Syntheses of Carbohydrate Antigens Expressed by Gastric-intestinal Bacteria and Conjugates Thereof

by

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Diarrhea is one of the most common and debilitating ailments in the world and gastro-intestinal microorganisms are responsible for a majority of cases. This thesis describes the creation of synthetic carbohydrate conjugate vaccines against two key diarrheal bacterial pathogens, *Clostridium difficile* (antibiotic-associated diarrhea) and *Campylobacter jejuni* (food-borne traveller’s diarrhea).

The synthesis of a conjugate containing two *C. difficile* antigens, the cell surface PS-I pentasaccharide repeating block, $\alpha$-L-Rhap-(1→3)-β-D-GlcP-(1→4)-[$\alpha$-L-Rhap-(1→3)]-α-D-GlcP-(1→2)-α-D-GlcP-(1→O(CH$_2$)$_5$NH$_2$, and a subunit peptide of exotoxin B (ToxB), the 248–262 fragment of the N-terminal NGESFNLYEQELVER, was achieved. Immunodetection studies using the aforementioned synthetic PS-I revealed that sera of healthy horses contained anti-PS-I IgG antibodies. This conjugate represents the first dual antigen *C. difficile* vaccine that has the potential to control colonization and disease by targeting a surface antigen and an exotoxin simultaneously.

In the past, we discovered that *C. jejuni* surface capsule polysaccharides (CPSs) are decorated with *O*-methyl-phosphoramidates (MeOPN) that serve as key structural markers responsible for *C. jejuni* serotype classification. Our previous *C. jejuni* studies
have also led to an efficacious CPS conjugate vaccine that is now in phase 1 human clinical trials. The immunization experiments with native *C. jejuni* CPS-based conjugates also revealed that the MeOPN-glycan linkages were highly immunogenic. Thus, we envisaged that conjugates rich in synthetic MeOPN-CPS epitopes could perhaps make more efficacious anti-*C. jejuni* conjugate vaccines. To this end, the main body of this thesis teaches the chemical syntheses of MeOPN-sugar antigens exposed by serotypes HS:23/36 and HS:1. The synthetic MeOPN-monosaccharide units were shown to react with *C. jejuni* whole-cell antisera, which indicated the presence of antibodies specific for these MeOPN-sugar linkages, especially against MeOPN→6-Gal. Interestingly, whole-cell sera raised by *C. jejuni* with CPSs with MeOPN at primary positions (C-6 of hexoses or C-7 of heptoses) were also seen to react with the synthetic MeOPN→6-Gal. Based on the observed heighten immunogenicity of MeOPN→6-Gal, a MeOPN→6-Gal-CRM$_{197}$ conjugate was synthesized, using an aminopentyl linker at C-1, and shown to be capable of raising antibodies against *C. jejuni* HS:23/36 cell surface with accompanying bactericidal activity. In order to increase the display of the low molecular weight MeOPN→6-Gal in a conjugate format, an approach involving the attachment of multiple MeOPN→6-Gal units to activated starch was also explored. The syntheses of *C. jejuni* CPS-associated MeOPN glycan antigens described in this thesis represent a significant advancement in the development of a next generation *C. jejuni* synthetic vaccine that in time may replace the native CPS-based vaccine.
Acknowledgement

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Abbreviations

Å angstrom
Ac acetyl
All allyl
aq aqueous
BCR B cell receptors
BF$_3$$\cdot$OEt$_2$ boron trifluoride etherate
Bn benzyl
Bz benzyol
BSA bovine serum albumin
C concentration
$^\circ$C degrees Celsius
CAN ceric ammonium nitrate
calcd calculated
CSA camphor sulphonic acid
Cbz carboxybenzyl
CDAD $C.\ difficile$-associated disease
COSY correlation spectroscopy
CPS capsular polysaccharides
CRM$_{197}$ cross-reactive-material 197
Da Dalton
CTL cytotoxic T lymphocytes
DBU 1,8-diazabicycloundec-7-ene
DMAP 4-dimethylaminopyridine
DMSO dimethyl sulfoxide
DMTST dimethyl(methylthio)sulfonium triflate
DT  diphtheria toxoid
EDC  1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA  enzyme-linked immunosorbent assay
Eq  equivalent
Et  ethyl
ETEC  enterotoxigenic E. coli
E  gram
GBS  Guillain-Barré Syndrome
GC  gas chromatography
h  hour
ESI  electrospray ionization
HMBC  heteronuclear multiple bond correlation
HRMS  high-resolution mass spectra
HS  heat-stable
HSQC  heteronuclear single quantum correlation
Hz  Hertz
Ig  immunoglobulin
K  kelvin
KDO  2-keto-3-deoxy-D-manno-2-octulosonic acid
KLH  keyhole limpet hemocyanin
L  liter
LT  heat-labile enterotoxins
LTA  lipoteichoic acids
LTB  enterotoxin B subunit
LOS  lipo-oligosaccharides
LPS  lipopolysaccharide
m/z  mass/charge
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<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MeOPN</td>
<td>O-methyl phosphoramidate</td>
</tr>
<tr>
<td>Me</td>
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<tr>
<td>MES</td>
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<tr>
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<tr>
<td>Troc</td>
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<tr>
<td>TT</td>
<td>tetanus toxoid</td>
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CHAPTER 1

Introduction
1.1 *Campylobacter jejuni* and *Clostridium difficile*

1.1.1 *Campylobacter jejuni* infection

*Campylobacter jejuni* is a spiral-shaped, motile, microaerophilic, Gram-negative bacterium that naturally resides in the digestive tracts of chicken and cattle.\(^1,2\) *C. jejuni* was first identified as the pathogenic cause of human gastroenteritis in the 1970s.\(^3\) Infection with *C. jejuni*, or campylobacteriosis, often occurs through the consumption of contaminated animal products or drinking water.\(^4,5\) Although the optimal growth temperature of *C. jejuni* is 42\(\,^\circ\mathrm{C}\), its minimal growth temperature allow it to thrive in the human digestive tract (>30\(\,^\circ\mathrm{C}\)).\(^6,7\) Ingestion of a dose as small as 500 viable cells is sufficient to cause disease.\(^8\) Infection cells surviving the acidic environment of the stomach reach the gut and establish itself for growth, with an average incubation period of 3.2 days. Since exposure to *C. jejuni* is often during travelling, campylobacteriosis is also commonly known as the traveller’s diarrhea.\(^9\) Infection rates are observed in 30% to 70% of travellers, depending on the destination and season of travel. The infection causes symptoms such as abdominal pain, acute diarrhea, fever, nausea, headaches, vomiting and sometime progresses to much more severe post-infectious complications including inflammatory bowel syndrome, a spondyloarthropathy known as Reiter’s Syndrome, and post-infectious polyneuropathy known as Guillain-Barré Syndrome (GBS).\(^10,11\) This food-borne pathogen is one of the leading causes of human gastroenteritis, causing more than 400 million cases per year worldwide. Although only about 50/100,000 people are infected with *C. jejuni* each year in the United States, incidents as high as 40,000/100,000
per year has been reported in some developing countries. An estimate of 40-60% of these is children under the age of 5.\textsuperscript{12-14}

1.1.2 \textit{Clostridium difficile}

\textit{Clostridium difficile} is a rod-shaped, anaerobic, spore-forming, Gram-positive bacterium first described in 1935 as a component of the intestinal flora in healthy newborn infants.\textsuperscript{15, 16} Today, it is identified as a nosocomial pathogen and one of the leading causes of antibiotic-associated diarrhea in hospitals and long-term care facilities.\textsuperscript{17} \textit{C. difficile} infection is commonly caused when a patient is exposed to broad-spectrum antibiotics, which disrupts other bacteria within a healthy intestinal flora and leaves \textit{C. difficile} producing toxins that inflames and damages the colon. \textit{C. difficile}-infected patients display symptoms including loss of appetite, nausea, fever up to 40.5 °C, abdominal pain, severe diarrhea and sometimes progress to life-threatening conditions such as toxic megacolon and bowel perforation.\textsuperscript{18, 19} In the United States, the estimated number of cases of \textit{C. difficile}-associated disease (CDAD) exceeds 250,000 per year.\textsuperscript{20} Outbreaks involving hypervirulent \textit{C. difficile} ribotypes have been reported in North America and several European countries with a mortality rate as high as 19%.\textsuperscript{21}

1.1.3 Treatment for \textit{C. jejuni} and \textit{C. difficile} infections

Campylobacteriosis is usually self-limiting lasting about a week. Fluids and electrolyte replacement is administered as needed.\textsuperscript{22} For more serious cases, antimicrobial
agents can be effective. Currently, the drugs of choice for *C. jejuni* infections are antibiotics such as erythromycin and ciprofloxacin. However, antibiotic treatments are often associated with many side effects. More importantly, many strains of *C. jejuni* are becoming more resistant against these antibiotics which limit the viable options for treatment. For instance, it was reported in England and Wales between the year 2000 and 2001 that 50% of *C. jejuni* infection cases were resistant to at least one antimicrobial agent while 8% were infected with multi-resistant strains.

Since *C. difficile* infection is triggered by the use of antibiotics, discontinuation of these treatments is sometimes sufficient to resolve the disease. Fluids and electrolyte replacement can be provided during this time. In severe cases of *C. difficile* infection, treatment is based on metronidazole or vancomycin. However, similarly to *C. jejuni*, reports of antibiotic resistance are common. In the United States, it is reported that 22% of the hospitalized patients failed to respond to the antibiotic treatments and infection relapses were observed in 28% of the treated patients. These failure rate increases dramatically in subsequent treatments.

An alternative solution to *C. jejuni* and *C. difficile* infections is vaccination. Vaccines utilize the human immune system to recognize and destroy the pathogen of infection and have the potential to prevent further infections for an extended period of time. The availability of vaccines against *C. jejuni* and *C. difficile* would greatly reduce these diarrheal diseases worldwide. Currently there is no licensed vaccine against *C. jejuni* or *C. difficile* infection on the market. The development of anti-*C. jejuni* and anti-*C.
difficile glycoconjugate vaccines is ongoing in the Monteiro research group and will be discussed in the future sections.

1.1.4 Basics of the human immune system

The human immune system evolved to protect the body from foreign materials potentially harmful to the body. These include bacteria, fungi, viruses, and also non-living substances such as toxins and chemicals. The immune system can be classified into two interconnected subsystems: the innate immunity and the adaptive immunity.

The innate immune system provides an immediate, but non-specific response as the first line of defense to a foreign pathogen that might cause infection. This includes physical and chemical barriers, as well as cellular-responses to many infectious agents, leading to their destruction. For example, the macrophage, a type of white blood cell belonging to this sophisticated immune system first identifies the foreign invader, then engulfs and kills the infecting pathogen through the activity of digestive enzymes. However, the innate immune system does not confer a long-lasting immunological memory against a pathogen, as illustrated in Figure 1.30

Unlike the innate immune system, the adaptive immune system is a much slower, but highly specific response to a foreign antigen. Studies have shown that molecules differing in the smallest detail, such as a single amino acid, could be distinguished by different antibodies. As the name implies, the adaptive immunity prepares the body's
immune system for future challenges. By creating an immunological memory after an initial response to a specific antigen, a much stronger, faster immune response can be released against the same antigen in the future.

![Figure 1](image)

**Figure 1.** Innate response occurs each time an antigen is encountered; its magnitude is unchanged regardless of how frequently this antigen has been encountered. On the other hand, the adaptive response allows the second exposure to the same antigen to produce a much faster and more antigen-specific response that is greater in magnitude than the primary response.

Two white blood cells of the acquired immune system are the T and B lymphocytes. The B lymphocytes (B cells) mature in stem cells of the bone marrow. They are the major cells involved in the creation of antibodies, also known as immunoglobulin (Ig), that circulate in blood plasma and lymph. B cell receptors (BCR) expressed on the surface of B cells specifically recognize an antigen. Upon recognition, B cells transform into plasma
cells which are formidable cellular factories that produce up to $10^5$ antibodies per second with antigen-binding sites identical to those on the BCR. These antibodies in turn identify and neutralize specific foreign antigen. This process is known as the humoral immunity.

On the other contrary, T lymphocytes (T cells) arise from the bone marrow but mature in the thymus gland. These cells are involved in cell-mediated immune responses. Unlike the BCR, T-cell receptor (TCR) is not released in a secreted form, and it is not able to recognize an antigen alone. The antigen needs to be fragmented and presented by antigen-presenting cells through the function of major histocompatibility complex (MHC). A major antigen-presenting cell in human is the dendritic cells, a type of white blood cell of the innate immune system. These cells utilize enzymes to chop pathogens into smaller fragments, and then display them (typically proteins) as a MHC-antigen complex on the cell surface.

T cells are divided into two major cell types based on the class of the MHC molecule recognized. T cells displaying CD8 membrane glycoproteins generally function as T cytotoxic ($T_c$) cells and recognize antigen in complex with MHC class I. Those displaying CD4 membrane glycoproteins on the surface generally function as T helper ($T_h$) cells and recognize antigen in complex with MHC class II. When $T_c$ cells bind to MHC class I, it differentiates into cytotoxic T lymphocytes (CTL), also known as killer T cells. CTL is able to recognize specific antigen with its TCR and kills damaged and infected cells, as well as cancer cells. On the other hand, $T_h$ cells “guide” the behavior and enable the activation of B cells, $T_c$ cells, macrophages, and various other cells that
participate in the immune response. This communication is achieved by both cell-cell interaction and soluble messengers known as cytokines. Once activated by cytokines, B cells develop into plasma cells, producing low affinity IgM antibodies. In addition, B cells and T cells can also be transformed into memory B cells and memory T cells, which persist for extended periods of time and produce high affinity IgG antibodies.

The goal of all vaccines is to elicit the development of specific and long-term adaptive immunity (via the production of IgG antibodies) so that the vaccinated individual will be protected in the future when the real pathogen comes along.

1.1.5 Glycoconjugate vaccines

The history of the study of glycoconjugates dates back to the 1920s when the pneumococcal cell-surface polysaccharide was identified as a target for antibodies and it was shown when conjugated to a protein, this carbohydrate-protein structure could induce the production of antibodies. Despite progress, this breakthrough was overshadowed by the discovery of antibiotics. The first licensed glycoconjugate vaccine was developed some 50 years later against *Haemophilus influenza* type b, which is still used today. Since then, glycoconjugate vaccines have also been developed and marketed for diseases caused by *Neisseria meningitidis* and *Streptococcus pneumonia*. Today, glycoconjugate vaccines are receiving much more attention and are being heavily investigated.

Glycoconjugate vaccine is created by attaching a carbohydrate structure, such as
bacterial polysaccharide, to a carrier protein to stimulate immunity. Polysaccharides expressed on bacterial cell surfaces are potentially immunogenic with an ability to trigger the human immune system and raise antibodies that recognize and kill the foreign pathogen.\textsuperscript{35-38} These polysaccharide structures are unique to each bacterium, making the resulting antibodies much more pathogen-specific. This specificity offers less risk in cross-reactivity with human tissue as well as the essential bacteria that reside within the body. However, carbohydrate structures alone do not enhance a T-cell dependent (TD) immune response that is necessary for inducing a long-term immunological memory and often poorly immunogenic in infants.\textsuperscript{39,40} One explanation for this phenomenon is that the hydrophilic carbohydrate molecules cannot dock into the cleft of the MHC on the surface of antigen-presenting cells and therefore cannot be presented to T cells (Figure 2A). Consequently, B cells that recognize polysaccharides cannot get help from T cells.\textsuperscript{41} This can be overcome by conjugating the weakly immunogenic carbohydrate to a suitable protein carrier. Antigen-presenting cells recognizing the carbohydrate are able to internalize and process the glycoconjugate as a whole, leading to the loading of class II MHC with peptides derived from the carrier protein that can then be recognized by the T cells, promote Th cell development and trigger the release of cytokines (Figure 2B).\textsuperscript{42} This TD immune response help B cells to proliferate and enables antibody class switching from IgM to IgG antibodies, and therefore offering a long-term immunological memory.\textsuperscript{43,44}
Figure 2. Immune response from (A) hydrophlic polysaccharide antigen, which cannot enter the MHC II, and therefore cannot be presented to T cells by MHC II; (B) polysacchar-protein glycoconjugate can be internalized and processed. Peptide epitope derived from the protein is presented by MHC II and recognized by a T cell, which elicits a TD immune response.

For glycoconjugates, the carrier protein itself must have a correspondingly high T-cell response and must have been rendered safe for administration. Tetanus toxoid (TT) and diphtheria toxoid (DT) are two protein carriers licensed for human use. Other proteins, such as *E. coli* heat-labile enterotoxins (LT), keyhole limpet hemocyanin (KLH),
bovine serum albumin (BSA) and a non-toxic mutant diphtheria toxoid mutant protein cross-reactive-material 197 (CRM197) are also commonly used.\textsuperscript{45}

\section*{1.2 Bacterial surface polysaccharides}

Bacteria produce a thick, mucous-like layer of polysaccharides on the outer cellular membrane as a protective barrier and it is involved in signaling and adhesion.\textsuperscript{46} This layer also has important roles during infections and it is often found associated with the virulence of the pathogenic bacteria by allowing them to adhere to host cells or by helping them to overcome host defense mechanisms.\textsuperscript{47} Bacteria can be classified as Gram-positive or Gram-negative by the Hans Christian Gram staining technique based on the difference in the structure of the bacterial cell wall.\textsuperscript{48}

\subsection*{1.2.1 Gram-positive bacteria}

Gram-positive bacteria contain a thick bacterial cell wall made up of multiple peptidoglycan layers (20–80 nm), which retain the purple dye (crystal violet) in the Gram-staining method.\textsuperscript{49} The peptidoglycan is a disaccharide-pentapeptide cross-linking structure that provides rigidity to the cell wall and protects the cell from osmotic lysis.\textsuperscript{50} The disaccharide repeating unit consists of $N$-acetylglucosamine (GlcNAc) and $N$-acetylmuramic acid (MurNAc) linked by a $\beta(1\rightarrow4)$ glycosidic bond.\textsuperscript{51} The pentapeptide consisting of five amino acids is attached to MurNAc through a lactyl group and links adjacent glycan strand by peptide cross-linkages. Gram-positive cell surface
polysaccharide components are either covalently attached to the membrane lipids like lipoteichoic acids (LTA) or to the peptidoglycan such as teichoic acids (TA) and teichuronic acids.\textsuperscript{52} Peptidoglycan-attached TAs are frequently formed by glycerol or ribitol groups that are connected by phosphodiester bonds while LTAs are connected directly to phospholipids or glycolipids.\textsuperscript{53} The presence of negatively charged phosphate within the polysaccharide repeating units is characteristic of TAs. With modifications containing free amino groups, most TAs have zwitterionic properties.\textsuperscript{54}

![Diagram of cell wall structure](image)

**Figure 3.** The cell wall structure of Gram-positive bacteria.

### 1.2.2 Gram-negative bacteria

Unlike Gram-positive bacteria, the cell envelope of Gram-negative bacteria comprises a much thinner layer of peptidoglycan (1-7 nm), which does not retain the purple dye in the Gram-staining method, and are stained by a counter-stain.
In addition of a cytoplasmic membrane, Gram-negative bacteria contain an outer membrane with a periplasmic space in between the two membranes (Figure 4). Lipoproteins embedded in the inner layer of the outer membrane covalently connect to the peptidoglycan layer.

**Figure 4.** The cell wall structure of Gram-negative bacteria.

One major type of bacterial surface polysaccharide commonly found in the outer membrane of Gram-negative bacteria is lipopolysaccharide (LPS). LPSs are comprised of 3 domains: O-antigen, core region, and lipid A (Figure 5A). The O-antigen region is composed of a long chain of oligosaccharide repeating units, each may contain up to seven different sugars units. The core region is composed of an inner conserved
oligosaccharide, containing usually 10 to 15 monosaccharide units. Phosphorylated heptoses and 2-keto-3-deoxy-D-manno-2-octulosonic acids (KDOs) are often found in the core region.\textsuperscript{60} The inner-most domain, lipid A, consists of a phosphorylated GlcNAc disaccharide unit linking to long chain fatty acids.\textsuperscript{61} The lipid A structure acts as the hydrophobic moiety that integrates LPS into the outer membrane of bacterial cell wall through hydrophobic interaction.\textsuperscript{62}

Some Gram-negative bacteria, such as \textit{C. jejuni}, express capsular polysaccharides (CPSs) and lipo-oligosaccharides (LOSs). These structures will be discussed in more detail in sections below.

1.2.3 \textit{C. jejuni} surface polysaccharides

For many years, researchers believed that the LPS is the main bacterial surface polysaccharide component in \textit{C. jejuni}, which contributes to its antigenicity and serospecificity.\textsuperscript{63} However, it is later recognized by Aspinall \textit{et. al.} that, unlike most other Gram-negative bacteria, \textit{C. jejuni} does not express LPS, and instead produce only LOS and CPS as its surface polysaccharides.\textsuperscript{64,65} This was later confirmed by genomic analysis, identifying determinants homologous to those genes found in related organisms that produce CPS.\textsuperscript{66}
Figure 5. Representation of general structures of (A) LPS, (B) LOS and (C) CPSs from Gram-negative bacteria.

1.2.3.1 LOS of *C. jejuni*

The LOS is a truncated version of LPS containing only the core oligosaccharide and lipid A, lacking an O-antigen polysaccharide (Figure 5B). The lipid A moiety is also known as the endotoxin, which can be found in all Gram-negative bacteria. Exposure to lipid A liberated from the bacterial cell wall can quickly lead to blood infection and
septic shock. Injection of a small amount of endotoxin in human volunteers produces fever, lowering of the blood pressure, activation of inflammation and coagulation.\(^6\)\(^7\) Moreover, the sialic acid containing outer core of LOSs of \textit{C. jejuni} structurally mimic human gangliosides, which is the only bacterial LOSs identified to date with this property. This structural similarity between core oligosaccharide and ganglioside glycoforms in the human brain leads to the production of anti-ganglioside antibodies causing auto-immune disease that affects peripheral nerves.\(^6\)\(^8\)\(^-\)\(^7\)\(^1\) Thus antibodies directed against LOS during \textit{C. jejuni} infection may sometimes result in post-infectious autoimmune disorders such as Guillain-Barré (GBS) and Miller–Fisher (MFS) neurological syndromes, both causes severe and sometimes fatal paralysis in patients.\(^7\)\(^2\),\(^7\)\(^3\) It has been demonstrated that the immunization of rabbits with purified \textit{C. jejuni} LOS mimicking the ganglioside GM1 resulted in high levels of anti-GM1 antibodies and symptoms of GBS in these animals.\(^7\)\(^4\),\(^7\)\(^5\)

Therefore, the LOS of \textit{C. jejuni} is not a suitable structure for the generation of a glycoconjugate vaccine. In addition, \textit{C. jejuni} polysaccharide vaccine must be screened for LOS absence for patient safety.\(^7\)\(^6\)
Table 1. Structural similarities between LOS core oligosaccharide of *C. jejuni* and human ganglioside

![Table](image.png)

1.2.3.2 CPS of *C. jejuni*

The CPS is a long chain polysaccharide consists of repeating oligosaccharide usually made up of 2 to 6 monosaccharide units (Figure 5C). This structure is anchored directly into the outer membrane by a phospholipid. This structure forms the outer-most layer surrounding the bacteria, mediates interaction with the host and environment, modulates the invasion of intestinal epithelial cells and is involved in serum resistance. It is demonstrated that non-encapsulated mutant of *C. jejuni* strain 81–176 showed a 10-fold reduction in invasion of intestinal epithelial cells.
**Figure 6** Characterized structures of CPSs of *C. jejuni* serotype HS:23/36, HS:1, HS:3 and HS:4. (R = H or MeOPN).

There is an enormous structural diversity and complexity in the structures of *C. jejuni* CPSs, where each serotype exhibits unique sugar composition and linkage type. A few characterized CPS structures of *C. jejuni* are shown in Figure 6. A total of 47 different serotypes of *C. jejuni* have been identified based on Penner heat-stable (HS)
antigen serotyping method, which is a passive slide hemaglutination technique based on the detection of the CPS structures by antibodies from the immune system.\textsuperscript{89-91}

As observed, the expression of heptoses of unusual configuration (i.e. \textit{altro}, \textit{ido}, \textit{gulo}, \textit{talo}) is common in the CPS of many serotypes.\textsuperscript{92-95} The structural complexity of the heptoses is further enhanced by 6-deoxy functions. Within a single CPS chain, it is common to observe the presence of the heptose and its complementary 6-deoxy-heptose. For example, both \textit{d-glycero-d-altro}-heptose and 6-deoxy-\textit{altro}-heptose can be found in \textit{C. jejuni} strains that belong to serotype HS:23/36.\textsuperscript{96,97}

\textit{C. jejuni} also decorate their CPS with phase-variable modifications including \textit{O}-methyl, ethanolamine and phosphates.\textsuperscript{98,99} The \textit{O}-methyl phosphoramidate (MeOPN) functional group is common among many serotypes. The importance of this modification will be discussed in the next section.

1.2.3.3 Biological importance of MeOPN in \textit{C. jejuni}

Phosphoramidates are rare in nature. However, MeOPN modification is found prevalent in \textit{C. jejuni}, where it is observed in about 70\% of strains.\textsuperscript{100} Although the exact role of MeOPN is not well understood, it has been shown that the biosynthesis of MeOPN is important for \textit{C. jejuni} cellular interactions and infection. For instance, Maue \textit{et al.} demonstrated that the disruption of a MeOPN biosynthetic gene in the virulent \textit{C. jejuni} strain 81–176 correlates to a significant reduction in both serum resistance and colonization in a mouse intestinal model.\textsuperscript{101} Another study showed that MeOPN acts as a
receptor for several C. jejuni lytic bacteriophages.\textsuperscript{102}

Studies by Szymanski’s group at Alberta Glycomics Centre identified four genes for the biosynthesis of MeOPN and two phase-variable genes that encode transferases in both C. jejuni strain 11168H and 81-176.\textsuperscript{103} The genetic expression is also located in related species such as Campylobacter lari and Campylobacter fetus. Previous structural analysis of MeOPN-containing CPS suggests that the expression of the MeOPN modification is non-stoichiometric, most likely due to phase variability. This suggests that MeOPN expression is desirable during certain stage of infection. This is supported by the study on C. jejuni 81–176 mutants lacking MeOPN expressions. These C. jejuni mutants became bacteriophage resistant and showed an increased invasiveness relative to the wild type.\textsuperscript{104} However, in a piglet infection model, loss of MeOPN showed a reduction in colonization relative to wild-type.\textsuperscript{103} Nevertheless, both results suggest that MeOPN has a contributory role in pathogenesis.

It is also speculated that MeOPN has insecticidal activity due to the structural similarities to some organo-phosphorous synthetic pesticides. Injection of three different strains of MeOPN-expressing C. jejuni into the larvae of the wax moth, Galleria mellonella, resulted in rapid killing.\textsuperscript{105} However, it was shown in a more recent study with a G. mellonella larvae model that the injection of synthetic MeOPN-linked monosaccharide as well as extracted MeOPN-containing CPS did not mediate insecticidal activity.\textsuperscript{103}
1.2.4 *C. difficile* surface polysaccharides

Three bacterial-surface polysaccharide structures were previously purified and characterized from *C. difficile* in the Monteiro research group, which were named PS-I, PS-II and PS-III. The strains responsible for the recent outbreak, *C. difficile* ribotype 027, or North American pulsortype 1 (NAP1), expressed two types of teichoic-acid-like cell wall polysaccharides, PS-I and PS-II. All other strains investigated were found to display only PS-II. A third water-insoluble lipoteichoic acid-like polysaccharide PS-III was recently identified from the pellet material collected during polysaccharide extractions.

1.2.4.1 Structure of *C. difficile* PS-I

The structure of PS-I identified from *C. difficile* ribotype 027 strain consists of a pentaglycosyl phosphate repeating unit \[\rightarrow 4\]-\(\alpha\)-L-Rhap-(1\(\rightarrow\)3)-\(\beta\)-D-Glcp-(1\(\rightarrow\)4)-[\(\alpha\)-L-Rhap-(1\(\rightarrow\)3]-\(\alpha\)-D-Glcp-(1\(\rightarrow\)2)-\(\alpha\)-D-Glcp-(1\(\rightarrow\)P], shown in Figure 7. The fact that this structure is only found expressed by *C. difficile* ribotype 027 suggests that it may be responsible for its hyper-virulence, giving it, for instance, an increased resistance to attack by phagocytes. However, analysis of additional *C. difficile* strains belonging to the ribotype 027 have failed to detect PS-I, suggesting that the PS-I structure is not a ribotype 027-unique antigen. Recently, analysis of *C. difficile* strain GV60 belonging to ribotype 053 indicated the presence of the characteristic PS-I units, rhamnose (Rha) and glucose (Glc), in small amounts. This finding suggests that *C. difficile* strains belonging to other
ribotypes may also possess the biosynthetic machinery to produce PS-I. The failure to reliably detect PS-I may suggest that this antigen is phase-variable and perhaps expressed only in vivo.\textsuperscript{108}

\textbf{Figure 7.} Structure of the \textit{C. difficile} PS-I pentaglycosyl phosphate repeating unit.

1.2.4.2 Structure of \textit{C. difficile} PS-II

The structure of PS-II has been identified in all of the strains of \textit{C. difficile} so far, which consists of a hexaglycosyl phosphate repeating unit: $[\rightarrow6]\text{-}\beta\text{-}\text{D-Glc}p-(1\rightarrow3]\text{-}\beta\text{-}\text{D-Gal}p\text{NAc}-(1\rightarrow4]\text{-}\alpha\text{-}\text{D-Glc}p-(1\rightarrow4]\text{-}[\beta\text{-}\text{D-Glc}p-(1\rightarrow1]\text{-}\beta\text{-}\text{D-Gal}p\text{NAc}-(1\rightarrow3]\text{-}\alpha\text{-}\text{D-Man}p-(1\rightarrowP]$, shown in Figure 8.\textsuperscript{107} This polysaccharide structure is heterogeneous in length that can range from two to ten repeating units, but with the most common being those with seven repeating units.\textsuperscript{109} The fact that this structure is found in all the strains analyzed suggests it to be a common antigen of \textit{C. difficile}.
1.3 *C. jejuni* and *C. difficile* polysaccharide glycoconjugates

1.3.1 Overview

Bacterial surface polysaccharide is a good candidate for the generation of a glycoconjugate vaccine as they are the outer-most structure on bacterial cell and potentially immunogenic. To generate glycoconjugates, bacterial surface polysaccharides are extracted from killed bacterial cultures and purified with a number of techniques such as dialysis, ultra-centrifugation and size-exclusion chromatography. These polysaccharide structures are then characterized using spectral techniques such as gas chromatography/mass spectrometry (GC/MS), electrospray ionization mass spectrometry (ESI-MS) and 1D/2D nuclear magnetic resonance spectroscopy (NMR). This can be a time-consuming process due to the complexity of these polysaccharide structures, which takes years to accomplish. Afterwards, based on the polysaccharide structure, a suitable conjugation strategy is developed to generate a potential glycoconjugate vaccine.
The generation of glycoconjugates with the polysaccharide structure of every serotype can be time-consuming. Also, despite the fact that over 47 Penner serotypes of *C. jejuni* having been identified, it is believed that most diarrheal disease is caused by only a fraction of these serotypes. Therefore, in the case of *C. jejuni*, CPSs from the most prevalent serotypes should be employed in a potential multivalent conjugate vaccine. For example, the currently licensed pneumococcal glycoconjugate vaccine is based on the CPSs of 23 most common serotypes from 90 identified serotypes of *Streptococcus pneumoniae*. This vaccine has an overall protective efficacy of about 60%–70%.\(^{111}\)

### 1.3.2 A prototype glycoconjugate vaccine against *C. jejuni* infection

In the past years, a number of *C. jejuni* CPS glycoconjugates have been generated by the Monteiro research group.\(^{112,113}\) One of the most successful CPS glycoconjugate by our lab was generated with the CPS extracted from *C. jejuni* serotype HS:23/36, strain 81-176.\(^{111}\) This CPS structure consists of a trisaccharide repeating unit made up of a Gal, GlcNAc and a 6-deoxy-3-OME-\textit{altro}-Hep with a terminal Gal at the non-reducing end. The polysaccharide is decorated with MeOPN groups non-stoichiometrically attached to the C-6 and C-2 positions of Gal. To conjugate, the purified CPS was first activated by periodate oxidation, giving aldehyde functionalities on the non-reducing end. A reductive amination with carrier protein CRM\textsubscript{197} then generates the corresponding glycoconjugate, as shown in Scheme 1.
Scheme 1. Conjugation of CPS from *C. jejuni* serotype HS:23/36 to protein carrier CRM$_{197}$.

