, J 7.2), 5.91 – 5.78 (1 H, m), 5.53 (2 H, d, J 8.4), 5.31 – 5.14 (4 H, m), 5.10 (1 H, d, J 8.8), 4.55 – 4.37 (2 H, m), 4.30 (1 H, d, J 11.4), 4.23 (1 H, d, J 3.3), 4.18 – 3.99 (5 H, m), 3.84 (1 H, q, J 9.8, 9.0), 3.51 (1 H, dd, J 10.0, 3.5), 3.37 (1 H, s), 2.36 (2 H, s), 2.12 (3 H, s), 2.09 (3 H, s), 2.06 (6 H, s), 1.57 (3 H, s); **^{13}C NMR (101 MHz,**

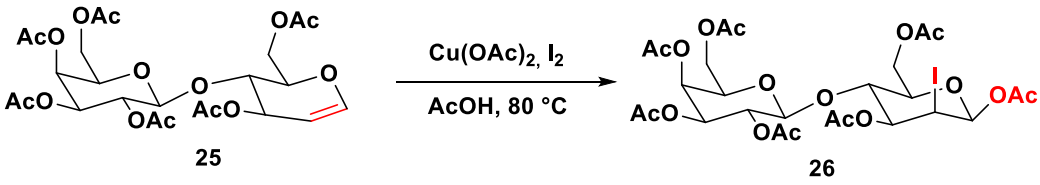
Chloroform-d) δ 190.7, 170.4, 170.1, 169.9, 168.9, 143.0, 137.3, 134.5, 129.2, 128.8, 128.2, 126.6, 124.6, 119.4, 117.3, 101.5, 101.2, 77.5, 77.3, 77.0, 76.7, 75.3, 73.1, 71.3, 69.6, 69.1, 68.7, 66.8, 61.7, 20.9, 20.7, 20.5, 1.0; **HRMS (ESI):** m/z calcd for $C_{38}H_{42}F_3NO_{15}$ $[M+Na]^+$– 832.2404, found 832.2398.

5.2.3 Synthesis of building block 31

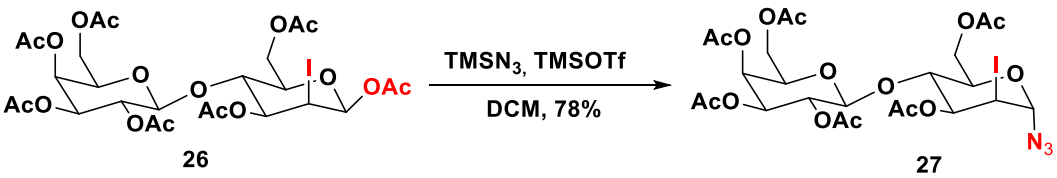


Synthesis of hexa-*O*-acetyl-D-lactal (25)[7]**:** To a suspension of D-lactose (1.00 g) in acetic anhydride (2.7 g, 9.0 mol equiv) was added 1.0 g of 31% HBr acetic acid at room temperature (the temperature was kept at room temperature while cooled by a water bath). Although the mixture was stirred at room temperature for 4 h, the solid lactose did not completely dissolve. Additional 31 % HBr/acetic acid (4.0 g, thus making an overall total of 6.7 mol equiv of HBr) was added and the resulting mixture was stirred overnight at room temperature, at which point all of the solid lactose went into solution. The reaction mixture was poured onto a suspension of pulverized $CuSO_4.5H_2O$ (0.18 g) and zinc (7.3 g) in a solution of water (10 mL) and acetic acid (15 mL) containing sodium acetate (5.5 g). The resultant reaction mixture was stirred vigorously at room temperature for 1.5 h in a bath of running tap water. The mixture was then filtered and the solid collected was washed with ethyl acetate (100 mL) and then with water (100 mL). The organic layer of the filtrate was washed successively with saturated aqueous $NaHCO_3$ (100 mL) and brine (100 mL), and then dried (Na_2SO_4). The solvent was removed under reduced pressure to provide a crude solid which was purified by silica gel flash column chromatography using 50% ethyl acetate in hexanes as the eluent to afford hexa-O-acetyl- D-lactal **(25)** (1.2 g, 86%) as a colourless solid:

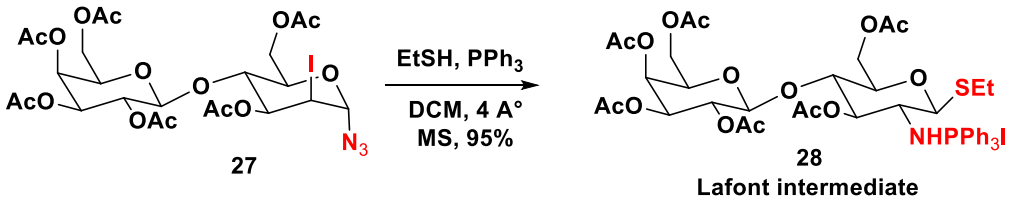
Spectroscopic data were in agreement with those reported in the literature.[7]



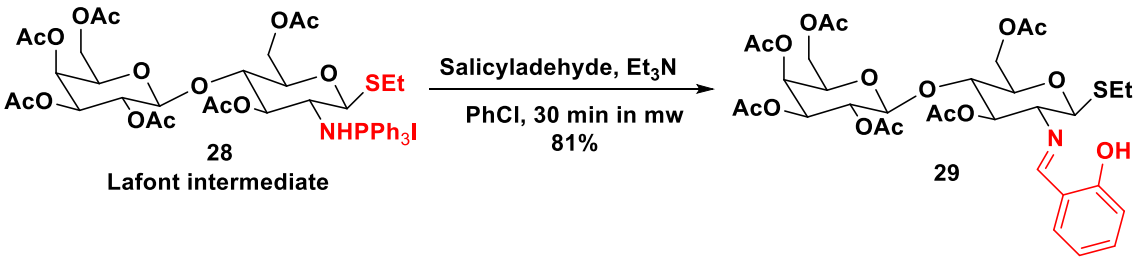
Synthesis of 1-*O*-acetyl-3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-gulcopyranosyl)-2-deoxy-2-iodo-α-D-mannopyranose (26)[8]: In a 200 mL acetic acid solution**,** glycal **25** (10.0 g, 17.8 mmol), $Cu(OAc)_2$ (3.6 g, 19.6 mmol), and I_2 (5.4 g, 21.4 mmol) were added in respective order. The resulting mixture was stirred at 80 °C overnight under argon atmosphere. Then the reaction was evaporated to dryness under reduced pressure and the obtained residue was diluted with DCM (500 mL). The organic layer was washed with aq. $NaHCO_3$ (150 mL), aq. $Na_2S_2O_3$ (150 mL) and brine (150 mL) and dried over Na_2SO_4, and concentrated. The crude residue was purified by silica gel column chromatography (*n*-hexane : ethyl acetate = 2/1) to yield the compound **X** as white solid (9.3 g, 70%). Spectroscopic data were in agreement with those reported in the literature.[8]



Synthesis of 3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-gulcopyranosyl)-2-deoxy-2-iodo-*α*-D-mannopyranosyl azide (27)[8]: To the solution of iodoacetate **26** (9.0 g, 12.0 mmol) and $TMSN_3$ (1.9 mL, 14.5 mmol) in dry DCM (100 mL), TMSOTf (435 µL, 2.4 mmol) was added at 0 °C under argon atmosphere. The reaction mixture was gradually warmed to room temperature and then stirred overnight. The resulting mixture was then diluted with DCM (400 mL) and the organic phase was washed with aq. $NaHCO_3$ (150 mL) dried over Na_2SO_4. The organic layer was concentrated on rotary evaporator and the resulting residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate = 1:1) to afford compound **X** as a white solid (7.0 g, 80%) as white solid. Spectroscopic data were in agreement with those reported in the literature.[8]

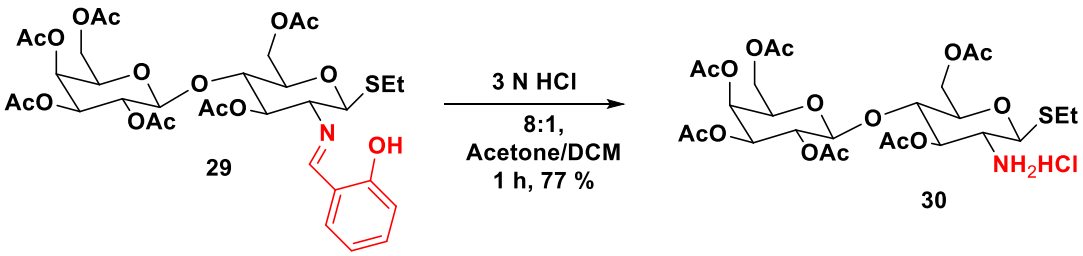


Synthesis of ethyl [3,6-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-2-aminotriphenylphosphonium-2-deoxy-1-thio-*β*-D-glucopyranoside] iodide (28)[8]: In 100 mL of dry DCM, compound **27** (7.0 g, 9.6 mmol) and ethanethiol (831 µL, 11.5 mmol) and 4 Å MS were added under argon. The reaction mixture was then cooled down to 0 °C and stirred for 30 min. Then a solution of PPh_3 (2.6 g 10.1 mmol) in DCM (5 mL) was added dropwise**.** The reaction mixture was allowed to warm gradually to room temperature and further stirred for additional 12 h. The molecular sieves was filtered off through a pad of Celite and the filtrate was concentrated under reduced pressure. The resulting crude residue was purified by silica gel column chromatography using ethyl acetate: ethanol (10/1) as eluent to yield the compound **28** (9.4 g, 95%) as yellow foam. The resulting Lanfont intermediate was stored and next steps were carried out in batches when required. Spectroscopic data were in agreement with those reported in the literature.[8]

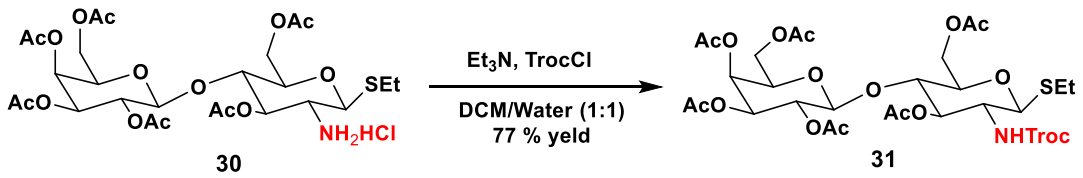


Synthesis of Ethyl 3,6-*O*-di-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-2-deoxy-2-*o*hydroxylbenzylideneamino-1-thio-*β*-D-glucopyranoside (29)[8]: To a mixture of compound **28** (1.0 g, 1.0 mmol) and salicylaldehyde (1 mL in 2 mL of chlorobenzene in a Microwave tube, Et_3N (2 mL) was added. Then the reaction was irradiated with 150 W of microwave energy at 140 °C for 30 min. The resulting reaction mixture was then transferred into a round bottom flask and concentrated *in vacuo*. Purification by silica gel column chromatography (*n*-hexane/ethyl acetate = 1:1) afforded compound **11** (0.6 g, 80 %) as foam.

The scale of the reaction was reduced due to the size limitation of microwave set-up. Spectroscopic data were in agreement with those reported in the literature.[8]

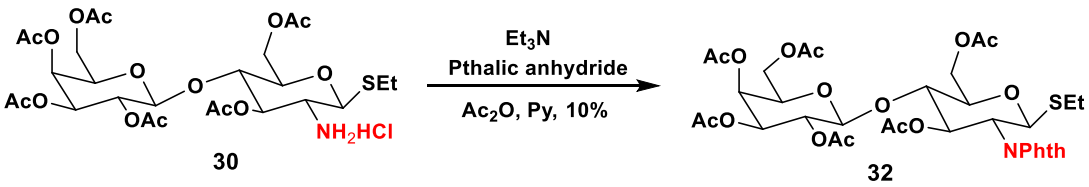


Synthesis of ethyl 3,6-*O*-di-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-2-deoxy-2-amino-1-thio-*β*-D-glucopyranoside hydrochloride (30)[8]: To a solution of compound **29** (3.0 g, 4.0 mmol) in a mixture of acetone/DCM (8:1, 10 mL), aq. 3 M HCl solution (1.4 mL, 4.1 mmol) was added. The reaction mixture was then stirred for 1 h at room temperature and the progress of the reaction was monitored using TLC. On complete conversion of the starting material, the solution was diluted with toluene (6 mL) and the solvent was removed under *vacuo*. The residue was purified by silica gel column chromatography using ethyl acetate/ethanol (20:1) to yield compound **12** (1.9 g, 72%) as foam. Spectroscopic data were in agreement with those reported in the literature.[8]



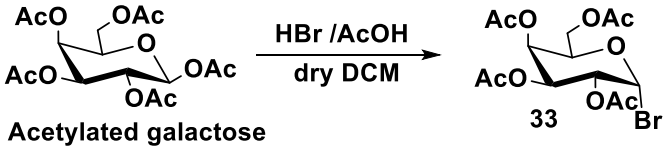
Synthesis of ethyl 3,6-*O*-di-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-2-deoxy-2-*N*-Troc-1- thio-*β*-D-glucopyranoside (31)[8]: To a solution of **30** (0.5 g, 0.8 mmol) and Et_3N (511 µL, 3.7 mmol) in DCM/H2O (1:1, 30 mL), TrocCl (1 mL, 7.9 mmol) was added. The reaction was stirred then overnight at room temperature. The resulting solution was diluted with DCM (150 mL) and washed with aq. $NaHCO_3$ (100 mL) and brine (100 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated over *vacuo*. The crude residue was

purified by silica gel column chromatography using hexane/ethyl acetate (1/1) to afford compound **14** (0.5 g, 77%) as foam. Spectroscopic data were in agreement with those reported in the literature.[8]

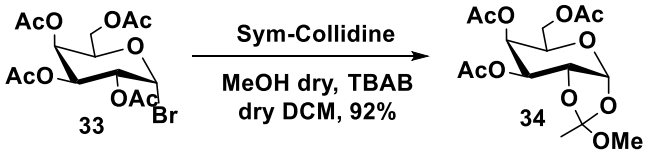


Synthesis of ethyl-3,6-*O*-di-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-2-deoxy-2-*N* phthalimido-1-thio-*β*-D-glucopyranoside (32)[8]: To a solution of **30** (0.95 g, 1.4 mmol) in pyridine (30mL), Et_3N (247 µL, 1.8 mmol) was added. The reaction mixture was stirred for 30 minutes followed by the addition of phthalic anhydride (0.2 g, 1.6 mmol). The resulting solution was stirred for 2 hours and then a second portion of phthalic anhydride (0.2 g, 1.6 mmol) and Et_3N (247 µL, 1.8 mmol) were added. Then the resulting reaction mixture was stirred for additional 2 hours and then the reaction was quickly moved to an preheated oil bath at 90 °C followed by the addition of Ac_2O (10 mL). The mixture was then stirred for another 30 min at 90 °C and after 30 minutes, the solution was concentrated *in vacuo*. The resulting residue was dissolved in DCM (100 mL) and the organic layer was washed with aq. 1 N HCl (70×3), water (60 mL), aq. $NaHCO_3$ (50 mL), and brine (50 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography using hexane/ethyl acetate (1:1) to yield compound **13** (0.96 g, 20%) as white solid. Spectroscopic data were in agreement with those reported in the literature.[8]

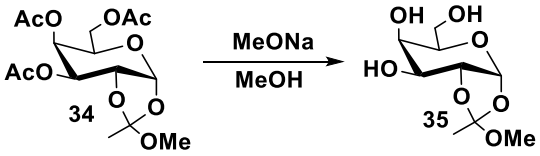
5.2.4 Synthesis of building block 38



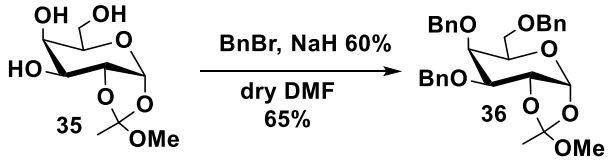
Synthesis of 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide (33)[9]**:** To the penta-acetyl galactose (2.5 g, 6.4mmol) was added 40 mL 33% HBr-AcOH solution and the resulting mixture was stirred at room temperature for 1h. Then the resulting solution was diluted with DCM (100 mL) and transferred to a separatory funnel containing ice. The ice was allowed to melt and thehe organic phase was washed with ice water until neutral pH was achieved. The organic phase was then dried over Na_2SO_4, filtered, and concentrated to give compound **33** as a syrup in good yield: 88% (2.3 g). The product was used directly in the next step without purification.



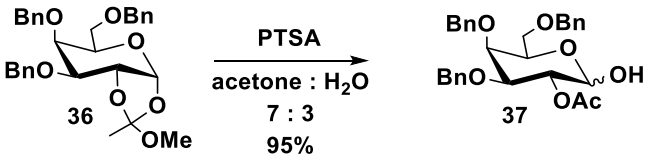
Synthesis of 3,4,6-tri-O-acetyl-1,2-O-(1-methoxyethylidene)-α-D-galactopyranoside (34)[9]**:** Compound **33** (2.0 g, 4.86 mmol) and 2,4,6-collidine (0.9 g, 7.3 mmol) were dissolved in dry DCM (20 mL). Anhydrous methanol (10 mL) was then added in one portion followed by followed by the addition of tetrabutylammonium bromide (0.8 g, 2.4 mmol). The resulting mixture was stirred at room temperature for 12 hour. The mixture was diluted with DCM and water. The aqueous portion was extracted with DCM (3x50 mL). The combined organic portion was dried over anhydrous Na_2SO_4, filtered, concentrated, and washed with hexanes to give compound **34** as a white solid in good yield (92%., 1.6 g). The compound was used for next steps without further purification.



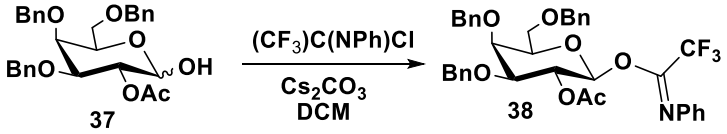
Synthesis of 1,2-O-(1-methoxyethylidene)-α-D-galactopyranoside (35)[10]**:** To a solution of **34** (1.00 g, 2.7 mmol) in dry MeOH (20 mL), 0.4 M NaOMe in MeOH (1.0 mL, 0.5 mmol) was added. After 30 min the reaction mixture was diluted with MeOH and neutralized with AMBERLITE® resin (H^+ form). The mixture was then filtered and the solvent was removed in *vacuo*, affording compound **35** as a white solid (0.6 g, quant).



Synthesis of 3,4,6-tri-O-benzyl-1,2-O-(1-methoxyethylidene)-α-D-galactopyranoside (36)[10]**:** To a solution of **35** (0.5 g, 2.1 mmol) and benzyl bromide (1.2 mL, 10.6 mmol) in dry DMF (50 mL), sodium hydride (0.25 g, 10.6 mmol) was slowly added. The reaction mixture was then stirred for 3 hours and then after 3 h the reaction mixture was carefully quenched by adding MeOH (10 mL). The reaction mixture was then concentrated in vacuo and the crude residue was diluted with H2O. The aqueous solution was extracted with DCM (3 x 20 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, concentrated in *vacuo*, and purified using flash chromatography Hexane:EtOAc (8:2 to 7:3 + 1% TEA) to obtain the **36** as a colourless oil (0.7 g, 65%).

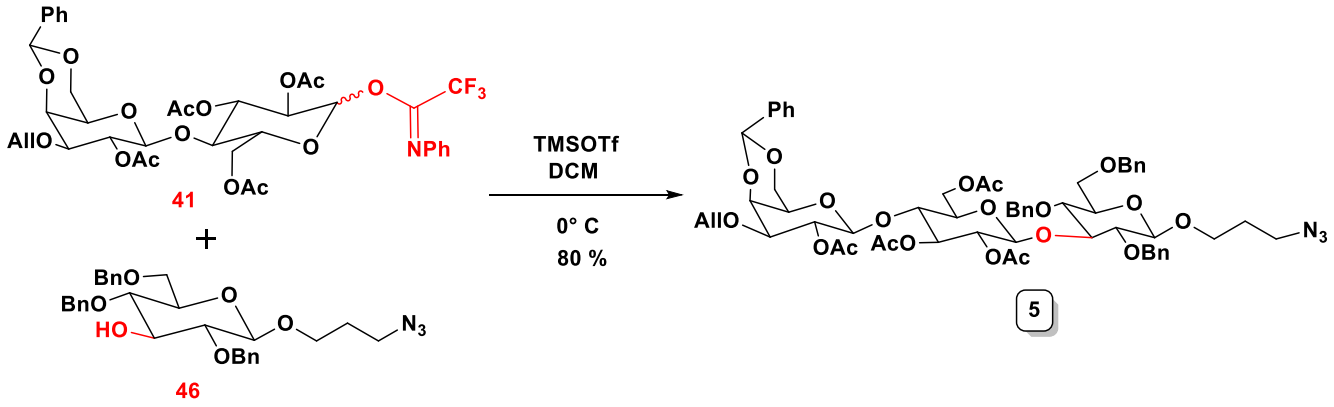


Synthesis of 2-O-acetyl-3,4,6-tri-O-benzyl-D-galactopyranose (37)[10]**:** Solution of **36** (0.7 g, 1.3 mmol) in acetone: H_2O (7:3, 10 mL) was cooled to 0 °C and p-TsOH (0.034 g, 0.4 mmol) was added to the flask. The reaction was then stirred for 2 h and then the reaction mixture was neutralized with triethyl amine and concentrated in *vacuo*. The crude residue was purified by flash chromatography (Hexane: AcOEt; 7:3) to obtain yield compound **37** (0.5 g, 95%).

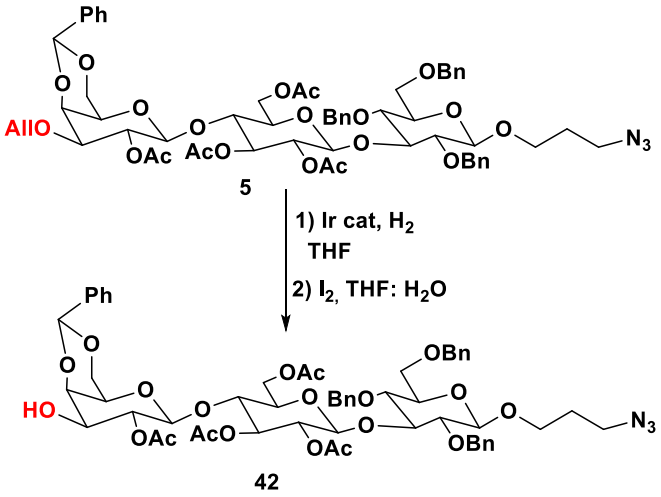


Synthesis of 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-galactopyranosyl trifluoro-*N*-phenylacetimidate (38)[9]**:** To a solution of 1-hydroxy sugar **37** (0.5 g, 1.0 mmol) in dry DCM (10 mL) was added 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride (0.42 g, 2.0 mmol). The reaction mixture was cooled to 0 ^oC and to the cool solution Cs_2CO_3 (0.66 g, 2.0 mmol) was added. The reaction mixture was allowed to warm up to room temperature and stirred for 2 hours. Reaction progress monitor by TLC and on completion of the reaction, the reaction mixture was filtered over celite and the filtrate was concentrated in *vacuo*. The product was purified by column chromatography using toluene/acetone (8:2) as eluent and obtained in 95% (0.6 g) yield as colourless oil.

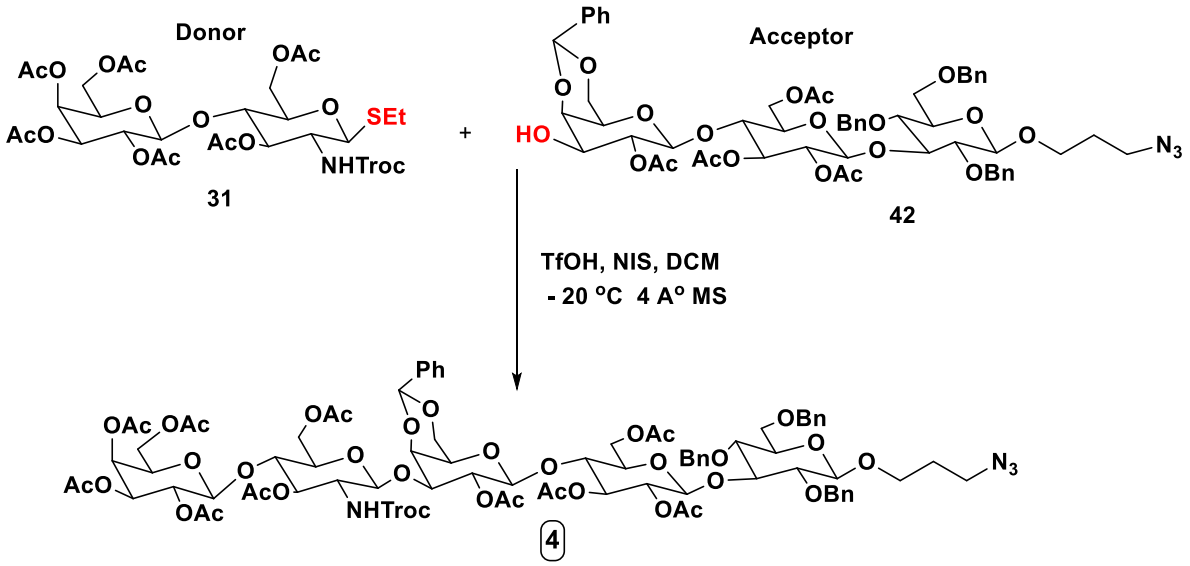
5.2.5 Combining the Fragments



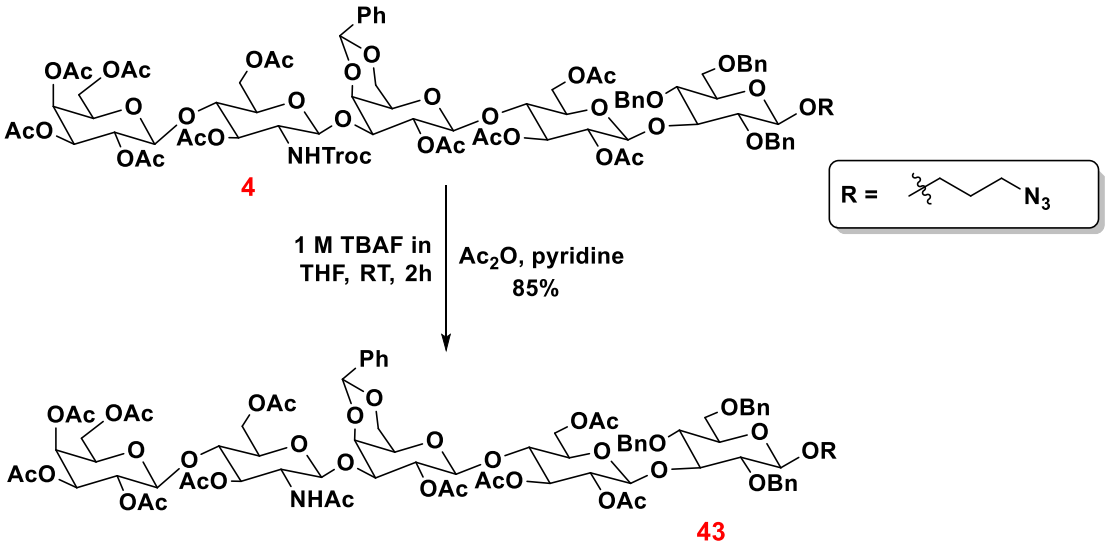
Synthesis of Trisaccharide (5): A solution of donor **41** (0.2 g, 0.26 mmol) and acceptor **46** (0.17 g, 0.31 mmol) in dry DCM (5 mL) was cooled to 0 °C and then TMSOTf (4.7 µL, 0.03 mmol) was added slowly to the reaction under argon atmosphere. The resulting reaction mixture was stirred at 0 °C and after completion of reaction; the reaction was quenched by triethyl amine. The reaction mixture was dried over *vacuo* and purification by silica get column chromatography using toluene: acetone (8/2) as eluent afforded trisaccharide **5** in 80 % yield (0.24 g).RF =0.5. **^{1}H NMR (400 MHz, Chloroform-d)** δ 7.57 (1 H, d, J 8.7), 7.51 – 7.43 (6 H, m), 7.42 – 7.29 (9 H, m), 7.22 (4 H, d, J 7.6), 5.83 (1 H, ddd, J 22.7, 10.7, 5.5), 5.50 (1 H, s), 5.26 (1 H, d, J 1.6), 5.24 – 5.13 (4 H, m), 5.02 – 4.94 (2 H, m), 4.89 (1 H, d, J 9.8), 4.62 (1 H, d, J 9.8), 4.52 (2 H, q, J 12.2), 4.43 (1 H, d, J 11.1), 4.37 – 4.23 (4 H, m), 4.20 (1 H, d, J 3.3), 4.15 – 3.94 (6 H, m), 3.74 (1 H, t, J 9.5), 3.70 – 3.55 (3 H, m), 3.54 – 3.33 (7 H, m), 3.31 (1 H, s), 2.08 (3 H, s), 2.03 (6 H, s), 1.92 (3 H, s), 1.63 (2 H, s); **HRMS (ESI):** m/z calcd for $C_{60}H_{71}N_3O_{20}$ $[M+Na]^+$– 1176.4529, found 1176.4534.



Synthesis of compound 42: The trisaccharide **5** (0.4 g, 0.3 mmol) was dissolved in dry THF (1.5 mL) under nitrogen and the reaction flask was evacuated. Iridium catalyst (0.03 g, 0.03 mmol) was added to the flask following the evacuation of the flask. The reaction mixture was stirred at room temperature. A balloon of H_2 gas was attached and this reaction was monitored until the red color of the solution turned to pale yellow. H_2 balloon was then removed and the flask was flushed with Argon gas and stirred for another 6 hours. The resulting solution was concentrated and dissolved in 80% THF in water (4.5 mL) followed by the addition of I_2 (0.17 g, 0.6 mmol). The resulting solution was stirred at room temperature for an hour, quenched by addition of saturated sodium thiosulfate solution, and washed with sodium bicarbonate, brine and water. The residue was then concentrated and evaporated then purified by Flash Chromatography (Tol/acetone-8:2; RF= 0.2) to afford **42** in 85% yield (0.3 g) over 2 steps. **^{1}H NMR (400 MHz, Chloroform-d)** δ 7.44-7.39 (6 H, m), 7.37 – 7.27 (5 H, m), 7.25 – 7.08 (9 H, m), 5.46 (1 H, s), 5.19 – 5.09 (2 H, m), 4.99 – 4.81 (4 H, m), 4.57 (1 H, d, J 9.8), 4.47 (2 H, q, J 12.2), 4.38 (1 H, d, J 11.1), 4.35 – 4.20 (4 H, m), 4.14 – 4.08 (1 H, m), 4.09 – 3.88 (4 H, m), 3.70 (1 H, t, J 9.5), 3.63 – 3.30 (11 H, m), 2.47 (1 H, d, J 11.2), 2.30 (1 H, s), 2.04 (6 H, s), 2.01 (3 H, s), 1.87 (3 H, s); **^{13}C NMR (101 MHz, Chloroform-*d*)** δ 170.4, 170.3, 170.2, 169.6, 138.7, 138.1, 137.7, 137.3, 129.5, 128.9, 128.6, 128.4, 128.2, 127.9, 127.8, 127.6, 127.5, 126.5, 103.2, 101.8, 100.9, 100.1, 82.8, 81.4, 77.4, 77.0, 76.7, 76.3, 75.7, 75.4, 75.1, 74.6, 74.4, 73.5, 72.8, 72.5, 72.5, 72.1, 72.0, 68.9, 68.5, 66.6, 66.6, 62.4, 48.3, 29.3, 20.9, 20.8, 20.7; **HRMS (ESI):** m/z calcd for $C_{57}H_{67}N_3O_{20}$ $[M+Na]^+$– 1136.4216, found 1136.4220.

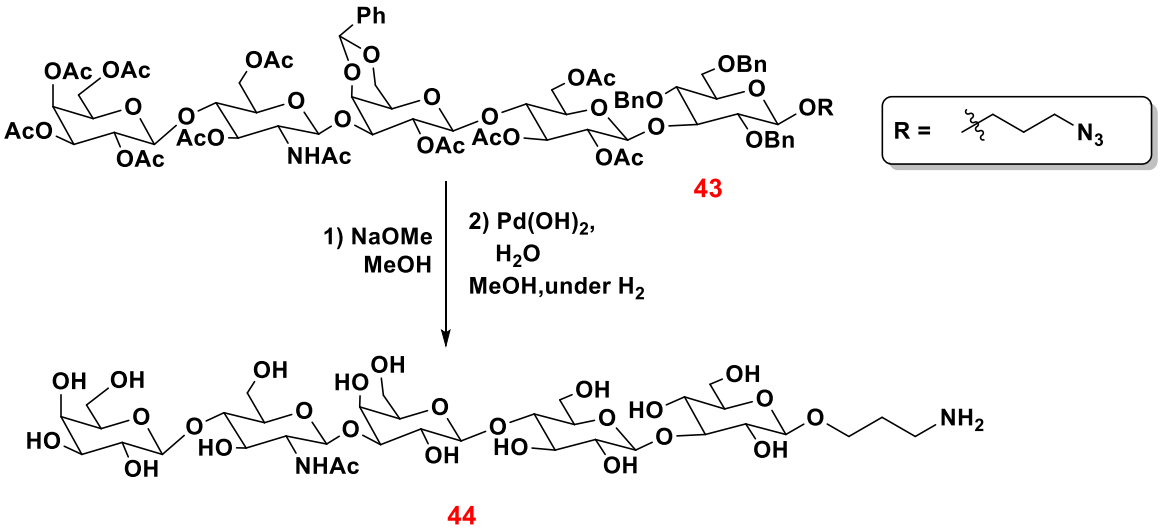


Synthesis of 4: Acceptor **42** (0.15 g, 0.13 mmol) and donor **31** (0.13 g, 0.16 mmol) were dissolved in dry CH_2Cl_2 (2 mL) together with 4 Å molecular sieves (0.2 g). The mixture was stirred for 1 hour, then NIS (0.06 g, 0.3 mmol) was added and the mixture was cooled to -20 °C. TfOH (9.5 µL, 0.1 mmol) was then dropped and the reaction was stirred for 30 minutes at the same temperature, then quenched with Et_3N, filtered over Celite and evaporated *in vacuo*. Purification by flash column chromatography (Tol/Acetone, 9:1 → 7:3, v/v) gave **4** (0.2 g, 80%) as a white foam. Rf = 0.34, Tol/Acetone 7:3. **^{1}H NMR (400 MHz, Chloroform-d)** δ 8.58 (1 H, s), 7.48 – 7.40 (6 H, m), 7.38-7.32 (4 H, m), 7.32 – 7.13 (10 H, m), 5.50 (1 H, s), 5.38 – 5.29 (2 H, m), 5.18 – 5.04 (5 H, m), 4.98 – 4.91 (3 H, m), 4.86 (1 H, d, J 9.9), 4.79 – 4.65 (3 H, m), 4.62 – 4.56 (2 H, m), 4.54 – 4.43 (4 H, m), 4.40 (1 H, d, J 11.1), 4.31 (1 H, d, J 7.8), 4.28 – 4.18 (4 H, m), 4.12 – 3.92 (8 H, m), 3.86 – 3.81 (1 H, m), 3.78 – 3.44 (10 H, m), 3.40 – 3.31 (4 H, m), 3.29 (1 H, s), 2.11 (3 H, s), 2.05 (6 H, d, J 2.3), 2.02 (9 H, s), 1.97 (6 H, q, J 5.9, 4.4), 1.94 (3 H, d, J 2.0), 1.90 (3 H, s), 1.86 – 1.81 (2 H, m), 1.23 (2 H, s); **^{13}C NMR (101 MHz, Chloroform-*d*)** δ 189.16, 177.69, 170.58, 170.38, 170.20, 170.14, 169.63, 169.06, 168.93, 153.86, 138.65, 138.14, 137.65, 129.18, 128.92, 128.53, 128.34, 128.15, 127.87, 127.75, 127.61, 127.48, 126.52, 103.22, 101.26, 101.08, 100.97, 99.96, 95.39, 82.74, 81.42, 79.17, 77.39, 77.07, 76.75, 75.74, 75.52, 75.08, 74.54, 74.36, 73.44, 72.72, 72.49, 72.08, 71.47, 70.90, 70.72, 69.75, 69.23, 68.90, 68.49, 66.68, 66.58, 62.36, 61.10, 60.86, 56.02, 31.64, 29.60, 29.31, 20.98, 20.88, 20.63; **HRMS (ESI):** m/z calcd for $C_{84}H_{101}Cl_3N_4O_{37}$ $[M+Na]^+$– 1885.5108, found 1885.5115.



Synthesis of 43: To the solution of pentasccharide **4** (0.2 g, 0.05 mmol) was dissolved in dry THF (2 mL) and then 1 M TBAF in THF (322 µl) was added to the flask. The mixture was stirred at room temperature for 2 hours and then diluted with EtOAc. The resulting solution was washed with water, dried over $MgSO_4$ and filtered. Solvent was removed under reduced pressure and the crude product was used for next step without further purification.

The crude residue was dissolved in 2 mL of acetic anhydride/pyridine (1:1) and the resulting solution was stirred for 5 hours. Then the solvent was removed in *vacuo*. Purification by flash column chromatography using toluene: Acetone (7: 3) as eluent afforded the *N*-acetyl product **43** as a white solid in 90 % yield (0.16 g). **^{1}H NMR (400 MHz, Chloroform-d)** δ 7.47 (6 H, d, J 6.2), 7.37 (4 H, d, J 6.6), 7.34 – 7.16 (10 H, m), 5.88 (1 H, d, J 8.1), 5.50 (1 H, s), 5.36 (1 H, d, J 3.4), 5.31 (1 H, d, J 8.2), 5.21 – 5.07 (4 H, m), 5.02 – 4.87 (5 H, m), 4.64 (2 H, t, J 10.5), 4.59 – 4.40 (4 H, m), 4.33 (3 H, dd, J 16.4, 7.7), 4.28 – 4.21 (2 H, m), 4.19-4.06 (4 H, m), 4.04 – 3.95 (3 H, m), 3.87 (1 H, t, J 6.9), 3.81 – 3.73 (2 H, m), 3.73 – 3.56 (5 H, m), 3.55 – 3.47 (2 H, m), 3.44-3.35 (5 H, m), 3.33 (1 H, s), 2.36 (1 H, s), 2.15 (3 H, s), 2.09 (6 H, d, J 7.4), 2.05 (12 H, d, J 3.0), 1.98 (6 H, s), 1.93 (4 H, s), 1.90 (3 H, s); **^{13}C NMR (101 MHz, Chloroform-*d*)** δ 170.5, 170.4, 170.3, 170.2, 170.1, 170.0, 169.8, 169.6, 169.2, 169.2, 138.6, 138.1, 137.8, 137.7, 129.1, 128.9, 128.5, 128.4, 128.3, 128.2, 127.9, 127.7, 127.6, 127.5, 126.5, 103.2, 101.3, 100.9, 100.7, 100.6, 99.9, 82.8, 81.4, 78.2, 77.5, 77.1, 76.8, 75.7, 75.4, 75.1, 74.5, 74.3, 73.4, 72.8, 72.5, 72.1, 70.8, 70.4, 70.4, 69.1, 68.9, 68.5, 66.7, 66.6, 62.3, 62.0, 60.8, 54.1, 48.3, 29.3, 23.2, 21.0, 20.9, 20.6, 20.5.