In animal studies, mice immunized with this glycoconjugate produced a significant amount of IgM and IgG antibodies.$^{114}$ These immunized mice were then challenged in an intranasal infection model with live *C. jejuni* serotype HS:23/36. The diarrheal symptoms were significantly reduced from the control. In primate studies with New World monkeys
(Aotus nancymaee), this vaccine showed a 100% protection rate against C. jejuni serotype HS:23/36, where all the immunized animals were protected from the diarrheal disease. In a later study, the monkeys were also challenged with a mutant strain 8421 that belongs to C. jejuni serotype complex HS:23/36. The CPS of this strain lacks the O-methyl modification on the 6-deoxy-altro-Hep. Monkeys vaccinated with the wild type 81-176 glycoconjugate vaccine were found to be also protected from the 8421 strain. This observation points to the fact that the antibody might not be raised from only the internal epitope of the polysaccharide repeating unit. Since this mutant strain contains no ganglioside-mimicking structures in its LOS, it can be used as a safer challenge model in later human trails. This anti-C. jejuni glycoconjugate vaccine is currently being tested in Phase 2 human trials.

1.3.3 Glycoconjugate vaccines against C. difficile infection

The PS-II hexaglycosyl phosphate repeating unit is the most abundantly expressed polysaccharide antigen and has been identified in all strains of C. difficile, making this structure an attractive antigen for vaccine development. Previously, a glycoconjugate was prepared with the extracted C. difficile PS-II with enterotoxin B subunit (LTB) of enterotoxigenic E. coli (ETEC) in the Monteiro research group. To conjugate, the purified PS-II was first activated by stoichiometric oxidation of the primary hydroxyls to carboxylic acids with 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO). This activated polysaccharide carries several carboxylic acids, which is conjugated to protein LTB
through an amide coupling reaction promoted by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to create a lattice-type conjugate (Scheme 2). It was determined that approximately 10% of PS-II was oxidized and the oxidation favored the C-6 position of the 3-substituted GalNACs.

Scheme 2. Conjugation of *C. difficile* PS-II to protein LTB.

Whole-rabbit antisera from rabbits immunized with this PS-II–LTB glycoconjugate recognized PS-II and cell-wall extracts of various *C. difficile* ribotypes. This study
demonstrated that glyconjugates containing partially TEMPO-oxidized PS-II is able to generate an immunogenic response against native PS-II.

In a separate study, TEMPO-oxidized PS-II was conjugated to BSA. Mice were immunized with this PS-II-BSA glycoconjugate admixed in subunit-KLH as an adjuvant.\textsuperscript{117} The derived polyclonal antibodies tested positive in an immunoblot analysis against su-KLH, unconjugated PS-II and PS-II-BSA conjugate. In contrast, mice immunized with unconjugated native PS-II in su-KLH showed no significant increase of anti-PSII titer, confirming the weak immunogenicity of PS-II in mice as compared to the same polysaccharide conjugated to BSA. Later, this PS-II-KLH conjugate was shown to protect 92\% of mice from a primary \textit{C. difficile} infection where only 40\% of unvaccinated mice survived.\textsuperscript{118} In a secondary challenge with a 10 fold higher spore count, all vaccinated animals survived where only 50\% of unvaccinated mice survived. These data suggest that the PS-II conjugate vaccine is effective against primary and recurring \textit{C. difficile} infection. Protection and colonization studies in other animal models are ongoing with the PS-II–KLH vaccine.

\subsection*{1.4 Introduction to carbohydrate synthesis}

\subsubsection{1.4.1 Importance of carbohydrate synthesis}

Carbohydrates are the most abundant group of natural organic molecules. Other than a structural material (e.g cellulose) and energy source (e.g starch) for living organisms, carbohydrates are also involved in a wide range of biological processes. As previously
discussed, bacterial cell surface polysaccharides play an important role in cell-cell recognition and are often found associated with the virulence of pathogenic bacteria. These carbohydrate structures are becoming increasingly important in studies surrounding their biological activities. Development of glycoconjugates vaccines against bacterial infections is also a growing field of research that utilizes the immunogenic properties of these carbohydrate structures. Clearly, these studies are dependent on the availability of large amounts of carbohydrate sample.

One of the challenges in the development of glycoconjugate vaccines is to obtain sufficient amount of bacterial carbohydrate antigen. Due to the relatively low expression level in bacteria, isolation of these polysaccharides from bacterial cultures can be low yielding, time-consuming and relatively expensive. Also, extracted carbohydrates need to be thoroughly purified as contaminants from a bacterial sample can potentially affect the desired immune response. Also, in the case of *C. jejuni*, it is extremely important to eliminate LOS containments from a CPS sample for glycoconjugate generation as LOS containment might result in serious side-effects due to its human ganglioside-mimicking property. Native bacterial samples are also structurally undefined as bacteria often change their carbohydrate make-up to adapt different growth environments. These include length of polysaccharide, phase-variable chemical modifications, and even sugar compositions, which all affect the immunogenicity of the resulting glycoconjugate. These polysaccharide structures can also be altered during extraction and purification procedures.
On the other hand, chemical synthesis of carbohydrates offers many advantages over the extraction method. First of all, chemical synthesis can be much more efficient. Once a synthetic strategy is developed, large amounts of carbohydrates can be produced in a relatively short period of time and often foregoes complicated purification procedures to rid of containments. More importantly, synthesis produces chemically well-defined structures. Expression of chemical modifications on the carbohydrate structure can be synthetically controlled, allowing these structures to generate more conclusive results in biological studies. Also, synthetic carbohydrate can be equipped with a conjugation site via linker/spacer molecules, thus controlling the point of attachment to the carrier protein, while leaving the internal sugar epitope intact.

Due to above reasons, efficient and rapid chemical synthesis is often favored over the complicated cultivation and isolation of polysaccharide from bacteria culture. This section will discuss the basics of chemical synthesis of carbohydrates.

1.4.2 Carbohydrate nomenclature

Carbohydrate is a term given to compounds that consist of carbon and water molecules with a general formula $C_x(H_2O)_y$. Today, the carbohydrate family also extends to molecules derived from these structures by replacement of one or more hydroxyl groups by hydrogen, amino or other heteroatomic functionality. Monosaccharide is the simplest type of carbohydrates which cannot be hydrolyzed into smaller carbohydrate units. Oligosaccharides and polysaccharides are polymers of monosaccharide molecules.
joined by glycosidic linkages. Oligosaccharides usually consist of 2 to 10 monosaccharide subunits while polysaccharides describe much larger structures. For example, amylose is a polysaccharide of a huge polymer of up to 3000 D-glucose joined by 1,4-α-O-glycosidic bonds.

Monosaccharides are divided into two main groups according to whether their acyclic forms contain an aldehyde, named aldose, or a keto group, named ketose. Aldose and ketose sugars in their acyclic form can exist as two enantiomers, dextro (D) and laevus (L). When represented in a Fischer projection, D-sugars have their most distant stereocenter from the carbonyl group on the right, whereas L-sugars have theirs on the left. These can be further classified according to the number of carbon atoms in the structure, such as pentoses (5 carbons), hexoses (6 carbons), heptoses (7 carbons), etc. Monosaccharide exists in solution as a mixture of isomers. Generally, the open-chain form is energetically unfavorable comparing to its cyclic hemiacetal counterpart. Using D-glucose as an example in Scheme 3, the six-membered pyranose ring is formed by an intramolecular nucleophilic attack of the C-5 hydroxyl group on the carbonyl carbon C-1, also termed the anomic position. Nucleophilic attack of the C-4 hydroxyl results in a less stable five-membered furanose ring.
**Scheme 3.** Different forms of D-glucose during mutarotation. Acyclic open chain form of glucose is represented in a Fischer projection. Glucopyranoses are represented in chair conformations while glucofuranoses are represented in Haworth projections.

After the nucleophilic attack, the resulting diastereoisomers are denoted as the α or β anomers depending on whether the C-1 substituent is in the same direction (cis) or in the opposite direction (trans) to the substituent on the highest-numbered stereocenter. For D-glucose, the α anomer has its C-1 substituent on the bottom (axial position) of the ring and the β anomer has its C-1 substituent on the top (equatorial position). In solution,
hemiacetal ring opens and closes to give products with different configurations, shown in Scheme 3. The pyranose, furanose, α and β forms have a characteristic optical rotation that changes until equilibrium is reached. This change in optical rotation is called mutarotation.\textsuperscript{122}

Carbohydrates can be projected in several ways. The Fischer projections are used to represent monosaccharides in its acyclic open-chain form. Carbon with the highest oxidation state is normally placed at the top, leaving the asymmetric centre at the bottom of the chain. The Haworth representation displays the sugar in a flat ring form, with ring substituents lying above or below the plane of the ring. As a more accurate depiction of these molecules, sugars are usually represented in the chair conformations. Most D-hexopyranoses exist in a \textsuperscript{4}C\textsubscript{1} chair conformation with the CH\textsubscript{2}OH group in a more energetically favorable equatorial position while most L-hexopyranoses adopt the \textsuperscript{1}C\textsubscript{4} conformation. The letter “C” designates for “chair” and the numbers designate for the ring atoms lying above or below the plane of the “chair” structure. On the other hand, the conformation of 5-membered furanoses can be much more complicated and can only be described by the concept of pseudorotation. For instance, X-ray structural analysis reveals that crystalline D-fructose exists mainly as the β-pyranose isomer, due to the anomeric effect and the equatorial arrangement of the hydroxymethyl (CH\textsubscript{2}OH) unit.\textsuperscript{123}

For D-fructofuranoses (in solution), conformation analysis shows that the most energetically favorable for β-fructofuranose are two “twist” and two “envelope”
conformations ($^2T_3$, $E_3$, $^4T_3$, $^4E$). For $\alpha$-fructofuranose, the energetically favorable conformations are one twist and two envelope conformations ($E_2$, $^0T_2$, $^0E$).\textsuperscript{124}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Chair conformation of $\beta$-D-glucopyranose and Haworth projection of $\beta$-D-fructofuranose}
\end{figure}

The numbering system for a sugar molecule starts from the right side with carbon-1 (C-1) to carbon-6 (C-6). The anomeric position for an aldose sugar is always the C-1 position. However, this is not the case for ketose sugar. For example, the anomeric centre for fructofuranose is at the C-2 position, shown in Figure 9. If a sugar molecule is functionalized at its anomeric position, it becomes a glycoside. Substituent at the anomeric position of a sugar is commonly known as the aglycone, whereas the heterocyclic ring is referred to as the glycone. The glycone moiety and the aglycone moiety are linked together via an $O$-glycosidic bond. A chair conformation of $\beta$-D-glucopyranose and a Haworth projection of $\beta$-D-fructofuranose are shown in Figure 9.
1.4.3 Protecting groups of carbohydrates synthesis

Carbohydrates contain multiple hydroxyl groups with different reactivity. These often need to be selectively blocked and freed when a selective chemical modification at a single position is desired. An ideal protecting group must be stable under all the conditions used during subsequent steps, must be easily removed in a selective manner and high yielding. Protecting groups can also affect the reactivity of other hydroxyl groups on a sugar molecule due to both electronic and steric factors. Generally, electron-donating substituents on a sugar structure increase nucleophilicity of other hydroxyl groups and increase the reactivity of the sugar molecule overall, while electron-withdrawing substituents decelerate such reactions. Modification with protecting group at certain position can also greatly affect the stereochemical outcome of a reaction on other locations, such as the neighboring-group-participation effect during glycoside syntheses. Therefore, the choice of protecting groups plays a decisive role in multi-step synthetic carbohydrate chemistry, and requires careful design. Some protecting groups used in this research thesis are shown in Figure 10.
1.4.4 Glycosylation

A glycosidic bond is formed when a nucleophilic hydroxyl group of a glycosyl acceptor attacks an electrophilic glycosyl donor equipped with a potential leaving group at its anomeric center. During glycosylation, the glycosyl donor is activated that allows the removal of the leaving group, generating an oxocarbenium ion. The glycosyl acceptor with a free hydroxyl group then attacks either from the axial or the equatorial position, generating either α or β anomer. The stereochemical outcome of glycosylations depends on many factors including solvent, reaction temperature, type of leaving group on the glycosyl donor and the choice of the protecting groups on both glycosyl donor and
acceptor. The stereoselectivity of glycosylations remains one of the greatest challenges in carbohydrate chemistry.\textsuperscript{129,130}

1.4.4.1 Trichloroacetimidates

The trichloroacetimidate protecting group was originally developed by Schmidt’s group in the 1980s and they are perhaps the most commonly used glycosyl donors.\textsuperscript{131,132} These donors are easily prepared with a hemiacetal by a base-catalyzed reaction, such as potassium carbonate or 1,8-diazabicycloundec-7-ene (DBU) in the presence of trichloroacetonitrile (CCl\textsubscript{3}CN).

\textbf{Scheme 4.} Synthesis of \(\alpha\)-, or \(\beta\)-trichloroacetimidate glycosyl donor from a hemiacetal.
Depending on the strength of the base, either $\alpha$-, or $\beta$-trichloroacetimidates can be produced. In the presence of a weak base, kinetic $\beta$-product is produced as the major product irreversibly. However, in the presence of a strong base, the initial formed $\beta$-trichloroacetimidate product can be converted back to the $\beta$-alkoxide, which anomerizes to the more stable $\alpha$-alkoxide. The $\alpha$-alkoxide reacts with trichloroacetonitrile forming the $\alpha$-trichloroacetimidate as the thermodynamic product (Scheme 4).

Scheme 4. Glycosylation reaction with a trichloroacetimidate glycosyl donor.

A trichloroacetimidate donor is easily activated during glycosylation by Lewis acid catalysts such as boron trifluoride etherate complex ($\text{BF}_3\cdot\text{OEt}_2$) or trimethylsilyl trifluoromethanesulfonate (TMSOTf). After generating the oxocarbenium ion, a nucleophilic glycosyl acceptor carrying a free hydroxyl group attacks either from the axial or the equatorial position, generating either $\alpha$ or $\beta$ anomer depending on the reaction condition (Scheme 5). Generally, trichloroacetimidates are not stable enough to endure
most protecting group manipulations, therefore they are introduced just before the glycosylation.

1.4.4.2 Thioglycosides

Another versatile glycosyl donor is the thioglycosides, first reported by E. Fischer.\textsuperscript{133} They can be easily prepared by a Lewis acid catalyzed reaction of an anomeric acetate with a thiol.\textsuperscript{134} Lewis acid promotes the formation of an oxocarbenium ion, which is followed by a nucleophilic attack by a thiol acceptor. Alternatively, thioglycosides can be generated with a nucleophilic substitution of a glycosyl bromide with a thiolate.\textsuperscript{135}

\begin{center}
\textbf{Scheme 6.} Glycosylation reaction with a thioglycoside glycosyl donor ($X^+ = \text{Me}^+$, $\text{Me}_2\text{S}^+\text{SMe}$, $\text{I}^+$).
\end{center}

One of the most attractive features of thioglycosides is their chemical stability, making them compatible with many reaction conditions during protecting group manipulation. It is possible to employ the thioglycoside as a temporary anomeric protection, and activate them directly during glycosylation. The most commonly used
activators for thioglycosides include methyl triflate (MeOTf), dimethyl(methylthio)-sulfonium triflate (DMTST), and N-iodosuccinimide (NIS)/triflic acid (TfOH).\textsuperscript{13,17} During glycosylation reactions, the lone pair on the sulfur atom reacts with the electrophilic activator forming a sulfonium intermediate, which is an excellent leaving group (Scheme 6). The resulting oxocarbenium ion is then attacked by the hydroxyl group of a glycosyl acceptor, generating α or β linkage depending on the reaction condition.

1.4.5 The anomeric effect

First described by Lemieux in 1958, the anomeric effect explains the tendency of anomeric substituents to prefer the axial orientation rather than the less sterically-hindered equatorial orientation in glycosidation and glycosylation reactions under thermodynamic conditions.\textsuperscript{138} The expected destabilization is analogous to the unfavourable 1,3-diaxial/flagpole interactions between axial-oriented substituents in a cyclohexane ring.

One explanation involves the interaction between two intramolecular dipoles, one due to the two lone pair electrons on the endocyclic oxygen and another due to the electronegative substituent attached to the anomeric center. For glucopyranose, when the substituent is in the equatorial or the β-configuration, the two dipoles reinforce each other, as illustrated in Figure 11. When the substituent is in the axial or the α-configuration, the
two dipoles partially cancel each other.\textsuperscript{139} The cancellation of the two dipoles is energetically favored making $\alpha$ the more stable anomer.

![Diagram of anomeric effect explained by dipole-dipole interaction](image)

**Figure 11.** The anomeric effect explained by dipole-dipole interaction. ($X =$ electronegative substituent, e.g. Br, Cl, N, O, S).

Another explanation of the anomeric effect involves the molecular orbital interaction between the anomeric position and the endocyclic oxygen. The lone pair of the non-bonding electrons from the endocyclic oxygen is able to transfer its electron density into the $\sigma^*$ anti-bonding orbital of the $\alpha$-substituent at the anomeric center, as illustrated in Figure 12.\textsuperscript{140} This effect is supported experimentally by the observation of a slight lengthening of the C$_1$-X bond and shortening of the C$_1$-O bond in $\alpha$-anomers. This delocalization, also called hyperconjugation, leads to additional stabilization in $\alpha$-anomers through resonance. Such stabilization in the $\beta$-configuration is not possible since these orbitals do not overlap, thus making the $\beta$-anomer the less stable configuration.
Figure 12. The anomeric effect explained by molecular orbital interaction. (X = electronegative substituent e.g. Br, Cl, N, O, S).

1.4.6 Neighboring group participation

The nature of the C-2 protecting group in the glycosyl donor greatly affects the stereochemical outcome during the synthesis of glycosidic bonds.\textsuperscript{141,142} 2-\textit{O}-Acyl protecting groups such as \textit{O}-acetyls and \textit{O}-benzoyls are capable of stabilizing the initially produced oxocarbenium ion by forming a more stable cyclic acyloxonium ion intermediate, shown in Scheme 7.\textsuperscript{143} Nucleophilic attack at the anomeric center by an acceptor then follows a S\textsubscript{N}2 opening mechanism which leads to a 1,2-\textit{trans} \textit{O}-glycosidic linkage. However, formation of orthoester as a side-product can be observed depending on the reaction conditions, where the acyloxonium ion intermediate is attacked by the nucleophile as opposed to at the anomeric cation. Usually, these orthoesters are unstable in acidic conditions and can rearrange into a glycosidic bond under these conditions.
Scheme 7. Neighboring group participation during the synthesis of 1,2-trans-glycosides.

1.5 Conjugation methods toward synthetic glycoconjugates

Carbohydrates are generally T-cell independent, and therefore cannot elicit a long-term immunological memory. To circumvent this, carbohydrates need to be covalently attached to a carrier protein, generating glycoconjugates. There is a variety of existing conjugation methods available for the generation of glycoproteins and glycoconjugates. To maintain an intact carbohydrate epitope necessary for antibody recognition, a suitable conjugation method needs to be carefully selected to suit the nature of each carbohydrate structure. A few existing conjugation methods will be discussed in this section.
1.5.1 Common conjugation methods for synthetic glycoconjugates

Although first introduced some 30 years ago, reductive amination is perhaps still the most common methodology applied in glycoprotein and glycoconjugate synthesis. This method involves the formation of an amine bond between a carbonyl functionality with an amino group. Typically, the carbonyl group can be generated within the carbohydrate moiety through “activation” and the activated structure is attached to the protein through lysine residues. For instance, during the synthesis of the *C. jejuni* HS:23/36 prototype vaccine, the extracted CPS is activated by periodate oxidation with sodium periodate in sodium acetate buffer. Since this activation strategy acts only on vicinal diol, which in the case of *C. jejuni* HS:23/36 CPS, is only located on the terminal Gal residue on the non-reducing end, the internal antigen epitope is retained. The activated CPS structure is conjugated to protein carrier CRM\(_{197}\) through the resulting aldehyde by reductive amination using sodium cyanoborohydride. As another example, reductive amination is used to generate glycoconjugate vaccine for *Neisseria meningitidis* serogroup X, as shown in Scheme 8.

Another commonly applied conjugation method is the amide coupling, which involves an amide bond formation between a carboxylic acid and an amino group. Sugars lacking the carboxylic acid function can be activated where the primary hydroxyl groups are selectively oxidized. This can be achieved by using an excess amount of TEMPO as oxidant. The activated carbohydrate structure containing carboxylic acids can be conjugated to a protein carrier in a coupling reaction using a promoter such as EDC.
strategy was applied to generate a \textit{C. difficile} PSII–LTB glycoconjugate as previously mentioned.

![Scheme 8. Synthesis of MenX–CRM\textsubscript{197} glycoconjugate via reductive amination.](image)

Activation and conjugation strategy needs to be carefully planned for each sugar structure, since derivatization of carbohydrate structures can potentially disrupt the antigen epitopes required for antibody recognition. Therefore, one of the major advantages with synthetic carbohydrate structures is the ability to include a conjugation site, thus controlling the point of attachment to the carrier protein, while leaving the sugar epitope intact. Typically this involves a linker molecule attached at the anomeric position on the reducing end. These linker molecules also act as spacers, keeping the sugar moiety well away from the protein, avoiding potential steric or other intermolecular interference during antibody binding. Many types of linker molecules have been applied in glycoconjugate formation, including amino-, azide-carrying alkyls, as well as thioglycosides and \textit{n}-pentenyl glycosides\textsuperscript{148,149} Squaric acid diesters and adipic...
acid-based diesters are also popular linker system that can be installed efficiently under mild conditions.\textsuperscript{150} As an example, an amino-functionalized aminopropyl linker, as well as a di-\textit{N}-hydroxy-succinimidyl adipate ester as a spacer and activator have been applied by Adamo \textit{et al}. to generate a synthetic \textit{C}. \textit{difficile} PSII-CRM\textsubscript{197} glycoconjugate vaccine (Scheme 9).\textsuperscript{151}

\begin{center}
\textbf{Scheme 9.} Generation of an anti-\textit{C}. \textit{difficile} synthetic PSII-CRM\textsubscript{197} glycoconjugate vaccine. Reagents and conditions: (a) di-\textit{N}-hydroxy-succinimidyl adipate ester, Et\textsubscript{3}N DMSO; (b) CRM\textsubscript{197}, NaPi buffer, pH 7
\end{center}
1.6 Biochemical evaluation of glycoconjugates

The success of a glyconjugation can be evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a separation and analytical technique for macromolecules such as DNA and proteins. First, SDS-denatured glycoconjugate sample (protein unfolded into a linear chain) are loaded onto a polyacrylamide gel. In the presence of an electric field, conjugate sample containing negatively charges created under acidic environment moves through the gel toward the end with positive charge. Sample is separated by mass-to-charge ratio where larger molecules move more slowly through the gel while smaller molecules move faster. The separation of the conjugate sample can be visualized by a stain such as Bromophenol or Coomassie blue. The different sized molecules form distinct bands on the gel which can be compared to a standard protein marker to determine its molecular weight (MW). The success of sugar-to-protein linkage can be determined by locating species of higher MW than the unconjugated carrier protein.

A common procedure to evaluate the antigenicity of the synthetic glycoconjugate is to perform a Western blot experiment. After a SDS-PAGE analysis, glycoconjugate sample (containing the antigen of interest) is transferred and immobilized onto a nitrocellulose sheet. Then, the immobilized glycoconjugate antigen is incubated with a primary antibody, usually isolated from serum of animals immunized with a glycoconjugate or infected with native bacterial cells. If the glycoconjugate contains the active antigen epitope of interest, these primary antibodies are able to recognize and bind
to the immobilized antigen. A secondary enzyme-containing antibody, which recognizes the immobilized antigen-primary-antibody complex is then introduced. The resulting complex is visualized through the secondary antibody by producing a color change upon addition of the proper substrate. The intensity of the color produced is proportional to the amount of antibodies bound to the immobilized antigen. Another way to visualize the primary antibodies recognition is through a secondary antibody equipped with a fluorescently labeled probe. Successful recognition results in fluorescence, which can be detected by a photosensor.

Immuno-dot blot analysis is a simplification of the Western blot technique to test the antigenicity of a glycoconjugate, or sometimes unconjugated carbohydrate structures. Samples are applied directly onto a nitrocellulose membrane in one spot, without being first separated by electrophoresis. Then, following the same concept of Western blot experiment, antigen-antibody recognition is evaluated.

1.6 Scope of thesis

Bacteria produce polysaccharide structures on its cell surface. These structures are potentially immunogenic and can be utilized to create glycoconjugate vaccines. However, the two polysaccharides of interest, \textit{C. jejuni} CPSs and \textit{C. difficile} PS-I often have a low extraction yield from cultivated bacterial cultures. Chemical synthesis is an efficient alternative to generate these carbohydrate structures on a large scale.
Since PS-II is a *C. difficile* common antigen produced in abundant quantities, it has attracted the most conjugate vaccine research effort. However glyconjugate of *C. difficile* PS-I structure has yet to be generated. The deprotection of a previously synthesized *C. difficile* PS-I pentasaccharide will be described in this thesis. Conjugation using this pentasaccharide epitope as well as the immunological analyses of the synthetic PS-I pentasaccharide will be discussed.

The MeOPN modification expressed on *C. jejuni* CPSs plays an important role in virulence and has shown to be highly immunogenic in CPS conjugate vaccines. However this modification can be an obstacle during biological analyses due its phase-viable expression and noticeable degradation during extraction and purification procedures. A chemically well-defined MeOPN-containing carbohydrate structure is therefore welcome for the study of these molecules. This thesis describes a new strategy to synthetically introduce MeOPN modifications onto carbohydrate structures. The preparation of several MeOPN-containing glycan epitopes found in the CPS repeating unit of *C. jejuni* serotypes HS:23/36 and HS:1 will be described. Some of these structures are equipped with linker molecules and are conjugated to carrier proteins generating glycoconjugates. Novel conjugation methods and the immunological analyses of these glycoconjugates will be discussed.

Finally, experimental procedures including all the characterization data for the synthesized compounds and references cited conclude this thesis.
CHAPTER 2

Global deprotection of synthetic *C. difficile* PS-I repeating unit and its glycoconjugate to a subunit of *C. difficile* exotoxins
2.1 Introduction

Since PS-II is a C. difficile common antigen produced in abundant quantities, this structure has attracted the attention of most conjugate vaccine research groups. A number of glycoconjugates, utilizing both native and synthetic structures of PS-II, have been reported in the literature. However, studies on C. difficile PS-I are lacking and to date, a PS-I glycoconjugate has yet to be generated. Due to the fact that PS-I is not greatly expressed in hyper-virulent C. difficile ribotype 027 and therefore having a low extraction yield, a synthetic structure is needed. The total chemical synthesis of the protected PS-I pentasaccharide repeating unit 1 was achieved previously in the lab from four monosaccharide building blocks, shown in Scheme 10.154 First, trisaccharide 2 was constructed using building blocks 4-6 in a linear fashion. Trichloroacetimidate donor 4 carried a 2-O-Bz group to provide neighboring group assistance to generate the 1,2-trans β(1-4) linkage. In trichloroacetimidate donor 5, 2-O-All protecting group was installed to avoid participation in order to generate the desired 1,2-cis α(1-2) linkage. Glucosyl acceptor 6 was equipped with an aminopentanylinker at the reducing end to provide a point of attachment for the conjugation to a protein carrier. After trisaccharide 2 was achieved, both chloroacetate groups on O-3 positions were removed in a single step, leaving both positions accessible for a double glycosylation with rhamnosyl donor 3. Acetyl group on O-2 position of rhamnosyl donor 3 was again chosen to provide the necessary neighboring group assistance to generate the 1,2-trans linkages.
Chapter 2: Global deprotection of synthetic \textit{C. difficile} PS-I repeating unit and its glycoconjugate to a subunit of \textit{C. difficile} exotoxins

Using the protected PS-I pentasaccharide at hand, we move onto generating its glycoconjugate. This chapter describes the global deprotection of the PS-I pentasaccharide 1. This deprotected PS-I pentasaccharide structure is conjugated to a

\textbf{Scheme 10.} Retrosynthetic scheme of protected PS-I pentasaccharide 1 equiped with an aminopentanyln linker.
protein carrier to generate the corresponding glycoconjugate. This chapter also discusses the immunological evaluations of this synthetic PS-I pentasaccharide structure.

2.2 Generation of synthetic PS-I glycoconjugate

2.2.1 Global deprotection of synthetic PS-I pentasaccharide

![Diagram of synthetic PS-I pentasaccharide deprotection](image)

**Scheme 11.** Global deprotection of PS-I pentasaccharide 1 under dissolving-metal condition.

With the synthetic pentasaccharide, we began generating its glycoconjugate. First, global deprotection of pentasaccharide 1 was achieved in dissolving metal conditions.
with sodium metal in liquid ammonia and THF at -78 °C (Scheme 11).\textsuperscript{155} After 1 hour, the reaction was quenched with MeOH. Evaporation of the solvent followed by purification with Bio-Gel P-2 column and dialysis (500 Da MW cutoff) gave the deprotected pentasaccharide 7 in reasonably high yield (74%). This method proved to be an efficient deprotection strategy which allowed the removal of benzoyl, acetyl, benzylidene and carboxybenzyl (Cbz) groups in one step.

![Figure 13. ¹H NMR spectrum of synthetic PS-I pentasaccharide 7 obtained after dialysis. *Recurring contaminants from dialysis.](image)

The ¹H NMR spectrum for PS-I pentasaccharide 7 revealed five anomeric signals as well as characteristic signals for aminopentanyl linker, shown in Figure 13. Chemical shifts are in accordance with those of native PS-I and previously synthesized PS-I pentasaccharide reported in literature.\textsuperscript{156}
2.2.2 Generation of PS-I glycoconjugate

The synthetic PS-I pentasaccharide 7 was conjugated to a subunit peptide of *C. difficile* exotoxin B (ToxB: 248–262 fragment of the N-terminal *C. difficile* Toxin B, 1826.9 Da) to create a potential dual-valent anti-*C. difficile* vaccine. This was achieved with an EDC-promoted amide coupling reaction between the amine on the linker of PS-I pentasaccharide 7 and carboxylic acid functional group on the peptide. After stirring the mixture in MES buffer (pH 5.5) at room temperature for 1 day and 37 °C for 2 days, the conjugate 8 was purified with dialysis (1 kDa MW cutoff).

Scheme 12. Conjugation of synthetic PS-I pentasaccharide 7 to subunit peptide ToxB.
Mass spectrometry analysis of the PSI–ToxB conjugate 8 revealed a major species between m/z 3500 and 4500, which pointed to conjugate product containing 2 to 3 PS-I pentasaccharide units per peptide (Figure 14). The species at m/z 2000 is most likely due to doubly-charged species. Gel electrophoresis of the PS-I–ToxB conjugate also confirmed the presence of the same conjugate species observed by mass spectrometry (Figure 14). However, the gel-electrophoresis also showed some high molecular weight species, probably resulted from peptide cross-linking. No free peptide was observed in the gel electrophoresis of the conjugate product. Thus far, this PSI–ToxB conjugate is the only PS-I glycoconjugate reported in literature.