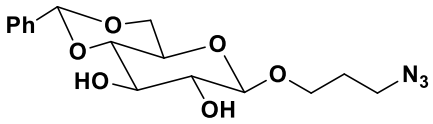


Synthesis of 44: The *N*-acetylated pentasaccharide **43** (0.05 g) was dissolved in CH_3OH (1 mL) and to the solution was added 1 M CH_3ONa in CH_3OH until pH reached 12. The mixture was then stirred at room temperature for 12 h and then neutralized with Amberlyst 15 H^+ resin, and filtered. The filtrate was concentrated under vacuum. The residue was purified on Sephadex LH-20 GEL column with CH_3OH as the eluent to give a white solid.

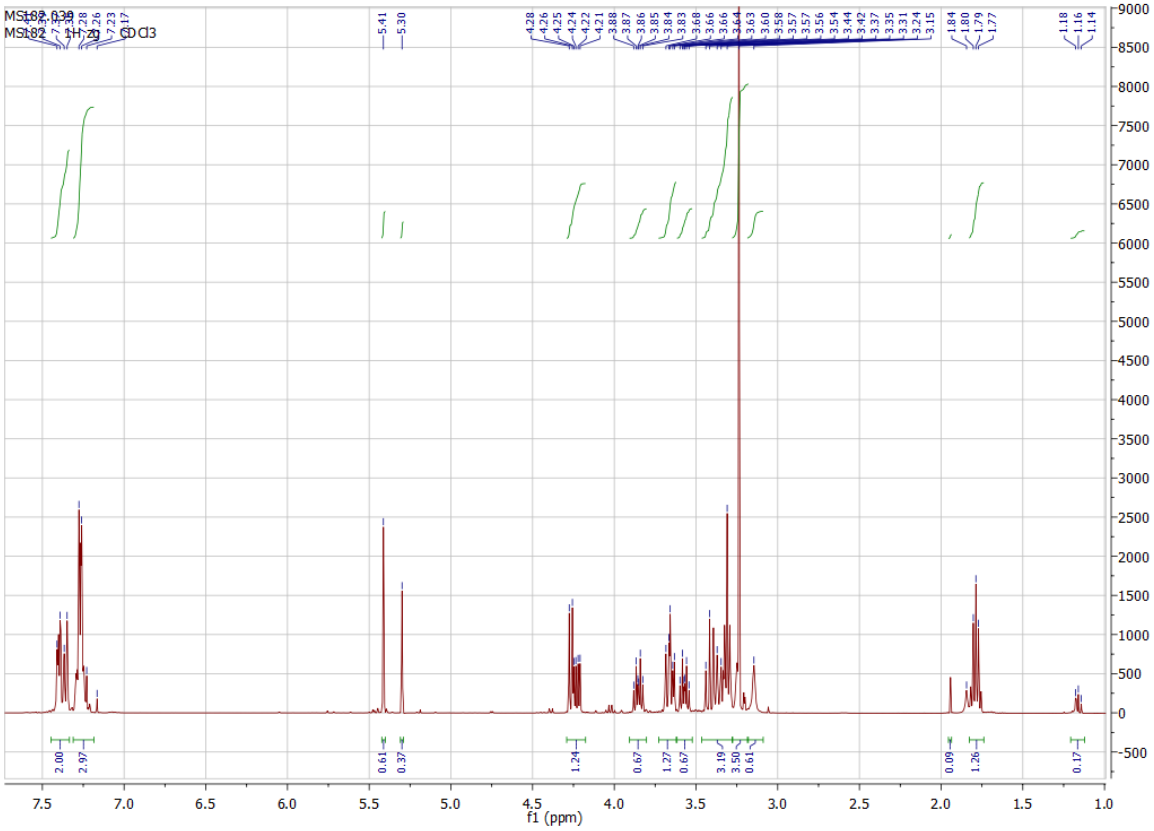
The obtained solid was dissolved in CH_3OH and H_2O (v/v, 4:1, 1 mL) and then mixed with $Pd(OH)_2$ (10 mol %). After the mixture was stirred under a H_2 atmosphere at 50 Psi for 36 h. After that, it was filtered, and concentrated to give a residue, which was purified on Sephadex G-25 gel column with H_2O as the eluent to produce the linear chain pentasaccharide **44** as a white solid. **^{1}H NMR (400 MHz, Deuterium Oxide)** δ 5.53 (3 H, d, J 3.6), 5.31 (1 H, t, J 7.7), 5.10 – 4.97 (3 H, m), 4.64 – 4.43 (18 H, m), 4.41 – 4.25 (24 H, m), 4.24 – 3.95 (23 H, m), 3.92-3.90 (6 H, m), 3.75 (1 H, q, J 7.4), 3.47 (1 H, s), 3.33 (1 H, d, J 19.2), 3.28 – 3.10 (1 H, m), 2.65 – 2.58 (3 H, m).