Figure 14. MALDI-TOF-MS and gel-electrophoresis of PS-I-ToxB glycoconjugate 8.
2.2.3 Immunological evaluation of PS-I glycoconjugate

Previous studies have shown that sera from healthy horses contain natural IgG antibodies against PS-II of *C. difficile*. Here, horse sera were analyzed for the presence of anti-PSI antibodies. The synthetic PS-I pentasaccharide 7 was used as the marker in an enzyme-linked immunosorbent assay (ELISA).

![Figure 15](image_url)

**Figure 15.** Detection of anti-PS-I IgG antibodies in horse serum against synthetic pentasaccharide 7 (black), and against native PS-I polysaccharide (gray). The results are displayed as a ratio of the optical density for the individual sera, divided by the optical density for a pooled positive control serum.

It was demonstrated that the horse sera containing IgG antibodies recognized both the synthetic PS-I pentasaccharide 7 and the native PS-I polysaccharide (Figure 15). With the exception of animals B and J, in which the response to the native PS-I was
significantly higher, the IgG antibody response to synthetic PS-I pentasaccharide and native PS-I polysaccharide was similar. These data indicate that healthy mature horses have circulating IgG antibodies that recognize non-phosphorylated PS-I pentasaccharide structure.

2.3 Concluding remarks

A previously synthesized PS-I pentasaccharide repeating unit of *C. difficile* ribotype 027 was globally deprotected in a single step under dissolving metal conditions. In an ELISA binding study, natural IgG antibodies in horse sera bound to the native PS-I and synthetic PS-I pentasaccharide 7, indicating the presence of antibodies specific for the non-phosphorylated regions of PS-I pentasaccharide structure. The conjugation of the synthetic pentasaccharide 7 to a subunit of *C. difficile* Toxin B was achieved through an EDC-mediated amide coupling between the aminopentanyl linker and the carboxylic acid on the peptide. This represents the first reported potential dual *C. difficile* PS-I glycoconjugate vaccine. This PS-I–ToxB glycoconjugate is now being evaluated in animals for immunogenicity.
CHAPTER 3

Synthesis of MeOPN-containing monosaccharide epitopes present in *C. jejuni*

serotype HS:23/36 and HS:1 CPS


3.1 Introduction

The MeOPN modification has been identified on most C. jejuni CPSs in non-stoichiometric amounts and has been shown to be a virulence factor during infection. It is noticed in the C. jejuni serotype HS:23/36 prototype glycoconjugate vaccine that even though MeOPN is not present in every CPS trisaccharide repeating block (due to non-stoichiometric expression), these MeOPN-containing monosaccharide epitopes are highly immunogenic. Also, the studies on mutant strain 8421 of serotype HS:23/36 point to the possibility that antibody might not be entirely raised from the internal epitope of the polysaccharide repeating unit but also raised from these MeOPN-containing epitopes. Therefore it is hypothesized that a synthetic conjugate containing only MeOPN monosaccharide epitopes could be immunogenic and potentially an effective antigen for a C. jejuni conjugate vaccine.

This chapter describes a new strategy to introduce MeOPN modification onto carbohydrate structures. The first synthetic target we designed is MeOPN→6-α-D-Galp, a monosaccharide epitope based on the non-reducing end of C. jejuni HS:23/36 CPS, as the 4-methoxyphenyl (OMP) galactoside (Figure 16). It is demonstrated that this synthetic epitope can be successfully recognized by C. jejuni CPS conjugate antisera of serotype HS:23/36, and cross-reacted with antisera from two other serotypes HS:1 and HS:4. For C. jejuni serotype HS:23/36, it has been reported that the MeOPN modifications is also detected non-stoichiometrically at C-2 of D-Gal in the CPS of strain 81-176. Recently, the MeOPN modification is also discovered
non-stoichiometrically at the C-4 position of D-Gal in the CPS of a *C. jejuni* mutant strain PG3718 of serotype HS:23/36. For *C. jejuni* serotype HS:1, MeOPN modification is located on C-6 position of D-Gal as well as C-3 of D-fructose. Therefore it is worthwhile to investigate whether if the MeOPN modifications on secondary positions offer similar antigenicity. With the newly developed strategy for the introduction of MeOPN modification, we attempted to synthetically generate these three MeOPN-containing monosaccharide epitopes, MeOPN→2-D-Gal, MeOPN→4-D-Gal and MeOPN→3-D-Fru. Chemical preparation and immunological studies of some of these structures will be discussed in this chapter.

![Structures of MeOPN epitopes](image)

**Figure 16.** Structures of MeOPN→6-α-D-Gal-OMP 9, MeOPN→2-β-D-Gal 10, MeOPN→4-β-D-Gal 11 and MeOPN→3-β-D-Fru 12.

### 3.2 Synthesis of MeOPN→6-Gal

#### 3.2.1 Purposed synthetic strategy to introduce MeOPN

Previous reports have described the preparation of *N*-protected alkyl and aryl phosphorimidates. However synthetic works on *N*-unsubstituted phosphorimidates
are rare in literature. The strategy for the introduction of MeOPN applied in this research thesis is inspired by a similar reaction performed by Mara et al. In this work, authors introduced \(N\)-substituted \(O\)-ethyl phosphoramidate units onto a series of phenol derivatives using the commercially available ethyl dichlorophosphate under basic conditions. This is followed by the displacement of the chlorine atom as a leaving group in the resulting compound with \(N\)-substituted primary and secondary amines. Although the authors, on one occasion, did report the generation of an \(N\)-unsubstituted \(O\)-ethyl phosphoramidate molecule, the yield of this reaction was extremely low (5%).

Scheme 13. Purposed synthesis strategy to introduce MeOPN moiety onto carbohydrate structures.

The strategy towards MeOPN-containing glycans applied in this research thesis involves a modified scheme from this approach which starts with a phosphorylation using the commercially available methyl dichlorophosphate as the phosphorylating agent in the presence of a base, such as \(\text{Et}_3\text{N}\) (Scheme 13). This generates a methyl-chlorophosphoryl sugar compound. Sequential displacement of the chlorine atom on this compound in situ by an ammonolysis reaction with ammonia gas generates the MeOPN modification.
This two-step-one-pot reaction strategy offers a simple and efficient way to introduce the MeOPN moiety onto carbohydrate structures.

3.2.2 Synthesis of MeOPN→6-α-D-galactoside

The first MeOPN-containing galactoside 9 is an epitope mimicking the terminal D-Gal expressed by *C. jejuni* serotype HS:23/36 CPS. This structure was prepared in 5 steps from a previously reported compound, 4-methoxyphenyl-α-D-galactopyranoside 13 (Scheme 14). Briefly, compound 13 was synthesized from D-Gal by acetylation, glycosidation with 4-methoxyphenol, followed by deacetylation under Zemplén conditions according to published methods.162 A trityl group was then selectively introduced to yield compound 14, distinguishing the O-6 position. Since the MeOPN functional group is unstable even in mild acidic conditions, suitable protecting groups circumventing these conditions need to be selected for the rest of the sugar hydroxyl groups. Thus, O-allyl groups were chosen to protect the C-2, C-3 and C-4 positions generating compound 15. These would be later removed with catalytic hydrogenolysis with palladium (II) chloride, which were proven to be compatible with the MeOPN modification. The trityl group was then removed generating compound 16, exposing 6-OH for modification.

The introduction of MeOPN onto compound 16 was first experimented in tetrahydrofuran (THF) with pyridine as base. Upon the complete depletion of the starting material (2 hours) as indicated by thin layer chromatography (TLC), ammonia gas was injected directly into the reaction mixture through a needle. However, after purification, a mixture of products was collected and the desired MeOPN product could not be obtained. It was speculated from the relatively rapid consumption of the starting material that pyridine was nucleophilic enough to displace both chlorine as well as the OMe group from the methyl dichlorophosphate reagent which led to the formation of undesired products.

As a second attempt, the reaction was performed with Et₃N as a base in THF. The starting material was consumed after stirring the reaction overnight at room temperature. As before, ammonia gas was injected directly into the reaction mixture through a needle.
Although the yield was low, the desired MeOPN product 17 was isolated. However, a gel-like substance formed during the reaction and remained after ammonia was introduced, which somewhat complicated the purification process. When the same reaction was performed in CH$_2$Cl$_2$, this problem was solved. Some solids formed during reaction in CH$_2$Cl$_2$ can be simply removed with filtration, and the concentrated residue can be directly loaded onto a silica gel column. Also, the yield of the reaction was improved slightly from previous attempts performed in THF. Therefore, future MeOPN introductions were mostly performed in CH$_2$Cl$_2$.

The $^{31}$P NMR spectrum of product 17 revealed two phosphorus signals in an approximately 1:1 ratio at 10.5 ppm. This is indicative of two diastereoisomers resulting from the chiral nature of the newly introduced MeOPN. The $^1$H NMR spectrum also indicated the presence of a mixture of two diastereoisomers with two sets of $^1$H signals. This is especially apparent with the anomic and POCH$_3$ resonances. The reaction also yielded a mixture of side products, the most abundant being that in which the POCH$_3$ group on the MeOPN was replaced by a second NH$_2$ group. NMR of this di-amino-phosphoramidate product 18 reveals one $^{31}$P signal due to the lost of the stereocenter at the O-6 position. No POCH$_3$ signal is detected by $^1$H NMR and two NH$_2$ signals can be observed which integrate into four protons (Figure 17).
Chapter 3: Synthesis of MeOPN-containing monosaccharide epitopes present in *C. jejuni* serotype HS:23/36 and HS:1 CPS

![Diagram](image)

**Figure 17.** $^{31}$P NMR (top) and $^1$H NMR spectrum (bottom) of side product di-amino-phosphoramidyl-$\alpha$-D-galactoside 18.

The formation of the di-amino-phosphoramidate product is tough to explain. It was observed that the reaction time and the amount of NH$_3$(g) introduced into the reaction did not affect the amount of MeOPN and di-amino-phosphoramidate product. These observations indicate that the -OMe group is not displaced during ammonolysis. Therefore it is hypothesized that -OMe group of the methyl dichlorophosphate reagent...
might not be stable under such basic conditions, and is displaced generating an unknown intermediate structure, and leads to the formation of the di-amino-phosphoramidate product during ammonolysis. These types of side-products will be further discussed in later sections.

Figure 18. $^{31}$P NMR (top) and $^1$H NMR spectrum (bottom) of MeOPN→6-α-D-galactoside 9.
Figure 19. 2D $^1$H-$^{31}$P HMBC NMR spectrum of MeOPN→6-α-D-galactoside 9.

Removal of the allyl groups with palladium (II) chloride generated MeOPN→6-α-D-galactoside 9. Similar to compound 17, a mixture of two diastereoisomers is observed by $^1$H and $^{31}$P NMR spectroscopy (Figure 18). The anomeric proton signal is observed as two doublets due to the two diastereoisomers. The POCH$_3$ signal also shows up as two sets of signals from the diastereoisomerism and splitting from the $^{31}$P nucleus transforms them into doublets. The $^{31}$P signals of this synthetic MeOPN-carrying galactoside (14.58 ppm and 14.43 ppm) are in accordance with the typical $^{31}$P signals produced by native MeOPN-containing polysaccharides (~14
ppm). A 2D $^{31}\text{H}-^{31}\text{P}$ HMBC (heteronuclear multiple bond correlation) NMR experiment was able to confirm that the MeOPN unit was introduced to the C-6 position through the observation of a correlation signal between the phosphorus of MeOPN and the H-6 resonances of Gal (Figure 19). The low yield of this deprotection might be due to the low concentration of palladium (II) chloride and short reaction time used during this reaction. Deprotection reaction with the same chemistry in later syntheses gave much higher yields.

3.2.3 Immunodetection of MeOPN→6-α-D-galactoside

In an immuno-dot-blot analysis, the synthetic MeOPN→6-α-D-Gal-(1→OMP 9 was tested for reactivity with antisera previously raised against native C. jejuni CPS conjugates of serotypes HS:1, HS:3, HS:4 and HS:23/36.\textsuperscript{163-165} Figure 20 shows that MeOPN-containing compound 9 was strongly recognized by HS:23/36 CPS conjugate antisera (CPS with MeOPN at C-6 of Gal). This is expected due to the immunodominance of the MeOPN epitope observed in the HS:23/36 CPS prototype vaccine. More interestingly, the synthetic galactoside was also recognized by CPS conjugate antisera from two other serotypes; HS:4 (CPS with MeOPN at C-7 of ido-heptose) elicited a response almost equivalent to anti-HS:23/36 CRM\textsubscript{197} conjugate in intensity, and HS:1 (CPS with MeOPN at C-3 of Fru and low amounts of MeOPN at C-6 of Gal) elicited a response with a lower intensity. The cross-reactivity observed, specially in the case of serotype HS:4, suggests that these antisera might contain antibodies that specifically
recognize MeOPN units at primary positions. The HS:3 CPS conjugate antisera (CPS with MeOPN at C-2 of ido-heptose) did not react with compound 9. As a control experiment, no reaction was observed between α-D-Gal-(1→OMP 13 (devoid of MeOPN) and native HS:23/36 CPS conjugate antisera (data not shown).

**Figure 20.** Immunodetection of MeOPN→6-α-D-Galp-(1→OMP 9 by native *C. jejuni* CPS conjugate antisera of serotypes HS:1 (final dilution 1:500), HS:3 (final dilution 1:500), HS:4 (final dilution 1:2000) and HS:23/36 (final dilution 1:2000).

The recognition of the synthetic MeOPN→6-α-D-Gal-OMP 9 by HS:23/36, HS:4 and HS:1 CPS conjugate antisera points to the fact that these polyclonal preparations might contain specific antibodies toward MeOPN units at primary positions. The observed immunoreactions of 9 with CPS conjugate antisera are even more significant due to the fact that presumably only half of the synthetic preparation contained the MeOPN stereoisomer present in the native *C. jejuni* CPSs. The stereochemistry of
MeOPN in the native *C. jejuni* CPSs is not yet known. The immunodetection of MeOPN→6-α-D-Gal 9 is an indication that the MeOPN antigenic determinant could be used as an effective vaccine antigen where a single MeOPN-containing epitope could cross-protect across multiple *C. jejuni* serotypes.

### 3.3 Synthesis of MeOPN→2-Gal

#### 3.3.1 Synthesis of MeOPN→2-β-D-galactoside

It has been reported that the MeOPN modification is also detected non-stoichiometrically at C-2 position of D-Gal in the CPS of *C. jejuni* strain 81-176 of serotype complex HS:23/36.\textsuperscript{157,158} The synthesis of this MeOPN-containing galactoside 10 began with a known compound 4-methoxyphenyl 3,4-O-isopropylidene-6-O-trityl-β-D-galactopyranoside 19, which was prepared in 5 steps from D-Gal following published procedures (Scheme 15).\textsuperscript{166}

To distinguish the C-2 position, *O*-allylation was performed generating compound 20 in excellent yield. Since MeOPN can be removed in acidic media, suitable protecting groups need to be installed. Therefore *O*-isopropylidene and *O*-trityl groups were removed in a single step here under acidic conditions, giving compound 21, which was then per-benzoylated generating compound 22. Next, allyl group was removed giving compound 23 with a free 2-OH for modification.

The introduction of MeOPN group onto compound 23 followed the same strategy previously described. The $^{31}$P NMR spectrum of MeOPN product 24 revealed two phosphorus signals in a roughly 1:1 ratio due to the formation of two diastereoisomers. This reaction also yielded the di-amino-phosphoramidate side-product 25.

The reactivity of 23 during MeOPN introduction is notably lower than what was observed during the synthesis of MeOPN→6-Gal. This is overcome with a greater equivalence of reagents (10 equivalents) combined with a longer reaction time at elevated temperature (40 °C). The reduced reactivity is expected from the secondary 2-OH than the primary hydroxyl group 6-OH. The nucleophilicity can be further decreased by the electron-withdrawing O-benzoyl groups.
Figure 21. $^1$H-$^{31}$P HMBC NMR experiment of 10* showing correlation between the phosphorus of MeOPN and H-2 resonance.

As the last step of the synthesis, compound 24 was de-benzoylated generating MeOPN-containing galactoside 10. The pH value needs to be carefully controlled during this reaction as a higher concentration of NaOMe was found to cleave the MeOPN modification. $^1$H-$^{31}$P HMBC NMR experiment was able to confirm that the MeOPN was introduced to the C-2 position through the correlation signal between the phosphorus of MeOPN and H-2 resonance of Gal (Figure 21). Interestingly, one of the diastereoisomers can be purified here using flash chromatography. $^{31}$P NMR spectrum of the mixture 10
reveals two signals at 14.27 ppm and 14.04 ppm, while the pure diastereoisomer 10* reveals a single signal at 14.27 ppm (Figure 22). The absolute configuration of this diastereoisomer is not yet assigned.

![Figure 22. 31P NMR spectrum of the mixture 10 and the pure diastereoisomer 10*.](image)

3.3.2 Immunodetection of MeOPN→2-β-D-galactoside

Both the mixture of diastereoisomers 10 and the pure diastereoisomer 10* of synthetic MeOPN→2-β-D-Gal were evaluated in an immunodot-blot analysis, shown in Figure 23. Both diastereoisomers were able to be recognized by antisera raised by C. jejuni HS:23/36 strain 81-176 wild-type cell (CPS with MeOPN at C-2 and C-6 of Gal). Although the recognition is not as strong as its MeOPN→6-Gal counterpart, this
observation demonstrates that the antisera of *C. jejuni* strain 81-176 wild-type cell contain antibodies that target MeOPN on the C-2 position. The immunodot-blot analysis also indicate that the antisera of HS:23/36 CPS conjugate (CPS with MeOPN at C-6 of Gal) was able to detect synthetic MeOPN→6-Gal structure but did not detect either diastereoisomers of MeOPN→2-Gal. This indicates that antisera of HS:23/36 CPS conjugate, containing antibodies that target MeOPN on primary O-6 position, does not cross-react with MeOPN on secondary O-2 position. These data further demonstrate the antibody specificity toward MeOPN on primary and secondary positions, as well as the immunodominance of MeOPN-6-Gal epitope over MeOPN-2-Gal.

![Detecting Antiserum](image)

**Figure 23.** Immunodetection of synthetic MeOPN→6-α-Gal 9, (A) synthetic MeOPN→2-β-D-Gal 10, and (B) pure diastereoisomer 10* with anti-HS:23/36 conjugate and anti-wild type 81-176 antibody.
Unfortunately, only one of the two diastereoisomers could be isolated, and therefore results were inconclusive on which diastereoisomer is immunoactive. This synthetic MeOPN→2-Gal is currently being tested for cross-reactivity against other antisera raised from CPS conjugates with MeOPN on secondary positions.

3.4 Synthesis of MeOPN→4-Gal

3.4.1 Synthesis of MeOPN→4-β-D-galactoside

Recently, the MeOPN modification is discovered non-stoichiometrically expressed at the C-4 position of D-Gal in the CPS of a *C. jejuni* mutant strain PG3718 that belongs to serotype complex HS:23/36.\(^{165}\) We therefore designed a MeOPN→4-β-D-Gal epitope \(\text{11}\) for immunological analysis. In addition, this scheme is designed to test the MeOPN introduction strategy on a relatively less reactive position, as the 4-OH is known to have the least reactivity in Gal structures.\(^{167}\) The synthesis of galactoside \(\text{11}\) began with a known compound 4-methoxyphenyl-β-D-galactopyranoside \(\text{26}\), obtained from published procedure. Then, a selective benzoylation with \(\sim 3\) equivalents of benzoyl chloride on galactoside \(\text{26}\) yielded the 2,3,6-tri-O-benzoylated product \(\text{27}\) (Scheme 16).

The selectivity can be explained by the difference in reactivity between the four hydroxyl groups in a galactoside. Hydroxyl groups in the axial orientation are expected to undergo acylation less rapidly than OH groups in the equatorial orientation, which are less sterically hindered and much more accessible. In addition, the 4-OH is further sterically hindered by the larger hydroxymethyl group on the C-5 position and therefore
has the lowest reactivity. However, the yield attained here is unexpectedly lower than anticipated, generating significant amount of 3,4,6-tri-\(O\)-benzoylated and the fully benzoylated product. The separation process by flash chromatography is also extremely tough. A different strategy approaching 4-\(OH\) galactopyranosides should be followed in the future.


The introduction of MeOPN modification onto compound 27 followed a similar strategy as before. After stirring the sugar with methyl dichlorophosphate in the presence of \(\text{Et}_3\text{N}\) (40 eq.) for 48 hours at 35 °C, the starting material was completely consumed, as indicated by TLC. The low reactivity is expected, as 4-\(OH\) has the least reactivity in a galactoside, and further decreased by electron-withdrawing \(O\)-Bz groups. After purification by flash chromatography, MeOPN product 28 was collected as two diastereoisomers in a roughly 2:1 ratio as indicated by \(\text{\textsuperscript{31}}\text{P}\) and \(\text{\textsuperscript{1}}\text{H}\) NMR. \(\text{\textsuperscript{1}}\text{H-\textsuperscript{31}}\text{P}\) HMBC NMR experiment was able to confirm that the MeOPN group was introduced to the C-4 position through the correlation between the phosphorus of MeOPN and the H-4.
resonance (Figure 24). The phosphorus signals also correlate to POCH₃ and NH₂ resonances. The yield of this reaction is much lower than similar reactions performed on 6-OH and 2-OH of Gal. This is likely correlated to the low reactivity of the 4-OH position. A large amount of the di-amino-phosphoramidate product 29 was also generated.

**Figure 24.** $^1$H-$^{31}$P HMBC NMR experiment of 28 showing correlation between the phosphorus of MeOPN and H-4 resonance.
Next, de-benzoylation of compound 28 was attempted. Same to the deprotection strategy of MeOPN-2-Gal, 0.01 M sodium methoxide (NaOMe) in MeOH was initially applied (Scheme 17). The starting material was found to be consumed quickly (1 hour) and produced one major spot as indicated by TLC. To our surprise, this was not the desired MeOPN product. $^1$H NMR confirmed the removal of O-Bz groups. However, $^{31}$P NMR analysis revealed that this product contained two phosphorus signals in a 2:1 ratio at 1.36 ppm and 0.93 ppm, instead of the typical MeOPN signals around 14 ppm. 2D heteronuclear single quantum correlation (HSQC) and $^1$H-$^{31}$P HMBC NMR experiments confirmed presence of POCH$_3$ signals, and revealed that phosphorus was attached to C-4. These data indicate that NH$_2$ group might be altered. ESI-MS data confirms that this spot contained the O-methyl phosphate product 30 (NH$_2$ replaced by OH).

![Scheme 17](image)

**Scheme 17.** Deprotection of compound 28 to generate O-methyl phosphate product 30 and MeOPN product 11. Reagents and conditions: (a) NaOMe, MeOH; (b) sat. NH$_3$(aq) in MeOH; (c) 7:2:1 Et$_3$N/MeOH/H$_2$O.

A second attempt was performed at a milder condition with 0.005 M of NaOMe in MeOH. Reaction proceeded much slower (~3 hours) but produced the same undesired
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*O*-methyl phosphate product 30. Longer reaction times results in the removal of MeOPN modification. These surprising results were not observed before during the deprotection of MeOPN-2-Gal 27.

The de-benzoylation reaction was also attempted with saturated NH₃(aq) in MeOH solution. Starting material 28 was consumed in ~6 hours but produced the same results as before with NaOMe, generating the *O*-methyl phosphate product 30.

Finally, the deprotection of 28 was attempted in a 7:2:1 mixture of MeOH/H₂O/Et₃N. Starting material 28 was completely consumed in ~5 hours producing the undesired *O*-methyl phosphate product 30, as indicated by TLC. Longer reaction time led to significant cleavage of the MeOPN group, generating OMP-galactoside as well as degraded products at TLC baseline. However, with an extremely low yield (14%), some deprotected MeOPN product 11 was able to be recovered. This deprotected product 11 was collected as a single diastereoisomer, producing a single phosphorus signal at 14.65 ppm. ¹H-¹H correlation spectroscopy (COSY), HSQC and ³¹H-³¹P HMBC NMR experiment were able to confirm that the MeOPN was located at the C-4 position through the correlation between the phosphorus of MeOPN and the H-4 resonance. ESI-MS result also confirms the identity of MeOPN product 11. This deprotection reaction was also attempted at milder conditions with a mixture of 16:3:1 MeOH/H₂O/Et₃N. Similar result as before was observed, generating a large amount of degraded products. A low amount of MeOPN-containing product 11 was collected as a single diastereoisomer (13%). The fact that a single diastereoisomer is observed might be simply due to the extremely low
yield. This deprotection strategy is still being optimized in the lab. This MeOPN-containing galactoside will be tested in immunological analysis.

3.5 Synthesis of MeOPN→3-Fru

3.5.1 Synthesis of MeOPN→3-D-fructoside

The MeOPN→3-β-D-fructoside is an epitope expressed by C. jejuni serotype HS:1. During immunostudies of the first MeOPN→6-α-D-galactoside, the synthetic epitope can be weakly recognized by HS:1 antisera. This surprising result is credited to the presence of low amounts of MeOPN at C-6 of Gal in the CPS. To verify, we attempted to generate this Fru epitope for immunological studies.

The first attempt toward MeOPN→3-D-fructoside began with thioglycoside donor 31 (Scheme 18), which was prepared according to procedures described in literature.168 Taking advantage of the unique reactivity of D-Fru, a selective benzoylation was able to yield a 1,3,4,6 tetra-O-benzoylated product, leaving the anomeric 2-OH free for modification. The simple conversion of this product into the 2-O-acetylated product then allowed the generation of the corresponding thioglycoside donor via a BF3•etherate-promoted reaction with ethanethiol. From the α/β mixture 31, an aminopentyl linker was introduced as a point of attachment to the carrier protein. Here, the pure α-anomer 32 was obtained, due to participation effect from the 3-O-benzoyl group. In order to distinguish the O-3 position, benzoyl protecting groups were removed generating compound 33, and then a subsequent selective benzoylation with ~3
equivalents of benzoyl chloride with pyridine generated a 1,4,6-tri-O-benzoylated product 34, leaving 3-OH for modification.

Scheme 18. Synthesis of building block 34 towards MeOPN→3-Fru.

The introduction of MeOPN onto the O-3 position of structure 34 was initially attempted with the same strategy as developed before with methyl dichlorophosphate in the presence of Et₃N. After 2 days of stirring at room temperature the starting material remained with no apparent change. A higher concentration of reagent and base (35-45 eq.) at elevated temperature (40 °C in CH₂Cl₂, 60 °C in THF, 60 °C in 1,2-dichloroethane and 80 °C in DMF) was able to push the reaction forward. Once the starting material was consumed (2-3 days), the reaction mixture was treated with ammonia gas. In most cases, TLC revealed one major product, which was purified with flash chromatography (Table...
2. However, NMR analysis on this product revealed only one phosphorus NMR signal and lacked of a POCH$_3$ signal on $^1$H NMR. Further NMR and MS data supports the fact that the OMe was replaced by an NH$_2$, giving a di-amino-phosphoramidate product 36 (Scheme 19). In the reaction performed in 1,2-dichloroethane, a small amount of MeOPN product 35 was isolated with an extremely low yield (5%) with the majority being the di-amino-phosphoramidate product 36. The location of the MeOPN modification can be confirmed by HMBC NMR experiment (Figure 25).


Table 2. Introduction of MeOPN onto compound 34

<table>
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<th>Temperature</th>
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<th>37</th>
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<td>Et$_3$N</td>
<td>40 °C</td>
<td>-</td>
<td>21%</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>CH$_2$Cl$_2$</td>
<td>pyridine</td>
<td>40 °C</td>
<td>-</td>
<td>10%</td>
<td>17%</td>
</tr>
<tr>
<td>3</td>
<td>THF</td>
<td>Et$_3$N</td>
<td>60 °C</td>
<td>-</td>
<td>20%</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>13%</td>
<td>-</td>
</tr>
<tr>
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<td>ClCH$_2$CH$_2$Cl</td>
<td>Et$_3$N</td>
<td>60 °C</td>
<td>5%</td>
<td>18%</td>
<td>-</td>
</tr>
</tbody>
</table>
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The successful acquisition of these MeOPN products in previous synthesis shows that the OMe group should be stable under basic conditions during ammonolysis, as well as subsequent aqueous work up procedures. Therefore the OMe group is not replaced during ammonolysis and must arise from the phosphorylation step through an unknown mechanism. It is speculated here that the excess reagent and elevated temperature used to push the reaction to completion promoted this side reaction. The reaction was also attempted with N-methylimidazole (NMI), pyridine, as well as the addition of 4-dimethylaminopyridine (DMAP), but could not generate the desired product. Reactions using pyridine also produced a phosphoramidate product 37, with the NH$_2$ group installed but lacked of OCH$_3$.

**Figure 25.** $^1$H-$^3$P HMBC NMR experiment of 35 showing correlation between the phosphorus of MeOPN and H-3 resonance of D-Fru.
The low reactivity of 3-OH of D-fructoside is likely due to steric reasons, as observed during the selective benzoylation of 33. The electron-withdrawing nature of O-benzoyl groups might further decrease the nucleophilicity of this position. It is thus hypothesized that when the reactivity of 3-OH is increased, MeOPN can be introduced as before at a milder condition, and therefore minimizes the side-reaction.

![Scheme 20](image)

**Scheme 20.** Synthesis towards building block 41 in attempt to increase O-3 reactivity.

To increase nucleophilicity of 3-OH, attempts were made to replace O-benzoyl groups with the smaller, slightly electron-donating O-allyl protecting groups, which were proven to be compatible with the MeOPN modification. First, a tert-butylidimethylsilyl (TBDMS) protecting group was introduced to the O-3 position, generating compound 38 (Scheme 20). O-Benzoyl groups were then removed generating compound 39. Allylation of O-1,4,6 positions would generate the tri-O-allylated product 40. TBDMS protecting
group could be removed with tetra-\textit{n}-butylammonium fluoride (TBAF), generating compound 41 for modification.

Unfortunately, allylation of 39 with NaH did not produce the desired product. A number of unexpected side products were isolated which were believed to be the outcome of side reactions between allyl bromide and phthalimide group promoted by NaH. This result is supported by literature according to Meng et al.\textsuperscript{169} The hydride ion generated by NaH attacks one of the carbonyl carbons on phthalimide forming a amide anion, which is in turn \textit{N}-allylated. The allylations were also attempted with NaOH in DMF to avoid these side reactions. However, the phthalimide group was removed under such basic conditions. Alternatively, the reaction was attempted under neutral condition with silver (I) oxide, but produced a similar result as observed before with NaH.

Therefore another attempt at building block 41 followed a slightly modified scheme (Scheme 21). Since phthalimide is not compatible with NaH, the linker molecule can be introduced after the installation of allyl groups. Starting from ethyl 2-thio-\alpha-D-fructofuranoside 42, prepared from published procedures,\textsuperscript{168} a selective benzylation was first conducted, generating a 1,4,6-tri-\textit{O}-benzoylated product 43. As before, TBDMS was chosen to temporarily protect the O-3 position, giving compound 44. Replacement of \textit{O}-benzoyl groups with \textit{O}-allyl groups was thought to generate a tri-\textit{O}-allylated product 46. This would be followed by the introduction of the aminopentyl linker molecule, generating compound 47. The removal of TBDMS would give product 40, ready for MeOPN introduction.
Scheme 21. An alternative strategy to synthesize building block 41.

During the glycosidation reaction of 46, some degradation of TBDMS was observed. Moreover, confusing NMR spectrum of the degradation product raised concerns about the identity of starting sample 46. To investigate, a de-silylation reaction was performed with TBAF on a fraction of compound 46. COSY NMR experiment revealed that the product was in fact thiofructoside 49 carrying a 1-OH group (Scheme 22). O-All ethers are not prone to migration and therefore the TBDMS protecting group must have migrated to the O-1 position in the starting material 46. This unexpected structure might
have been the result of a base-promoted 1→3-silyl migration during allyl introduction (Scheme 23). The migration is likely to take place since O-1 is less sterically crowded than the O-3 position.

**Scheme 22.** Unexpected product 49 isolated after TBDMS removal.

In place of TBDMS, 4-methyloxybenzyl group (PMB) was also tested on compound 45 to prevent migration. However extensive degradation and migration of O-Bz groups under basic conditions (NaH) forced us to discontinue this strategy.

**Scheme 23.** Formation of compound 50 explained via a 1→3-silyl migration.
As an interesting side note, when a selective benzylation with ~3 equivalent of BzCl was performed on ethyl 2-thio-β-D-fructofuranoside 51, a 1,3,6-tri-O-benzyolated compound 52 was isolated as the major product. This result was surprising since a 1,4,6-O-selectivity was observed previously in the α-anomer (Scheme 24), suggesting that the 3-OH is least reactive position in α-furanosides. This is due to the quaternary carbon centre at the anomeric position causing a steric effect. However the formation of 1,3,6-tri-O-benzyolated β-product suggests that the reactivity of 3-OH can be largely affected by the orientation of the anomeric substituents. When the hydroxymethyl (CH₂OH) is oriented on the top face in the α-anomer, O-3 position is more sterically crowded due to the unfavourable flagpole/diakial interaction and thus less reactive. In the β-anomer, O-3 position is much more reactive, even more than the O-4 position.

With this 1,3,6-tri-O-benzyolated β-product 52, we designed a scheme to test the introduction of MeOPN unit onto the C-3 position, without the linker molecule (Scheme 24). Compound 53 was first allylated at the O-4 position, generating compound 54. Extensive migration of O-Bz groups was observed during this reaction, causing an extremely low reaction yield. Removal of the benzoyl groups gave compound 54. A second selective benzylation with ~2 equivalent of BzCl generated product 55, with a free 3-OH for modification.
Scheme 24. Synthesis of β-thiofructoside 55 to test the introduction of MeOPN modification

The introduction of MeOPN was attempted on compound 55 as before with methyl dichlorophosphate in the presence of Et₃N. The reactivity of 55 did not improve significantly during MeOPN introduction from structure 34, judging by the reaction rate. After stirring for 3 days with methyl dichlorophosphate in the presence of Et₃N (45 eq.) at 40 °C in CH₂Cl₂, starting material was consumed and the reaction mixture was treated with ammonia gas. However, this time MeOPN product 56 was successfully obtained in 34% yield (Scheme 25). Sadly, deprotection of this product could not be carried out due to the incompatibility between allyl and ethanethiol group. This result further suggests that the reactivity of the modification site greatly affects the success of this one-pot MeOPN introduction method.


Scheme 25. Formation of MeOPN product 56 and di-amino-phosphoramidate product 57 during MeOPN introduction.

3.6 Concluding remarks

In summary, a new synthetic strategy to introduce MeOPN moiety onto carbohydrate structures is successfully developed. This strategy involves a two-step-one-pot reaction that starts with a phosphorylation using commercially available methyl dichlorophosphate in the presence of a base, such as Et$_3$N. This is followed by an ammonolysis reaction with ammonia gas. This introduction strategy is first applied in the synthesis of the MeOPN→6-α-D-Gal-OMP 9, a monosaccharide epitope from the C. jejuni HS:23/36 CPS repeating unit. Although our one-pot approach to introduce MeOPN gives generally low yields, it offers a relatively inexpensive and straight-forward method to generate MeOPN-containing glycans. In a dot-blot immunological study, it is demonstrated that the MeOPN→6-α-D-Gal-OMP 9 structure can be recognized by antisera raised by conjugates of three different serotypes with native CPSs containing
MeOPN at primary positions. This result points to the presence of antibody specifically targets MeOPN on primary locations.

The MeOPN-carrying monosaccharide epitopes expressed by *C. jejuni* strain 81-176 of serotype HS:23/36 is successfully synthesized. Interestingly, one of the two diastereoisomers of MeOPN→2-β-D-Gal-OMP 10 can be purified with flash chromatography. Both diastereoisomers have been tested with dot-blot immunological study. It is demonstrated that this structure can be recognized by antisera raised by *C. jejuni* strain 81-176 wild type cell and does not cross-react with antisera raised by native HS:23/26 CPS conjugate. This synthetic galactoside is currently being tested for cross-reactivity against antisera from other CPS conjugates containing MeOPN on secondary positions. The next step would be to generate the corresponding glycoconjugate using this monosaccharide epitope for immunological testing.

The MeOPN introduction strategy is applied on a relatively more sterically hindered 4-OH position to generate the MeOPN→4-β-D-Gal-OMP epitope 11, expressed by *C. jejuni* mutant strain PG3718 belonging to serotype complex HS:23/36. The removal of O-Bz of the protected MeOPN product proves to be tricky as methanolic NaOMe and sat. NH₃ deprotection strategies generate the O-methyl phosphate product 30. MeOPN product 11 is obtained using a mixture of MeOH-H₂O-Et₃N. This deprotection strategy is being optimized in the lab.

Unfortunately, synthesis toward MeOPN→3-D-fructoside using our one-pot introduction strategy generates the desired product with extremely low yield. Introduction
of MeOPN onto structure 34 yields mainly the di-amino-phosphoramidate product 36. Attempts to replace of O-Bz with O-All groups to increase reactivity of 3-OH position are unsuccessful due to side-reactions with phthalimide group on the linker arm in the presence of NaH. Alternative synthetic routes are also unsuccessful due to degradations and migrations of O-Bz and O-TBDMS protecting groups. The MeOPN introduction strategy is also tested on a slightly more reactive β-D-fructofuranoside 55 and afforded desired MeOPN product in moderate yield. This result suggests that the reactivity of the modification site greatly affects the success of this MeOPN introduction method.

Although unpromising, the synthesis endeavour towards MeOPN→3-fructoside offers insight into the unique reactivity of D-fructosides, protecting group manipulation, and mechanism of MeOPN introduction. The reason for the formation the di-amino-phosphoramidate product is still unclear. Although not proven experimentally, we speculate that the amount of the di-amino-phosphoramidate product may be correlated to the reactivity of the site of modification. Base on the data collected in this thesis, as the reactivity of the modification site decreases, the yield of the corresponding MeOPN product decreases while the amount of di-amino-phosphoramidate product increases. This MeOPN→3-D-fructoside structure is also generated with with an alternative introduction method, which is discussed in Chapter 5.
CHAPTER 4

Synthesis of the first MeOPN-containing monosaccharide conjugate against \textit{C. jejuni}

serotype HS:23/36
4.1 Introduction

Immunological studies of the synthetic epitopes indicate that the MeOPN-containing epitopes can be recognized by antisera raised by native \textit{C. jejuni} CPS conjugates. Therefore it is hypothesized that a synthetic conjugate containing only MeOPN monosaccharide epitopes could be immunogenic and potentially an effective \textit{C. jejuni} vaccine. With the development of the MeOPN introduction method, here we describe the first attempt in developing such a synthetic \textit{C. jejuni} glycoconjugate vaccine based on a synthetic MeOPN-containing monosaccharide epitope, MeOPN→6-Gal. This MeOPN-containing epitope resembles the non-reducing end of \textit{C. jejuni} HS:23/36 CPS, and is equipped with a linker molecule for conjugation. This chapter also describes a novel conjugation scheme involving an oxidized-starch structure, as well as the immunological evaluations of these glycoconjugates.

4.2 Synthesis of MeOPN→6-Gal conjugate

4.2.1 Synthesis of MeOPN→6-Gal conjugate

Starting from galactoside 15, described previously, the OMP protecting group at the anomeric position was removed. The corresponding hemiacetal was then converted into trichloroacetimidate donor 58 in the presence of potassium carbonate (Scheme 26). As a site of attachment for the carrier protein, 5-amino-\textit{N}-phthalimido-pentanyl linker was introduced with TMSOTf as an activator at 0 °C. A mixture of two anomers was
collected with 29% as the β anomer 59 and 65% as the α anomer 60. The α/β ratio was found to be largely affected by temperature, where a higher temperature generated more α anomer as the thermodynamic product.

Scheme 26. Synthesis of MeOPN→6-D-Gal-linker 67 and 68.
As a way to study the importance of the anomeric orientation of the monosaccharide epitope in glycoconjugates, both α and β anomers were converted into the corresponding MeOPN-galactoside and glycoconjugate. Thus, the removal of trityl group gave free 6-OH groups on both anomers 61 and 62 for the introduction of MeOPN. Using the same strategy as described before, MeOPN-containing galactoside 63 and 64 were collected each as a mixture of two diastereoisomers in a 1:1 ratio. Allyl and phthalimido protecting groups were removed subsequently giving 67 and 68 with acceptable yields.

As an attempt to increase the yield, introductions of MeOPN modification onto compounds 61 and 62 were also performed with the base NMI. Both compounds 61 and 62 were treated with methyl dichlorophosphate in the presence of NMI at room temperature. The reaction proceeds much more rapidly than using Et₃N. Upon complete consumption of starting material (4 hours) as indicated by TLC, ammonia gas was introduced. Although MeOPN products were obtained, the yield did not vary significantly (21% and 23% respectively for 61 and 62) from using Et₃N, and both cases yielded a mixture of two diastereoisomers in a 1:1 ratio.

However it is worthy to note that the reactions with NMI did not produce the di-amino-phosphoramidate product, one of the major side-product observed during the introduction of MeOPN with Et₃N. Instead, reactions in the presence of NMI generated a large amount of O-methyl phosphate by-products, carrying a -OH group instead of an -NH₂ group, with the -OMe group intact. It is hypothesized here that the nucleophilic substitution with methyl dichlorophosphate in the presence of NMI first undergoes a
methylimidazolium ion. The relative high reactivity of this complex in the presence of trace amount of water introduced during the reaction promotes the formation of O-methyl phosphate products. However, it seems that NMI is not basic enough to remove the -OMe group from the reagent. The successful acquisition of the MeOPN and O-methyl phosphate products here again supports the fact that the -OMe group or -OH is not replaced during ammonolysis. It is hypothesized that -OMe group of the methyl dichlorophosphate reagent is not stable under such basic conditions in Et$_3$N, which is replaced generating an unknown intermediate structure, and leads to the formation of the di-amino phosphoramidate product during ammonolysis.

**Scheme 27.** Conjugation of MeOPN→6-β-D-Gal-linker 67 to protein carrier CRM$_{197}$. 

![Scheme 27](image-url)
Conjugation was first performed on the linker-equipped β anomer 67. Compound 67 was first treated with an excess of di-\textit{N}-hydroxysuccinimidyl adipate in dimethyl sulfoxide (DMSO) in the presence of Et\textsubscript{3}N (Scheme 27). The resulting half ester 69 was condensed with the amino groups on the protein CRM\textsubscript{197} in phosphate buffer (NaPi buffer, pH 7.0). After purification with dialysis (25 kDa MW cutoff), glycoconjugate 70 was obtained.

**Figure 26.** (A) Gel electrophoresis (Coomassie stain) of protein CRM\textsubscript{197} and MeOPN→6-β-D-Gal CRM\textsubscript{197} conjugate 70; and (B) Western blot of MeOPN→6-β-D-Gal CRM\textsubscript{197} conjugate 70 with \textit{C. jejuni} HS:23/36 whole cell antisera. (C) MALDI-TOF MS shows the molecular weight of the glycoconjugate. Native CRM\textsubscript{197} was 57,967 Da determined in a similar MALDI experiment (not shown).
The success of conjugation of sugar moiety 67 to protein CRM\textsubscript{197} was confirmed by NMR analysis by locating signals characteristic of the linker and the sugar epitope. Surprisingly, conjugate 70 did not produce any $^{31}\text{P}$ NMR signals. This suggests that the MeOPN on the sugar epitope might be masked by the much larger CRM\textsubscript{197} protein. The success of conjugation was further confirmed by gel electrophoresis (Figure 26A), Western blot (Figure 26B) and MALDI-TOF mass spectrometry (Figure 26C) using conventional methods. MS data pointed that the major conjugate produced a major peak of 61,781 Da, representing a single CRM\textsubscript{197} protein carrying eight synthetic MeOPN-containing Gal moieties.

4.2.3 Immunodetection of MeOPN→6-Gal conjugate

MeOPN→6-β-D-Gal\textsubscript{p}(1→O(CH\textsubscript{2})\textsubscript{5}NH\textsubscript{2} 67 as well as the corresponding conjugate 70 (data not shown) were studied in an immuno-dot-blot analysis in the same fashion as MeOPN→6-α-D-galactoside 9 (Figure 27). No dissimilarity in antisera reactivity was observed between these molecules, which imply that the recognition of MeOPN at the exocyclic C-6 position of Gal is not dependent on the anomeric configuration. These observations further points to the presence of antibody specifically targets primary MeOPN.

Flow cytometry analysis detects antibody-binding to cells with the use of fluorescently-labeled antisera. Analysis showed that \textit{C. jejuni} HS:23/36 cells successfully bound with rabbit antisera raised by synthetic MeOPN→6-β-D-Gal→CRM\textsubscript{197} conjugate
70 (Figure 28). A larger numbers of *C. jejuni* HS:23/36 cells were recognized by antibodies raised by the native HS:23/36 CPS conjugate. The intensity of binding of the synthetic MeOPN→6-D-Gal conjugate is somewhat lesser than this response. A portion of the *C. jejuni* HS:23/36 cells did not react with MeOPN→6-D-Gal antibodies.

**Figure 27.** Immuno-recognition of (A) MeOPN→6-α-D-Gal-(1→OMP 9 compared to (B) MeOPN→6-β-D-Gal-(1→O(CH2)5NH2 67.

This glycoconjugate is undergoing farther immunological testing and is being studied in animal models. It has been shown that this MeOPN-6-Gal-CRM197 glycoconjugate is effective in rabbits, where serum from rabbits immunized with this conjugate showed an increase in the resistance against *C. jejuni* serotype HS:23/36 by 16 fold.
Chapter 4: Synthesis of the first MeOPN-containing monosaccharide conjugate against C. jejuni serotype HS:23/36

Figure 28. Flow cytometry analysis of C. jejuni HS:23/36 cells with antisera raised by HS:23/36 native CPS conjugate (red) and synthetic MeOPN→6-β-D-Gal→CRM\textsubscript{197} conjugate 70 (blue). Yellow represents the binding of secondary antibody as negative control.

4.3 Novel conjugation strategy via an oxidized-starch structure

4.3.1 Preparation of a glycoconjugate containing multiple MeOPN→6-Gal epitopes via an oxidized-starch structure

One of the greatest challenges of glycoconjugates is its ability to generate an adequate amount of antigen-specific antibodies. Typically, it is desired to maximize the sugar antigen:protein ratio, thus maximizing antigen-specific antibody production. Here, a novel conjugation method involving the use of an oxidized-starch structure is proposed by our lab where water-soluble starch is first activated by periodate oxidation, generating
aldehyde functional groups. Carbohydrate antigen can then be attached to the activated starch structure through reductive amination (Scheme 28). This cascade of sugar epitope is then conjugated to the carrier protein, increasing antigen presentation by several magnitudes. Also, in theory it is possible to generate a sugar-starch complex using multiple antigens from several C. jejuni serotypes to generate a multi-valent glycoconjugate vaccine against C. jejuni.

Scheme 28. Conjugation of MeOPN→6-D-Gal via an oxidized-starch structure.
This strategy was first attempted to create a MeOPN→6-α-D-Gal-CRM\textsubscript{197} conjugate. Lintner starch was oxidized with sodium periodate in sodium acetate buffer, pH 4. The resulting oxidized structure was purified with ultracentrifugation, dialysis (1 kDa MW cutoff), and size-exclusion P-2 gel column. The conjugation was then performed on MeOPN→6-α-D-Gal-linker \textit{68} with purified oxidized-starch in a reductive amination with sodium cyanoborohydride. The reaction was stirred at 37 °C for 2 days, and then purified with dialysis (2 kDa MW cutoff). \textit{1}H NMR analysis confirms the success of conjugation revealing signals characteristic of the oxidized-starch and the Gal epitope (Figure 29). However, \textit{31}P NMR revealed two phosphorus peaks at 9.8 and 16.2 ppm,
devoid of MeOPN signals (~14 ppm). This indicated that the MeOPN modification could not withstand the strong condition of reductive amination, and was altered during the reaction.

Nevertheless, NMR results indicate that the conjugation took place since the phosphorus signals and the linker signals can only come from the sugar moeity. A second attempt was performed on MeOPN→6-α-D-Gal-linker 68 with purified oxidized-starch in a similar fashion. This reaction was stirred at room temperature for 3 hours, as an attempt to limit the degradation of MeOPN. This conjugate was again purified with dialysis (2 kDa MW cutoff) but was found unsuccessful. No conjugate was obtained and unconjugated sugar epitope was found in the dialysate. $^{31}$P NMR analysis reveals the presence of MeOPN modification at 14 ppm from intact sugar and also signs of degradation with two phosphorus peaks at 9 and 16 ppm. This result farther indicates that MeOPN is not stable under reducing condition of reductive amination. A third conjugation attempt was performed in DMSO at room temperature for 2 days. No conjugation product was detected as $^{31}$P NMR gave no phosphorus signals.

The oxidized-starch-Gal construct (devoid of MeOPN) was conjugated to protein CRM$_{197}$. The conjugation method was performed in a similar fashion as before to generate this oxidized-starch-Gal construct. Reductive amination reaction in the presence of sodium cyanoborohydride was stirred at 37 °C for 2 days, and then the resulting conjugate purified with dialysis (25 kDa MW cutoff). Conjugation was confirmed by NMR revealing signals characteristic of Gal epitope. Gel electrophoresis revealed
products of higher molecular weight than CRM\textsubscript{197} (Figure 30). Western blot of this oxidized-starch-Gal-CRM\textsubscript{197} construct with \textit{C. jejuni} HS:23/36 whole cell antisera did not exhibit antibody recognition. This negative result is expected, pointing to the fact that MeOPN unit is a crucial epitope for antibody recognition.

Figure 30. (A) Gel electrophoresis of protein CRM\textsubscript{197} and oxidized-starch-Gal-CRM\textsubscript{197} construct; and (B) Western blot of oxidized-starch-Gal-CRM\textsubscript{197} construct with \textit{C. jejuni} HS:23/36 whole cell antisera.

4.4 Concluding remarks

Linker-carrying monosaccharide epitopes from the \textit{C. jejuni} HS:23/36 CPS repeating unit, MeOPN→6-D-Gal-linker 67 and 68, are generated in this chapter. Five
MeOPN-containing Gal epitopes in total have been reported in this thesis. The chemical shifts of $^{31}\text{P}$ and $^1\text{H}$ resonances of POCH$_3$ are tabulated in Table 3. This data matches the NMR resonance of existing native MeOPN-containing CPS in literature.

**Table 3.** $^{31}\text{P}$ and $^1\text{H}$ NMR chemical shift of MeOPN-containing Gal epitopes synthesized in this thesis

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^{31}\text{P}$ signals (ppm)</th>
<th>$^1\text{H}$ signal for POCH$_3$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOPN→6-α-D-Gal-OMP 9</td>
<td>14.58, 14.43</td>
<td>3.41, 3.33</td>
</tr>
<tr>
<td>MeOPN→6-β-D-Gal-linker 67</td>
<td>14.79, 14.75</td>
<td>3.61, 3.57</td>
</tr>
<tr>
<td>MeOPN→6-α-D-Gal-linker 68</td>
<td>14.80, 14.74</td>
<td>3.67, 3.64</td>
</tr>
<tr>
<td>MeOPN→2-β-D-Gal-OMP 10</td>
<td>14.27, 14.04</td>
<td>3.59, 3.56</td>
</tr>
<tr>
<td>MeOPN→4-β-D-Gal-OMP 11</td>
<td>14.65</td>
<td>3.56</td>
</tr>
</tbody>
</table>

The corresponding glycoconjugates from the β-anomer 67 is generated by attaching this sugar structure to protein carrier CRM$_{197}$. In a dot-blot immunological study, it is demonstrated that the MeOPN→6-β-D-Gal-linker 67 and the corresponding glycoconjugate 70 displays the same antisera recognition as MeOPN→6-α-D-Gal-OMP 9 structure. These results point to the presence of antibody specifically targets MeOPN on primary locations. The fact that no dissimilarity in antisera reactivity was observed between α and β anomers suggests that the recognition of MeOPN at the exocyclic C-6 position of Gal is not dependent on the anomic configuration. Immunological study has revealed that this glycoconjugate is effective in rabbits against live *C. jejuni* challenges.
Attempts using the oxidized-starch conjugation strategy on MeOPN→6-α-D-Gal-linker 68 are unsuccessful. Even though conjugation of sugar to oxidized-starch takes place, the MeOPN group is altered during reductive amination. This construct is conjugated protein CRM197 and as expected, Western blot of this glycoconjugate with *C. jejuni* HS:23/36 whole cell antisera does not exhibit antibody recognition. This conjugation concept is still being tested in the lab. The success of this conjugation strategy will be a big advance in the development of a multi-valent glycoconjugate vaccine against *C. jejuni* infection.
CHAPTER 5

Synthesis of MeOPN-containing monosaccharide epitopes using other MeOPN introduction strategies
5.1 Introduction

Described previously, a new synthetic strategy to introduce MeOPN moiety onto carbohydrate structures has been successfully developed in this thesis. This strategy utilizes methyl dichlorophosphate as the phosphorylating agent in the presence of a base, followed by an ammonolysis with ammonia gas in situ. This strategy has been applied to successfully generate four MeOPN-containing monosaccharides: MeOPN→6-D-Gal, MeOPN→2-D-Gal, MeOPN→4-D-Gal and MeOPN→3-D-Fru. The MeOPN→6-D-Gal epitope has been conjugated to a protein carrier and this conjugate is recognized by antisera previously generated against multiple native CPS conjugates. Although our one-pot approach offers a relatively inexpensive and simple method to introduce MeOPN group, it gives low yields (5-34%). This is especially apparent when modifying relatively unreactive sites. For instance, when modifying the C-3 position of D-Fru, a low yield of 5% is achieved. During the course of this research project, two other MeOPN introduction strategies are published. This chapter describes attempts to generate MeOPN→6-D-Gal and MeOPN→3-D-Fru structures using these strategies.

5.2 Alternative strategies to introduce MeOPN

5.2.1 Ashmus and Lowary’s synthesis of MeOPN

During the course of this research project (2014), Ashmus and Lowary at the Alberta Glycomics Centre published their strategy for the introduction of MeOPN moieties onto carbohydrate structures. The installation involves the use of methyl pivolyl
Using other MeOPN introduction strategies, employing $H$-phosphonate as the phosphorylating agent. Starting from $H$–phosphonate ammonium salt, this reagent is converted into its tetra-$N$-butylammonium and then pivoly derivative (Scheme 29). This phosphorylating agent is then used to generate the sugar $H$-phosphonate intermediate, which is followed by a Todd-Atherton oxidation with benzylamine, $p$-methoxybenzylamine, or $o$-nitrobenzylamine as the oxidant in combination with bromotrichloromethane (CBrCl$_3$) and Et$_3$N. In each case, the MeOPN product is produced as a 1:1 mixture of two diastereoisomers, which are inseparable by flash chromatography most of the time. These reaction conditions are shown to be compatible with a variety of protecting groups including benzylidene and isopropylidene acetals, benzoates and acetate esters, and silyl and benzyl ethers. However the strategy seems to be incompatible with azide and NAc functional groups.

Scheme 29. Ashmus and Lowary’s strategy to introduce MeOPN moiety onto carbohydrates. Reagents and conditions: (a) Bu$_4$NHSO$_4$, 20% NaOH; (b) PivCl, CH$_2$Cl$_2$, pyridine; (c) CH$_2$Cl$_2$, pyridine; (d) Bn-NH$_2$, Et$_3$N, CBrCl$_3$, CH$_2$Cl$_2$; (e) H$_2$, Pd/C; (f) NaBrO$_3$, Na$_2$S$_2$O$_4$. 

\[
\begin{array}{c}
\text{MeO} \quad \text{P} \quad \text{OR} \\
\text{H} \\
\text{a} \\
\text{b} \\
e \quad \text{or} \quad f \\
d \\
\text{MeO} \quad \text{P} \quad \text{O} \\
\text{NH} \text{Bn} \\
\text{MeO} \quad \text{P} \quad \text{O} \\
\text{NH}_2 \\
\end{array}
\]
Subsequent deprotection of the $N$-protected phosphoramide yields the MeOPN group, which is stable under such conditions including standard hydrogenation with hydrogen with palladium on carbon, ceric ammonium nitrate (CAN) and photolysis. A number of deprotection strategies for sugar protecting groups are shown to be compatible, except acid hydrolysis, which leads to the removal of the MeOPN moiety.

5.2.2 Wang’s one-step direct synthesis of MeOPN

More recently, towards the end of this Ph.D research project (2015), another synthetic strategy for the introduction of MeOPN unit was developed by Cheng-Chung Wang’s group in Taiwan. The strategy involves a direct installation of the MeOPN moiety to carbohydrate structures by using methyl benzylphosphoramidochloridate as the phosphorylating agent. This reagent can be prepared in one step by using the commercially available methyl dichlorophosphate in the presence of benzylamine and $\text{Et}_3\text{N}$, shown in Scheme 30.\textsuperscript{172} This phosphorylating agent is only stable for 5–6 hours, and needs to be introduced onto the sugar immediately, using NMI and DMAP as base. Authors are able to demonstrate that protecting groups such as benzyl ether, acetoxy, azide, trimethylsilyloxy and isopropylidene, are well tolerated under these reaction conditions. The $N$-benzyl protecting group on methyl phosphoramidates can be removed under hydrogenolytic conditions using hydrogen with palladium or palladium hydroxide on carbon and also under biphasic conditions using sodium bromate and sodium.
dithionite (NaBrO₃/Na₂S₂O₄). The reaction strategy generally gives acceptable yields about 50-70% over 2 steps.

Scheme 30. Introduction of MeOPN moiety onto carbohydrate structures using methyl benzylphosphoramidochloridate as phosphorylating agent. Reagents and conditions: (a) BnNH₂, Et₃N, CH₂Cl₂; (b) NMI, DMAP, CH₂Cl₂; (c) H₂, Pd/C, EtOH; (d) H₂, Pd(OH)₂/C, EtOH; (e) NaBrO₃, Na₂S₂O₄, EtOAc, H₂O.

In most of the cases, this strategy produces an inseparable mixture of two MeOPN diastereoisomers, although a few sugar structures produce a single diastereoisomer. Authors credit this observed diastereoselectivity to the steric environment around the reaction centre. The exact diastereochemistry of the diastereomers formed is not determined.
5.3 Synthesis towards MeOPN→6-β-D-Gal with other strategies

5.3.1 Wang’s one-step direct synthesis towards MeOPN→6-Gal

First, we applied Wang’s one-step direct synthesis strategy in an attempt to generate the MeOPN→6-Gal structure. Starting with compound 61, obtained previously, methyl benzylphosphoramidochloridate, which was generated according to published procedure, was installed using NMI with addition of DMAP (Scheme 31). The starting material was found to be consumed quickly (4 hours) generating the expected N-benzyl-protected-MeOPN product 71. $^{31}$P NMR experiment revealed that product 71 was a mixture of 1:1 diastereoisomers, producing two $^{31}$P signals at ppm at 10.21 and 10.05 ppm.

![Scheme 31](image)

*Scheme 31.* Synthesis MeOPN→6-D-Gal-linker 63 with Wang’s one-step direct synthesis.

Deprotection of the Bn-protected MeOPN product 71 was attempted with NaBrO$_3$/Na$_2$S$_2$O$_4$ system. However, this condition was found to remove allyl groups instead.
5.3.2 Ashmus and Lowary’s synthetic strategy towards MeOPN→6-Gal

Next we attempted to generate the MeOPN→6-Gal structure with Ashmus and Lowary’s synthetic strategy. First, tetra-N-butylammonium methyl-H-phosphonate reagent was generated as described in published procedure. This phosphorylating agent was then turned into its pivoly derivative and was installed onto compound with Et3N (Scheme 32). As expected, starting material was consumed quickly, generating the H-phosphonate product 72. After a quick work-up, the thoroughly dried residue containing product 72 is subjected to a Todd-Atherton oxidation with benzylamine or p-methoxybenzylamine in the presence of CBrCl3 and Et3N, generating products 71 and 73. The yields of these reactions were much lower than expected. Both reactions resulted in a mixture of 1:1 diastereoisomers.

Scheme 32. Synthesis of MeOPN→6-D-Gal-linker with Ashmus and Lowary’s synthetic strategy.

Deprotection of PMB-protected MeOPN product 73 was achieved with CAN in MeCN:H2O mixture, generating MeOPN product 63 in good yield. Spectroscopy data of
product 63 is identical to the MeOPN product generated with our one-pot approach in previous chapter.

5.4 Synthesis towards MeOPN→3-D-Fru with other strategies

5.4.1 Wang’s one-step direct synthesis towards MeOPN→3-D-Fru

After successfully generating the Bn-protected MeOPN→6-D-Gal structure 71 with Wang’s one-step direct synthesis strategy, attempts were made to generate the MeOPN→3-Fru structure using the same approach.

As before, compound 34 was treated with methyl benzylphosphoramidochloridate in the presence of NMI (8 eq) with addition of DMAP (1 eq), as shown in Scheme 33. However, starting material was resistant to modification after stirring for 2 days at room temperature. Other attempts with excess reagent (up to 50 eq.) at elevated temperature (40 °C in CH₂Cl₂, ClCH₂CH₂Cl in 60 °C) had no effect. The low reactivity of C-3 position of D-Fru compound 34 is expected due to steric reasons, as observed previously.
Methyl benzylphosphoramidochloridate as the phosphorylating agent is likely too bulky for modifying this site.

5.4.2 Ashmus and Lowary’s synthetic strategy towards MeOPN→3-D-Fru

Ashmus and Lowary’s MeOPN introduction strategy was applied to MeOPN→3-D-Fru structure 34. The tetra-N-butylammonium methyl-$H$-phosphonate phosphorylating agent was turned into its pivolyl derivative in situ and installed onto compound 34 in the presence of Et$_3$N (Scheme 34).

Scheme 34. Generation of MeOPN→3-Fru-linker 35 with Ashmus and Lowary’s strategy.

To our delight, the starting material 34 was consumed relatively quickly as indicated by TLC, fully converting into product 75 within 1 hour. After work-up, product 75 is
followed by Todd-Atherton oxidation with \( p \)-methoxybenzylamine, generating the desired PMB-protected product 76 as a mixture of 1:1 diastereoisomers. However, the yields of these reactions were much lower than expected. Removal of PMB protecting group in the presence CAN was accomplished generating MeOPN product 35 in good yield. Spectroscopy data of MeOPN product 35 is in agreement as previously reported.

Deprotection of MeOPN→3-D-Fruc-linker 35 began with de-benzoylation. Initially, 0.01 M NaOMe in MeOH was attempted. The starting material was consumed in 4 hours and produced one major spot as indicated by TLC. However this was not the desired MeOPN product. \( ^1H \) NMR on this product confirmed the removal of OBz groups and \( ^{31}P \) NMR confirmed the presence of phosphorus signal. However \( ^1H-^{13}C \) HSQC and \( ^1H-^{31}P \) HMBC NMR experiments failed to detect the presence of POCH\(_3\) signals. This surprising outcome was not observed during removal of OBz from Gal-2 and Gal-4 structures.

Next, the deprotection was attempted in a 7:2:1 mixture of MeOH/H\(_2\)O/Et\(_3\)N. Reaction proceeds much more slowly. After 14 hours, starting material was fully consumed as indicated by TLC. Unexpectedly, NMR on the product revealed that Phth protecting group was removed under these condition while one OBz group remained. Longer reaction time altered the MeOPN modification but did not remove the last OBz group.
**Scheme 35.** De-benzoylation of MeOPN→3-Fru 35 resulted in a 3→1 MeOPN migration.