5.3 NMR Spectra

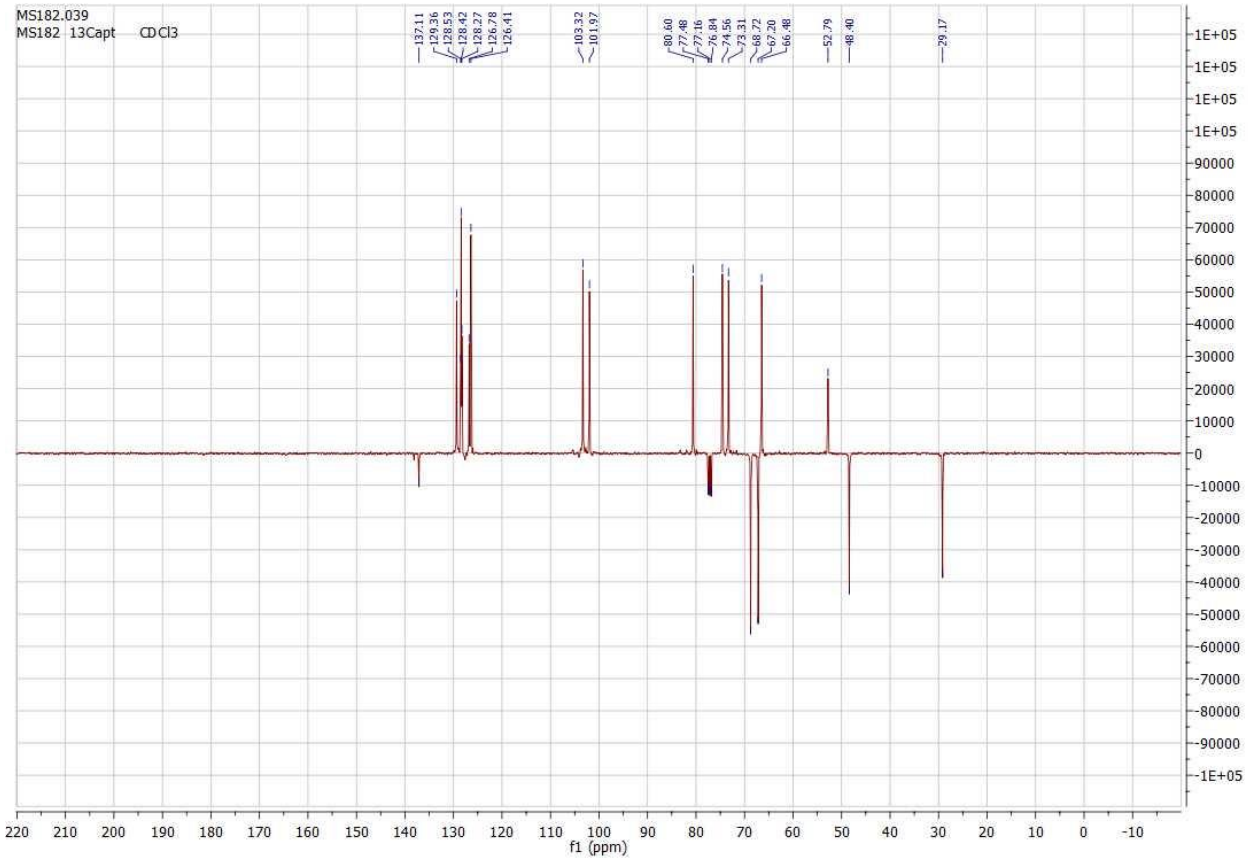
NMR Spectra of compound 15



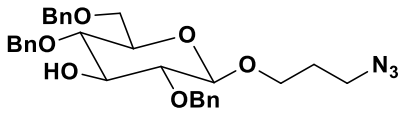
^{1}H NMR of compound 15



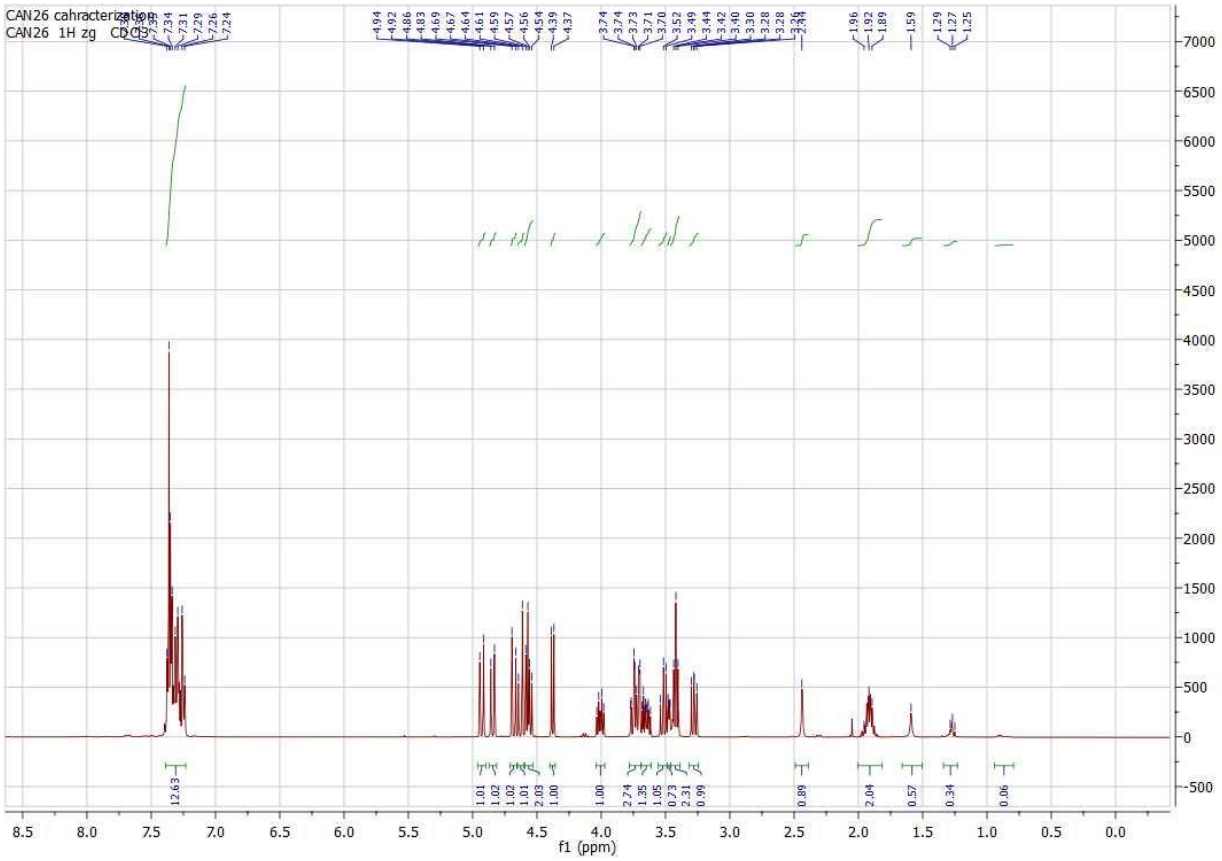
^{13}C NMR of compound 15



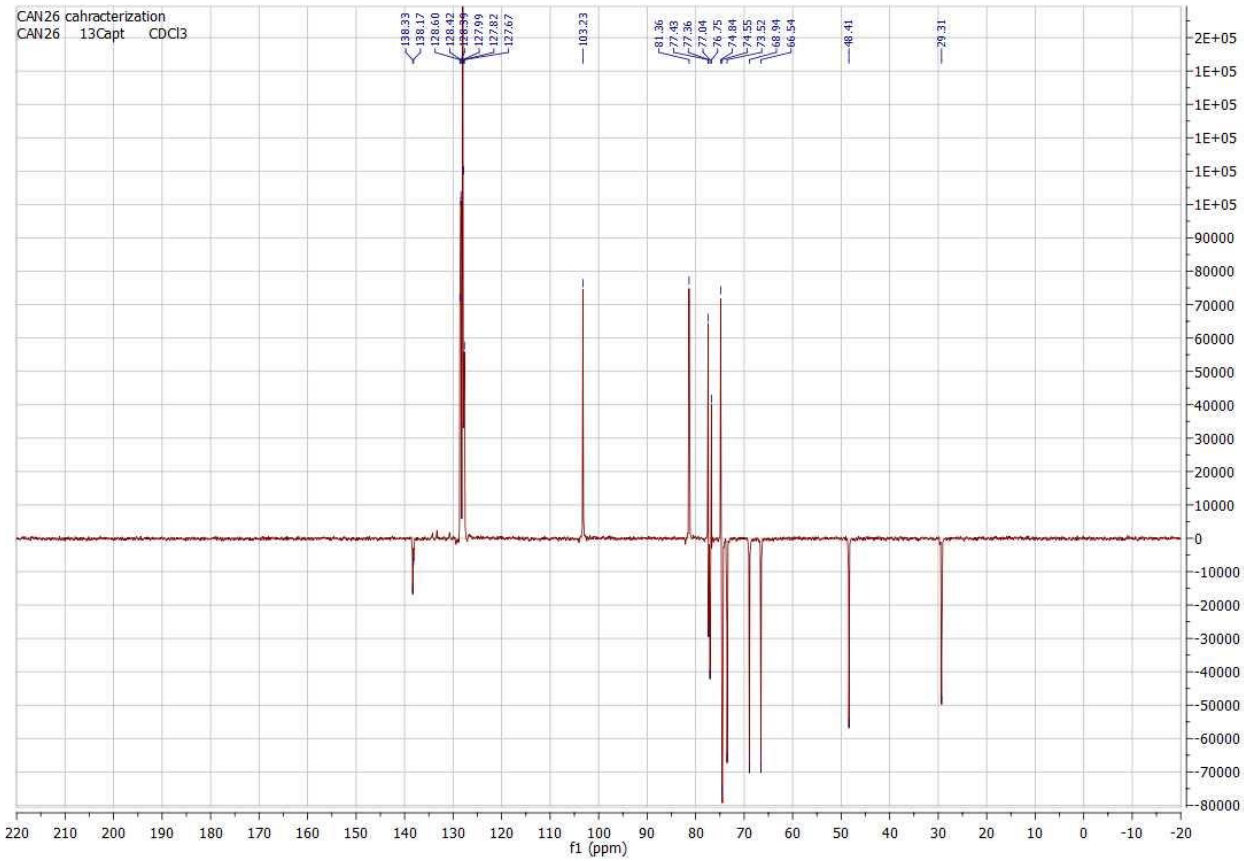
NMR Spectra of compound 46



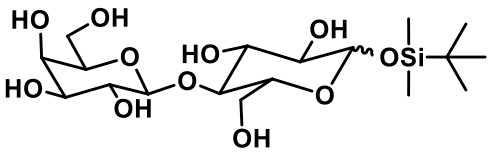
^{1}H NMR of compound 46



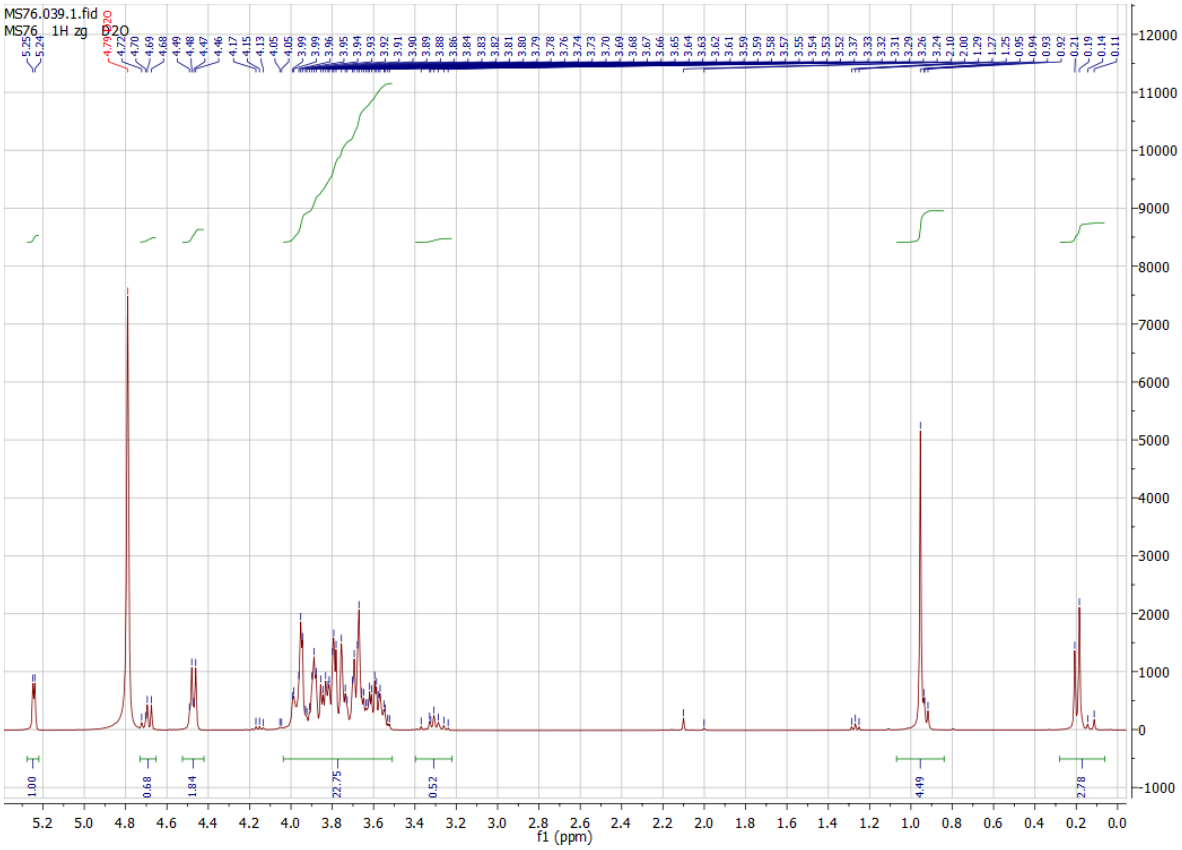
^{13}C NMR of compound 46



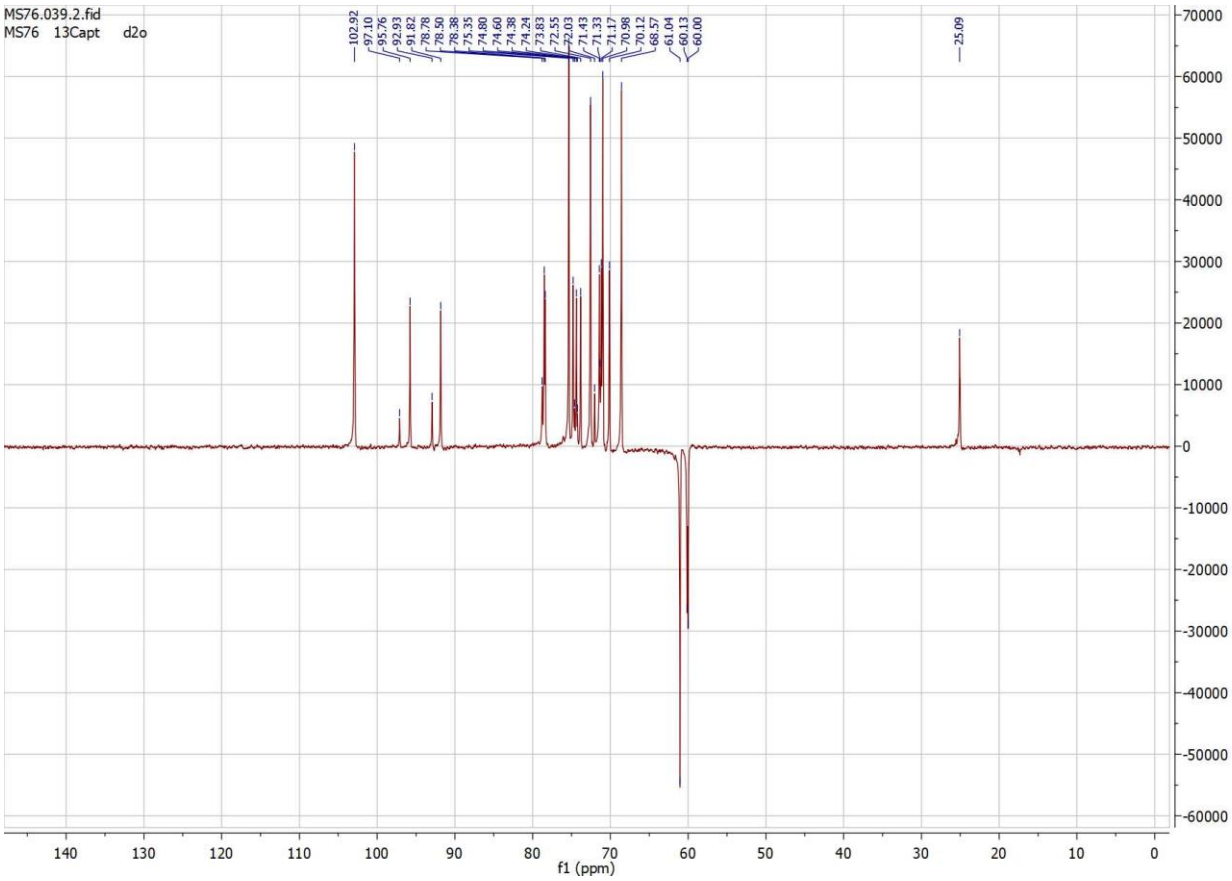
NMR Spectra of compound 21



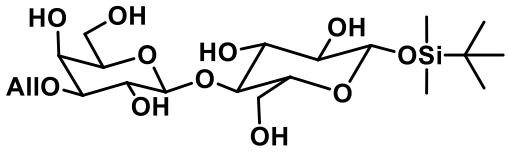
^{1}H NMR of compound 21



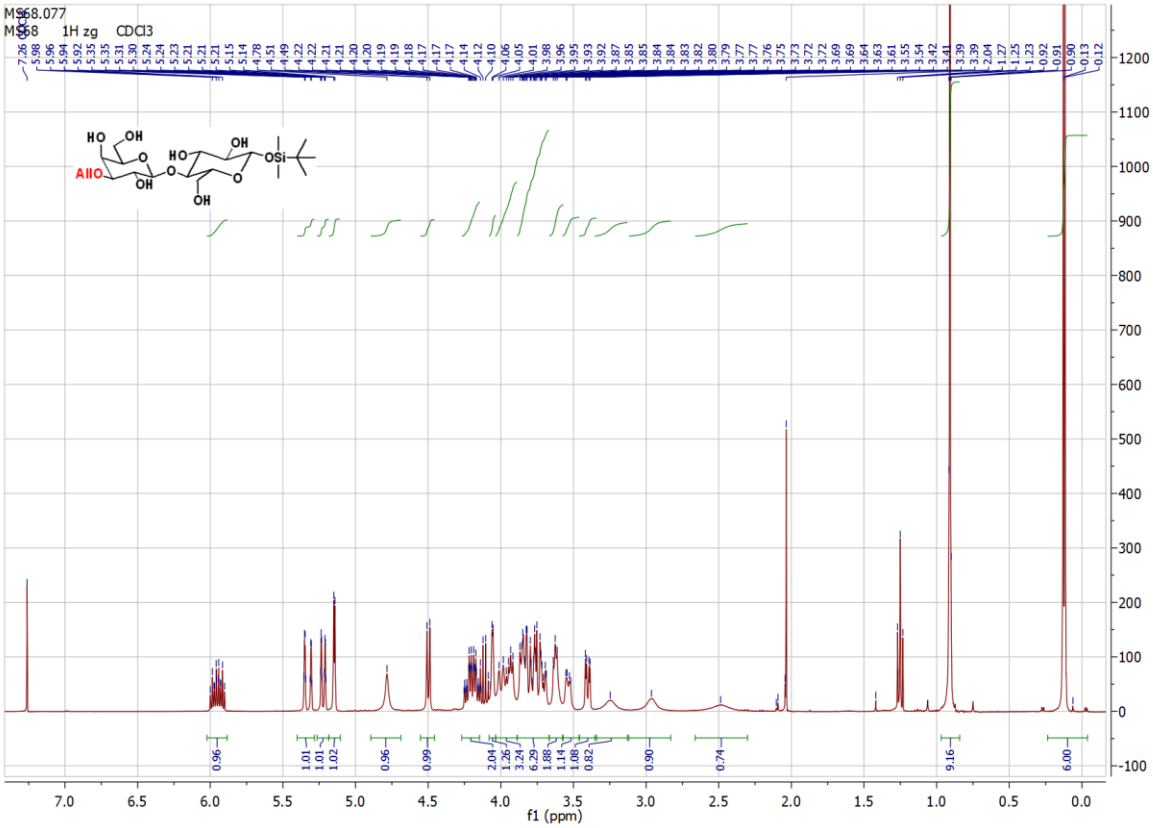
^{13}C NMR of compound 21



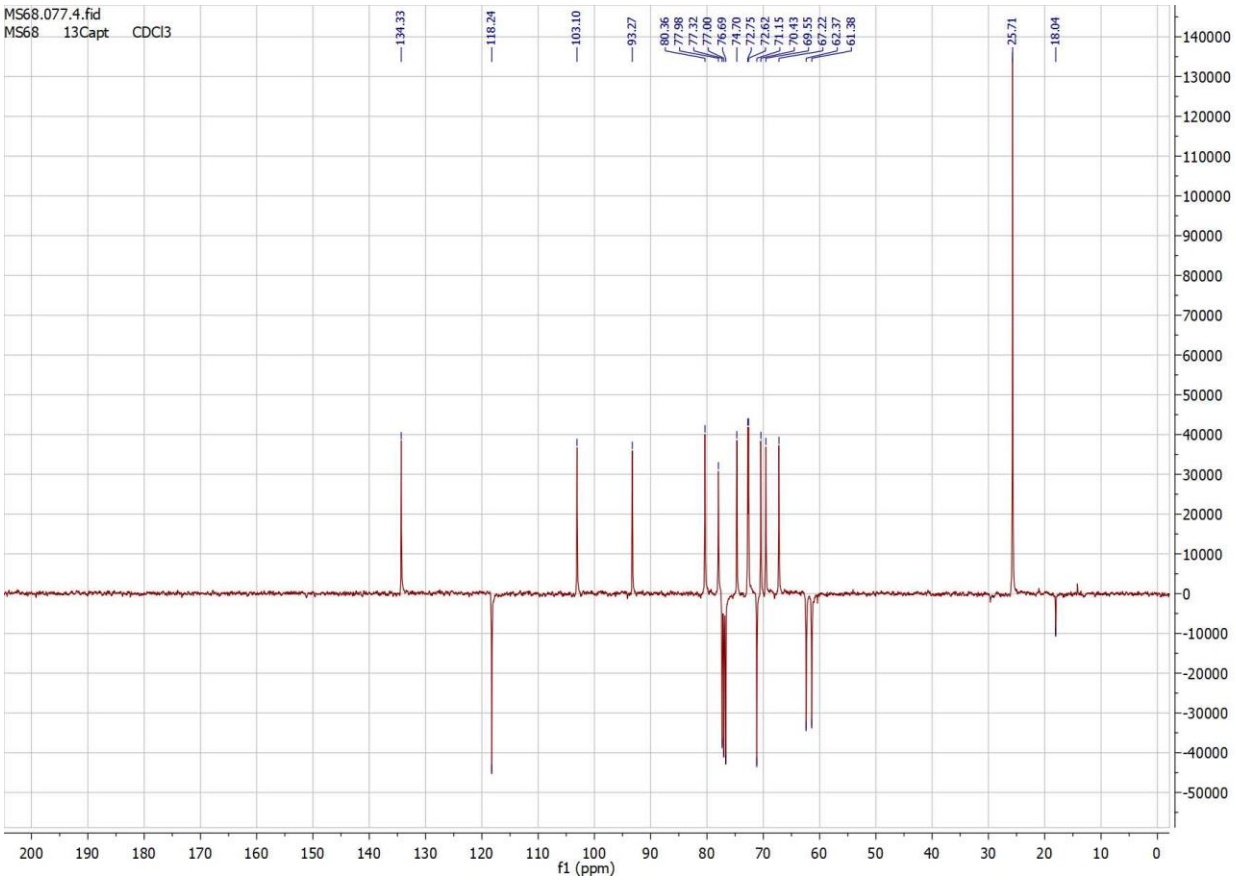
NMR Spectra of compound 22



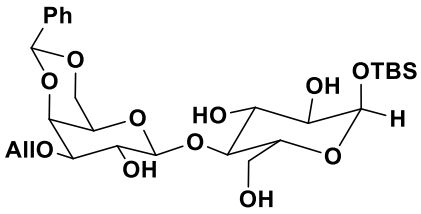
^{1}H NMR of compound 22



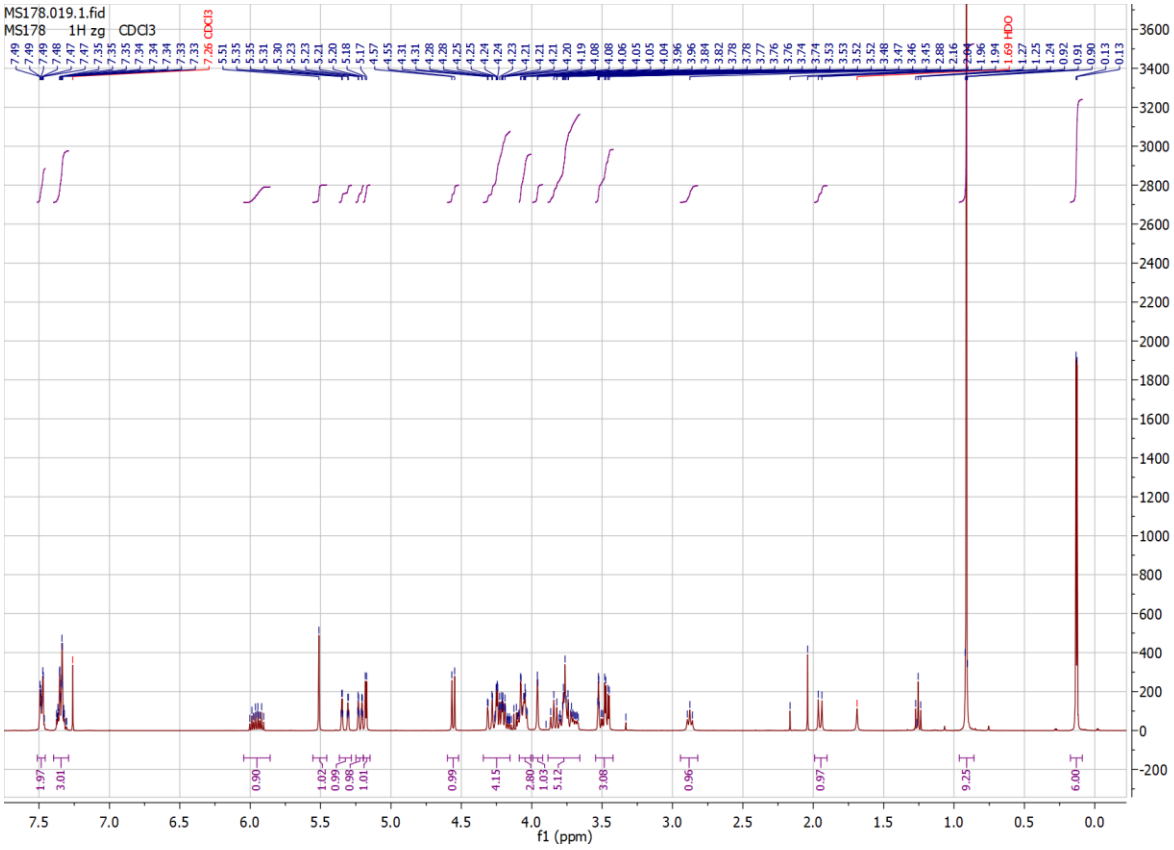
^{13}C NMR of compound 22



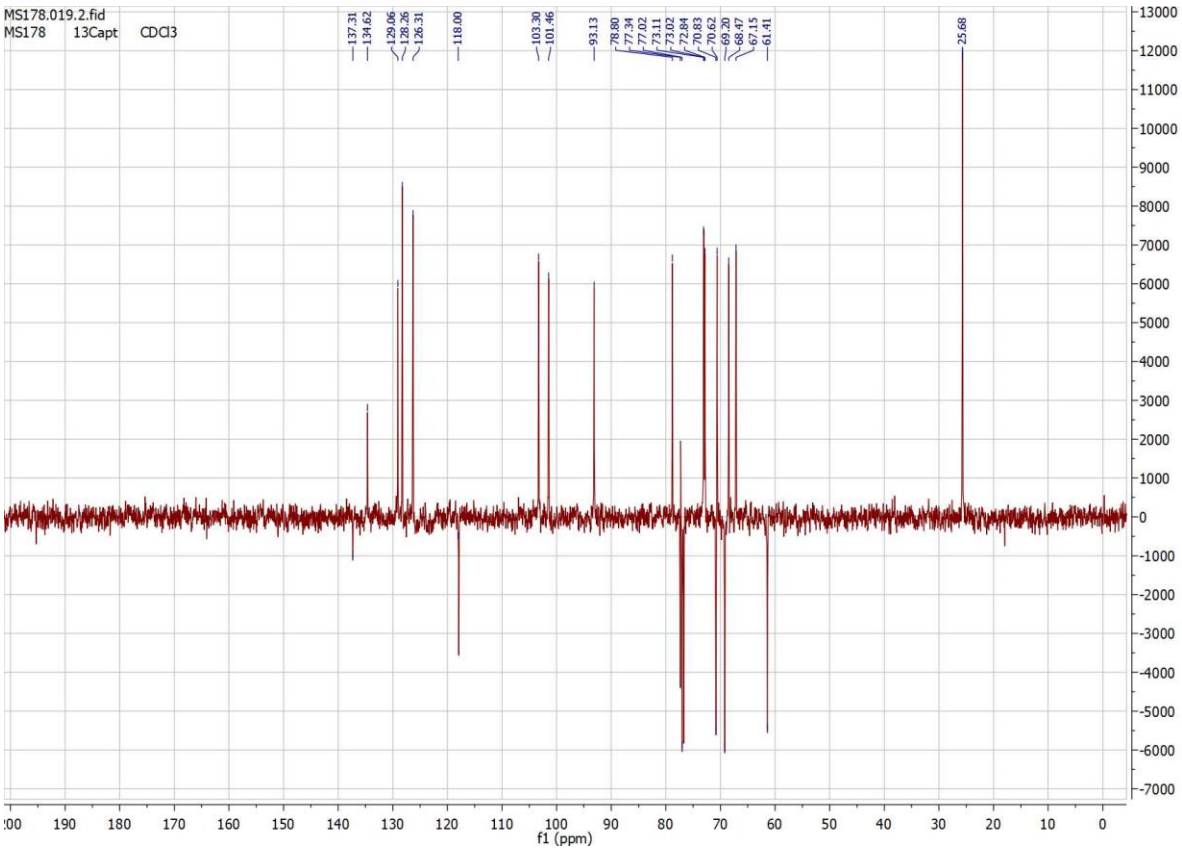
NMR Spectra of compound 23



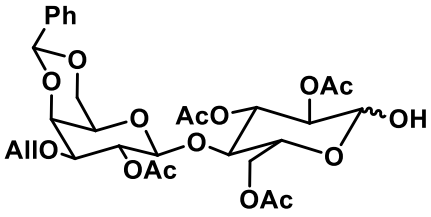
^{1}H NMR of compound 23



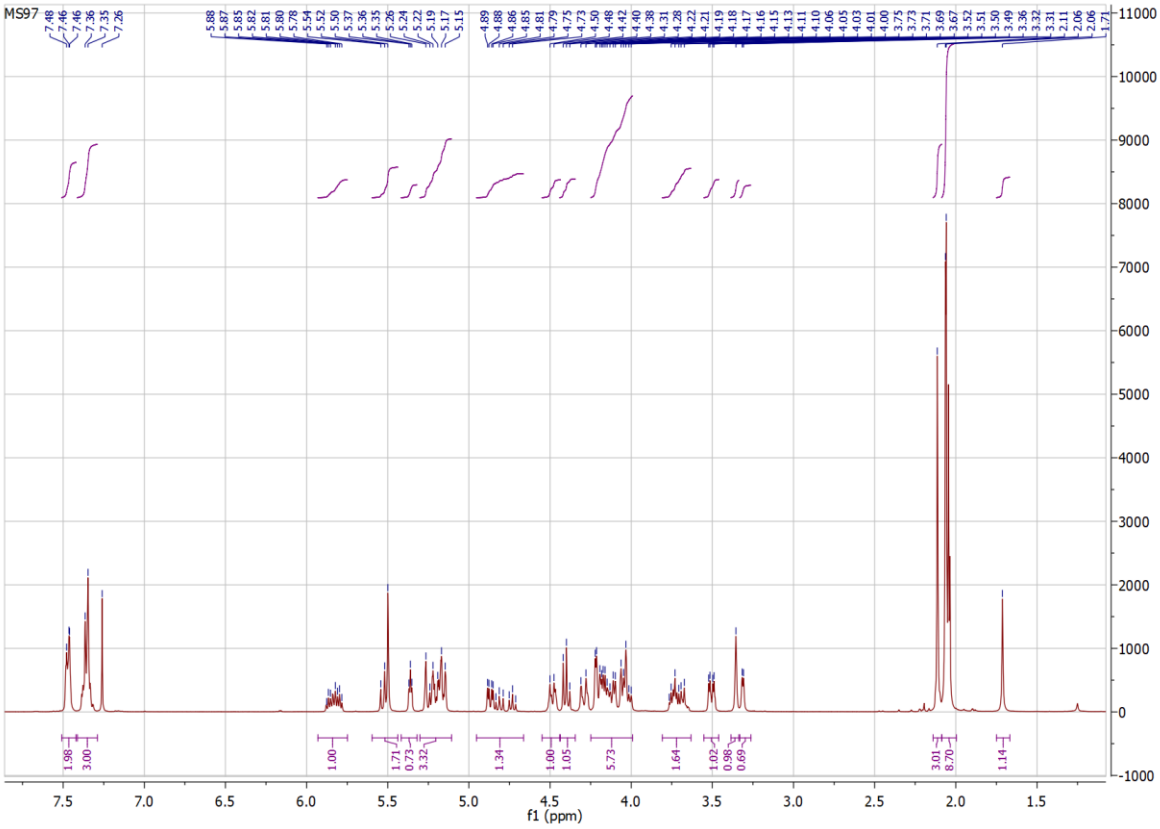
^{13}C NMR of compound 23



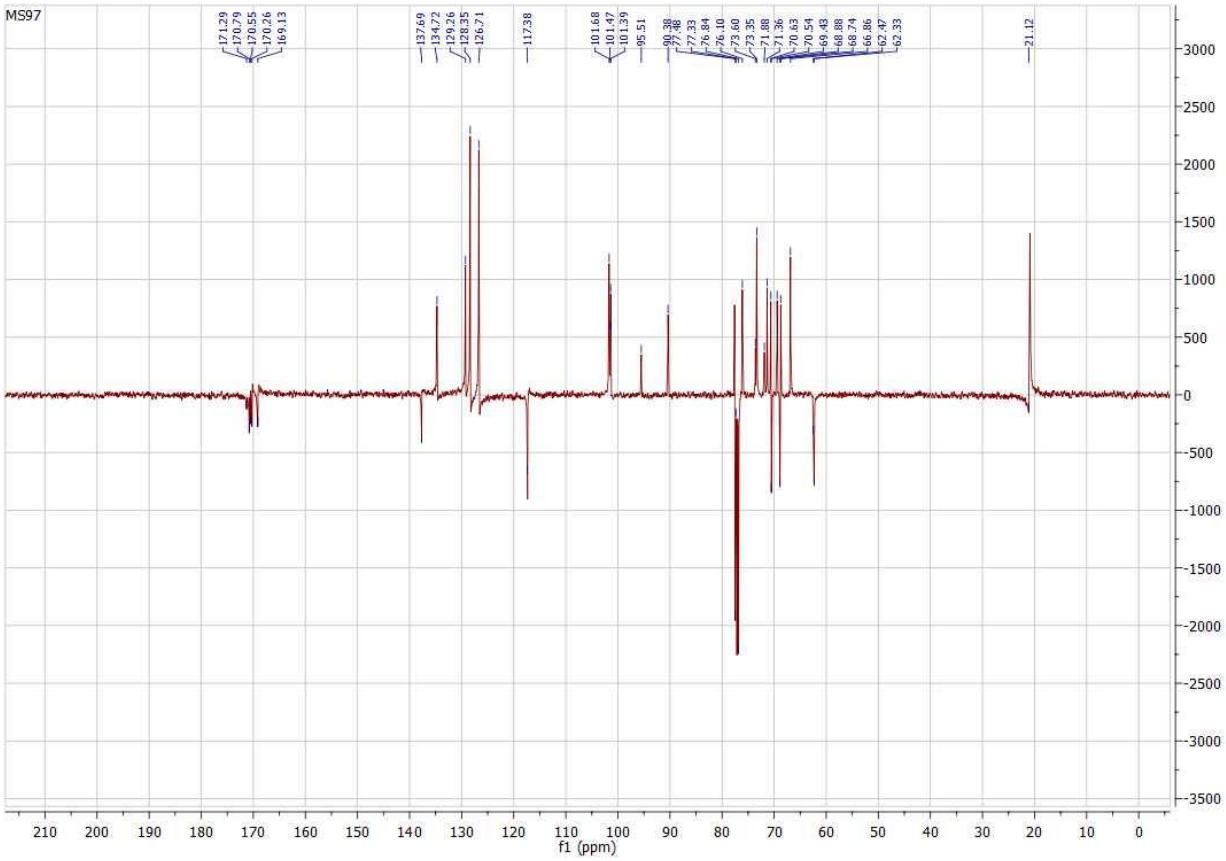
NMR Spectra of compound 39



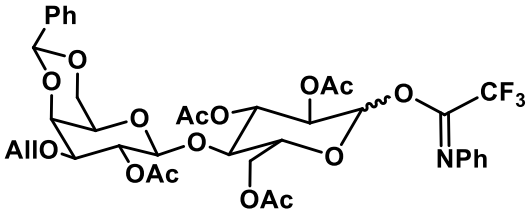
^{1}H NMR of compound 39



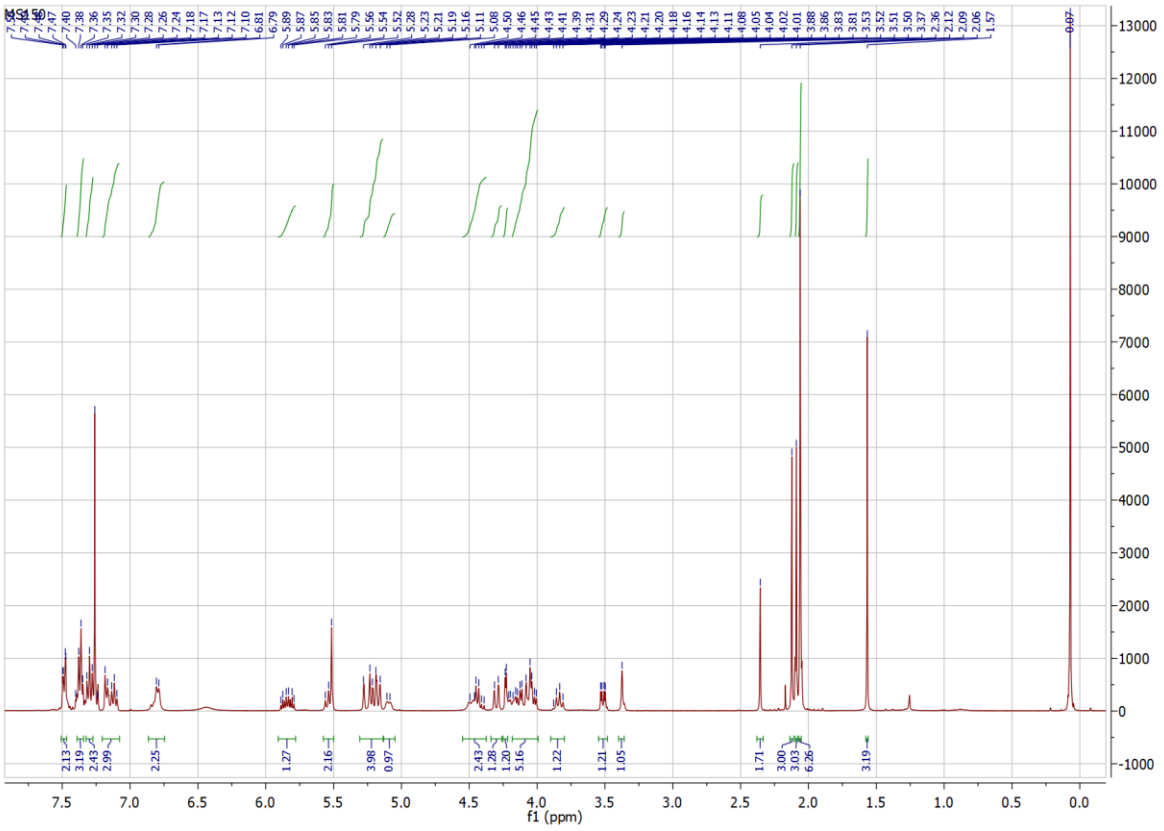
^{13}C NMR of compound 39



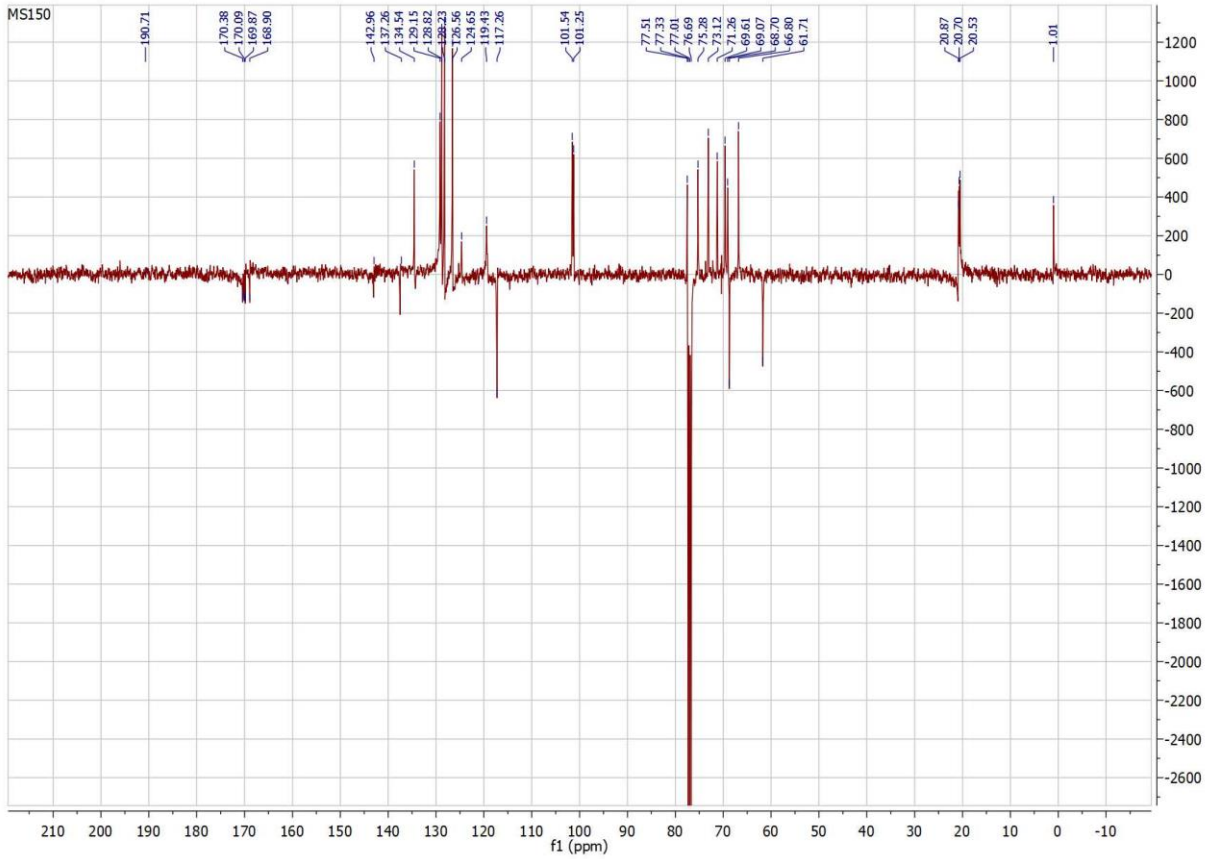
NMR Spectra of compound 41



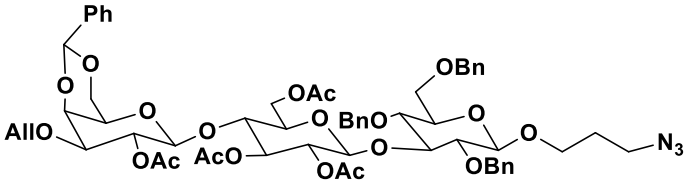
^{1}H NMR of compound 41