Finally deprotection of compound 35 was performed with 0.0033 M NaOMe in MeOH to avoid MeOPN degradation. Reaction proceeded much more slowly than before. After 10 hours, debenzoylated MeOPN product was obtained. However, the NMR analysis of this product revealed surprising results. On the HMBC NMR, phosphorus signal from the MeOPN functional group demonstrated the expected correlation to POCH$_3$ but two correlation signals to H-1 of D-Fru, instead of H-3 (Figure 31). This result sadly suggests that the MeOPN group might have migrated to the C-1 position. As mentioned in previous chapter, silyl groups are also capable of 3→1 migration under basic conditions. These observations suggest that position 3 of Fru is exceptionally susceptible to migration. These results might also explain the difficulty in the removal of Bz groups, leading to degradation of the MeOPN group. Synthetic schemes involving modifications of these positions need to be carefully planned in future synthesis. The immunodot analysis of this structure did not produce any recognition with antisera HS:1.
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**Figure 31.** $^1$H-$^{31}$P HMBC NMR experiment of 77 showing correlation between the phosphorus of MeOPN and H-1 resonance of D-Fru.

5.5 Concluding remarks

Two alternative MeOPN introduction strategies are attempted to synthesize MeOPN→6-D-Gal-linker and MeOPN→3-D-Fru-linker structures. Wang’s one-step direct synthesis strategy is able to achieve the Bn-protected MeOPN→6-D-Gal-linker structure 71 with acceptable yield. Ashmus and Lowary’s synthetic strategy is also able to achieve the Bn-protected and PMB-protected MeOPN→6-D-Gal-linker structures, 71 and 73. PMB-protected MeOPN compound 73 was deprotected, successfully generating
MeOPN→6-D-Gal-linker 63. Spectroscopy data is in agreement with the MeOPN product generated with our one-pot approach in previous chapter.

In the case of Fru-3 epitope, Wang’s method is ineffective towards compound 34, likely due to steric reasons. Ashmus and Lowary’s synthetic strategy is able to form the desired MeOPN product 35, although yields are low. Removal of Bz protecting group from this MeOPN product is more complicated than expected as the product is found to be more sensitive to NaOMe condition than observed in previous MeOPN-containing compounds. NMR experiments on the deprotected product suggest that the MeOPN group has migrated to the C-1 position. This synthesized MeOPN-carrying structure did not produce any recognition with antisera HS:1.
CHAPTER 6

Synthesis of MeOPN-containing GlcNAc-Gal disaccharide epitope from \textit{C. jejuni}

serotype HS:23/36
6.1 Introduction

Two transferases, CJJ1420 and CJJ1435, are responsible for the MeOPN modifications on C-6 and C-2 position of D-Gal in C. jejuni serotype HS:23/36. Recently, it is discovered that when the genes of these two transferases are disrupted in C. jejuni mutant strain PG3718 of serotype complex HS:23/36, a new MeOPN linkage arises at C-4 of D-Gal (Figure 32).\textsuperscript{158} Human sera contain natural antibodies against the GlcNAc-β-(1→3)-Gal linkage, which give us some natural resistance against C. jejuni serotype HS:23/36. However, when the MeOPN modification is located on the C-4 position, this recognition is lost making this mutant strain highly resistant to human sera.

![Chemical structure of MeOPN modification on C. jejuni CPS](image)

**Figure 32.** C. jejuni mutant strain PG3718 carries a MeOPN modification at C-4 of Gal in the CPS.

For immunological studies, the MeOPN-4-Gal epitope was synthesized during this project. However, immunoactivity of this epitope was poor. This suggests that the internal sugar backbone might be involved in recognition. To investigate, a
MeOPN-containing GlcNAc-\(\beta\)-(1→3)-Gal disaccharide is envisaged for additional immunological tests. This chapter describes the synthesis toward the GlcNAc-\(\beta\)-(1→3)-Gal disaccharide 78 via a D-galactosyl acceptor 79 and an N-acetyl-D-glucosaminosyl donor 80, as well as the introduction of MeOPN onto the C-4 position (Scheme 36).

![Scheme 36. Retrosynthetic scheme of GlcNAc-\(\beta\)-(1→3)-Gal disaccharide 78.](image)

6.2 Synthesis of GlcNAc-Gal disaccharide

6.2.1 Synthesis of D-galactosyl acceptor

Synthesis toward D-galactosyl acceptor 80 began with the previously reported \(\alpha\)-galactoside 13 (Scheme 37). Isopropylidene was used to selectively protect O-3 and O-4 position generating compound 81. Both O-2 and O-6 positions were then protected
with allyl groups, generating compound 82. To distinguish between O-3 and O-4 positions, isopropylidene was first removed generating compound 83. Position 4 was then selectively protected with OAc group through orthoacetate chemistry, generating acceptor 80, with a free 3-OH for glycosylation.

Scheme 37. Synthesis of D-galactosyl acceptor 80.

6.2.2 Synthesis of N-acetyl-D-glucosaminosyl donor

Synthesis of N-acetyl-D-glucosaminosyl donor 79 started with per-acetylated GlcNAc 84. Anomeric acetate was first replaced with ethanethiol generating thioglycoside 85 (Scheme 38). BF₃•OEt₂ as promoter was attempted at first. However the reaction was extremely slow and was unable to form the desired product. A stronger Lewis acid tin (IV) chloride was used to overcome the low reactivity of compound 84. After refluxing for 24 hours, product 85 was collected with a yield of 58% as a roughly
2:1 β/α mixture. Unreacted starting material could be recovered. This reaction favored β product due to the neighbouring participation effect of the -NHAc group at C-2.

To distinguish O-4 position of acceptor from other hydroxyl groups on the disaccharide and for the ease of deprotection later, acetyl groups on compound 85 were removed under Zemplén conditions generating compound 86 and replaced with allyl protecting groups generating donor 79.

Scheme 38. Synthesis of N-acetyl-D-glucosaminosyl donor 79.

6.2.3 Synthesis of MeOPN-GlcNAc-Gal disaccharide

To overcome the low reactivity of the N-acetyl-D-glucosaminosyl donor 79, glycosylation reactions were typically attempted at relatively high temperature with large amount of Lewis acid (Table 4). After multiple attempts, it was determined that the NIS/TfOH promoter system works best for the glycosylation. It was found that the ratio of acceptor 80 and donor 79 did not affect the yield greatly. Higher equivalent of Lewis
acid promoter caused degradation to OMP protecting group on the D-galactosyl residue (Entry 4). Reaction with 2 eq. of donor 80 and 0.9 eq. of TfOH promoter in CH$_2$Cl$_2$ at room temperature for 48 hours was able to yield the best result, generating the β(1,3)-linked disaccharide 87 with 54% yield (Entry 2). The β-selectivity is due to participation effect from the NHAc group. Interestingly, reactions performed with TMSOTf as the promoter in ClCH$_2$CH$_2$Cl generated the α-linkage (8%) with no β-linked product. The low yield of this glycosylation reaction was credited to the low reactivity of donor 79. This is reflected by the long reaction times. Amino protecting groups such as 2,2,2-trichloroethyl (Troc) or phthlimidates could be explored in future synthesis.

Scheme 39. Synthesis towards the MeOPN-containing GlcNAc-β-(1→3)-Gal disaccharide 78.
Table 4. Glycosylation between D-galactosyl acceptor acceptor 80 and N-acetyl-D-glucosaminosyl donor 79

<table>
<thead>
<tr>
<th>Entry</th>
<th>Donor:Acceptor</th>
<th>Promoter</th>
<th>Promoter eq</th>
<th>Temperature</th>
<th>Time</th>
<th>β</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2:1</td>
<td>NIS/TfOH</td>
<td>1.0</td>
<td>0 °C - RT</td>
<td>10 h</td>
<td>50%</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2:1</td>
<td>NIS/TfOH</td>
<td>0.9</td>
<td>RT</td>
<td>48h</td>
<td>54%</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>1.2:1</td>
<td>NIS/TfOH</td>
<td>1.0</td>
<td>RT</td>
<td>24 h</td>
<td>51%</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2.5:1</td>
<td>NIS/TfOH</td>
<td>1.5</td>
<td>RT</td>
<td>60 h</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>2:1</td>
<td>TMSOTf</td>
<td>2.0</td>
<td>RT - 50 °C</td>
<td>34 h</td>
<td>-</td>
<td>8%</td>
</tr>
</tbody>
</table>

After disaccharide 87 was obtained, acetyl group was then selectively removed giving compound 88 with a free 4-OH for MeOPN introduction.

Our one-pot MeOPN introduction strategy was first attempted on disaccharide 88. The disaccharide was found to be extremely unreactive. The starting material could be consumed after stirring the sugar with methyl dichlorophosphate in the presence of excess reagents and Et$_3$N (40 eq.) for 48 hours at 40 °C. However, after ammonolysis and purification by flash chromatography, the desired MeOPN product could not be obtained.

Lowary’s synthetic method was also attempted. However this method was found incompatible with the NHAc group, as mentioned in the publication. After the Todd–Atherton oxidation, these reactions produced an unknown structure which seemed to have the NHAc and PMB groups modified instead.

Wang’s strategy is currently being tested on this disaccharide structure. After MeOPN-containing disaccharide 89 is achieved, allyl protecting groups can be removed.
generating MeOPN-containing disaccharide 78. This MeOPN-containing disaccharide will be tested for immunological activities.

### 6.3 Concluding remarks

To further investigate the MeOPN-4-Gal epitope in immunological studies, we attempted to synthesize a MeOPN-containing GlcNAc-β-(1→3)-Gal disaccharide from D-galactosyl acceptor 80 and N-acetyl-D-glucosaminosyl donor 79. Due to the low reactivity of N-acetyl-D-glucosaminosyl donor 79, glycosylation reaction is performed under relatively harsh conditions, and is able to yield the desired disaccharide with acceptable yield.

Our one-pot MeOPN introduction strategy is attempted on disaccharide 88, but is unable to modify this structure. Lowary’s synthetic method was found incompatible with the NHAc group. Other MeOPN introduction strategies are being tested. If successful, the fully deprotected MeOPN-containing disaccharide will be tested for immunological activities. These studies will offer insight into the role of MeOPN modifications in virulence and antibody interactions.
CHAPTER 7

EXPERIMENTAL
7.1 General Method

All chemicals were purchased from commercial suppliers and used as received. Molecular sieves were activated by heating with heating mantle under reduced pressure. Thin layer chromatography (TLC) was carried out on TLC silica gel F254. TLC plates were visualized under UV light and/or by charring with 10% H₂SO₄ in ethanol. Flash chromatography was performed with silica gel P60, (43-60 μm, 230-400 mesh). Size exclusion chromatography was performed with a Bio-Gel P-2 gel column. Dialyses were performed with Spectra/Por® Biotech dialysis membrane from Spectrum Labs. ¹H NMR, ¹³C NMR and ³¹P NMR spectra were recorded with Bruker Avance 400 or 600 MHz spectrometers. ¹H NMR, ¹³C NMR and ³¹P NMR chemical shifts are reported in part per million (ppm). The proton signal of residual, non-deuterated solvent (δ 7.24 ppm for CHCl₃) was used as internal reference for ¹H spectra. For ¹³C NMR spectra, the chemical shifts are reported relative to the solvent (δ 77.1 ppm for CDCl₃). For ³¹P NMR spectra, the chemical shifts are reported relative to a phosphate H₃PO₄ external standard (0 ppm). For ¹H NMR data, diastereomer proton signals were indicated by an asterisk (*) when clearly resolved. Coupling constants (J) were obtained from a first-order analysis of one-dimensional spectra and are reported in Hertz (Hz). ¹H NMR data are reported using standard abbreviations: singlet (s), doublet (d), triplet (t), doublet of doublet (dd), doublet of doublet of doublet (ddd). Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter at room temperature (25 °C) and concentration (c) is expressed in g/100 ml. High-resolution mass spectra (HRMS) for the synthetic
compounds were recorded by electron spray ionization (ESI) mass spectroscopy (time-of-flight analyzer) at the Mass Spectrometry Facility, University of Guelph, Ontario.

7.2 Experimental procedures

7.2.1 Experimental procedures for the preparation of PS-I glycoconjugate

5-Amino-pentanyl-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)→α-D-glucopyranosyl-(1→2)-α-D-glucopyranoside 7

\[
\text{C}_{35}\text{H}_{63}\text{NO}_{24}: 881.37 \text{ g/mol}
\]

Liquid ammonia (20 mL) was condensed into a round bottom flask at −78 °C and a piece of sodium metal (50.0 mg, 2.17 mmol) was added. A solution of the protected pentasaccharide 1 (15.0 mg, 0.00783 mmol) in anhydrous THF (4 mL) was added to the deep blue solution and the mixture was stirred for 1.5 h at −78 °C. The reaction was then quenched with MeOH (5 mL) and ammonia was allowed to evaporate at room
temperature for 3 h. The remaining solution was neutralized with AcOH (150 μL) and evaporated. The resulting residue was passed through a Bio-Gel P-2 size exclusion column (45-90 μm), yielding PS-I pentasaccharide 7 but also showed the presence of sodium acetate contamination. To remove the acetate contaminant, pentasaccharide sample was dialyzed against running water (500 Da MW cut-off). Retentate was lyophilized, giving pure pentasaccharide 7 (5.1 mg, 74%). $^1$H NMR (600 MHz, D$_2$O): δ 5.22 (s, 1H, H-$^1$Rha-$^D'$); 5.17 (d, 1H, $J$ = 3.4 Hz, H-$^1$Glc-$^A'$); 5.12 (s, 1H, H-$^1$Rha-$^D'$); 5.06 (d, 1H, $J$ = 3.8 Hz, H-$^1$Glc-$^B'$); 4.52 (d, 1H, $J$ = 8.1 Hz, H-$^1$Glc-$^C'$); 4.42-3.31 (ring proton, linker-H); 3.01, 1.69, 1.48 (m, 8H, linker-H), 1.25 (m, 6H, Rha-CH$_3$). $^{13}$C NMR (150 MHz, D$_2$O): δC-1 101.3 (C-$^1$Glc-$^C'$); 100.9 (C-$^1$Glc-$^A'$); 100.7 (C-$^1$Rha-$^D'$); 95.6 (C-$^1$Glc-$^B'$); 95.0 (C-$^1$Rha-$^D'$); 39.1, 26.5, 28.0 (linker); 16.5 (C-$^6$Rha-$^D,D'$).

Conjugation of synthetic PS-I pentasaccharide to *C. difficile* ToxB peptide 8

The linker-equipped PS-I pentasaccharide 7 (3.0 mg) and *C. difficile* exotoxin B (1 mg) were dissolved in a solution of MES buffer (2 mL, pH 5.5). EDC (20 μL) was added and the reaction mixture was stirred at room temperature for 1 day and 37 °C for 2 days. The reaction was then purified with dialysis (1 kDa MW cutoff) against running water for 2 days. Retentate was lyophilized to give PSI–ToxB glycoconjugate 8 (2 mg).
7.2.2 Experimental procedures for the preparation of MeOPN→6-Gal

**4-Methoxyphenyl 6-O-trityl-α-D-galactopyranoside 14**

![Chemical Structure](image)

C₃₂H₃₂O₇: 528.21 g/mol

To a solution of galactoside 13 (2.7 g, 9.3 mmol) dissolved in pyridine (40 mL), trityl chloride (3.1 g, 11 mmol) was added and the reaction mixture was stirred at 60 °C for 3 days. The reaction mixture was then concentrated under reduced pressure. Purification with flash chromatography (1:1 EtOAc-hexanes) gave product 14 (4.7 g, 95%). [α]D²⁵ = +91.2 (c = 0.21, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 7.44-7.20 (m, 15H, Ar-H); 7.11-6.83 (m, 4H, MeOC₆H₄); 5.51 (d, 1H, J = 3.6 Hz, H-1); 4.05-3.93 (m, 4H, H-2, H-3, H-4, H-5); 3.79 (s, 3H, OCH₃); 3.54-3.32 (m, 2H, H-6). ¹³C NMR (100 MHz, CDCl₃): δ 155.3, 151.2, 150.6, 144.3, 143.8, 143.7, 143.6, 129.1, 128.6, 128.0, 127.9, 127.8, 127.5, 127.3, 127.1, 127.0, 118.5, 117.9, 114.6, 114.5, 114.4 (Ar); 98.4 (C-1); 87.0, 71.2, 70.0, 69.3 (C-2, C-3, C-4, C-5); 63.6 (C-6); 55.6 (CH₃). HRMS (ESI): Calcd for C₃₂H₃₂NaO₇ [M+Na]+: 551.2046, found: 551.2021.
4-Methoxyphenyl 2,3,4-tri-O-allyl-6-O-trityl-α-D-galactopyranoside 15

A solution of compound 14 (4.7 g, 8.8 mmol) dissolved in DMF (60 mL) with allyl bromide (4.6 mL, 53 mmol) was cooled to 0 °C. Sodium hydride, 60% dispersion in mineral oil (1.2 g, 29 mmol) was added and the reaction mixture was stirred for 1 h at 0 °C. The reaction was then quenched with MeOH (10 mL), poured into ice-cold water (100 mL) and extracted with EtOAc (3 × 100 mL). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. Purification by flash chromatography eluting with 1:7 EtOAc-hexanes gave product 15 (5.1 g, 89%). \([\alpha]_D^{25} = +132.6 \text{ (c = 0.1, CHCl}_3\); \(^1\)H NMR (400 MHz, CDCl₃): \(\delta 7.38-7.18 \text{ (m, 15H, Ar-H); 7.10-6.75 \text{ (m, 4H, MeOC}_6\text{H}_4\); 6.00-5.53 \text{ (m, 3H, CH}_2\text–CH=CH}_2\); 5.42 \text{ (d, 1H, J = 3.2 Hz, H-1); 5.33-4.98 \text{ (m, 6H, CH}_2\text–CH=CH}_2\); 4.37-3.72 \text{ (m, 13H, CH}_2\text–CH=CH}_2, H-2, H-3, H-4, H-5, OCH}_3\); 3.38 \text{ (m, 1H, H-6a); 3.01 \text{ (m, 1H, H-6b).} \(^{13}\)C NMR (100 MHz, CDCl₃): \(\delta 155.0, 151.0, 143.9 \text{ (Ar); 135.2, 135.1, 135.0 (CH}_2\text–CH=CH}_2\); 128.6, 127.8, 127.0, 119.0, 117.4, 117.3, 116.4, 114.4 (CH₂–CH=CH₂, Ar); 97.5 (C-1); 86.8; 78.2 (C-2); 77.4 (C-4); 76.1 (C-5); 73.9, 72.5, 71.9 (CH₂–CH=CH₂); 70.4 (C-3) 63.3 (C-6); 55.6 (OCH₃). HRMS (ESI): Calcd for C₄₁H₄₄NaO₇ [M+Na]^+: 671.2985, found: 671.2970.
Chapter 7: EXPERIMENTAL

4-Methoxyphenyl 2,3,4-tri-O-allyl-α-D-galactopyranoside 16

A solution of compound 15 (300 mg, 0.46 mmol) in 80% aqueous AcOH (5 mL) was stirred at 80 °C for 1.5 h. The reaction mixture was concentrated under reduced pressure before purification by flash chromatography (1:6 EtOAc-hexanes) giving product 16 (147 mg, 78%). [α]D25 = +68.0 (c = 0.1, CHCl3); 1H NMR (400 MHz, CDCl3): δ 7.02-6.78 (m, 4H, MeOC6H4); 5.95-5.89 (m, 3H, CH2–CH=CH2); 5.50 (d, 1H, J = 3.5 Hz, H-1); 5.35-5.12 (m, 6H, CH2–CH=CH2); 4.42 (dd, 1H, J1 = 3.2 Hz, J2 = 9.3 Hz, H-3); 4.27-3.89 (m, 10H, CH2–CH=CH2, H-2, H-4, H-5, OH); 3.81 (m, 1H, H-6a); 3.74 (s, 3H, OCH3); 3.70 (m, 1H, H-6b). 13C NMR (100 MHz, CDCl3): δ 155.1, 150.9 (Ar); 135.0, 134.9 (CH2–CH=CH2); 118.6, 118.0, 117.4, 116.6, 114.5 (CH2–CH=CH2, Ar); 97.5 (C-1); 78.2, 75.9, 74.0, 72.6, 72.0, 71.0 (CH2–CH=CH2, C-2, C-3, C-4, C-5); 62.7 (C-6); 55.6 (OCH3). HRMS (ESI): Calcd for C22H30NaO7 [M+Na]+: 429.1890, found: 429.1891.
4-Methoxyphenyl 2,3,4-tri-O-allyl-6-O-methyl-phosphoramidyl-α-D-galactopyranoside 17 and 4-Methoxyphenyl 2,3,4-tri-O-allyl-6-O-di-amino-phosphoramidyl-α-D-galactopyranoside 18

![Chemical Structure 17](image1.png)  
C$_{23}$H$_{34}$NO$_9$P: 499.20 g/mol

![Chemical Structure 18](image2.png)  
C$_{22}$H$_{33}$N$_2$O$_8$P: 484.20 g/mol

To a solution of compound 16 (65 mg, 0.16 mmol) and methyl dichlorophosphate (150 µL, 1.3 mmol) dissolved in anhydrous CH$_2$Cl$_2$ (3 mL) with crushed molecular sieves (4 Å), Et$_3$N (175 µL, 1.3 mmol) was added drop-wise at 0 °C. The reaction mixture was stirred at room temperature for 5 hours. Upon completion of the reaction as judged by TLC, ammonia gas was injected into the reaction mixture through a needle. After 10 min, the reaction mixture was filtered and concentrated under reduced pressure.
Purification with column chromatography (1:1 EtOAc-hexanes) yielded MeOPN product 17 (15 mg, 19%) and di-amino-phosphoramidate product 18 (20 mg, 25%).

17: $[\alpha]_D^{25} = +100.5$ (c = 0.2, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.04-6.77 (m, 8H, MeOC$_6$H$_4$); 5.99-5.85 (m, 6H, CH$_2$–CH=CH$_2$); 5.48 (d, 1H, $J$ = 3.6 Hz, H-1); 5.47 (d, 1H, $J$ = 3.5 Hz, H-1*); 5.36-5.10 (m, 12H, CH$_2$–C=CH$_2$); 4.41 (m, 2H, H-3, H-3*); 4.29-4.10 (m, 16H, CH$_2$–CH=CH$_2$, H-2, H-2*, H-4, H-4*); 3.95-3.86 (m, 6H, H-5, H-5*, H-6a, H-6a*, H-6b H-6b*); 3.73 (s, 6H, OCH$_3$); 3.62 (d, 3H, $^3$J$_{PH}$ = 11.4 Hz, POCH$_3$); 3.53 (d, 3H, $^3$J$_{PH}$ = 11.4 Hz, POCH$_3$*); 2.75 (d, 2H, NH$_2$); 2.56 (d, 2H, NH$_2$*). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 155.2, 155.0, 150.9 (Ar); 135.0, 134.9 (CH$_2$–CH=CH$_2$); 128.9, 128.3, 118.8, 118.5, 118.7, 117.5, 117.4, 116.6, 114.5, 114.4 (CH$_2$–CH=CH$_2$, Ar); 97.6, 97.2 (C-1); 78.1, 75.8, 74.4, 74.0, 72.7, 71.9, 70.5, 70.4, 70.0, 69.9, 68.5, 65.5, (CH$_2$–CH=CH$_2$, C-2, C-3, C-4, C-5, C-6); 55.7, 53.3, 53.2 (OCH$_3$). $^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 10.58, 10.35. HRMS (ESI): Calcd for C$_{23}$H$_{35}$NO$_9$P [M+H]$^+$: 500.2049, found: 500.2035.

18: $[\alpha]_D^{25} = +105.3$ (c = 0.7, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.04-6.75 (m, 4H, MeOC$_6$H$_4$); 5.98-5.83 (m, 3H, CH$_2$–CH=CH$_2$); 5.48 (d, 1H, $J$ = 3.6 Hz, H-1); 5.36-5.10 (m, 6H, CH$_2$–CH=CH$_2$); 4.42 (m, 1H, H-3); 4.29-3.84 (m, 11H, CH$_2$–CH=CH$_2$, H-2, H-4, H-5, H-6a, H-6b); 3.71 (s, 3H, OCH$_3$); 2.71, 2.51 (2s, 4H, NH$_2$). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 153.2, 155.1, 151.3 (Ar); 135.3, 135.2, 135.0 (CH$_2$–CH=CH$_2$); 128.9, 128.3,
119.3, 119.2, 117.5, 117.0, 116.3, 114.7 (CH₂–CH=CH₂, Ar); 98.3 (C-1); 78.1, 75.9, 74.4, 73.9, 72.4, 71.6, 70.2 (CH₂–CH=CH₂, C-2, C-3, C-4, C-5, C-6); 55.6 (OCH₃). 

³¹P NMR (162 MHz, CDCl₃): δ 15.79. HRMS (MALDI): Calcd. For C₂₂H₃₃N₂NaO₈P [M+Na]+: 507.1872, found: 507.00.

4-Methoxyphenyl 6-0-methyl-phosphoramidyl-α-D-galactopyranoside 9

![Structure Image]

C₁₄H₂₂N₂O₈P: 379.10 g/mol

To a solution of compound 17 (17.0 mg) dissolved in MeOH (1 mL), PdCl₂ (5.0 mg) was added and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was then filtered through Celite® and concentrated under reduced pressure. Purification with column chromatography (EtOAc) yielded product 9 (5.1 mg, 39%). [α]D²⁵ = +63.5 (c = 0.1, H₂O); ¹H NMR (400 MHz, D₂O): δ 6.98–6.80 (m, 8H, MeOC₆H₄); 5.41 (d, 1H, J = 3.6 Hz, H-1); 5.38 (d, 1H, J = 3.6 Hz, H-1*); 4.13 (m, 2H, H-3, H-3*); 4.01–3.85 (m, 8H, H-4, H-4*, H-5, H-5*, H-6a, H-6a*, H-6b, H-6b*); 3.78 (m, 2H, H-2, H-2*); 3.63 (s, 6H, OCH₃); 3.41 (d, 3H, ³JPH = 11.4 Hz, POCH₃); 3.33 (d, 3H, ³JPH = 11.4 Hz, POCH₃*). ¹³C NMR (100 MHz, D₂O): δ 154.6, 150.0, 149.9, 119.3, 119.1, 114.9 (Ar); 98.1, 97.9 (C-1); 70.3, 70.2, 70.0, 69.1, 68.8, 67.8, 65.8 (C-2, C-3, C-4, C-5,
C-6); 55.6 (OCH₃); 53.6, 53.5, 53.4 (OCH₃). ³¹P NMR (162 MHz, D₂O): δ 14.58, 14.43.

7.2.3 Experimental procedures for the preparation of MeOPN→2-Gal

4-Methoxyphenyl 2-O-allyl-3,4-O-isopropylidene-6-O-trityl-β-D-galactopyranoside

A solution of compound 19 (0.68 g, 1.2 mmol) dissolved in DMF (18 mL) with allyl bromide (0.16 mL, 1.8 mmol) was cooled to 0 °C. Sodium hydride, 60% dispersion in mineral oil (57 mg, 1.4 mmol) was added and the reaction mixture was stirred for 1 h at 0 °C. The reaction was then quenched with MeOH (2 mL), poured into ice-cold water (40 mL) and extracted with CH₂Cl₂ (3 × 50 mL). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. Purification by flash chromatography eluting with 1:7 EtOAc-hexanes gave product 20 (0.69 g, 95%). [α]D²⁵ = +40.2 (c = 0.05, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 7.46-7.19 (m, 15H, Ar); 7.10-6.75 (m, 4H, MeOC₆H₄); 5.92 (m, 1H, CH₂=CH=CH₂); 5.34-5.19 (m, 2H, CH₂=CH=CH₂); 4.67 (d, 1H, J = 8.1 Hz, H-1); 4.36 (m, 2H, CH₂=CH=CH₂); 4.08 (m, 2H, H-3, H-4); 3.73 (s, 3H, OCH₃); 3.61-3.53 (m, 3H, H-2, H-5, H-6a); 3.34 (m, 1H, H-6b); 1.47 (s, 3H, CH₃); 1.29 (s, 3H,
4-Methoxyphenyl 2-O-allyl-β-D-galactopyranoside 21

![Structural formula of 4-Methoxyphenyl 2-O-allyl-β-D-galactopyranoside 21]

C₁₆H₂₂O₇: 326.13 g/mol

A solution of compound 20 (0.69 g, 1.1 mmol) dissolved in 80% aqueous AcOH (10 mL) was stirred at 80 °C for 1 h. The reaction mixture was concentrated under reduced pressure. Purification by flash chromatography (1:1 EtOAc-hexanes) gave product 21 (0.35 g, 94%). \([\alpha]D^25 = +90.2 \text{ (c = 0.2, CHCl}_3\); \]H NMR (400 MHz, CDCl₃): \(\delta 7.01-7.78 \text{ (m, 4H, MeOC}_6H_4\); 5.91 (m, 1H, CH₂–CH=CH₂); 5.19 (m, 2H, CH₂–CH=CH₂); 4.83 (d, 1H, \(J = 7.5 \text{ Hz, H-1}); 4.53-4.25 (m, 2H, CH₂–CH=CH₂); 4.14 (m, 1H, H-5); 3.96 (m, 1H, H-6a); 3.85 (m, 1H, H-6b); 3.76 (s, 3H, OCH₃); 3.62 (m, 3H, H-2, H-3, H-4). \(^{13}C \text{ NMR (100 MHz, CDCl}_3\): \(\delta 155.4, 151.1 \text{ (Ar); 134.5 (CH}_2–\text{CH=CH}_2\); 118.5, 118.2, 118.0, 114.6, 114.6 (CH₂–CH=CH₂, Ar); 102.6 (C-1); 78.4 (C-3); 75.9 (C-4); 73.7

CH₃). \(^{13}C \text{ NMR (100 MHz, CDCl}_3\): \(\delta 155.2, 151.5, 144.0, 143.9 \text{ (Ar); 134.9 (CH}_2–\text{CH=CH}_2\); 128.8, 127.9, 127.8, 127.0, 126.9, 118.6, 118.3, 117.7, 117.4, 114.5, 114.4, 110.2, 109.3 (CH₂–CH=CH₂, Ar); 102.2 (C-1); 86.8 (CMe₂) 79.4 (C-2); 79.2; (C-3); 73.8 (C-4); 72.9 (CH₂–CH=CH₂); 72.6 (C-5); 63.0 (C-6); 55.6 (OCH₃); 27.9, 26.3 (CH₃). HRMS (ESI): Calcd. For C₃₈H₄₀NaO₇ [M+Na]⁺: 631.2672, found: 631.2670.
(CH$_2$–CH=CH$_2$); 73.0 (C-2); 68.9 (C-5); 62.8 (C-6); 55.7 (OCH$_3$). HRMS (ESI): Calcd. For C$_{16}$H$_{23}$O$_7$ [M+H]$^+$: 327.1445, found: 327.1422.

4-Methoxyphenyl 2-O-allyl-3,4,6-tri-O-benzoyl-β-D-galactopyranoside 22

$$\text{O}$$

$$\text{OBz}$$

$$\text{OBz}$$

$$\text{BzO}$$

$$\text{OAll}$$

$$\text{OMP}$$

$\text{C}_{37}\text{H}_{34}\text{O}_{10}$: 638.21 g/mol

To a solution of compound 21 (27 mg, 0.83 mmol) in CH$_2$Cl$_2$ (1 mL) and pyridine (65 μL, 8.3 mmol), BzCl (100 μL, 8.3 mmol) was added and the reaction mixture was stirred at room temperature for 18 hours. MeOH (1 mL) was added and the reaction mixture was concentrated under reduced pressure. Purification with flash chromatography (1:3 EtOAc-hexanes) gave product 22 (51 mg, 97%). [α]$_D^{25}$ = +48.6 (c = 0.1, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): δ 8.07-7.29 (m, 15H, Ar); 7.06-6.71 (m, 4H, MeOC$_6$H$_4$); 5.89 (d, 1H, $J$ = 2.7 Hz, H-4); 5.74 (m, 1H, CH$_2$–CH=CH$_2$); 5.42 (dd, 1H, $J_1$ = 3.5, $J_2$ = 10.0 Hz, H-3); 5.21-5.01 (m, 3H, CH$_2$–CH=CH$_2$, H-1); 4.57 (m, 1H, H-6a); 4.39-4.06 (m, 5H, CH$_2$–CH=CH$_2$, H-6b, H-5, H-2); 3.73 (s, 3H, OCH$_3$). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 171.2, 166.0, 165.7, 155.6, 151.2, 134.3, 133.8, 133.5, 133.2, 133.1, 132.9, 130.6, 130.2, 129.8, 129.7, 129.6, 129.4, 128.8, 128.5, 128.4, 118.8, 114.6 (Ar); 117.7 (CH$_2$–CH=CH$_2$); 102.8 (C-1); 78.7 (C-2); 74.0 (C-3); 73.6 (CH$_2$–CH=CH$_2$); 72.2
(C-5); 69.9 (C-4); 63.5 (C-6); 55.6 (CH$_3$). HRMS (ESI): Calcd. For C$_{37}$H$_{34}$NaO$_{10}$ [M+Na]$^+$: 661.2050, found: 661.2041.