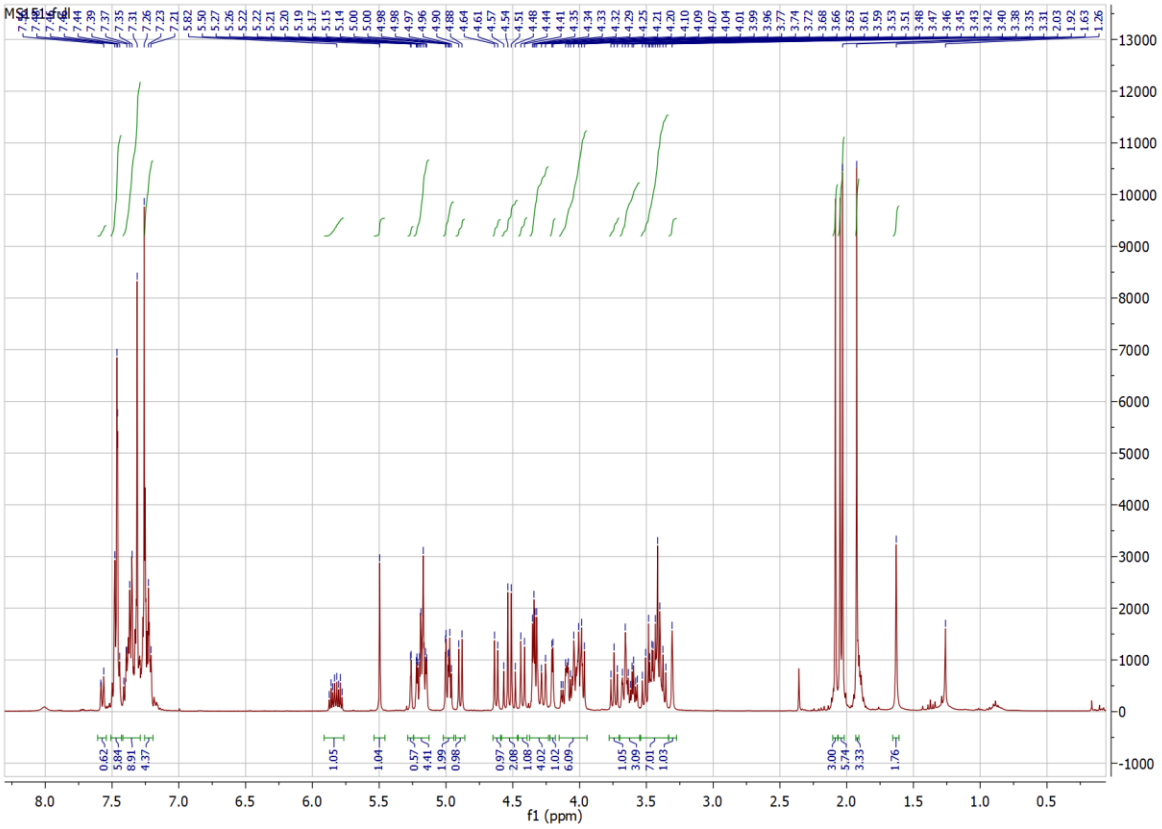
^{13}C NMR of compound 41



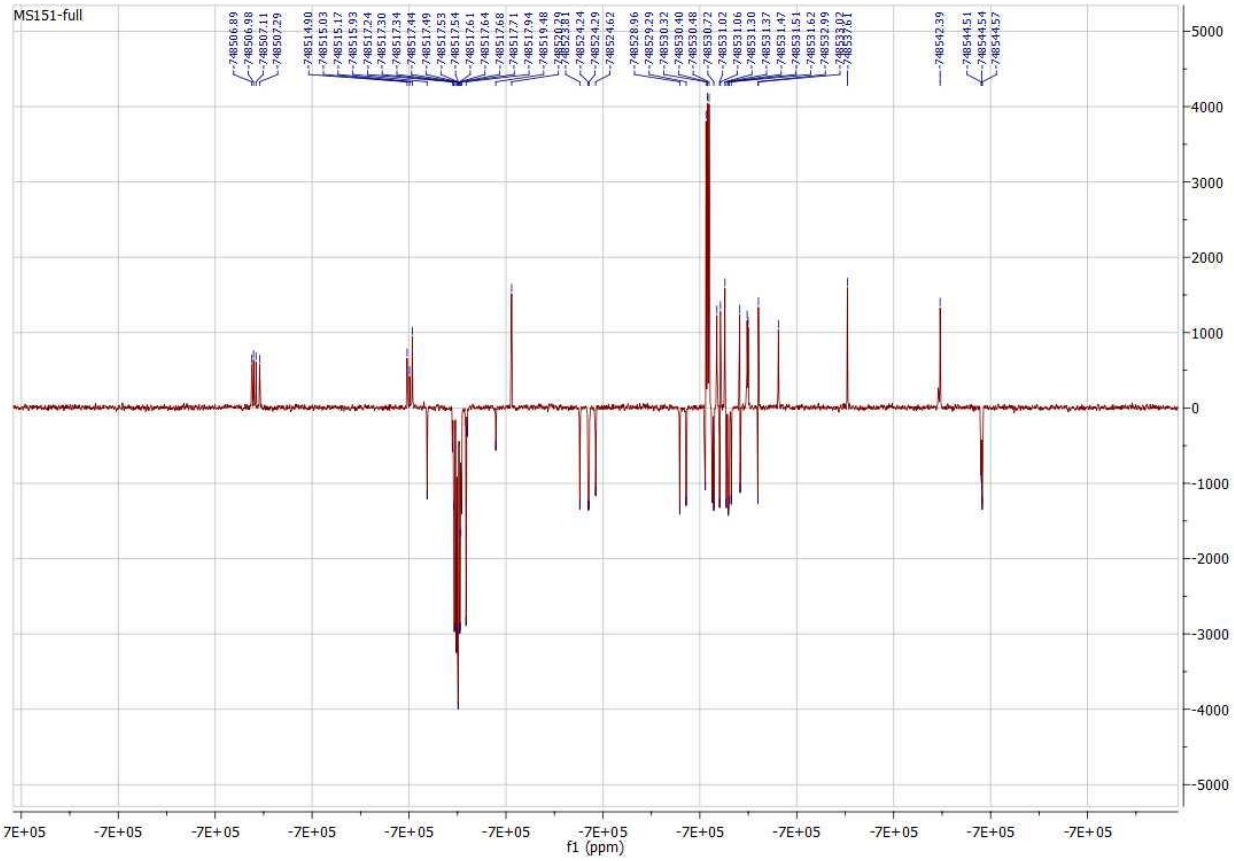
NMR Spectra of compound 5



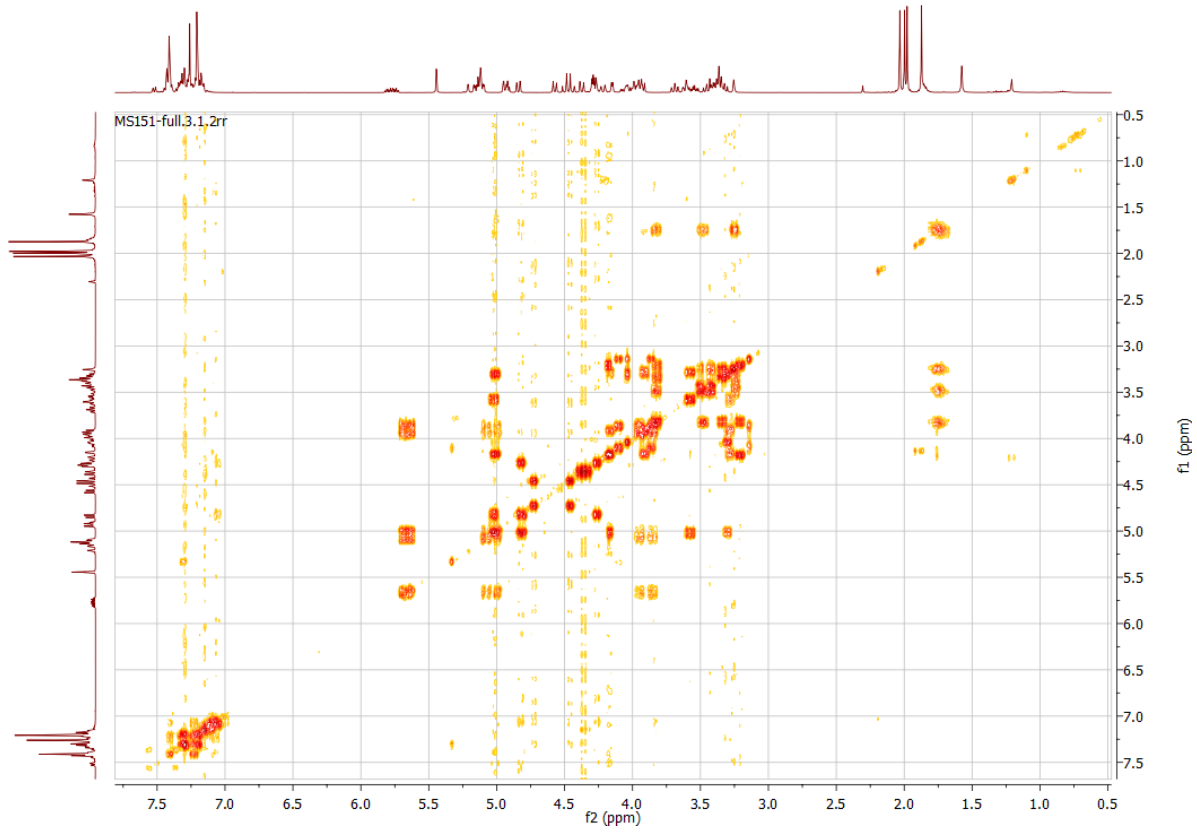
^{1}H NMR of compound 5



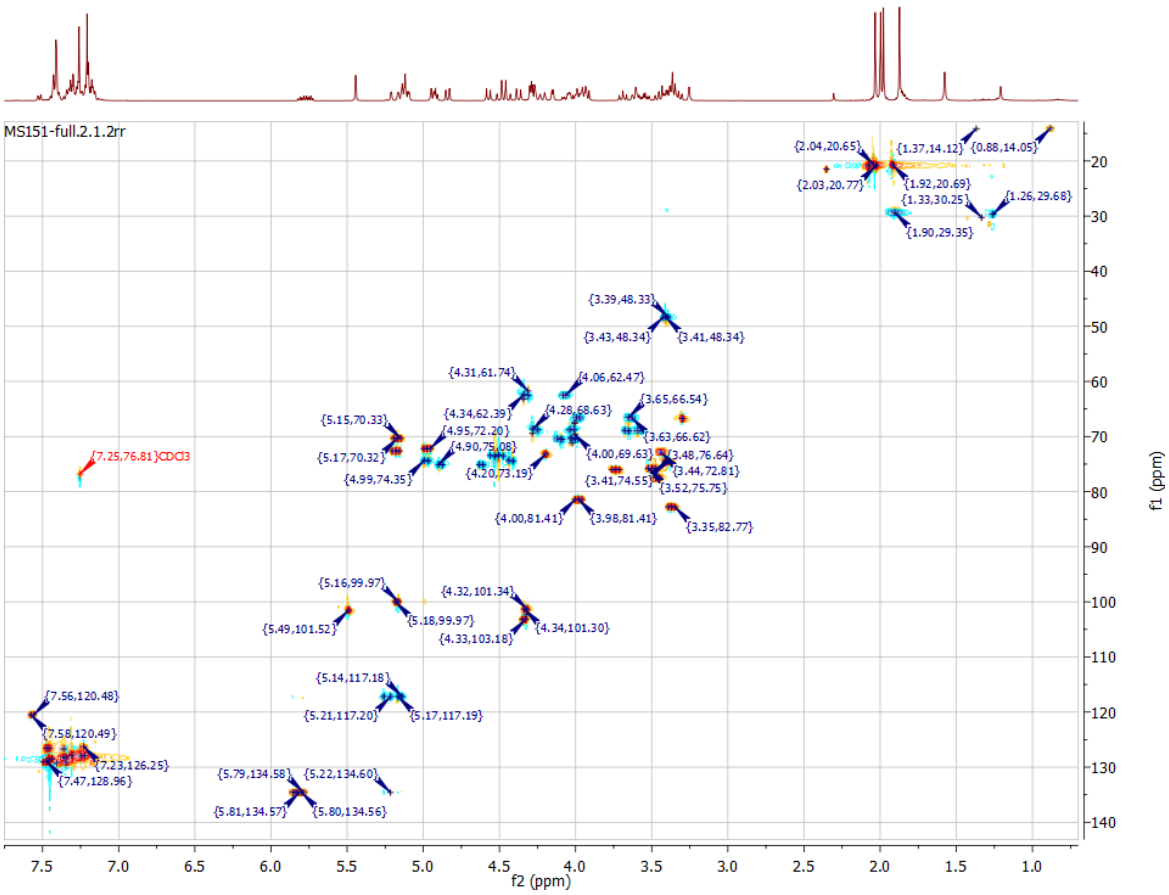
^{13}C NMR of compound 5



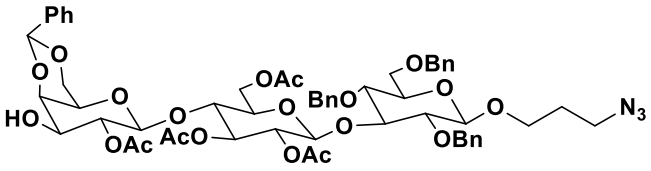
^{1}H-^{1}H COSY NMR of compound 5



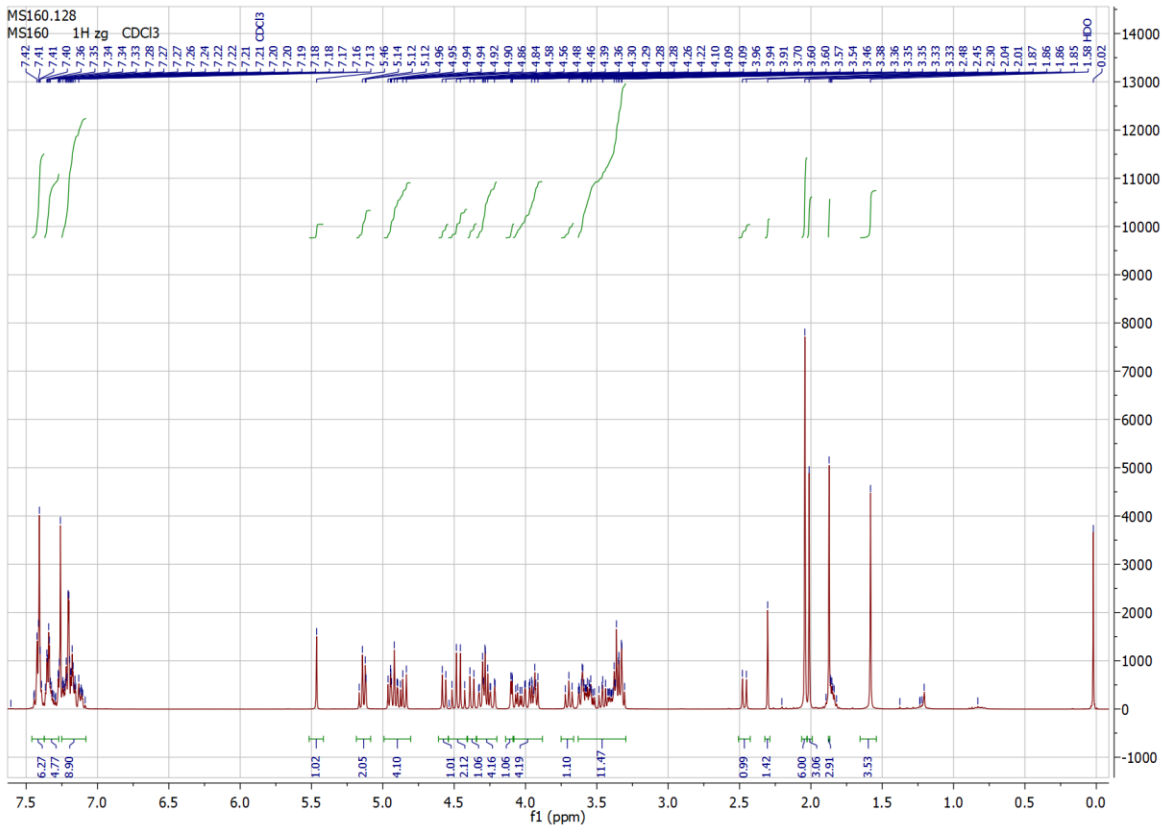
^{1}H-^{13}C HSQC NMR of compound 5



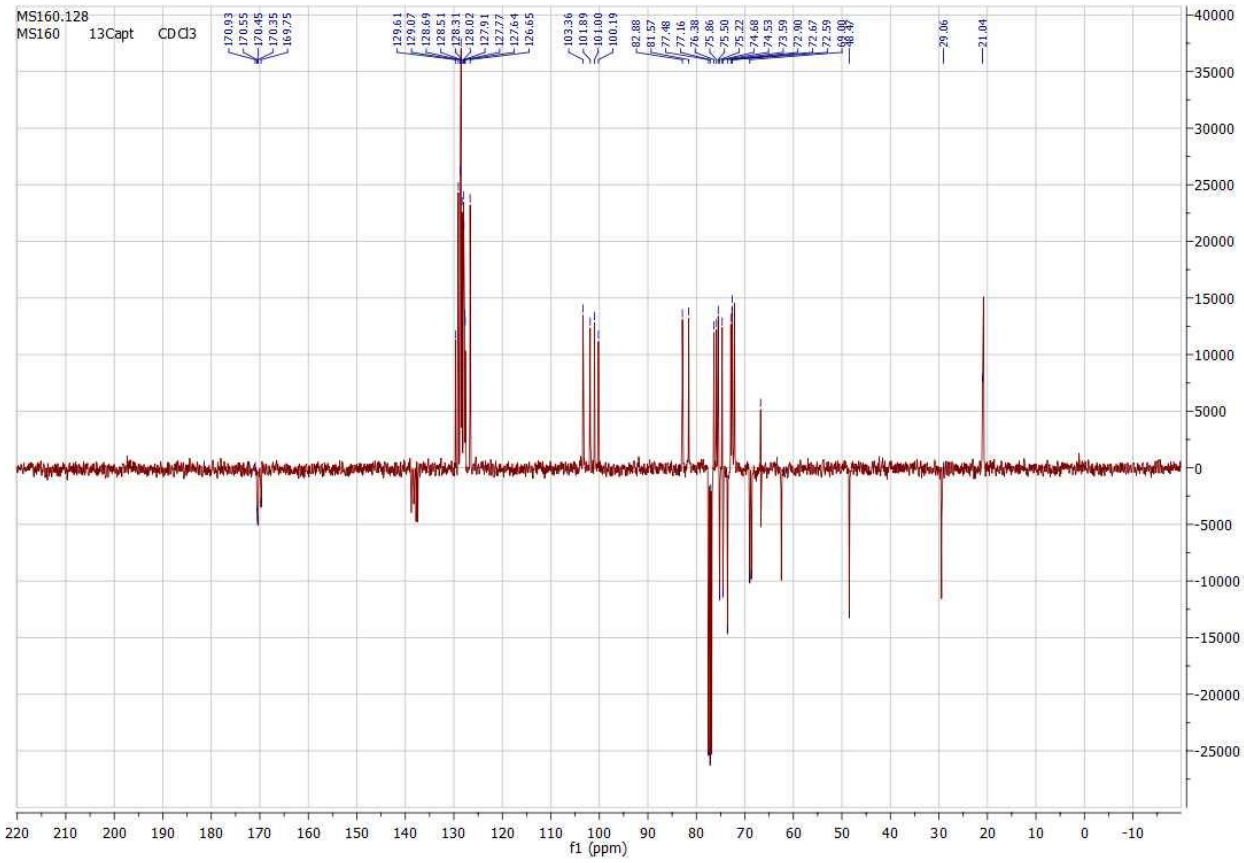
NMR Spectra of compound 42



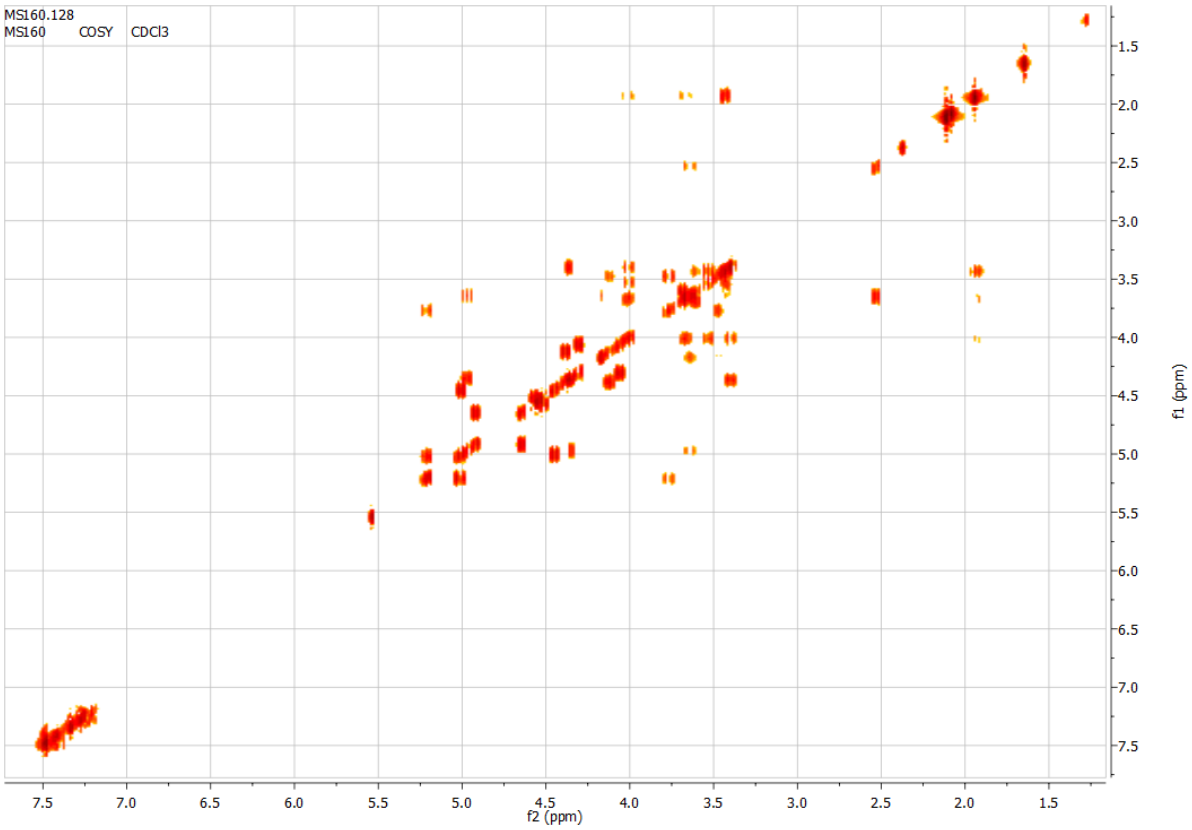
^{1}H NMR of compound 42



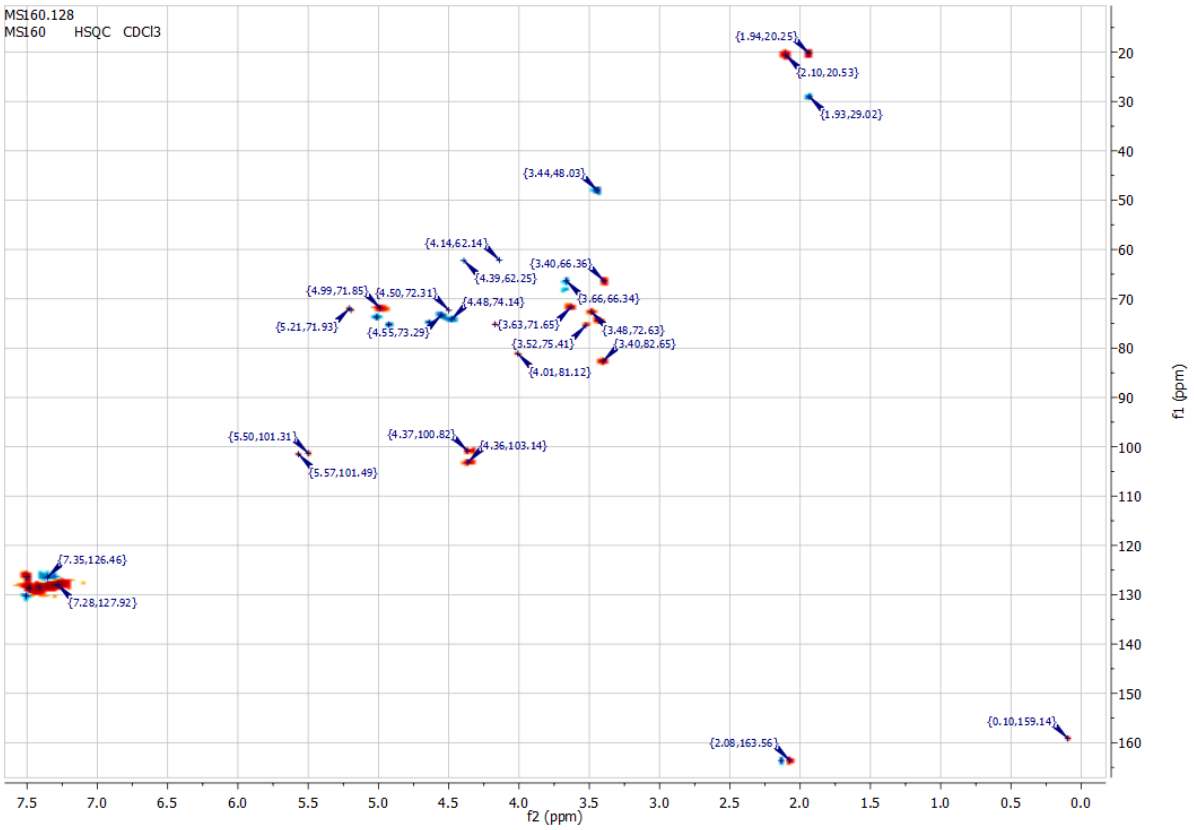
^{13}C NMR of compound 42



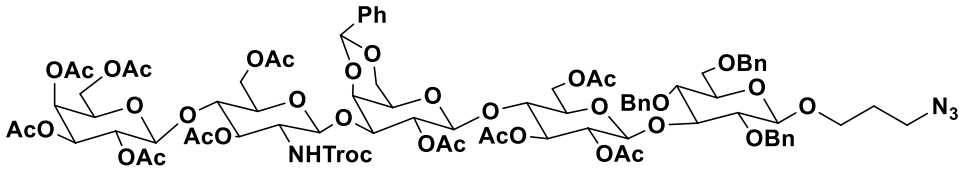
^{1}H-^{1}H COSY NMR of compound 42



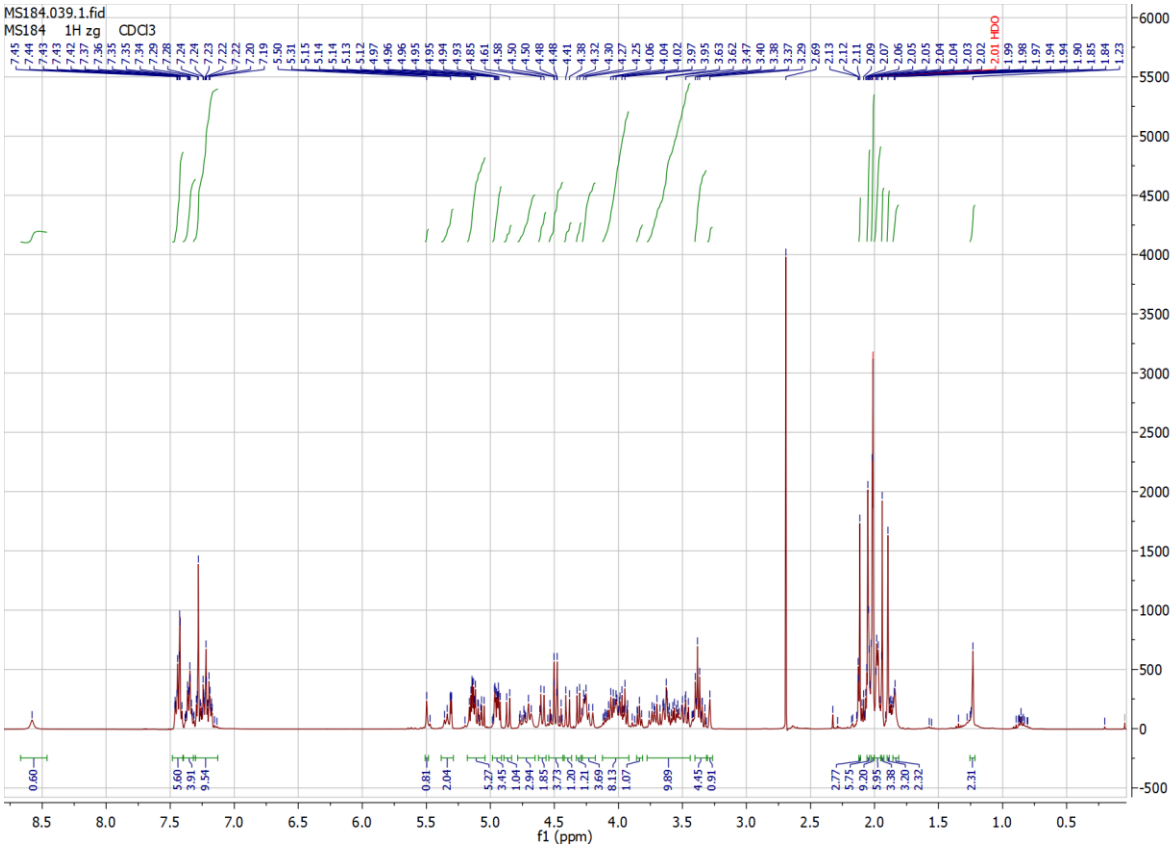
^{1}H-^{13}C HSQC NMR of compound 42



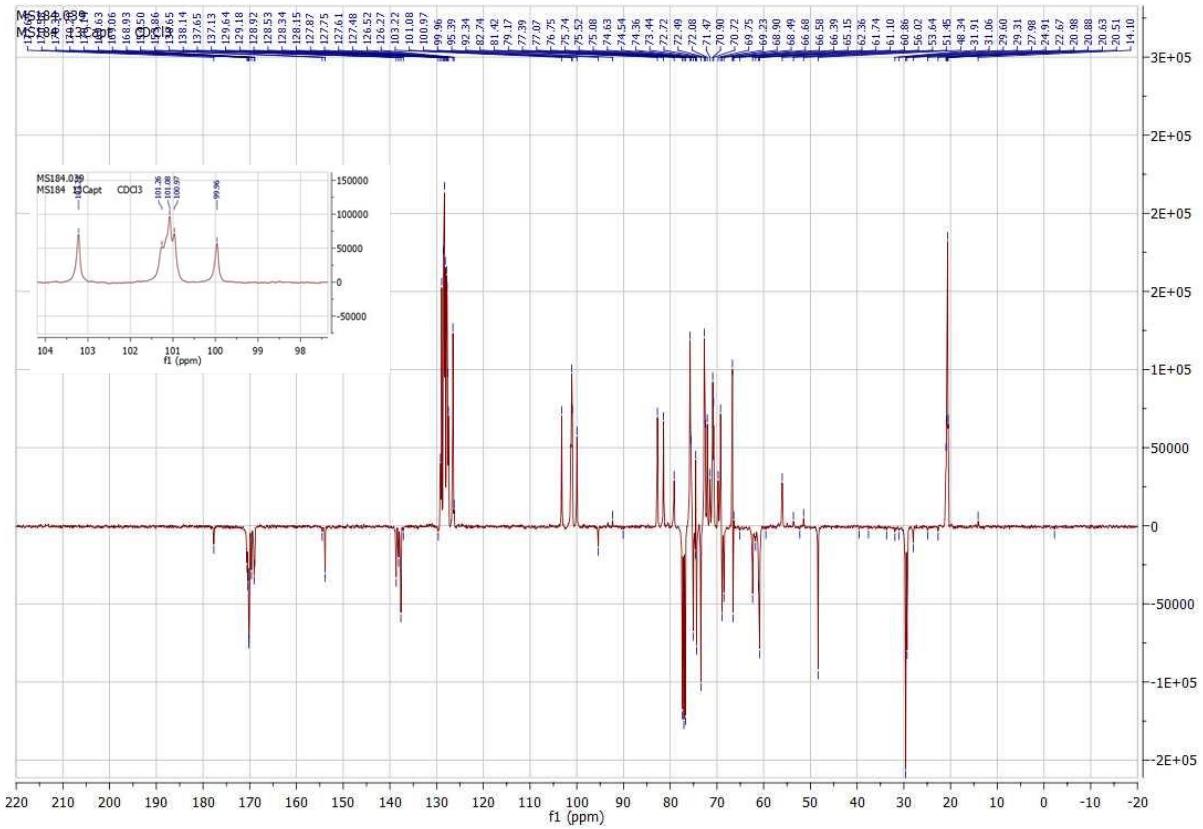
NMR Spectra of compound 4



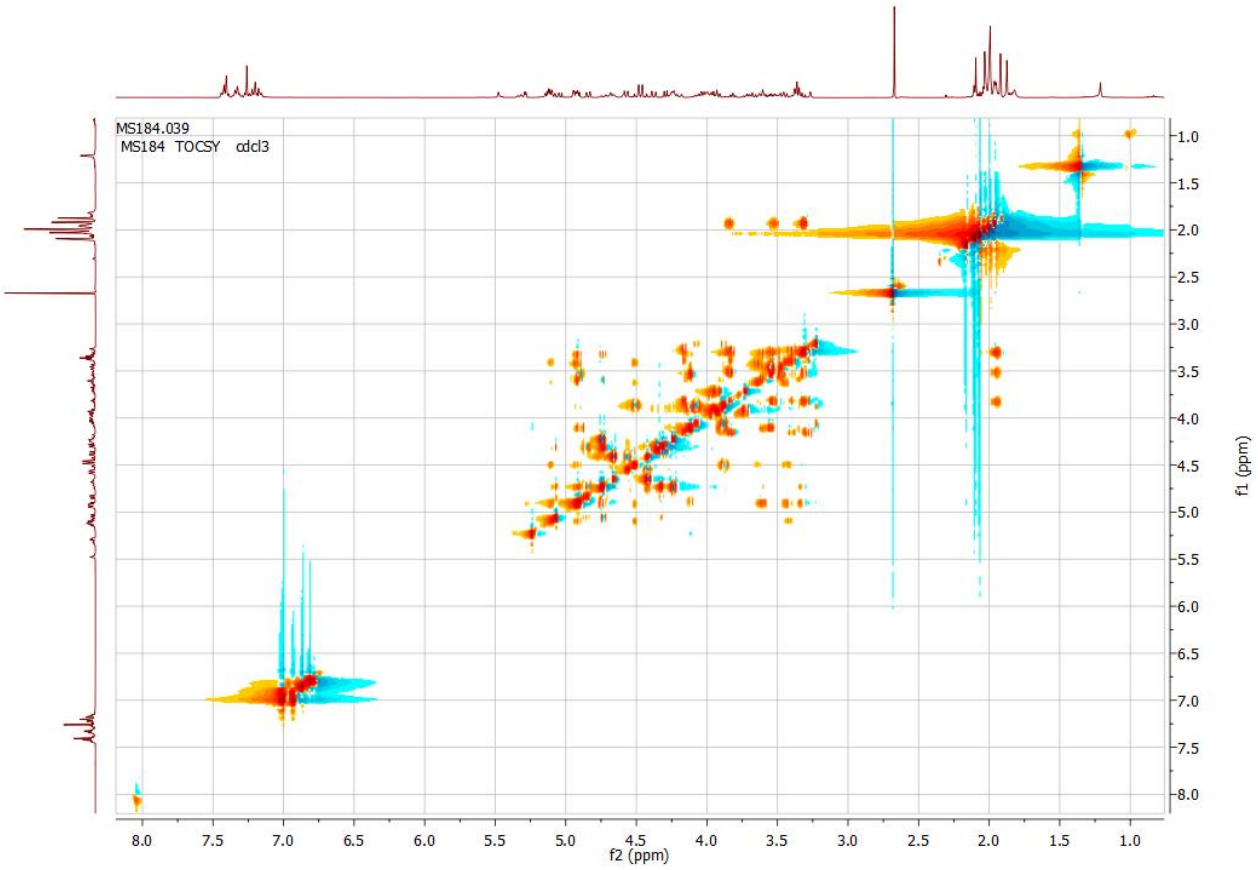
^{1}H NMR of compound 4



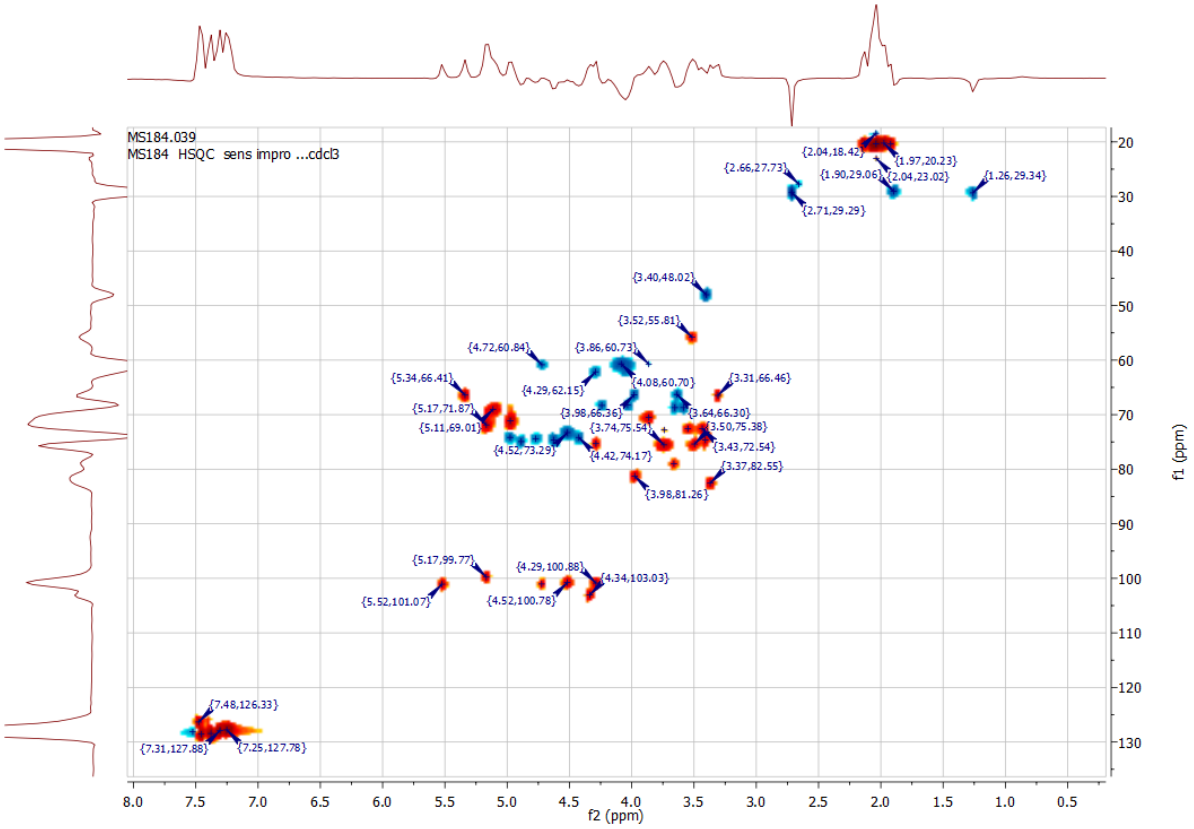
^{13}C NMR of compound 4



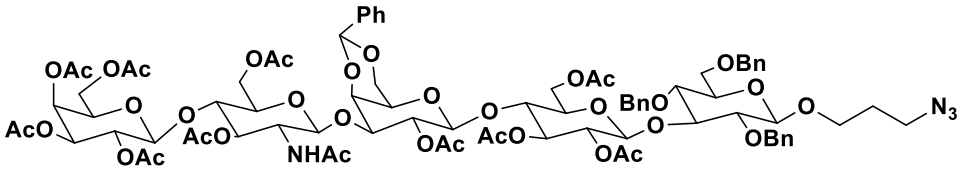
^{1}H-^{1}H TOCSY NMR of compound 4



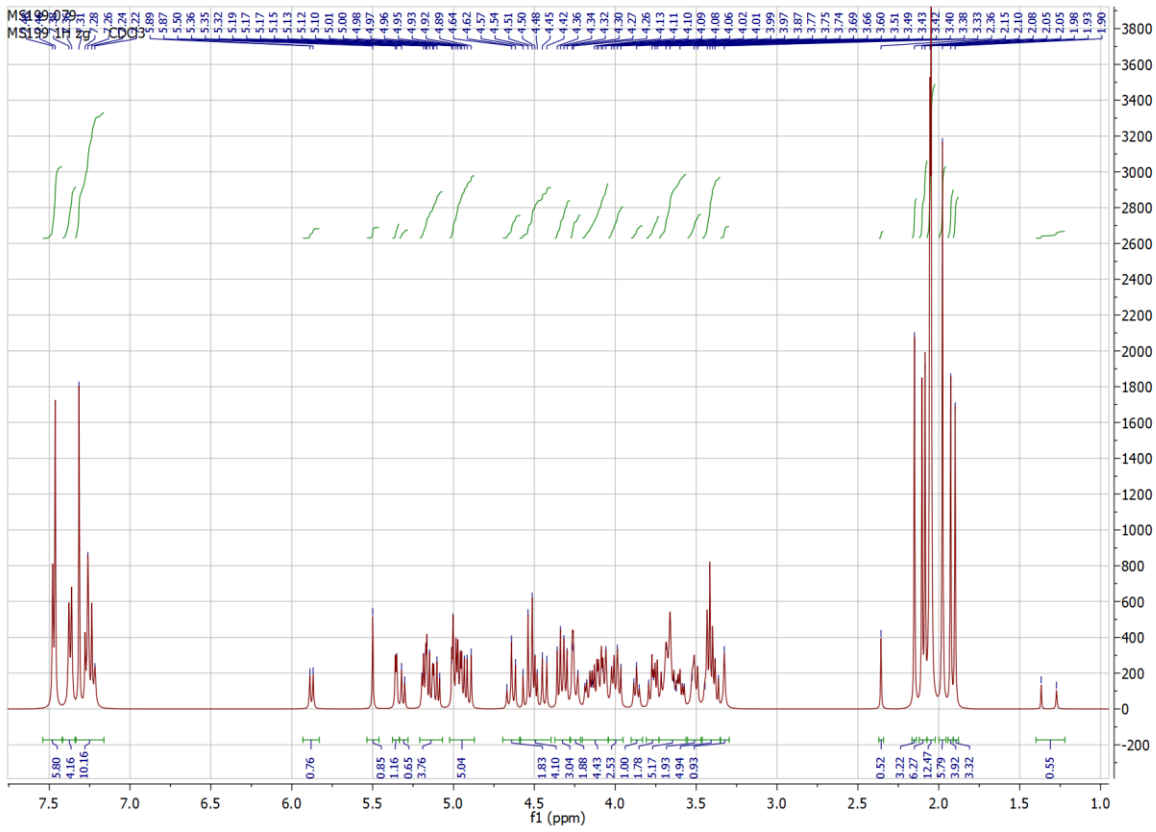
^{1}H-^{13}C HSQC NMR of compound 4



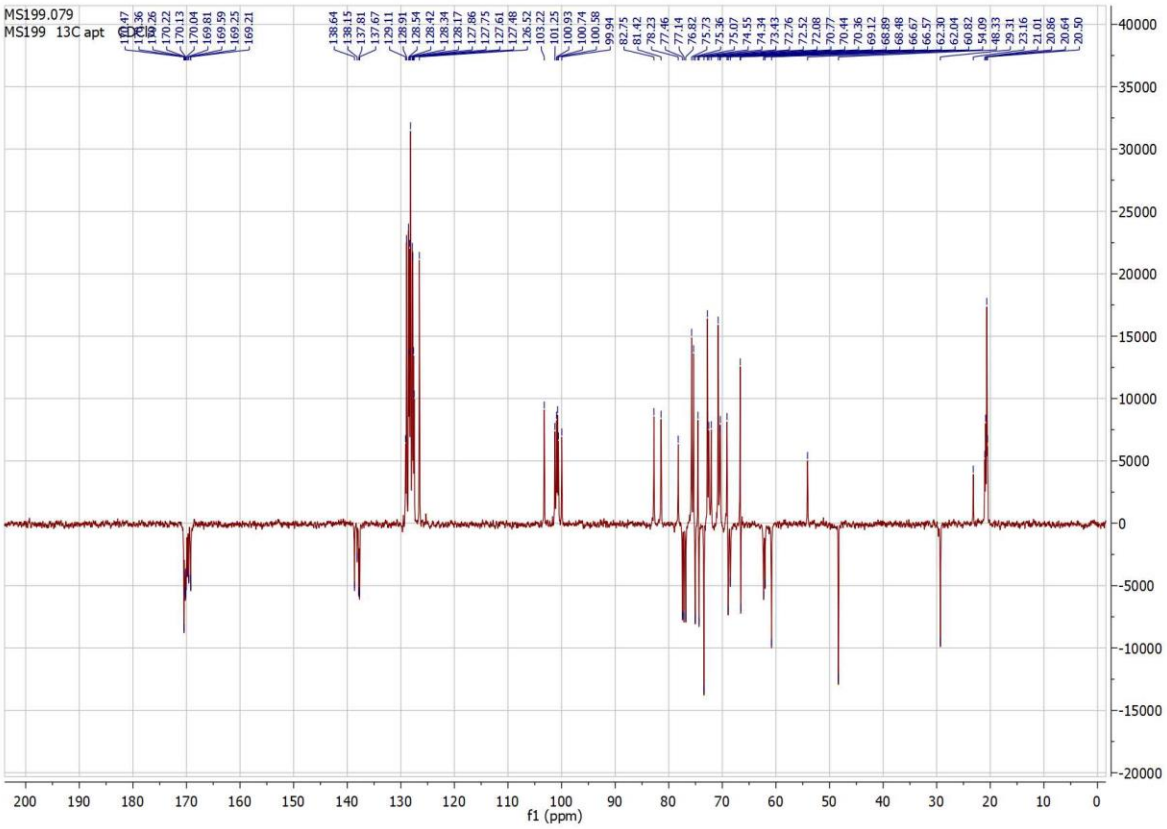
NMR spectra of compound 43



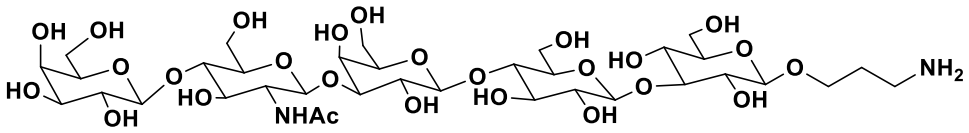
^{1}H NMR of compound 43



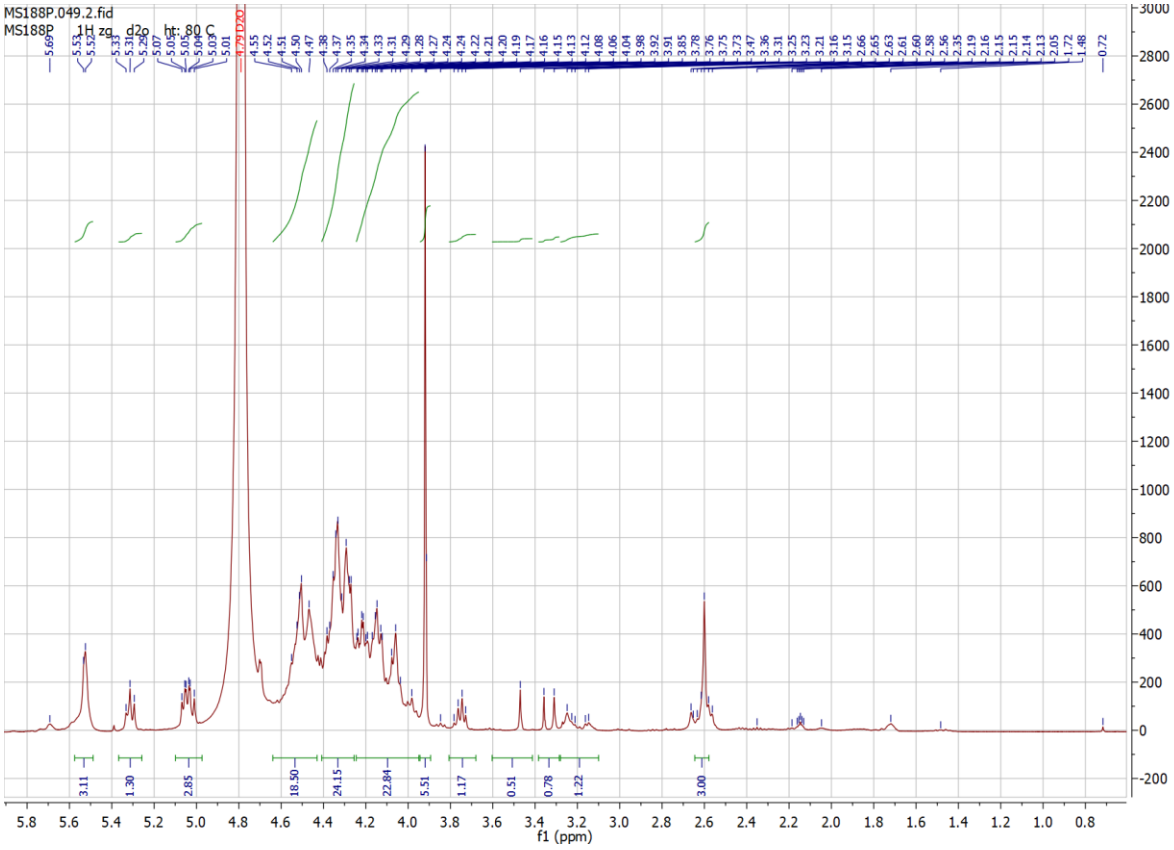
^{13}C NMR of compound 43



NMR Spectra of compound 44

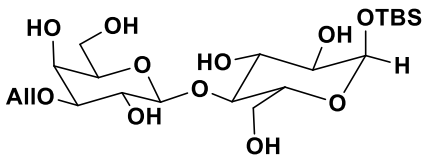