4-Methoxyphenyl 3,4,6-tri-O-benzyl-β-D-galactopyranoside 23

\[
\begin{align*}
\text{OBz} & \quad \text{OBz} \\
\text{BzO} & \quad \text{OMP} \\
\text{OH} & \\
\end{align*}
\]

C$_{34}$H$_{30}$O$_{10}$: 598.18 g/mol

To a solution of compound 22 (45 mg, 70 μmol) dissolved in MeOH (1 mL), PdCl$_2$ (2 mg) was added and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then filtered and concentrated. Purification with column chromatography (1:3 EtOAc-hexanes) gave product 23 (39 mg, 92%). $[\alpha]_D^{25} = +78.2$ (c = 0.1, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): δ 8.08-7.28 (m, 15H, Ar); 7.07-6.72 (m, 4H, MeOC$_6$H$_4$); 5.91 (d, 1H, $J = 3.5$ Hz, H-4); 5.45 (dd, 1H, $J_1 = 3.5$ Hz, $J_2 = 10.1$ Hz, H-3); 5.00 (d, 1H, $J = 7.8$ Hz, H-1); 4.60 (m, 1H, H-6a); 4.44 (m, 1H, H-6b); 4.34 (m, 2H, H-5, H-2); 3.73 (s, 3H, OCH$_3$); $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.0, 165.5, 155.7, 150.9, 133.7, 133.4, 130.0, 129.9, 129.8, 129.4, 129.2, 129.1, 128.5, 128.4, 118.6, 114.5 (Ar); 102.6 (C-1); 73.2 (C-3); 71.6 (C-5); 69.7 (C-2); 68.1 (C-4); 62.3 (C-6); 55.6 (OCH$_3$). HRMS (ESI): Calcd. For C$_{34}$H$_{30}$NaO$_{10}$ [M+Na]$^+$: 621.1737, found: 621.1723.
To a solution of compound 23 (18 mg, 0.030 mmol) and methyl dichlorophosphate (70 µL, 0.30 mmol) dissolved in anhydrous CH₂Cl₂ (1 mL) with crushed molecular sieves 4 Å. Et₃N (85 µL, 0.30 mmol) was then introduced drop-wise at 0 °C. The reaction mixture was stirred at 40 °C for 14 hours. Upon completion of the reaction as judged by TLC, ammonia gas was injected into the reaction mixture through a needle. After 5 min, the reaction mixture was filtered and concentrated under reduced pressure. Purification
with column chromatography (EtOAc) yielded MeOPN product 24 (5.4 mg, 26%) and
di-amino phosphoramidate product 25 (9.2 mg, 45%).

24: $[\alpha]_D^{25} = +68.5$ (c = 0.05, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.06-7.31 (m, 30H, Ar); 7.07-6.72 (m, 8H, MeOC$_6$H$_4$); 5.94 (m, 2H, H-4, H-4*); 5.54 (m, 2H, H-3, H-3*); 5.10 (m, 4H, H-1, H-1*, H-2, H-2*); 4.58 (m, 2H, H-6a, H-6a*); 4.45 (m, 2H, H-6b, H-6b*); 4.35 (m, 2H, H-5, H-5*); 3.73 (s, 6H, OCH$_3$); 3.67 (d, 3H, $^3J$_PH = 11.6 Hz, POCH$_3$); 3.41 (d, 3H, $^3J$_PH = 11.5 Hz, POCH$_3$); 2.92 (d, 2H, NH$_2$); 2.51 (d, 2H, NH$_2$*).

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 166.0, 165.7, 165.6, 165.5, 155.8, 155.7, 150.8, 150.6, 133.8, 133.6, 133.5, 133.4, 130.1, 130.0, 129.9, 129.8, 129.4, 128.9, 128.8, 128.7, 128.6, 128.5, 128.4, 118.6, 114.7, 114.6 (Ar); 101.2, 101.1 (C-1); 73.9, 73.6 (C-2); 72.5, 72.4 (C-3); 71.7, 71.5 (C-5); 68.0 (C-4); 62.1 (C-6); 55.6 (OCH$_3$); 53.6, 53.3 (POCH$_3$). $^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 10.97, 10.43. HRMS (ESI): Calcd. For C$_{35}$H$_{35}$NO$_{12}$P $[M+H]^+$: 692.1897, found: 692.1815.

25: $[\alpha]_D^{25} = +150.3$ (c = 0.1, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.10-7.29 (m, 15H, Ar); 7.07-6.71 (m, 4H, MeOC$_6$H$_4$); 5.93 (d, 1H, $J = 3.7$ Hz, H-4); 5.53 (dd, 1H, $J_1 = 3.6$ Hz, $J_2 = 10.4$ Hz, H-3); 5.10 (m, 2H, H-1, H-2); 4.60 (m, 1H, H-6a); 4.43 (m, 1H, H-6b); 4.35 (m, 1H, H-5); 3.71 (s, 3H, OCH$_3$); 2.80 (s, 2H, NH$_2$); 2.61 (s, 2H, NH$_2$). $^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 15.11.
4-Methoxyphenyl 2-\textit{O}-methyl-phosphoramidyl-\textit{\textbeta}-\textit{D}-galactopyranoside 10

\begin{center}
\includegraphics[width=0.3\textwidth]{structure}
\end{center}

C_{14}H_{22}NO_{9}P: 379.10 g/mol

Compound 24 (2.5 mg, 3.6 μmol) was dissolved in 0.01 M methanolic MeONa solution (1 mL) and the mixture was stirred for 1 h at room temperature before it was neutralized with acetic acid and concentrated under reduced pressure. Purification by flash chromatography eluting with 1:1 EtOAc-MeOH gave product 10 as a mixture of two diastereomers and the pure diastereomer 10\textsuperscript{*} (1.0 mg, 73%).

\textbf{10:} δ \textsuperscript{1}H NMR (400 MHz, D\textsubscript{2}O): δ 6.97-6.83 (m, 8H, MeOC\textsubscript{6}H\textsubscript{4}); 5.05 (2d, 2H, H-1, H-1\textsuperscript{*}); 4.28 (m, 2H, H-2, H-2\textsuperscript{*}); 3.91 (m, 2H, H-4, H-4\textsuperscript{*}); 3.77-3.72 (m, 4H, H-3, H-3\textsuperscript{*}, H-5, H-5\textsuperscript{*}); 3.67-3.60 (m, 10H, H-6, H-6\textsuperscript{*}, OCH\textsubscript{3}); 3.59 (d, 3H, \textsuperscript{3}J\textsubscript{PH} = 11.5 Hz, POCH\textsubscript{3}). 3.56 (d, 3H, \textsuperscript{3}J\textsubscript{PH} = 11.5 Hz, POCH\textsubscript{3}\textsuperscript{*}). \textsuperscript{13}C NMR (100 MHz, D\textsubscript{2}O): δ 154.5, 150.7, 117.7, 114.9 (Ar); 99.7 (C-1); 77.0 (C-2); 75.3 (C-5); 71.6 (C-3); 68.6 (C-4); 60.5 (C-6); 55.6 (OCH\textsubscript{3}); 53.9 (POCH\textsubscript{3}). \textsuperscript{31}P NMR (162 MHz, D\textsubscript{2}O): δ 14.27, 14.04. HRMS (ESI): Calcd. For C_{14}H_{23}NO_{9}P [M+H]\textsuperscript{+}: 380.1111, found: 380.1085.
10*: [α]D^25 = -11.0 (c = 0.01, H2O); 1H NMR (400 MHz, D₂O): δ 6.97-6.83 (m, 4H, MeOC₆H₄); 5.05 (d, 1H, J = 7.8 Hz, H-1); 4.28 (m, 1H, H-2); 3.91 (d, 1H, J =3.5 Hz, H-4); 3.77 (dd, 1H, J₁ =3.5 Hz, J₂= 9.8 Hz, H-3); 3.72 (m, 1H, H-5); 3.67-3.60 (m, 5H, H-6, H-6', OCH₃); 3.56 (d, 3H, JₚΗ = 11.5 Hz, POCH₃). 13C NMR (100 MHz, D₂O): δ 154.5, 150.7, 117.7, 114.9 (Ar); 99.7 (C-1); 77.0 (C-2); 75.3 (C-5); 71.6 (C-3); 68.6 (C-4); 60.5 (C-6); 55.6 (OCH₃); 53.9 (POCH₃). 31P NMR (162 MHz, D₂O): δ 14.27. HRMS (ESI): Calcd. For C₁₄H₂₃NO₉P [M+H]^+: 380.1111, found: 380.1085.

7.2.4 Experimental procedures for the preparation of MeOPN→4-Gal

**4-Methoxyphenyl 2,3,6-tri-O-benzoyl-β-D-galactopyranoside 27**

![Chemical Structure](image)

C₃₄H₃₀O₁₀: 598.18 g/mol

To a solution of 4-methoxyphenyl β-D-galactopyranoside 26 (1.92 g, 67.1 mmol) dissolved in CH₂Cl₂ (50 mL), DMF (4 mL) and pyridine (2.15 mL, 268 mmol), BzCl (2.31 mL, 201 mmol) was then added over 1 h at -20 °C. The reaction mixture was stirred at 0 °C for 3 hours before MeOH (5 mL) was added and the reaction mixture was concentrated under reduced pressure. Purification with flash chromatography (1:4 EtOAc-hexanes) gave product 27 (1.53 g, 38%). [α]D^25 = +124.0 (c = 0.1, CHCl₃); 1H
NMR (400 MHz, CDCl₃): δ 8.04-7.32 (m, 15H, Ar); 7.00-6.66 (m, 4H, MeOC₆H₄); 6.00 (dd, 1H, J₁ = 8.0 Hz, J₂ = 10.3 Hz, H-2); 5.39 (dd, 1H, J₁ = 3.2 Hz, J₂ = 10.3 Hz, H-3); 5.12 (d, 1H, J = 8.0 Hz, H-1); 4.71 (m, 1H, H-6a); 4.61 (m, 1H, H-6b); 4.39 (m, 1H, H-4); 4.13 (m, 1H, H-5); 3.69 (s, 3H, OCH₃); ¹³C NMR (100 MHz, CDCl₃): δ 166.4, 165.8, 165.4, 155.7, 151.2, 133.6, 133.4, 133.3, 129.9, 129.8, 129.4, 128.9, 128.6, 128.5, 128.4, 119.0, 114.4 (Ar); 101.2 (C-1); 74.1 (C-3); 72.6 (C-5); 69.3 (C-2); 67.3 (C-4); 62.8 (C-6); 55.6 (OCH₃). HRMS (ESI): Calcd. For C₃₄H₃₀NaO₁₀ [M+Na]⁺: 621.1737, found: 621.1733.

4-Methoxyphenyl 2,3,6-tri-O-benzoyl-4-O-methyl-phosphoramidyl-β-D-galactopyranoside 28 and 4-Methoxyphenyl 4-O-di-amino-phosphoramidyl-β-D-galactopyranoside 29

![Diagram of compound 28](image)

C₃₅H₃₄NO₁₂P: 691.18 g/mol

![Diagram of compound 29](image)

C₃₄H₃₃N₂O₁₁P: 676.18 g/mol
To a solution of compound 27 (94.1 mg, 0.157 mmol) and methyl dichlorophosphate (0.57 mL, 4.6 mmol) dissolved in anhydrous CH₂Cl₂ (4 mL) with crushed molecular sieves 4 Å, Et₃N (0.64 mL, 4.6 mmol) was added drop-wise at 0 °C. The reaction mixture was stirred at 35 °C for 48 hours. Upon completion of the reaction as judged by TLC, ammonia gas was injected into the reaction mixture through a needle. After 3 min, the reaction mixture was filtered and concentrated under reduced pressure. Purification with column chromatography (1:1 EtOAc-hexanes) yielded MeOPN product 28 (16.1 mg, 15%) and di-amino phosphoramidate product 29 (21.6 mg, 20%).

28: ¹H NMR (400 MHz, CDCl₃): δ 8.10-7.36 (m, 30H, Ar); 6.92-6.60 (m, 8H, MeOC₆H₄); 6.00 (m, 2H, H-2, H-2*); 5.15 (dd, 1H, J₁ = 2.3 Hz, J₂ = 10.6 Hz, H-3); 5.12 (dd, 1H, J₁ = 2.3 Hz, J₂ = 10.6 Hz, H-3*); 5.19 (2dd, 2H, J₁ = 3.1 Hz, J₂ = 10.0 Hz, H-4, H-4*); 5.15 (2d, 2H, J = 8.0 Hz, H-1, H-1*); 4.70 (m, 4H, H-6a, H-6a*, H-6b, H-6b*); 4.35 (m, 2H, H-5, H-5*); 3.72 (d, 3H, JPH = 11.4, POCH₃); 3.68 (s, 6H, OCH₃); 3.52 3.50 (d, 3H, JPH = 11.4 Hz, POCH₃*); 2.87 (d, 2H, J = 4.7 Hz, NH₂); 2.71 (d, 2H, J = 4.6 Hz, NH₂*). ¹³C NMR (100 MHz, CDCl₃): δ 166.1, 165.7, 165.5, 155.7, 151.1, 133.5, 133.4, 133.3, 130.1, 129.8, 129.7, 129.6, 129.3, 129.2, 128.7, 128.5, 128.4, 126.3, 119.0, 118.9, 114.4 (Ar); 101.1 (C-1); 72.7 (C-5); 72.1, 72.0 (C-3); 71.5 71.4 (C-4); 69.0, 68.9 (C-2); 62.8, 62.7 (C-6); 55.6 (OCH₃); 53.8, 53.7 (POCH₃). ³¹P NMR (162 MHz, CDCl₃): δ 11.27, 10.79. HRMS (ESI): Calcd. For C₃₅H₃₅NO₁₂P [M+H]⁺: 692.1897, found: 692.1868.
29: $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.10-7.34 (m, 15H, Ar); 6.92-6.62 (m, 4H, MeOC$_6$H$_4$); 5.98 (dd, 1H, $J_1$ = 8.0 Hz, $J_2$ = 10.4 Hz, H-2); 5.43 (dd, 1H, $J_1$ = 3.0 Hz, $J_2$ = 10.4 Hz, H-3); 5.23 (dd, H, $J_1$ = 3.0 Hz, $J_2$ = 10.6 Hz, H-4); 5.14 (d, H, $J$ = 8.0 Hz, H-1); 4.69 (m, 2H, H-6a, H-6b); 4.28 (m, 1H, H-5); 3.69 (s, 6H, OCH$_3$); 2.92, 2.72 (2d, 4H, NH$_2$); $^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 16.15. HRMS (ESI): Calcd. For C$_{35}$H$_{35}$NNaO$_{12}$P [M+Na]$^+$: 699.1720, found: 699.1717.

4-Methoxyphenyl 4-O-methyl-phosphoryl-β-D-galactopyranoside 30

![Structure of 4-Methoxyphenyl 4-O-methyl-phosphoryl-β-D-galactopyranoside 30](image)

\[
C_{14}H_{21}O_{10}P: 380.09 \text{ g/mol}
\]

Compound 28 (5.0 mg, 7.2 μmol) was dissolved in 0.01 M methanolic MeONa solution (1.8 mL) and the mixture was stirred for 1 h at room temperature before it was neutralized with acetic acid and concentrated under reduced pressure. Purification by flash chromatography eluting with 5:1 EtOAc-MeOH gave product 30 as a mixture of two diastereomers (1.4 mg, 51%). $^{31}$P NMR (162 MHz, D$_2$O): $\delta$ 1.36, 0.93. HRMS (ESI): Calcd. For C$_{14}$H$_{20}$O$_{10}$P [M-H]$^-$: 379.0794, found: 379.0799.
4-Methoxyphenyl 4-O-methyl-phosphoramidyl-β-D-galactopyranoside 11

![Chemical Structure](image)

C_{14}H_{22}NO_{9}P: 379.10 g/mol

Compound 28 (4.0 mg, 5.8 μmol) was dissolved in a solution of 7:2:1 mixture of MeOH-H_{2}O-Et_{3}N (1.5 mL). The mixture was stirred for 6 h at room temperature before it was neutralized with acetic acid and concentrated. Purification by flash chromatography eluting with 5:1 EtOAc-MeOH gave product 11 as a single diastereomers (0.3 mg, 14%).

δ\^1H NMR (600 MHz, D_{2}O): δ 7.02-6.83 (m, 4H, MeOC_6H_4); 4.81 (d, 1H, H-1); 4.11 (m, 1H, H-4); 3.92 (m, 2H, H-3, H-5); 3.75-3.65 (m, 5H, H-2, H-6a, OCH_3); 3.61-3.55 (m, 4H, H-6b, POCH_3)

δ\(^{13}\)C NMR (150 MHz, D_{2}O): δ 118.0, 115.0 (Ar); 101.3 (C-1); 73.6 (C-3); 72.4 (C-2); 70.4 (C-5); 68.1 (C-4); 58.9 (C-6); 55.7 (OCH_3); 53.9 (POCH_3)

δ\(^{31}\)P NMR (162 MHz, D_{2}O): δ 14.66. HRMS (ESI): Calcd. For C_{14}H_{21}NO_{9}P [M-H]^-: 378.0954, found: 378.0954.
5.2.5  Experimental procedures for the preparation of MeOPN→3-Fru

5-Amino-N-phthalimido-pentanyl 1,3,4,6-tetra-O-benzoyl-α-D-fructofuranoside 32

![Diagram of the compound structure]

C_{47}H_{41}NO_{12}: 811.26 g/mol

Thiofructofuranoside 31 (790 mg, 1.2 mmol) and 5-amino-N-phthalimido- pentanol (430 mg, 1.8 mmol) were dissolved in anhydrous CH_{2}Cl_{2} (12 mL). N-Iodosuccinimide (420 mg, 1.8 mmol) was introduced and the reaction mixture was cooled to 0 °C. TfOH (110 μL, 1.2 mmol) was added drop-wise and the reaction mixture was stirred for 30 min at 0 °C. The reaction mixture was then neutralized with Et_{3}N (~0.2 mL) and filtered through Celite. The residue was then concentrated and purified with flash chromatography (1:3 EtOAc-hexanes), which gave product 32 (810 mg, 81%). [α]_{D}^{25} = +10.3 (c = 0.2, CHCl_{3}); ¹H NMR (400 MHz, CDCl_{3}): δ 8.08-7.17 (m, 24H, Ar-H); 5.84 (d, 1H, J = 1.2 Hz, H-3); 5.53 (dd, 1H, J₁ = 1.2, J₂ = 4.6 Hz, H-4); 4.92 (d, 1H, J = 12.2 Hz, H-1a); 4.82 (dd, 1H, J₁ = 3.3 Hz, J₂ = 12.1 Hz, H-6a); 4.70 (dd, 1H, J₁ = 4.7 Hz, J₂ = 12.0 Hz, H-6b); 4.51 (m, 1H, H-5); 4.38 (d, 1H, J = 12.2 Hz, H-1b); 3.71-3.50 (m, 4H, linker-H); 1.64-1.39 (m, 6H, linker-H). ¹³C NMR (100 MHz, CDCl_{3}): δ 168.4, 166.2, 165.8, 164.8, 133.9, 133.6, 133.5, 133.2, 133.1, 132.1 129.9, 129.8, 129.7, 129.6, 129.3, 129.1, 129.0, 128.6, 128.5, 128.4, 128.3, 123.2 (Ar); 107.2 (C-2); 81.7 (C-5); 81.0 (C-3);
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78.8 (C-4); 63.7 (C-6); 60.9 (linker); 59.4 (C-1); 37.7, 29.2, 28.4, 14.2 (linker). HRMS (ESI): Calcd. For C_{47}H_{41}NO_{12} 811.2629.

5-Amino-N-phthalimido-pentanyl α-D-fructofuranoside 33

\[
\begin{align*}
\text{C}_{19}\text{H}_{25}\text{NO}_8: 395.16 \text{ g/mol} \\
\end{align*}
\]

Compound 32 (5.8 g, 7.2 mmol) was dissolved in a mixture of CH\(_2\)Cl\(_2\) (25 mL) and MeOH (85 mL). 0.8 M methanolic MeONa solution (5 mL) was slowly introduced and the mixture was stirred for 1 h at room temperature before it was neutralized with acetic acid (~0.24 mL). The mixture was concentrated under reduced pressure and purification by flash chromatography eluting with 9:1 EtOAc-MeOH gave product 33 (2.8 g, 99%). \([\alpha]_D^{25} = +80.5 \; (c = 0.2, \text{ CHCl}_3)\); \(^1\)H NMR (400 MHz, D\(_2\)O): \(\delta\) 7.70 (m, 4H, phthalimido-H); 3.95 (d, 1H, \(J = 3.6\) Hz, H-3); 3.78 (m, 2H, \(J = 1.2\) Hz, H-4, H-5); 3.65-3.45 (m, 8H, H-1a, H-1b, H-6a, H-6b, linker-H); 1.56-1.50 (m, 6H, linker-H). \(^{13}\)C NMR (100 MHz, D\(_2\)O): \(\delta\) 170.9, 134.5, 131.3, 128.7, 123.1 (Ar); 107.7 (C-2) 82.4 (C-5); 80.6 (C-3); 76.8 (C-4); 60.9 (C-1); 58.6 (C-6); 37.7, 28.4, 27.3, 22.7 (linker). HRMS (ESI): Calcd. For C\(_{19}\)H\(_{25}\)NNaO\(_8\) [M+Na]\(^+\): 418.1478, found: 418.1460.
5-Amino-N-phthalimido-pentanyl 1,4,6-tri-O-benzoyl-α-D-fructofuranoside 34

![Chemical structure](image)

C$_{40}$H$_{37}$NO$_{11}$: 707.24 g/mol

To a solution of compound 33 (2.82 g, 7.10 mmol) dissolved in CH$_2$Cl$_2$ (70 mL) and pyridine (2.9 mL, 35 mmol), BzCl (2.6 mL, 22 mmol) was added over 30 min at 0 °C. The reaction mixture was stirred at 0 °C for 1 hour before MeOH (2 mL) was added and the reaction mixture was concentrated under reduced pressure. Purification with flash chromatography (1:4 EtOAc-hexanes) gave product 34 (2.80 g, 55%). $[\alpha]_D^{25} = +88.7$ (c = 0.2, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.10-7.38 (m, 19H, Ar-H); 5.28 (dd, 1H, $J_1$ = 0.8 Hz, $J_2$ = 4.0 Hz, H-4); 5.27-4.63 (m, 3H, H-1a, H-1b, H-6a); 4.48-4.25 (m, 2H, H-5, H-6b); 4.25 (d, 1H, $J$ = 2.9 Hz, H-3); 3.93 (d, 1H, $J$ = 3.6 Hz, OH); 3.74-3.48 (m, 4H, linker-H); 1.63-1.38 (m, 6H, linker-H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 168.4, 167.1, 166.4, 166.3, 133.9, 133.6, 133.5, 133.1, 132.1, 129.9, 129.4, 129.3, 128.6, 128.5, 128.4, 123.2 (Ar); 107.9 (C-2); 81.4 (C-4); 80.7 (C-5); 79.8 (C-3); 64.3 (C-1); 60.9 (linker); 59.6 (C-6); 37.7, 29.4, 28.4 (linker). HRMS (ESI): Calcd. For C$_{40}$H$_{37}$NNaO$_{11}$ [M+Na]$^+$: 730.2264, found: 730.2268.
5-Amino-N-phthalimido-pentanyl

1,4,6-tri-O-benzoyl-3-O-methylphosphoramidyl-α-D-fructofuranoside 35

To a solution of compound 34 (31 mg, 0.211 mmol) and methyl dichlorophosphate (0.200 mL, 8.44 mmol) dissolved in anhydrous CH₂Cl₂CH₂Cl (2 mL) with crushed molecular sieves 4 Å, Et₃N (0.275 mL, 9.50 mmol) was added drop-wise. The reaction mixture was stirred at 60 °C for 40 hours. Upon completion of the reaction as judged by TLC, ammonia gas was injected into the reaction mixture through a needle. After 10 min, the reaction mixture was filtered and concentrated. Purification with column chromatography (1:1 EtOAc-Hexanes) yielded product 35 (1.8 mg, 5%). ¹H NMR (400 MHz, CDCl₃): δ 8.14-7.98 (m, 12H, Ar); 7.78-7.62 (m, 8H, phthalimido-H); 7.57-7.36 (m, 18H, Ar); 5.55 (d, 1H, J = 3.8 Hz, H-4); 5.51 (d, 1H, J = 3.8 Hz, H-4*); 5.03 (2d, 2H, J = 10.0 Hz, H-3, H-3*); 4.83-4.63 (m, 6H, H-1a, H-1a*, H-6a, H-6b, H-6a*, H-6b*); 4.51-4.42 (m, 4H, H-5,H-5*, H-1b, H-1b*), 3.70-3.60 (m, 7H, OCH₃, linker-H); 3.48 (m, 7H, OCH₃*, linker-H*); 2.93 (m, 4H, NH₂); 1.63 (m, 8H, linker-CH₂); 1.45 (m, 4H, linker-CH₂). ¹³C NMR (100 MHz, CDCl₃): δ 168, 166.4, 166.3, 166.1, 166.0, 133.9, 133.7, 133.3, 132.0, 130.0, 129.9, 129.6, 129.0, 128.6, 128.5, 128.4, 123.2, 107.8, 107.6
(Ar); 82.8, 82.7 (C-3); 81.9, 81.8 (C-5); 79.3 (C-4); 63.8, 63.7 (C-6); 61.0, 60.9 (linker); 58.9, 58.7 (C-1); 53.6, 53.5 (OCH3); 37.7, 29.7, 29.2, 28.4, 23.8 (linker). $^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 10.00, 9.57.

5-Amino-N-phthalimido-pentanyl 3-O-di-amino-phosphoramidyl-1,4,6-tri-O-benzoyl-\(\alpha\)-D-fructofuranoside 36

To a solution of compound 34 (43 mg, 0.061 mmol) and methyl dichlorophosphate (0.14 mL, 1.2 mmol) dissolved in anhydrous CH$_2$Cl$_2$ (3 mL) with molecular sieves 4 Å, Et$_3$N (0.17 mL, 1.2 mmol) was added drop-wise at 0 °C. The reaction mixture was stirred at 40 °C for 14 hours. Upon completion of the reaction as judged by TLC, ammonia gas was injected into the reaction mixture through a needle. After 5 min, the reaction mixture was filtered and concentrated under reduced pressure. Purification with column chromatography (EtOAc) yielded product 36 (9.7 mg, 20%). $[\alpha]_D^{25} = +56.5$ (c = 0.2, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.09-7.36 (m, 19H, Ar); 5.49 (d, 1H, $J = 3.9$ Hz, H-4); 5.03 (d, 1H, $J = 11.0$ Hz, H-3); 4.72-4.51 (m, 5H, H-5, H-1a, H-1b, H-6a, H-6b); 3.71-3.48 (m, 6H, linker-H); 2.95 (s, 4H, NH$_2$); 1.61 (m, 4H, linker-CH$_2$); 1.40 (m, 2H,
linker-CH₂). $^{13}$C NMR (100 MHz, CDCl₃): δ 168.3, 166.3, 133.9, 133.6, 133.3, 132.1, 131.6, 131.1, 129.9, 129.6, 129.0, 128.6, 127.3, 123.2 (Ar); 107.7 (C-2); 82.3 (C-3); 81.1 (C-5); 79.9 (C-4); 63.8 (C-1); 61.1 (linker); 59.1 (C-6); 37.7, 29.3, 28.4, 23.7 (linker). $^{31}$P NMR (162 MHz, CDCl₃): δ 15.10. HRMS (ESI): Calcd. For C₄₀H₄₀N₃O₁₂P [M+H]⁺: 785.2112, found: 785.2138.

5-Amino-N-phthalimido-pentanyl 1,4,6-tri-O-benzoyl-3-O-amino-phosphoryl-α-D-fructofuranoside 37

![Chemical structure](image)

C₄₀H₃₉N₃O₁₃P: 786.22 g/mol

To a solution of compound 34 (42 mg, 0.059 mmol) and methyl dichlorophosphate (0.065 mL, 0.55 mmol) dissolved in anhydrous CH₂Cl₂ (1.5 mL) with molecular sieves 4 Å, pyridine (0.060 mL, 0.74 mmol) was added drop-wise at 0 °C. The reaction mixture was then stirred at 40 °C for 20 hours. Upon completion of the reaction as judged by TLC, ammonia gas was injected into the reaction mixture through a needle. After 5 min, the reaction mixture was filtered and concentrated under reduced pressure. Purification with column chromatography (EtOAc) yielded product 37 (8.0 mg, 17%). $^1$H NMR (400 MHz,
CDCl₃: δ 8.10-7.98 (m, 6H, Ar); 7.75-7.62 (m, 4H, phthalimido-H); 7.57-7.37 (m, 9H, Ar); 5.47 (d, 1H, J = 3.9 Hz, H-4); 5.03 (d, 1H, J = 11.0 Hz, H-3); 4.72-4.50 (m, 5H, H-5, H-1a, H-1b, H-6a, H-6b); 3.71-3.48 (m, 4H, linker-H); 2.91 (m, 3H, NH₂, OH); 1.63 (m, 4H, linker-CH₂); 1.45 (m, 2H, linker-CH₂). ³¹P NMR (162 MHz, CDCl₃): δ 15.10, 15.08.

5-Amino-N-phthalimido-pentanyl

1,4,6-tri-O-benzoyl-3-O-(tert-butyldimethylsilyl)-α-D-fructofuranoside 38

![Chemical Structure](image)

C₄₀H₅₁NNaO₁₁: 821.32 g/mol

To a solution of compound 34 (1.27 g, 1.80 mmol) and imidazole (0.82 g, 12.0 mmol) in DMF (30 mL) was added TBDMSCl (1.28 g, 8.5 mmol) at room temperature. After stirring for 3 days, the reaction was then quenched by H₂O (30 mL). The reaction mixture was extracted with EtOAc (3 x 50 mL) and concentrated under reduced pressure. Purification with flash chromatography (1:4 EtOAc-hexanes) gave product 38 (1.29 g, 87%). [α]D²⁵ = +55.9 (c = 0.3, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 8.04-7.40 (m, 19H, Ar-H); 5.21 (d, 1H, J = 2.8 Hz, H-4); 4.70 (d, 1H, J = 11.9 Hz, H-6a); 4.60 (m, 2H, H-1a, H-1b); 4.50 (m, 1H, H-5); 4.42 (m, 2H, H-3, H-6b); 3.72-3.51 (m, 4H, linker-H);
1.62-1.43 (m, 6H, linker-H); 0.80 (s, 9H, C(CH$_3$)$_3$; 0.14 (s, 3H, CH$_3$; 0.04 (s, 3H, CH$_3$).

$^{13}$C NMR (100 MHz, CDCl$_3$): δ 168.3, 166.3, 166.0, 165.8, 133.8, 133.4, 133.1, 132.1, 129.9, 129.8, 129.7, 129.5, 128.5, 128.4, 123.1 (Ar); 108.7 (C-2); 81.3 (C-3); 81.0 (C-5); 80.0 (C-4); 64.9 (C-1); 61.1 (linker); 59.8 (C-6); 37.7, 29.5, 25.6 (linker); 23.8 C(CH$_3$)$_3$; 17.9 (C(CH$_3$)$_3$); -4.5, -5.1 (SiCH$_3$). HRMS (ESI): Calcd. For C$_{46}$H$_{51}$NNaO$_{11}$Si [M+Na]$^+$: 844.3129, found: 844.3106.