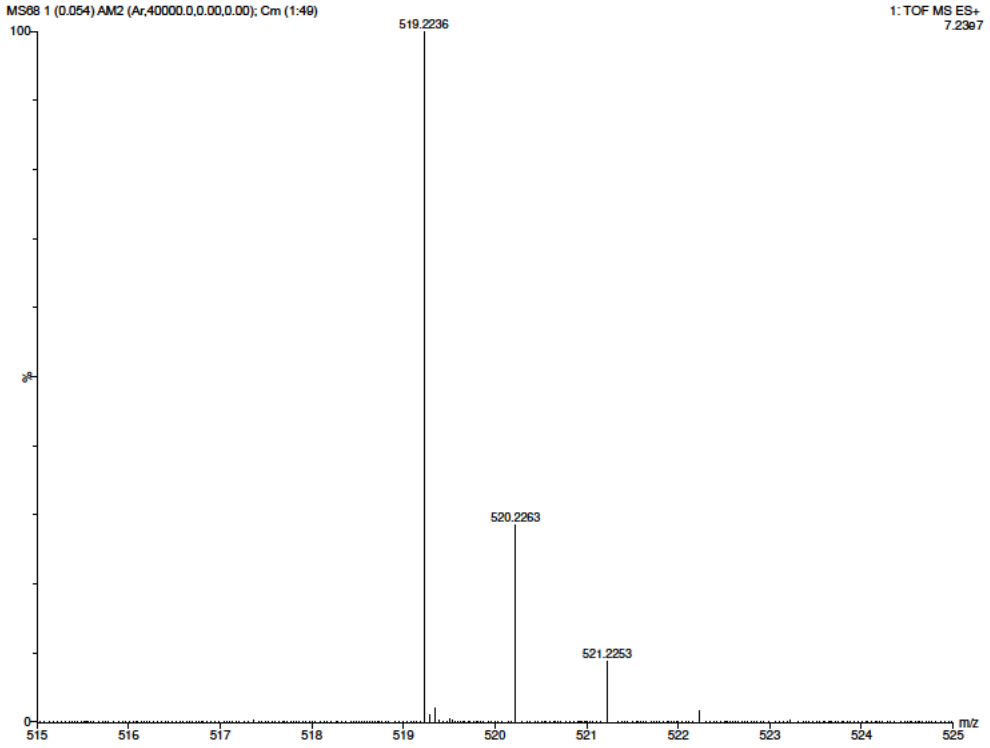
^{1}H NMR of compound 44



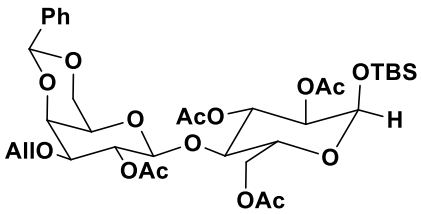
5.4 Mass Spectra

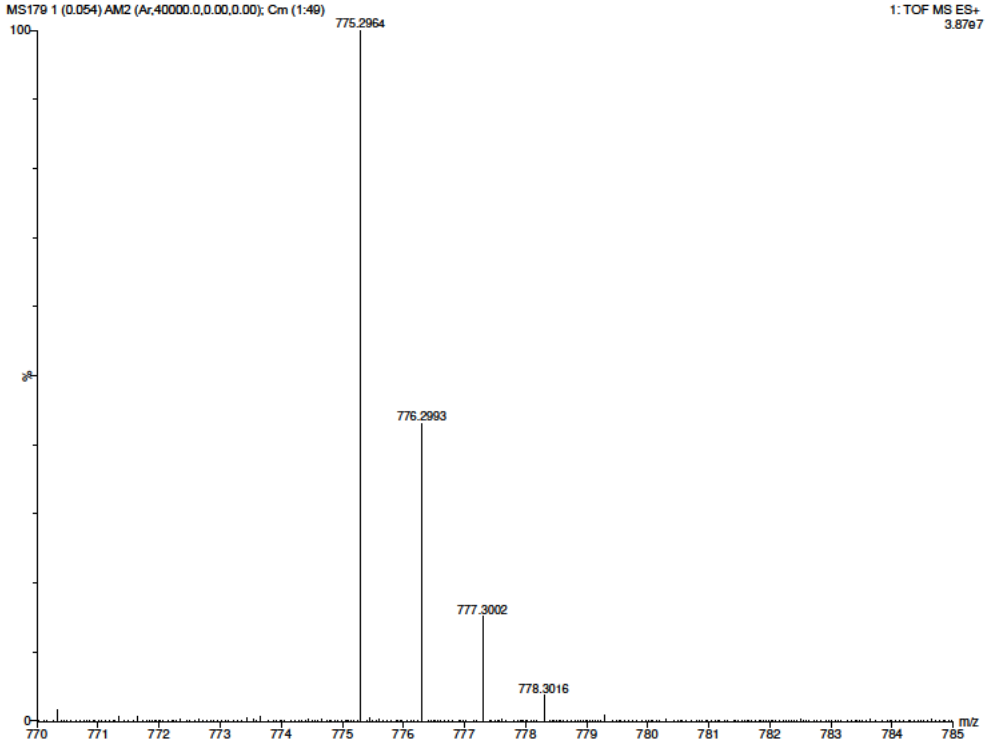
HRMS of compound 22



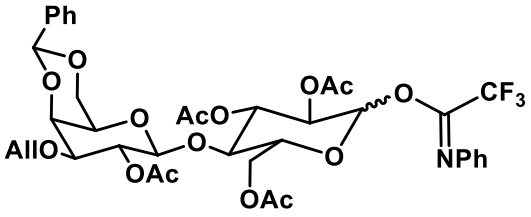


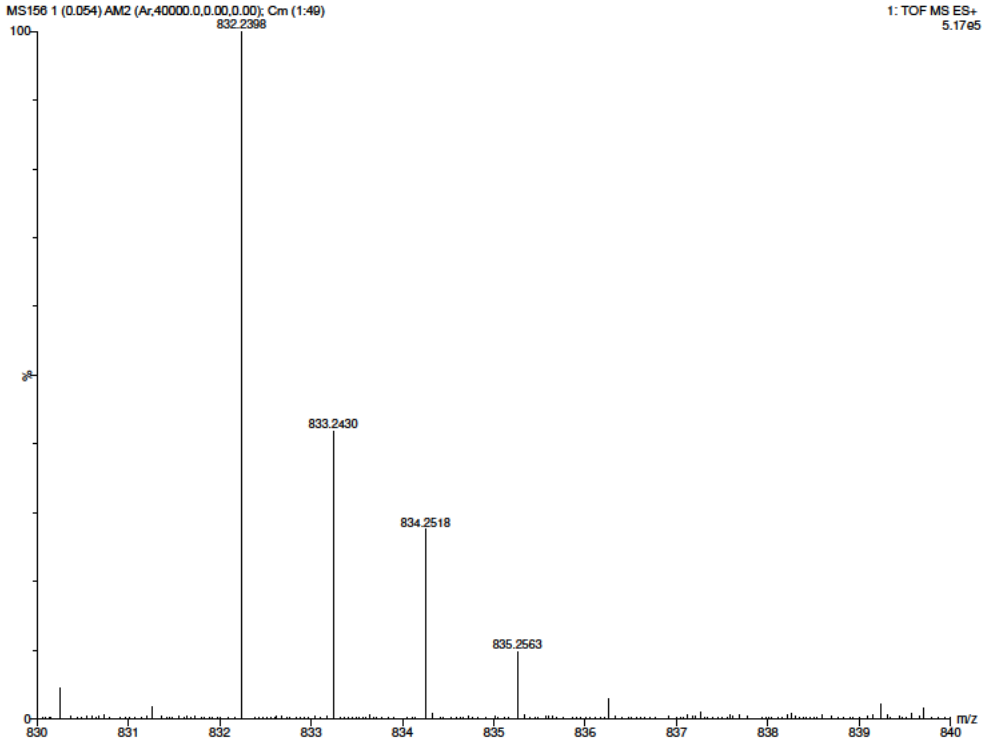
HRMS of compound 24



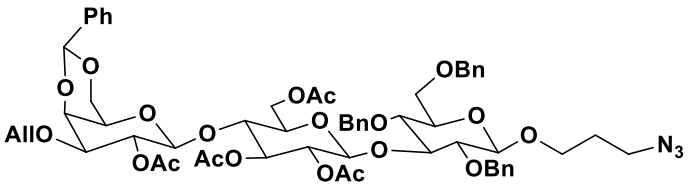


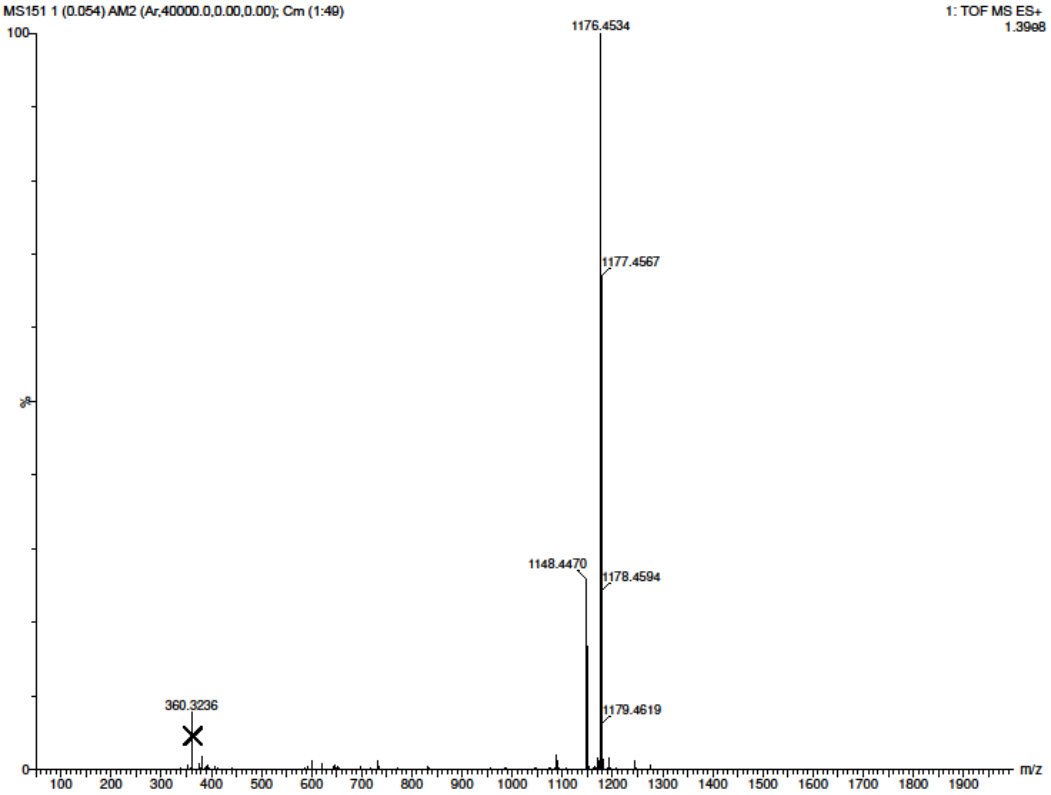
HRMS of compound 41



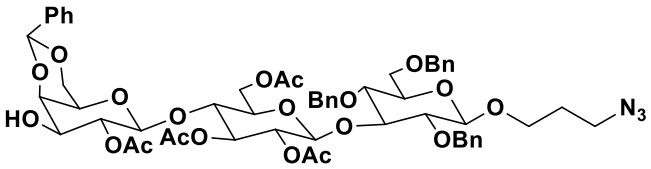


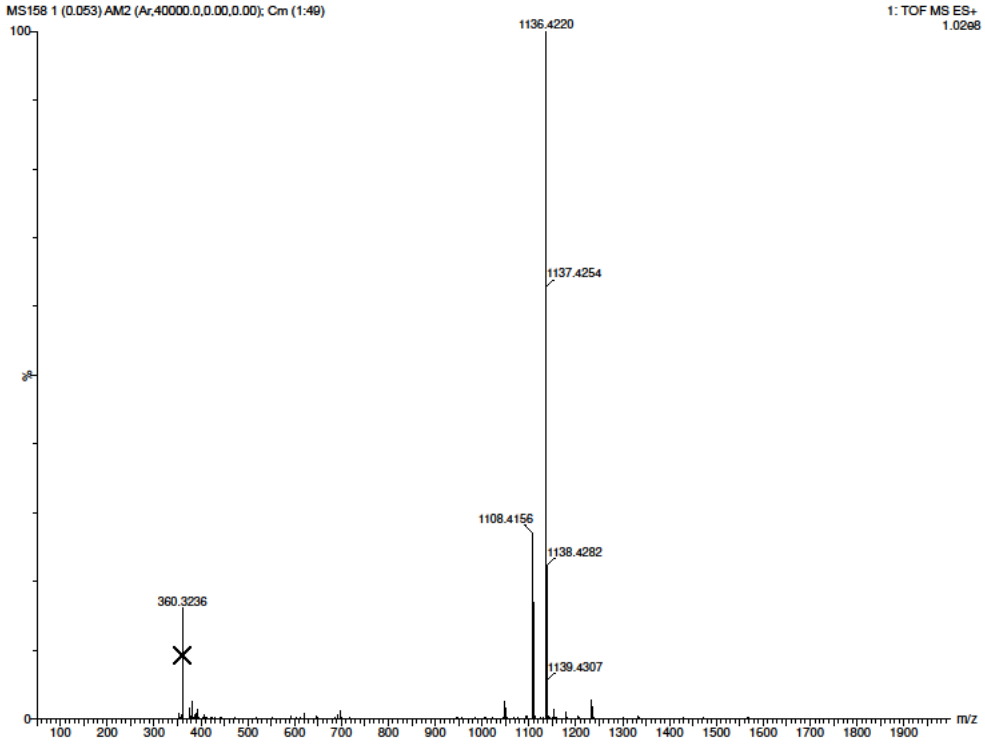
HRMS of compound 5



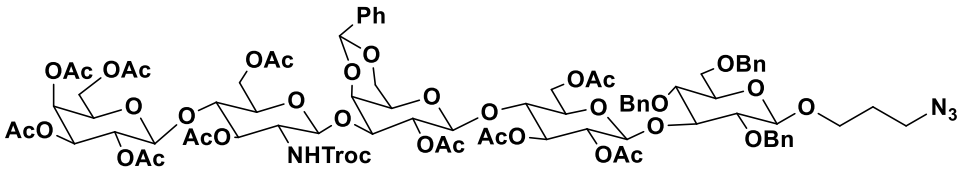


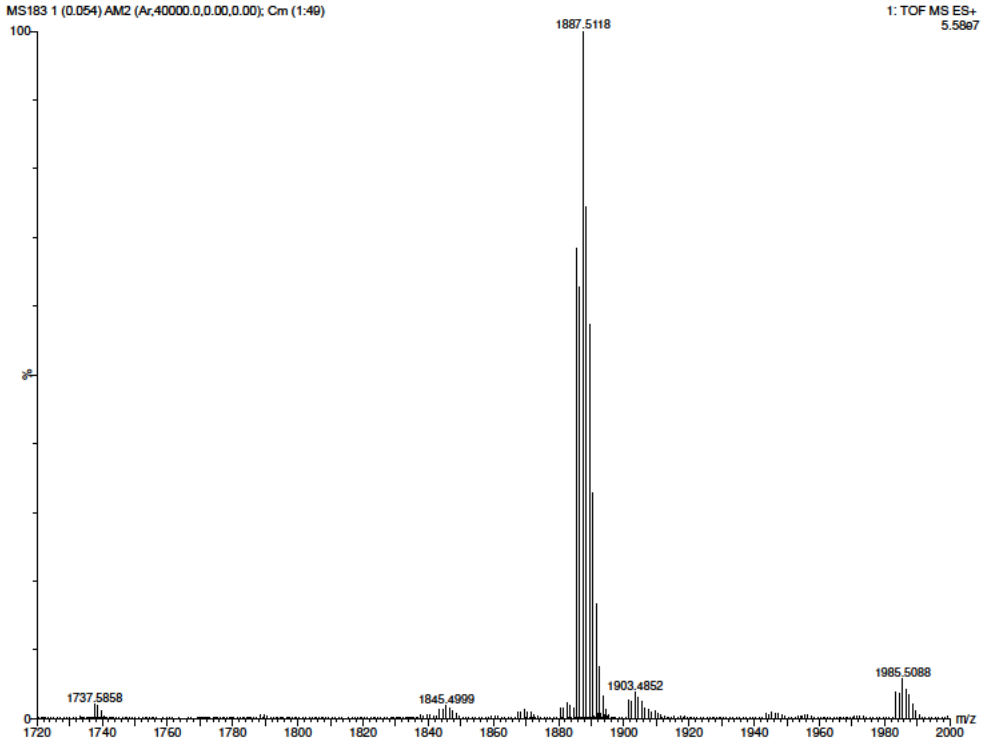
HRMS of compound 42





HRMS of compound 4





5.5 References

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