5-Amino-N-phthalimido-pentany

3-O-(tert-butyldimethylsilyl)-α-D-fructofuranoside 39

![Structure of 3-O-(tert-butyldimethylsilyl)-α-D-fructofuranoside 39]

C$_{25}$H$_{39}$NO$_8$Si: 509.24 g/mol

Compound 38 (1.28 g, 1.56 mmol) was dissolved in a mixture of CH$_2$Cl$_2$ (5 mL) and MeOH (10 mL). 0.5 M methanolic MeONa solution (1 mL) was slowly introduced and the mixture was stirred for 8 h at room temperature before it was neutralized with acetic acid (~0.035 mL). The mixture was concentrated under reduced pressure and purification by flash chromatography eluting with 1:2 EtOAc-hexanes gave product 39 (0.43 g, 54%). $[\alpha]_D^{25} = +14.3$ (c = 0.1, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): 7.81-7.65 (m, 4H, Ar-H); 4.06 (m, 2H, C-3, C-4); 3.91 (m, 1H, C-5); 3.80-3.43 (H-1a, H-1b, H-6a, H-6b, linker-H);
2.79 (d, 1H, J = 10.3 Hz, 4-OH); 2.30 (s, 1H, 1-OH); 1.96 (d, 1H, 6-OH); 1.72-1.32 (m, 6H, linker-H); 0.89 (s, 9H, C(CH$_3$)$_3$); 0.13 (s, 6H, CH$_3$). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 168.6, 134.0, 132.1, 123.3, 123.2 (Ar); 109.4 (C-2); 86.4 (C-3); 81.5 (C-4); 78.7 (C-5); 62.6, 60.9, 59.6 (C-1, C-6, linker); 37.7, 29.3, 28.2 (linker); 25.7 C(CH$_3$)$_3$; 23.5 (linker); 18.0 (C(CH$_3$)$_3$); -4.1, -5.6 (SiCH$_3$). HRMS (ESI): Calcd. For C$_{26}$H$_{40}$NO$_{10}$Si [M+COOH]$^-$: 554.2422, found: 554.2429.

Ethyl 1,4,6-tri-O-benzoyl-2-thio-α-D-fructofuranoside 43

![Structural diagram of Ethyl 1,4,6-tri-O-benzoyl-2-thio-α-D-fructofuranoside 43](image)

C$_{29}$H$_{38}$O$_5$S: 552.15 g/mol

To a solution of compound 42 (4.07 g, 18.1 mmol) dissolved in CH$_2$Cl$_2$ (100 mL) and pyridine (5.8 mL, 72 mmol), BzCl (6.4 mL, 55 mmol) was added over 1 h at 0 °C. The reaction mixture was stirred at 0 °C for another hour before MeOH (5 mL) was added and the reaction mixture was concentrated under reduced pressure. Purification with flash chromatography (1:9 EtOAc-hexanes) gave product 43 (4.84 g, 51%). $[^\alpha]_D^{25}$ = +68.5 (c = 0.1, CHCl$_3$); $^{1}$H NMR (400 MHz, CDCl$_3$): δ 8.05-7.34 (m, 15H, Ar-H); 5.27 (dd, 1H, $J_1$ = 3.8 Hz, $J_2$ = 6.4 Hz, H-4); 4.81-4.54 (m, 5H, H-1a, H-1b, H-5, H-6a, H-6b); 4.29 (t, 1H, $J$ = 2.6 Hz, H-3); 3.79 (d, 1H, $J$ = 3.8 Hz, 3-OH); 2.76 (m, 2H, -SCH$_2$CH$_3$); 1.29 (t, 3H, $J$ = 7.5 Hz, -SCH$_2$CH$_3$). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.8, 166.6, 166.3,
133.7, 133.4, 133.2, 130.0, 129.8, 129.7, 129.6, 129.0, 128.5, 128.4 (Ar); 95.0 (C-2); 82.6 (C-3); 82.2 (C-4); 78.3 (C-5); 63.6 (C-1); 62.7 (C-6). HRMS (ESI): Calcd. For C₃₀H₂₉O₁₁S [M+COOH]⁺: 597.1431, found: 597.1447.

**Ethyl 1,4,6-tri-O-benzoyl-3-O-(tert-butyldimethylsilyl)-2-thio-D-fructofuranoside 44**

![Diagram of the compound](image)

C₃₅H₄₂O₈Si: 650.237 g/mol

To a solution of compound 43 (7.74 g, 14.5 mmol) and imidazole (7.90 g, 116 mmol) in DMF (100 mL) was added TBDMSCl (13.1 g, 87.0 mmol) at room temperature. After stirring for 3 days, the reaction was then quenched by H₂O (200 mL). The reaction mixture was extracted with EtOAc (3 x 200 mL) and the organic layer was concentrated. Purification with flash chromatography (1:7 EtOAc-hexanes) gave product 44 (8.40 g, 91%). [α]_D²⁵ = +58.6 (c = 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 8.10-7.28 (m, 15H, Ar-H); 5.50 (dd, 1H, J₁ = 3.6 Hz, J₂ = 6.1 Hz, H-4); 4.66-4.51 (m, 5H, H-5, H-1a, H-1b, H-6a, H-6b); 4.42 (d, 1H, J = 3.6 Hz, H-3); 2.73 (m, 2H, -SCH₂CH₃); 1.29 (t, 3H, J = 7.5 Hz; SCH₂CH₃); 0.79 (s, 9H, C(CH₃)₃); 0.07 (s, 6H, CH₃). ¹³C NMR (100 MHz, CDCl₃): 215.9, 215.5, 167.2, 166.6, 134.3, 133.9, 133.8, 130.8, 130.7, 130.5, 130.0, 129.3, 129.1 (Ar); 94.5 (C-2); 83.3 (C-3); 80.8 (C-4); 78.7 (C-5); 64.5 (C-1); 64.3 (C-6); 25.7
(C(CH₃)₃); 22.8 (-SCH₂); 18.0 (C(CH₃)₃); 15.3 (-SCH₂CH₃); -4.5, -6.3 (SiCH₃). HRMS (ESI): Calcd. For C₃₅H₄₂O₈SSi: 650.237.

Ethyl 3-O-(tert-butyldimethylsilyl)-2-thio-D-fructofuranoside 45

![Chemical Structure](image)

C₁₄H₃₀O₅SSi: 338.16 g/mol

Compound 44 (8.40 g, 12.9 mmol) was dissolved in a mixture of CH₂Cl₂ (30 mL) and MeOH (60 mL). 0.5 M methanolic NaOMe solution (8 mL) was slowly introduced and the mixture was stirred for 3 h at room temperature before it was neutralized with acetic acid (~0.24 mL). The mixture was concentrated under reduced pressure. Purification by flash chromatography (1:1 EtOAc-hexanes) gave product 45 (3.04 g, 70 %). \([\alpha]_D^{25} = +156.2\) (c = 0.2, CHCl₃); \(^1\)H NMR (400 MHz, CDCl₃): δ 4.08-4.00 (m, 3H, H-3, H-4, H-5); 3.88-3.71 (m, 4H, H-1a, H-1b, H-6a, H-6b); 2.76 (d, 1H, J = 8.5 Hz, 4-OH); 2.68-2.58 (m, 3H, 1-OH, -SCH₂CH₃); 2.38 (s, 1H, 6-OH); 1.22 (t, 3H, J = 7.5 Hz, -SCH₂CH₃); 0.90 (s, 9H, C(CH₃)₃); 0.13 (s, 6H, CH₃). \(^{13}\)C NMR (100 MHz, CDCl₃): δ 96.1 (C-2); 85.9 (C-4); 84.7 (C-3); 79.7 (C-5); 64.4 (C-1); 62.3 (C-6); 25.8 (-SCH₂); 22.3 (CH₃); 18.1 (C(CH₃)₃); 15.4 (C(CH₃)₃); -4.7 (SiCH₃). HRMS (ESI): Calcd. For C₁₅H₃₁O₇SSi [M+COOH]⁺: 383.1560, found: 383.1572.
**Ethyl 3,4,6-tri-O- Allyl-2-thio-D-fructofuranoside 49**

\[
\begin{align*}
\text{C}_{17}\text{H}_{28}\text{O}_{5}\text{S} & : 344.17 \text{ g/mol} \\
\end{align*}
\]

To a solution of compound 50 (89 mg, 0.194 mmol) in THF (5 ml) at 0 °C was added tetra-n-butylammonium fluoride (50 mg, 0.192 mmol). After stirring for 3 h at room temperature, the reaction was quenched with sat. NH₄Cl. The aqueous layer was extracted with EtOAc (3 × 5 mL), and the organic layer was concentrated under reduced pressure. Purification by flash chromatography eluting with 1:5 EtOAc-hexanes gave compound 49 (52 mg, 79%). \([\alpha]_D^{25} = +109.0 \text{ (c = 0.1, CHCl}_3\text{)}; \] \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta \) 5.88 (m, 3H, CH\(_2\)–CH=CH\(_2\)); 5.32-5.17 (m, 6H, CH\(_2\)–CH=CH\(_2\)); 4.22-3.95 (m, 9H, CH\(_2\)=CH=CH\(_2\), H-3, H-4, H-5); 3.80-3.60 (m, 4H, H-1a, H-1b, H-6a, H-6b); 3.01 (t, 1H, \(J = 6.8 \text{ Hz, 1-OH}\)); 2.63 (m, 2H, -SCH\(_2\)CH\(_3\)); 1.21 (t, 3H, \(J = 7.5 \text{ Hz, }-\text{SCH}_2\text{CH}_3\)). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta \) 135.2, 135.1, 134.7 (CH\(_2\)=CH=CH\(_2\)); 118.8, 118.3, 118.2 (CH\(_2\)=CH=CH\(_2\)); 94.1 (C-2); 90.5 (C-3); 83.2 (C-5); 79.4 (C-4); 72.9, 72.1 (CH\(_2\)=CH=CH\(_2\)); 69.2 (C-6); 64.7 (C-1); 22.5 (-SCH\(_2\)); 15.3 (-SCH\(_2\)CH\(_3\)). HRMS (ESI): Calcd. For C\(_{17}\)H\(_{28}\)NaO\(_5\)S [M+Na]\(^+\): 367.1555, found: 367.1528.
Ethyl 1,4,6-tri-O-Allyl-3-O-(tert-butylidemethylsilyl)-2-thio-D-fructofuranoside 50

A solution of compound 45 (2.80 g, 8.26 mmol) dissolved in DMF (100 mL) with allyl bromide (4.28 mL, 49.6 mmol) was cooled to 0 °C. Sodium hydride, 60% dispersion in mineral oil (1.02 g, 25.6 mmol) was added and the reaction mixture was stirred for 3 h at 0 °C. The reaction was then poured into ice-cold water (100 mL) and extracted with EtOAc (3 × 100 mL). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. Purification by flash chromatography eluting with 1:16 EtOAc-hexanes gave compound 50 (3.00 g, 79%). [α]D²⁵ = +82.7 (c = 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 5.86 (m, 3H, CH₂–CH=CH₂); 5.30-5.11 (m, 6H, CH₂–CH=CH₂); 4.23-4.00 (m, 7H, CH₂–CH=CH₂, H-3); 3.89-3.58 (m, 6H, H-1a, H-1b, H-4, H-5, H-6a, H-6b); 2.63 (m, 2H, -SCH₂CH₃); 1.21 (t, 3H, J = 7.5 Hz, -SCH₂CH₃); 0.89 (s, 9H, C(CH₃)₃); 0.05 (2s, 6H, CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 135.7, 135.6, 135.4 (CH₂–CH=CH₂); 117.9, 117.7, 117.5 (CH₂–CH=CH₂); 94.8 (C-2); 86.8 (C-4); 83.3 (C-3); 79.5 (C-5); 72.8, 72.7, 72.3 (CH₂–CH=CH₂); 71.0 (C-6); 70.9 (C-1); 25.9 (-SCH₂); 22.3 C(CH₃)₃; 18.5 (C(CH₃)₃); 14.9 (-SCH₂CH₃); -4.9 (SiCH₃).
**Ethyl 1,3,6-tri-O-benzoyl-2-thio-β-D-fructofuranoside 52**

To a solution of ethyl 2-thio-β-D-fructofuranoside 51 (3.50 g, 15.6 mmol) dissolved in CH$_2$Cl$_2$ (80 mL), DMF (4 mL) and pyridine (4.40 mL, 54.4 mmol), BzCl (5.42 mL, 46.7 mmol) was added over 1 h at -20 °C. The reaction mixture was stirred at 0 °C for another hour before MeOH (5 mL) was added and the reaction mixture was concentrated under reduced pressure. Purification with flash chromatography (1:8 EtOAc-hexanes) gave product 52 (5.30 g, 63%). [α]$_D^{25}$ = -48.5 (c = 0.2, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): δ 8.12-7.31 (m, 15H, Ar-H); 5.65 (d, 1H, $J = 7.2$ Hz, H-3); 4.84-4.70 (m, 2H, H-4, H-6a); 4.63 (m, 2H, H-1a, H-1b); 4.51-4.42 (m, 2H, H-5, H-6b); 3.81 (s, 1H, 4-OH); 2.71 (m, 2H, -SCH$_2$CH$_3$); 1.22 (t. 3H, $J = 7.5$ Hz, -SCH$_2$CH$_3$). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 168.4, 167.5, 166.9, 134.9, 134.6, 134.1, 134.0, 131.0, 130.8, 130.7, 130.6, 130.5, 130.4, 129.5, 129.4, 129.3, 129.2 (Ar); 92.4 (C-2); 83.4 (C-3); 82.2 (C-5); 76.3 (C-4); 65.9 (C-6); 64.7 (C-1); 21.7 (-SCH$_2$); 14.6 (CH$_3$). HRMS (ESI): Calcd. For C$_{30}$H$_{29}$O$_{11}$S [M+COOH]$^-$: 597.1431, found: 597.1449.
Ethyl 4-O-Allyl-1,3,6-tri-O-benzoyl-2-thio-β-D-fructofuranoside 53

A solution of compound 52 (5.30 g, 9.87 mmol) dissolved in DMF (80 mL) with allyl bromide (1.71 mL, 19.7 mmol) was cooled to 0 °C. Sodium hydride, 60% dispersion in mineral oil (475 mg, 11.8 mmol) was added and the reaction mixture was stirred for 1 h at 0 °C. The reaction was then quenched with MeOH (1 mL), poured into ice-cold water (100 mL) and extracted with EtOAc (3 × 100 mL). The organic layer was dried over Na₂SO₄ and concentrated. Purification by flash chromatography eluting with 1:6 EtOAc-hexanes gave product 53 (2.00 g, 28%, estimated from H-3 resonance of ¹H NMR of a mixture of 3-O-All and 4-O-All product). ¹H NMR (400 MHz, CDCl₃): δ 8.12-7.36 (m, 15H, Ar-H); 6.08 (d, 1H, J = 6.7 Hz, H-3); 5.82 (m, 1H, CH₂–CH=CH₂); 5.28-5.17 (m, 2H, CH₂–CH=CH₂); 4.81-4.50 (m, 6H, CH₂–CH=CH₂, H-4, H-5, H-6a, H-6b); 4.12 (m, 2H, H-1a, H-1b); 2.72 (m, 2H, -SCH₂CH₃); 1.20 (m, 3H, -SCH₂CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 118.1 (CH₂–CH=CH₂); 92.5 (C-2); 83.3 (C-3); 79.7 (C-5); 79.3 (C-4); 71.8 (C-1); 65.8 (CH₂–CH=CH₂); 64.4 (C-6); 21.1 (-SCH₂); 15.1 (CH₃). HRMS (ESI): Calcd. For C₃₂H₃₂O₈S [M+COOH]: 621.1794, found: 621.1798.
**Ethyl 4-O- Allyl-2-thio-\(\beta\)-D-fructofuranoside 54**

![Chemical structure](image)

C\(_{11}\)H\(_{20}\)O\(_5\)S: 264.10 g/mol

Compound 53 (2.00 g, 3.47 mmol) was dissolved in a mixture of CH\(_2\)Cl\(_2\) (3 mL) and MeOH (12 mL). MeONa (0.12 g) dissolved in methanol (5 mL) was slowly introduced and the mixture was stirred for 1 h at room temperature before it was neutralized with acetic acid (~0.13 mL). The mixture was concentrated under reduced pressure and purification by flash chromatography (EtOAc) gave product 54 (0.815 g, 89%). \([\alpha]_D^{25} = -77.0\) (c = 0.4, CHCl\(_3\)); \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 5.89 (m, 1H, CH\(_2\)--CH=CH\(_2\)); 5.31-5.15 (m, 2H, CH\(_2\)--CH=CH\(_2\)); 4.43 (m, 1H, H-3); 4.24-4.06 (m, 4H, CH\(_2\)--CH=CH\(_2\), H-4, H-5); 3.83-3.68 (m, 4H, H-1a, H-1b, H-6a, H-6b); 3.01 (s, 1H, 3-OH); 2.61 (m, 2H, -SCH\(_2\)CH\(_3\)); 2.40-2.22 (2s, 2H, 1-OH, 6-OH); 1.25 (t, 3H, \(J = 7.5\) Hz, -SCH\(_2\)CH\(_3\)). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 135.0 (CH\(_2\)--CH=CH\(_2\)); 118.4 (CH\(_2\)--CH=CH\(_2\)); 97.5 (C-2); 85.5 (C-4); 85.0 (C-5); 80.0 (C-3); 71.8 (CH\(_2\)--CH=CH\(_2\)); 65.2 (C-6); 64.0 (C-1); 21.1 (-SCH\(_2\)); 15.1 (CH\(_3\)). HRMS (ESI): Calcd. For C\(_{12}\)H\(_{21}\)O\(_7\)S \([M+COOH]^+\): 309.1008, found: 309.1016.
Ethyl 4-O- Allyl-1,6-di-O-benzoyl-2-thio-β-D-fructofuranoside 55

\[
\text{C}_{25}\text{H}_{28}\text{O}_{7}\text{S} : 472.16 \text{ g/mol}
\]

To a solution of compound 54 (0.446 mg, 1.68 mmol) dissolved in CH\(_2\)Cl\(_2\) (10 mL) and pyridine (0.34 mL, 4.21 mmol), BzCl (0.39 mL, 3.36 mmol) was added over 1 h at 0 °C. The reaction mixture was stirred at 0 °C for another hour before MeOH (1 mL) was added and the reaction mixture was concentrated under reduced pressure. Purification with flash chromatography (1:5 EtOAc-hexanes) gave product 55 (0.488 g, 61%). \([\alpha]_D^{25} = -176.5 \text{ (c = 0.1, CHCl}_3\); \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.12-7.40 (m, 10H, Ar-H); 5.87 (m, 1H, CH\(_2\)-CH=CH\(_2\)); 5.30-5.12 (m, 2H, CH\(_2\)-CH=CH\(_2\)); 4.64 (m, 5H, CH\(_2\)-CH=CH\(_2\), H-6a, H-6b, H-4); 4.33 (t, 1H, \(J = 7.2\) Hz, H-3); 4.26-4.12 (m, 3H, H-5, H-1a, H-1b); 2.69 (m, 2H, -SCH\(_2\)CH\(_3\)); 1.21 (t, 3H, \(J = 7.5\) Hz, -SCH\(_2\)CH\(_3\)). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 167.7 167.4 135.1 134.3 134.0 130.7 130.6 130.3 129.4 129.2 (Ar, CH\(_2\)-CH=CH\(_2\)); 118.6 (CH\(_2\)-CH=CH\(_2\)); 94.2 (C-2); 83.6 (C-3); 79.8 (C-5); 79.7 (C-4); 72.3 (C-1); 66.2 (C-6); 65.2 (CH\(_2\)-CH=CH\(_2\)); 21.6 (-SCH\(_2\)); 14.8 (SCH\(_2\)CH\(_3\)). HRMS (ESI): Calcd. For C\(_{25}\)H\(_{28}\)NaO\(_7\)S [M+Na]\(^+\): 495.1453, found: 495.1454.
To a solution of 55 (100 mg, 0.211 mmol) and methyl dichlorophosphate (1.26 mL, 10.2 mmol) dissolved in anhydrous CH₂Cl₂ (4 mL) with crushed molecular sieves 4 Å, Et₃N (1.5 mL, 10.8 mmol) was added drop-wise. The reaction mixture was stirred at 40 °C for 72 hours. Upon completion of the reaction as judged by TLC, ammonia gas was injected into the reaction mixture through a needle. After 10 min, the reaction mixture was filtered and concentrated. Purification with column chromatography (9:1
EtOAc-MeOH) yielded MeOPN product 56 (41 mg, 34%) and di-amino-phosphoramidate product 57 (29 mg, 20%).

56: ^1^H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.13-7.40 (m, 20H, Ar); 5.83 (m, 2H, CH\(_2\)–CH=CH\(_2\)); 5.30-5.13 (m, 6H, H-3, H-3*, CH\(_2\)–CH=CH\(_2\)); 4.74 (m, 2H, H-1a, H-1a*); 4.68-4.50 (m, 8H, H-1b, H-1b*, H-6a, H-6a*, H-6b, H-6b*, H-4, H-4*); 4.35-4.10 (m, 8H, H-1b, H-1b*, H-6a, H-6a*, H-6b, H-6b*, H-4, H-4*); 3.78 (d, 3H, OCH\(_3\)); 3.73 (d, 3H, OCH\(_3\)*); 2.93 (dd, 4H, NH\(_2\)); 2.70 (q, 4H, SCH\(_2\)CH\(_3\)); 1.19 (t, 6H, SCH\(_2\)CH\(_3\)). ^1^C NMR (100 MHz, CDCl\(_3\)): 166.3, 166.0 (Ar); 133.9 (CH\(_2\)–CH=CH\(_2\)); 133.3, 129.9, 129.7, 128.5, 128.5 (Ar); 118.1 (CH\(_2\)–CH=CH\(_2\)); 92.1, 91.7 (C-2); 82.6, 82.2 (C-4); 81.3, 80.7 (C-3); 79.7, 79.4 (C-5); 72.1, 72.0 (CH\(_2\)–CH=CH\(_2\)); 65.0, 64.7, 64.4 (C-1, C-6); 53.8 (OCH\(_3\)); 21.6, 21.5 (SCH\(_2\)CH\(_3\)); 14.6 (SCH\(_2\)CH\(_3\)). ^3^P NMR (162 MHz, CDCl\(_3\)): \(\delta\) 9.81, 9.67. Calcd. For C\(_{26}\)H\(_{33}\)NO\(_9\)PS: [M+H]^+: 566.1614, found: 566.1568.

57: ^1^H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.05-7.36 (Ar); 5.84 (m, 1H, CH\(_2\)–CH=CH\(_2\)); 5.30-5.14 (m, 3H, CH\(_2\)–CH=CH\(_2\), H-3); 4.71-4.58 (m, 3H, CH\(_2\)–CH=CH\(_2\), H-6a); 4.50 (m, 2H, H-4, H-6a); 4.32-4.11 (m, 3H, H-5, H-1a, H-1b); 2.95 (d, 4H, J = 17.0 Hz, NH\(_2\)); 2.70 (m, 2H, -SCH\(_2\)CH\(_3\)); 1.16 (t, 3H, J = 7.5 Hz, -SCH\(_2\)CH\(_3\)). ^1^C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 166.3, 166.1 (Ar); 133.9 (CH\(_2\)–CH=CH\(_2\)); 133.3, 129.9, 129.8, 129.7, 128.5 (Ar); 118.1 (CH\(_2\)–CH=CH\(_2\)); 92.1 (C-2); 82.7 (C-4); 82.6 (C-3); 80.4 (C-5); 71.9 (C-1); 64.8 (CH\(_2\)–CH=CH\(_2\)); 64.3 (C-6); 21.6, (-SCH\(_2\)); 14.6 (SCH\(_2\)CH\(_3\)). ^3^P NMR (162 MHz,

7.2.6 Experimental procedures for the preparation of MeOPN→6-Gal conjugate

2,3,4-Tri-O-allyl-6-O-trityl-α,β-D-galactopyranosyl trichloroacetimidate 58

\[
\text{C₃₆H₃₅Cl₃NO₆: 685.18 g/mol}
\]

To a solution of compound 15 (5.0 g, 7.7 mmol) dissolved in a mixture of CH₃CN (480 mL) and H₂O (120 mL), cerium ammonium nitrate (12.8 g, 23 mmol) was added and the reaction mixture was stirred for 20 min at 0 °C. The mixture was then diluted with brine (200 mL) and extracted with EtOAc (3 × 300 mL). The organic layer was washed with saturated aq. Na₂CO₃ and water, dried over Na₂SO₄, concentrated under reduced pressure and purified with column chromatography (1:6 EtOAc-hexanes). The
resulting hemiacetal (3.3 g, 6.1 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (120 ml). CCl$_3$CN (310 μL, 30 mmol) and K$_2$CO$_3$ (420 mg, 30 mmol) were added. The reaction mixture was stirred at room temperature overnight before it was filtered through Celite® and concentrated under reduced pressure. Purification with flash chromatography (1:4 EtOAc-hexanes with 1% Et$_3$N by volume) gave product 58 as an α, β-mixture (3.6 g, 57% over 2 steps).

58A: [α]$_D^{25}$ = +89.2 (c = 0.05, CHCl$_3$); \(^1\)H NMR (400 MHz, CDCl$_3$): δ 8.52 (s, 1H, NH); 7.42-7.18 (m, 15H, Ar-H); 6.46 (d, 1H, $J$ = 3.6 Hz, H-1); 6.00-5.61 (m, 3H, CH$_2$–CH=CH$_2$); 5.39-4.98 (m, 6H, CH$_2$–CH=CH$_2$, H-2, H-3, H-4, H-5); 3.35 (m, 1H, H-6a); 3.09 (m, 1H, H-6b); \(^1^3\)C NMR (100 MHz, CDCl$_3$): δ 161.3, 160.8, 143.9, 143.7, 135.2, 135.0, 134.9, 134.8, 134.1, 133.8, 128.8, 128.6, 127.8, 127.1, 127.0 (Ar, CH$_2$–CH=CH$_2$); 117.9, 117.4, 117.3, 116.7, 116.5 (CH$_2$–CH=CH$_2$); 104.0 (C-1); 86.8 (C-3); 86.7 (C-2); 83.8 (C-3); 82.6; 76.7 (C-4); 75.3, 74.1, 72.5, 72.2, 71.8, 71.0 (CH$_2$–CH=CH$_2$, C-5); 51.9 (C-6). HRMS (ESI): Calcd. For C$_{36}$H$_{38}$Cl$_3$NNaO$_6$ [M+Na]$^+$: 708.1663, found: 708.1673.

58B: [α]$_D^{25}$ = +108.4 (c = 0.05, CHCl$_3$); \(^1\)H NMR (400 MHz, CDCl$_3$): δ 8.59 (s, 1H, NH); 7.41-7.18 (m, 15H, Ar-H); 5.90 (m, 2H, CH$_2$–CH=CH$_2$); 5.62 (m, 2H, H-1, CH$_2$–CH=CH$_2$); 5.35-5.01 (m, 6H, CH$_2$–CH=CH$_2$); 4.31-3.83 (m, 6H CH$_2$–CH=CH$_2$); 3.83 (m, 1H, H-5); 3.76 (dd, 1H, $J$_1 = 8.2 Hz, $J$_2 = 9.7 Hz, H-3); 3.62 (t, 1H, $J$ = 5.9 Hz,
H-2), 3.48-3.39 (m, 2H, H-4, H-6a); 3.12 (dd, 1H, \( J_1 = 7.2 \) Hz, \( J_2 = 9.3 \) Hz, H-6b). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 161.5, 143.8 (Ar); 135.4, 134.9, 134.8 (CH\(_2\)–CH=CH\(_2\)); 128.7, 128.6, 128.0, 127.9, 127.1 (Ar); 117.3, 117.0, 116.8 (CH\(_2\)–CH=CH\(_2\)); 98.5 (C-1); 86.8 (C-2); 81.6 (C-3); 77.8 (C-5); 74.6 (C-3) 74.2, 73.8, 73.3, 72.0 (CH\(_2\)–CH=CH\(_2\), C-4); 62.4 (C-6). HRMS (ESI): Calcd. For C\(_{36}\)H\(_{38}\)Cl\(_3\)NNaO\(_6\) \([\text{M+Na}]^+\): 708.1663, found: 708.1673.

5-Amino-N-phthalimido-pentanyl 2,3,4-tri-O-allyl-6-O-trityl-\( \beta \)-D-galactopyranoside 59 and 5-Amino-N-phthalimido-pentanyl 2,3,4-tri-O-allyl-6-O-trityl-\( \alpha \)-D-galactopyranoside 60

![Chemical Structure 59](image)

C\(_{47}\)H\(_{51}\)NO\(_8\): 757.36 g/mol

![Chemical Structure 60](image)

C\(_{47}\)H\(_{51}\)NO\(_8\): 757.36 g/mol

Trichloroacetimidate 58 (1.10 g, 1.6 mmol) and 5-amino-N-phthalimido-pentanol (560 mg, 2.4 mmol) were dissolved in anhydrous CH\(_2\)Cl\(_2\) (25 mL) and the reaction
mixture was cooled to 0 °C. TMSOTf (15 μL, 0.080 mmol) was added drop-wise and the reaction mixture was stirred for 15 min at 0 °C. The reaction was then neutralized with Et₃N (15 μL) and concentrated under reduced pressure. Purification with flash chromatography (1:8 EtOAc-hexanes) gave compound 59 (350 mg, 29%) and 60 (783 mg, 65%).

59: [α]D²⁵ = +67.5 (c = 0.05, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 7.80-7.67 (m, 4H, phthalimido protons); 7.41-7.19 (m, 15H, Ar-H); 5.98-5.59 (m, 3H, CH₂–C=CH₂); 5.33-4.94 (m, 6H, CH₂–C=CH₂); 4.30-3.84 (m, 8H, CH₂–C=CH₂, H-1, linker–CH₂); 3.77 (d, 1H, J = 2.9 Hz, H-5); 3.62 (t, 2H, J = 7.3 Hz, linker-CH₂); 3.45-3.35 (m, 4H, H-2, H-4, H-6a, linker-CHH); 3.29 (dd, 1H, J₁ = 3.0 Hz, J₂ = 9.8 Hz, H-3); 3.13 (dd, 1H, J₁ = 9.4 Hz, J₂ = 10.1 Hz, H-6b); 1.65 (m, 4H, linker-CH₂); 1.40 (m, 2H, linker-CH₂). ¹³C NMR (100 MHz, CDCl₃): δ 168.4, 143.8 (Ar); 135.7, 135.3, 135.2 (CH₂–C=CH₂); 133.9, 132.1, 128.7, 127.9, 127.1, 123.2 (Ar); 116.8, 116.5 (CH₂–C=CH₂); 103.7 (C-1); 86.8; 81.5 (C-1); 79.2 (C-2); 73.9, 73.6, 73.4, 73.3 (C-5, C-4, CH₂–C=CH₂); 71.9, 69.4 (linker); 62.5 (C-6); 37.9, 29.2, 28.4, 23.4 (linker). HRMS (ESI): Calcd. For C₄⁷H₅₁NNaO₈ [M+Na]⁺: 780.3513, found: 780.3489.

60: [α]D²⁵ = +35.0 (c = 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 7.81-7.66 (m, 4H, phthalimido protons); 7.51-7.15 (m, 15H, Ar-H); 5.97-5.55 (m, 3H, CH₂–C=CH₂); 5.33-4.93 (m, 6H, CH₂–C=CH₂); 4.83 (d, 1H, J = 3.5 Hz, H-1); 4.21-3.80 (m, 8H,
$CH_2-CH=CH_2$, H-5, H-2); 3.75-3.60 (m, 5H, H-3, H-4, linker-CH$_2$, linker-CHH); 3.48-3.09 (m, 3H, H-6a, H-6b, linker-CHH); 1.69 (m, 4H, linker-CH$_2$); 1.40 (m, 2H, linker-CH$_2$). $^{13}$C NMR (100 MHz, CDCl$_3$): 168.4, 144.0 (Ar); 135.5, 135.4, 135.2 (CH$_2$–CH=CH$_2$); 134.6, 133.9, 132.2, 128.8, 127.8, 127.0, 123.2 (Ar); 117.6, 117.0, 116.5 (CH$_2$–CH=CH$_2$); 97.4 (C-1); 78.3 (C-2); 74.9 (C-4); 73.9 (C-2); 72.8, 72.4, 71.8 (CH$_2$–CH=CH$_2$); 71.1 (C-5); 67.7 (linker); 62.9 (C-6); 37.9, 29.1, 28.5, 23.7 (linker).

Calcd. For C$_{47}$H$_{51}$NNaO$_8$ [M+Na]$^+$: 780.3513, found: 780.3490.

5-Amino-N-phthalimido-pentanyl 2,3,4-tri-O-allyl-β-D-galactopyranoside 61

![Structural formula](image)

$C_{28}H_{37}NO_8$: 515.25 g/mol

A solution of compound 59 (493 mg, 0.65 mmol) dissolved in 80% aqueous AcOH (10 mL) was stirred at 80 °C for 1 h. The reaction mixture was concentrated under reduced pressure before purification by flash chromatography (1:1 EtOAc-hexanes), giving product 61 (260 mg, 78%). $[\alpha]_D^{25} = +72.1$ (c = 0.05, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.81-7.66 (m, 4H, phthalimido protons); 5.92-5.82 (m, 3H, CH$_2$–CH=CH$_2$); 5.30-5.10 (m, 6H, CH$_2$–CH=CH$_2$); 4.37-4.02 (m, 6H, CH$_2$–CH=CH$_2$); 4.22 (d, 1H, $J =$ 7.7 Hz, H-1); 3.88 (m, 2H, H-6a, linker-CHH); 3.69-3.60 (m, 4H, H-4, H-6b, linker-CH$_2$); 3.51-3.42 (m, 2H, H-2, linker-CHH); 3.39 (m, 1H, H-5); 3.28 (dd, 1H, $J_1 =$ 3.0 Hz, $J_2 =$...
9.8 Hz, H-3; 2.09 (m, 1H, 6-OH); 1.65 (m, 4H, linker-CH₂); 1.40 (m, 2H, linker-CH₂).

$^{13}$C NMR (100 MHz, CDCl₃): δ 168.5 (phthalimido C=O); 135.3, 135.0, 133.9 (CH₂–CH=CH₂); 132.1, 123.2 (phthalimido); 117.8, 116.7, 116.6 (CH₂–CH=CH₂); 103.9 (C-1); 81.6 (C-3); 79.1 (C-2); 74.6 (C-5); 74.0 (C-4); 73.7, 73.6 (CH₂–CH=CH₂); 72.1, 69.6 (linker); 62.5 (C-6); 37.8, 29.2, 28.3, 23.3 (linker). HRMS (ESI): Calcd. For C$_{28}$H$_{37}$NNaO$_8$ [M+Na$^+$]: 538.2417, found: 538.2403.

5-Amino-N-phthalimido-pentanyl 2,3,4-tri-O-allyl-α-D-galactopyranoside 62

![Structural formula](image)

A solution of compound 60 (783 mg, 1.03 mmol) dissolved in 80% aqueous AcOH (16 mL) was stirred at 80 °C for 1 h. The reaction mixture was concentrated under reduced pressure before purification by flash chromatography (1:5 EtOAc-hexanes), giving product 62 (425 mg, 80%). [α]$_D^{25}$ = +25.7 (c = 0.3, CHCl₃); $^1$H NMR (400 MHz, CDCl₃): δ 5.30-5.10 (m, 6H, CH₂–CH=CH₂); 4.89 (d, 1H, J = 3.7 Hz, H-1); 4.40-4.05 (m, 6H, CH₂–CH=CH₂); 3.84-3.59 (m, 9H, H-2, H-3, H-4, H-5, H-6a, linker-CH₂); 3.47 (m, 1H, H-6b); 2.59 (m, 1H, 6-OH); 1.65 (m, 4H, linker-CH₂); 1.40 (m, 2H, linker-CH₂). $^{13}$C NMR (100 MHz, CDCl₃): δ 169.7 (phthalimido C=O); 136.1, 136.0, 135.9 (CH₂–CH=CH₂); 134.8, 132.8, 124.0 (phthalimido); 118.4, 117.8, 117.0 (CH₂–CH=CH₂);
98.2 (C-1); 78.9 (C-3); 75.7 (C-2); 74.4 (C-4); 72.9 (C-5); 72.2, 71.7, 70.9 (CH2–CH=CH2); 68.1 (linker); 62.7 (C-6); 38.2, 29.0, 28.5, 23.9 (linker). HRMS (ESI): Calcd. For C29H38NO10 [M+ COOH]: 560.2496, found: 560.2505.

**5-Amino-N-phthalimido-pentanyl**

**2,3,4-tri-O-allyl-6-O-methyl-phosphoramidyl-β-D-galactopyranoside 63**

![Chemical Structure](image)

C29H41N2O10P: 608.24 g/mol

To a solution of compound 61 (400 mg, 0.78 mmol) and methyl dichlorophosphate (0.70 mL, 6.0 mmol) dissolved in anhydrous CH2Cl2 (15 mL) with crushed molecular sieves 4 Å, Et3N (0.70 mL, 5.0 mmol) was added drop-wise at 0 °C. The reaction mixture was stirred at room temperature for 12 hours. Upon completion of the reaction as judged by TLC, ammonia gas was injected into the reaction mixture through a needle. After 10 min, the reaction mixture was filtered and concentrated under reduced pressure. Purification with column chromatography (9:1 EtOAc-MeOH) yielded product 63 (129 mg, 27%). 1H NMR (400 MHz, CDCl3): 7.80-7.68 (phthalimido protons); 5.88 (m, 3H, CH2–CH=CH2); 5.30-5.10 (m, 6H, CH2–CH=CH2); 4.23-4.10 (m, 9H, CH2–CH=CH2, H-1, linker-CH2); 3.82 (m, 1H, H-5); 3.71-3.39 (m, 9H, OCH3, H-4, H-2, H-6a, H-6b,
linker-CH$_2$); 3.28 (m, 1H, H-3); 2.87 (dd, 2H, $J_1 = 5.3$ Hz, $J_2 = 13.0$ Hz, NH$_2$); 1.66 (m, 4H, linker-CH$_2$); 1.38 (m, 2H, linker-CH$_2$). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 168.5 (Ar); 135.4, 135.2, 134.9 (CH$_2$–CH=CH$_2$); 133.9, 132.1, 123.2 (Ar); 117.5, 117.2, 116.8, 116.7, 116.6 (CH$_2$–CH=CH$_2$); 103.8 (C-1); 81.4 (C-3); 78.9 (C-2); 74.0, 73.8, 73.3, 73.2, 73.0, 72.9, 72.1 (CH$_2$–CH=CH$_2$, C-5, C-4); 69.8, 69.7 (C-6) 65.3; 65.0, 64.9 (linker); 53.4, 53.3 (POCH$_3$); 37.9, 29.7, 29.2, 28.3 (linker). $^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 10.81, 10.46. HRMS (ESI): Calcd. For C$_{29}$H$_{42}$N$_2$O$_{10}$P [M+H]$^+$: 609.2578, found: 609.2585.

5-Amino- $N$-phthalimido-pentanyl

2,3,4-tri- $O$-allyl-6- $O$-methyl-phoraramidyl- $\alpha$-D-galactopyranoside 64

![Image of structure 64]

C$_{29}$H$_{41}$N$_2$O$_{10}$P: 608.24 g/mol

To a solution of compound 62 (160 mg, 0.31 mmol) and methyl dichlorophosphate (0.26 mL, 2.2 mmol) dissolved in anhydrous CH$_2$Cl$_2$ (6 mL) with molecular sieves 4 Å, Et$_3$N (0.26 mL, 1.8 mmol) was added drop-wise at 0 °C. The reaction mixture was stirred at room temperature for 14 hours. Upon completion of the reaction as judged by TLC, ammonia gas was injected into the reaction mixture through a needle. After 10 min, the
reaction mixture was filtered and concentrated under reduced pressure. Purification with column chromatography (9:1 EtOAc-MeOH) yielded product 64 (50 mg, 26%). $^1$H NMR (400 MHz, CDCl$_3$): 7.79-7.66 (m, 4H, phthalimido-H); 5.88 (m, 3H, CH$_2$–CH=CH$_2$); 5.29-5.06 (m, 6H, CH$_2$–C=C=CH$_2$); 4.86 (d, 1H, $J_1 = 3.6$ Hz, H-1); 4.34 (m, 1H, H-2); 4.20-4.02 (m, 7H, CH$_2$–C=C=CH$_2$, H-4); 3.90 (m, 1H, H-6a); 3.79-3.58 (m, 9H, OCH$_3$, H-5, H-6b, linker-CH$_2$); 3.44 (m, 1H, H-3); 3.03 (m, 2H, NH$_2$); 1.63 (m, 4H, linker-CH$_2$); 1.35 (m, 2H, linker-CH$_2$). $^{13}$C NMR (100 MHz, CDCl$_3$): 168.5 (Ar); 134.8, 134.7, 134.6, 132.1 (CH$_2$–CH=CH$_2$); 117.7, 117.4, 117.3, 117.2, 117.1, 116.3 (Ar, CH$_2$–CH=CH$_2$); 100.1 (C-1); 84.0 (C-3); 79.2 (C-2); 79.0 (C-5); 78.9 (C-4); 74.0, 72.5, 72.3 (CH$_2$–CH=CH$_2$); 72.2, 72.1 (C-6); 71.4, 71.3 (linker); 68.1, 67.8 (OCH$_3$); 65.6, 65.5, 64.9, 37.9, 29.7, 29.0, 28.4, 23.6 (linker). $^{31}$P NMR (162 MHz, CDCl$_3$): δ 10.97, 10.68. HRMS (ESI): Calcd. For C$_{29}$H$_{42}$N$_2$O$_{10}$P [M+H]$^+$: 609.2577, found: 609.2594.

5-Amino-N-phthalimido-pentanyl

6-O-methyl-phosphoramidyl-β-D-galactopyranoside 65

\[
\text{C}_{20}\text{H}_{29}\text{N}_{2}\text{O}_{10}\text{P}: 488.16 \text{ g/mol}
\]
To a solution of compound 63 (95 mg, 0.16 μmol) dissolved in MeOH (4 mL), PdCl₂ (20 mg) was added and the reaction mixture was stirred at room temperature for 4 h. The reaction mixture was then filtered through Celite® and concentrated under reduced pressure. Purification with column chromatography (9:1 EtOAc-MeOH) gave product 65 (57 mg, 75%). ¹H NMR (400 MHz, D₂O): δ 7.64 (m, 4H, phthalimido-H); 4.23 (d, 1H, J = 8.0 Hz, H-1); 4.01 (m, 2H, H-6a, H-6b); 3.78-3.70 (m, 3H, H-4, H-5, linker-CH₂); 3.59-3.45 (m, 7H, OCH₃, linker-CH₂ linker-CHH, H-3); 3.33 (dd, 1H, J₁ = 8.0 Hz, J₂ = 9.8 Hz, H-2); 1.51 (m, 4H, linker-CH₂); 1.22 (m, 2H, linker-CH₂). ¹³C NMR (100 MHz, D₂O): 170.9, 134.5, 133.9, 131.3, 126.0, 123.1 (Ar); 102.6 (C-1); 73.2 (C-5); 72.5 (C-3); 71.9 (C-2); 70.3, 70.2 (linker); 68.1 (C-4); 65.4 (C-6); 53.6 (OCH₃); 48.7; 37.6 (linker); 28.2; 27.2, 22.3 (linker). ³¹P NMR (162 MHz, D₂O): δ 14.60, 14.53. HRMS (ESI): Calcd. For C₂₀H₃₀N₂O₁₀P [M+H]⁺: 489.1638, found: 489.1624.

5-Amino-N-phthalimido-pentany1

6-O-methyl-phosphoramidyl-α-D-galactopyranoside 66

\[\text{C}_{20}\text{H}_{29}\text{N}_2\text{O}_{10}\text{P}: 488.16 \text{ g/mol}\]
To a solution of compound 64 (140 mg, 0.23 mmol) dissolved in MeOH (5 mL), PdCl₂ (30 mg) was added and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then filtered through Celite® and concentrated under reduced pressure. Purification with column chromatography (9:1 EtOAc-MeOH) gave product 66 (53 mg, 48%). 1H NMR (400 MHz, D₂O): δ 7.78 (m, 4H, phthalimido protons); 4.86 (d, 1H, J = 3.1 Hz, H-1); 4.10-3.89 (m, 3H, H-4, H-6a, H-6b.); 3.81 (dd, 1H, J₁ = 2.4 Hz, J₂ = 9.2 Hz, H-3); 3.73-3.59 (m, 8H, OCH₃, linker-CH₂ linker-CHH, H-5, H-4, H-2); 3.48 (m, 1H, linker-CHH); 1.61 (m, 4H, linker-CH₂); 1.34 (m, 2H, linker-CH₂). 13C NMR (100 MHz, D₂O): 170.8, 134.6, 134.5, 131.2, 131,2 123.2 (Ar); 98.2 (C-1); 70.5 (C-5); 69.4 (C-3); 69.2 (C-4); 68.8 (C-2); 68.0 (linker); 65.9 (C-6); 53.6, 53.5 (OCH₃) 46.5, 37.7 (linker); 28.0, 27.2, 23.0, 22.9 (linker). 31P NMR (162 MHz, D₂O): δ 14.27, 14.22. HRMS (ESI): Calcd. For C₂₀H₃₀N₂O₁₀P [M+H]⁺: 489.1638, found: 489.1600.

5-Amino-pentanyl 6-O-methyl-phosphoramidyl-β-D-galactopyranoside 67

To a solution of compound 65 (23 mg, 0.047 μmol) dissolved in 95% EtOH (1 mL), hydrazine monohydrate (16 μL, 0.33 μmol) was added and the reaction mixture was
stirred at room temperature overnight. The reaction mixture was then concentrated under reduced pressure. Purification with column chromatography (3:1 EtOAc-MeOH) gave product 67 (14 mg, 83%). $^1$H NMR (400 MHz, D$_2$O): $\delta$ 4.27 (d, 1H, $J = 7.1$ Hz, H-1); 4.03 (m, 2H, linker-CH$_2$); 3.81-3.75 (m, 3H, H-4, H-5, H-6a); 3.61-3.48 (m, 5H, OCH$_3$, H-3, H-6b); 3.36 (dd, 1H, $J_1 = 7.9$, $J_2 = 9.9$ Hz, H-2); 2.82 (t, 2H, $J = 7.5$ Hz, linker-H); 1.52 (m, 4H, linker-H); 1.30 (m, 2H, linker-H). $^{13}$C NMR (100 MHz, D$_2$O): $\delta$ 102.6 (C-1); 73.2 (C-5); 72.5 (C-3); 70.5 (C-2); 70.1 (C-6); 68.1 (C-4); 60.0 ( linker); 48.7 (OCH$_3$); 39.2, 28.0, 26.3, 22.0, 21.9 (linker). $^{31}$P NMR (162 MHz, D$_2$O): $\delta$ 14.79, 14.75. HRMS (ESI): Calcd. For C$_{12}$H$_{28}$N$_2$O$_8$P [M+H]$^+$: 359.1584, found: 359.1587.

5-Amino-pentanyl 6-O-methyl-phosphoramidyl-α-D-galactopyranoside 68

To a solution of compound 66 (25 mg, 51 μmol) dissolved in 95% EtOH (1 mL), hydrazine monohydrate (17 μL, 360 μmol) was added and the reaction mixture was stirred at room temperature overnight. The reaction mixture was then concentrated under reduced pressure. Purification with column chromatography (3:1 EtOAc-MeOH) gave product 68 (10 mg, 55%). $^1$H NMR (400 MHz, D$_2$O): $\delta$ 4.87 (s, 1H, H-1); 4.12-4.01 (m,
3H, H-4, H-6a, H-6b); 3.91 (s, 1H, H-3); 3.78 (m, 2H, H-2, linker-H); 3.73-3.62 (m, 4H, OCH₃, linker-H); 3.46 (m, 1H, H-5); 2.85 (t, 2H, linker-CH₂); 1.59 (m, 4H, linker-CH₂); 1.39 (m, 2H, linker-CH₂). ¹³C NMR (100 MHz, D₂O): δ 98.8 (C-1); 69.8 (C-5); 69.2(C-3); 68.4 (C-2); 66.3 (C-6); 68.1 (C-4); 59.2 (linker); 53.9 (OCH₃); 39.5, 28.1, 26.7, 22.5 (linker). ³¹P NMR (162 MHz, D₂O): δ 14.77, 14.72. HRMS (ESI): Calcd. For C₁₂H₂₈N₂O₈P [M+H]⁺: 359.1584, found: 359.1556.

**Conjugation of MeOPN→6-β-D-Galp-(1→O(CH₂)₅NH₂ to CRM₁₉₇ protein**

The linker-equipped galactoside 67 (4.5 mg) and an excess of adipic acid N-hydroxysuccinimido diester (10 equiv.) was dissolved in DMSO (1 ml). Et₃N (60 μl), was added drop-wise and the reaction mixture was stirred at room temperature for 4 h. After concentration under reduced pressure, the residue was washed with H₂O and concentrated. Purification with column chromatography (3:1 EtOAc-Hexanes) gave the activated monosaccharide 69. This resulting half ester was then condensed onto the amino groups of the protein CRM₁₉₇ at a molar ratio of 100:1 (moles of active ester per moles of protein) in 70 mM phosphate buffer, pH 7.0. After stirring 3 days at room temperature, the reaction mixture was dialyzed against running water to yield conjugate 70.
**MeOPN→6-α-D-Gal-CRM\textsubscript{197} conjugate via oxidized-starch conjugation strategy**

Lintner starch (100 mg) was activated with 0.04 M NaIO\textsubscript{4} in 0.1 M NaOAc buffer (100 ml) pH 4, at 4 °C for 3 days. After 2 days, reaction mixture was dialyzed against running water (1 kDa molecular cutoff). The retentate was centrifuged and the supernatant was separated, lyophilized and purified on a Bio-Gel P-2 column.

Conjugation was carried out with the activated starch (8 mg) with MeOPN→6-α-D-Galp-(1→O(CH\textsubscript{2})\textsubscript{5}NH\textsubscript{2} \textbf{68} (4 mg) in 0.1 M borate buffer (5 ml), pH 9. Sodium cyanoborohydride (40 mg) was added and the reaction mixture was stirred for 1 day at room temperature and another 2 days at 37 °C. The conjugate was then dialyzed against running water (2000 Da). After 2 days, the retentate was lyophilized yielding starch-sugar conjugate (4 mg).

Conjugation with CRM\textsubscript{197} was carried out with the starch-sugar conjugate (4 mg) with CRM\textsubscript{197} (4 mg) in 0.1 M borate buffer (5 ml), pH 9. Sodium cyanoborohydride (40 mg) was added and the reaction mixture was stirred for 1 day at room temperature and another 2 days at 37 °C. The reaction mixture was then dialyzed against running water (2000 Da). After 2 days, the retentate was lyophilized yielding oxidized-starch-sugar-CRM\textsubscript{197} conjugate (4 mg).
7.2.7 Experimental procedures for the preparation of MeOPN→6-Gal and MeOPN→3-Fruc using alternative methods

5-Amino-\(N\)-phthalimido-pentanyl \(\rightarrow\) 2,3,4-tri-\(O\)-allyl-6-\(O\)-(methyl \(N\)-benzylphosphoramidyl-\(\beta\)-D-galactopyranoside \(\rightarrow\) 71

\[
\begin{align*}
\text{C}_{36}\text{H}_{47}\text{O}_{10}\text{N}_{2}\text{P}: & \quad 698.30 \text{ g/mol} \\
\end{align*}
\]

Wang’s method\textsuperscript{172}: To a solution of compound 61 (50 mg, 0.097 mmol) and methyl benzylphosphoramidochloridate\textsuperscript{171} (85 mg, 0.39 mmol) dissolved in anhydrous CH\(_2\)Cl\(_2\) (1.5 mL) was added NMI (0.062 mL, 0.78 mmol). The reaction mixture was stirred at room temperature for 4 hours before the reaction was concentrated under reduced pressure. Purification by flash chromatography eluting with 1:1.5 EtOAc-hexanes gave product 71 (20 mg, 30%).

Lowary’s method\textsuperscript{170}: To tetra-\(N\)-butylammonium methyl-\(H\)-phosphonate (100 mg, 0.30 mmol) in an anhydrous CH\(_2\)Cl\(_2\)-pyridine solution (2.5 mL), PivCl (0.040 mL, 0.36 mmol) was added. After stirring for 30 min, compound 61 (100 mg, 0.19 mmol) was added and the stirring continued for 1 hour. The reaction mixture was then poured into a saturated aqueous NaHCO\(_3\) solution (10 mL) and extracted with CH\(_2\)Cl\(_2\) (10 mL x 3).
The organic layers were combined and washed with brine (20 mL), dried over Na$_2$SO$_4$ and concentrated. The dried mixture was dissolved in anhydrous CH$_2$Cl$_2$ (3 mL). To this solution, CBrCl$_3$ (0.19 mL, 1.9 mmol), Et$_3$N (0.080 mL, 0.57 mmol), and benzylamine (0.032 mL, 0.28 mmol) were added and the reaction mixture was stirred for 2 hours. The reaction mixture was then poured into a saturated aqueous NaHCO$_3$ solution (5 mL) and extracted with CH$_2$Cl$_2$ (5 mL x 3). The organic layers were combined and washed with brine (410 mL), dried over Na$_2$SO$_4$ and concentrated. Purification by flash chromatography eluting with 1:1 EtOAc-hexanes gave product 71 (27 mg, 20%).

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.79-7.66 (m, 4H, phthalimido protons); 7.30 (m, 4H, Ph); 5.88 (m, 3H, CH$_2$–CH=CH$_2$); 5.28-5.03 (m, 6H, CH$_2$–CH=CH$_2$); 4.33-4.00 (m, 11H, CH$_2$–CH=CH$_2$, H-1, PhCH$_2$, linker-CH$_2$); 3.76 (m, 1H, H-6a); 3.70-3.58 (m, 6H, POCH$_3$, H-4, linker-CH$_2$); 3.49-3.31 (m, 3H, H-5, H-6b); 3.22 (dd, 1H, $J_1 = 3.0$ Hz, $J_2 = 9.7$ Hz, H-3); 2.93 (m, 1H, NH); 1.60 (m, 4H, linker-CH$_2$); 1.34 (m, 2H, linker-CH$_2$). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 168.4 (Ar); 135.4, 135.3, 135.0 (CH$_2$–CH=CH$_2$); 133.9, 132.1, 128.7, 127.6, 127.5, 127.3, 123.2, 117.3, 117.1 (Ar); 116.7, 116.6, 116.5 (CH$_2$–CH=CH$_2$); 103.8, (C-1); 81.4 (C-3); 79.0 (C-2); 73.9, 73.8, 73.1, 73.0, 72.9, 72.1 (CH$_2$–CH=CH$_2$, C-5, C-4); 69.6 (C-6); 64.9 (PhCH$_2$); 53.3, 53.2 (POCH$_3$); 45.4, 37.9, 29.2, 28.4, 23.4 (linker). $^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 10.21, 10.05. HRMS (ESI): Calcd. For C$_{36}$H$_{48}$N$_2$O$_{10}$P: [M+H]$^+$: 699.3047, found: 699.3004.
5-Amino-N-phthalimido-pentanyl  2,3,4-tri-O-allyl-6-O-(methyl N-p-methoxybenzylphosphoramidyl)-β-D-galactopyranoside 73

Lowary's method: To a solution of tetra-N-butylammonium methyl-H-phosphonate (100 mg, 0.30 mmol) dissolved in anhydrous 9:1 CH₂Cl₂-pyridine (2.5 mL), PivCl (0.040 mL, 0.36 mmol) was added. After stirring for 30 min, compound 61 (100 mg, 0.19 mmol) was added and the stirring continued for 1 hour. The reaction mixture was then poured into a saturated aqueous NaHCO₃ solution (10 mL) and extracted with CH₂Cl₂ (10 mL x 3). The organic layers were combined and washed with brine (20 mL), dried over Na₂SO₄ and concentrated. The dried mixture was dissolved in anhydrous CH₂Cl₂ (3 mL). To this solution, CBrCl₃ (0.19 mL, 1.9 mmol), Et₃N (0.080 mL, 0.57 mmol), and p-methoxybenzylamine (0.038 mL, 0.28 mmol) were added and the reaction mixture was stirred for 2 hours. The reaction mixture was then poured into a saturated aqueous NaHCO₃ solution (5 mL) and extracted with CH₂Cl₂ (5 mL x 3). The organic layers were combined and washed with brine (410 mL), dried over Na₂SO₄ and concentrated. Purification by flash chromatography eluting with 1:1 EtOAc-hexanes gave product 73 (21 mg, 15%). ¹H NMR (400 MHz, CDCl₃): δ 7.79-7.66 (m, 4H, phthalimido...
protons); 7.24-6.78 (m, 4H, MeOC₆H₄); 5.88 (m, 3H, CH₂–CH=CH₂); 5.29-5.03 (m, 6H, CH₂–CH=CH₂); 4.35-4.00 (m, 11H, CH₂–CH=CH₂, H-1, PhCH₂, linker-CH₂); 3.85-3.75 (m, 4H, H-6a, PhOCH₃); 3.71-3.60 (m, 6H, POCH₃, H-4, linker-CH₂); 3.54-3.45 (m, 3H, H-2, H-5, H-6b); 3.28 (dd, 1H, J₁ = 3.0 Hz, J₂ = 9.8 Hz, H-3); 2.91 (m, 1H, NH); 1.66 (m, 4H, linker-CH₂); 1.38 (m, 2H, linker-CH₂). ¹³C NMR (100 MHz, CDCl₃): δ 168.4, 159.0 (Ar); 135.4, 135.3, 135.0 (CH₂–CH=CH₂); 133.9, 132.1, 131.5, 131.4, 128.8, 128.6, 123.2, 117.3, 117.1 (Ar); 116.7, 116.6, 116.5 (CH₂–CH=CH₂); 114.0 (Ar); 103.8 (C-1); 81.4 (C-3); 79.0 (C-2); 73.9, 73.8, 73.1, 73.0, 72.9, 72.8, 72.1 (CH₂–CH=CH₂, C-5, C-4); 69.6 (C-6); 64.9, 64.6, 64.5 (PhCH₂); 55.3, 53.2 (POCH₃); 44.8, 44.2, 37.9, 29.2, 28.4, 23.4 (linker). ³¹P NMR (162 MHz, CDCl₃): δ 10.19, 10.06. HRMS (ESI): Calcd. For C₃₇H₄₈N₂O₁₁P: [M-H]⁻: 727.2996, found: 727.2957.

5-Amino-N-phthalimido-pentanyl

2,3,4-tri-O-allyl-6-O-methylphosphoramidyl-α-D-galactopyranoside 63

\[
\begin{align*}
\text{C}_{29}\text{H}_{41}\text{N}_{2}\text{O}_{10}\text{P} & : 608.24 \text{ g/mol} \\
\end{align*}
\]

* p-Methoxybenzyl deprotection: To a solution of compound 73 (19 mg, 0.026 mmol) dissolved in a 9:1 MeCN:H₂O solution (2 mL), CAN (43 mg, 0.078 mmol) was added.
The reaction mixture was stirred at room temperature for 1 hour before the mixture was poured into H$_2$O (5 mL) and extracted with EtOAc (5 mL x 3). The organic layer was concentrated and purification by flash chromatography eluting with 1:1 EtOAc-hexanes gave MeOPN product 63 (15 mg, 93%). Spectroscopy data is in agreement as previously reported.

5-Amino-N-phthalimido-pentanyl 1,4,6-tri-O-benzoyl-3-O-(methyl $N$-$p$-methoxylbenzylphosphoramidyl)-$\alpha$-D-fructofuranoside 76

![Chemical structure]

C$_{48}$H$_{47}$N$_2$O$_{14}$P: 906.28 g/mol

Lowary’s method$^{170}$: To a solution of tetra-$N$-butylammonium methyl-$H$-phosphonate (750 mg, 2.32 mmol) dissolved in anhydrous 9:1 CH$_2$Cl$_2$-pyridine (10 mL), PivCl (0.342 mL, 2.78 mmol) was added. After stirring for 30 min, compound 34 (327 mg, 0.463 mmol) was added and the stirring continued for 1 hour. The reaction mixture was then poured into a saturated aqueous NaHCO$_3$ solution (10 mL) and extracted with CH$_2$Cl$_2$ (10 mL x 3). The organic layers were combined and washed with brine (20 mL), dried over Na$_2$SO$_4$ and concentrated. The dried mixture was dissolved in anhydrous
CH₂Cl₂ (10 mL). To this solution, CBrCl₃ (0.450 mL, 4.63 mmol), Et₃N (0.195 mL, 1.40 mmol), and p-methoxybenzylamine (0.090 mL, 0.7 mmol) were added and the reaction mixture was stirred for 2 hours. The reaction mixture was then poured into a saturated aqueous NaHCO₃ solution (10 mL) and extracted with CH₂Cl₂ (10 mL x 3). The organic layers were combined and washed with brine (20 mL), dried over Na₂SO₄ and concentrated. Purification by flash chromatography eluting with 1:1 EtOAc-hexanes gave product 76 (44 mg, 10%). ¹H NMR (400 MHz, CDCl₃): δ 8.13-7.99 (m, 12H, Ar); 7.78-7.63 (m, 8H, phthalimido protons); 7.57-7.34 (m, 18H, Ar); 7.05-6.69 (m, 8H, Ar); 5.54 (d, 1H, J = 4.1 Hz, H-4); 5.50 (d, 1H, J = 4.0 Hz, H-4*); 5.09 (d, 1H, J = 1.2 Hz, H-3); 5.06 d, 1H, J = 1.3 Hz, H-3*); 4.82 (m, 2H, H-1); 4.72 (m, 2H, H-6a, H-6a*); 4.65 (m, 2H, H-6b, H-6b*); 4.50 (m, 2H, H-5, H-5*); 4.38 (m, 2H, H-1*); 3.90 (m, 4H, linker-H); 3.77 (s, 6H, OCH₃); 3.72 (s, 4H, CH₂N); 3.60 (m, 3H, POCH₃); 3.48 (m, 7H, linker-H, POCH₃); 1.66-1.32 (m, 12H, linker-H). ¹³C NMR (100 MHz, CDCl₃): δ 168.3, 166.0, 158.9, 133.8, 133.6, 133.2, 133.1, 132.1, 131.0, 130.0, 129.1, 128.8, 128.5, 128.3, 123.2, 114.0, 113.9 (Ar); 107.4 (C-2); 82.6 (C-3); 82.1 (C-5); 79.0 (C-4); 63.9 (C-6); 61.0 (linker); 59.2 (C-1); 55.3 (OCH₃); 53.0 (POCH₃); 44.7, 44.4, 44.2, 41.2, 37.7, 29.3, 28.4, 23.7 (linker). ³¹P NMR (162 MHz, CDCl₃): δ 9.65, 9.42. HRMS (ESI): Calcd. For C₄₈H₄₈N₂O₁₄P: [M+H]⁺: 907.2843, found: 907.2812.
5-Amino-N-phthalimido-pentanyl

1,4,6-tri-O-benzoyl-3-O-methylphosphoramidyl-α-D-fructofuranoside 35

\[ \text{C}_41\text{H}_{41}\text{N}_2\text{O}_{13}\text{P}: 800.23 \text{ g/mol} \]

\(p\)-Methoxybenzyl deprotection: To a solution of compound 76 (43 mg, 0.047 mmol) dissolved in a 9:1 MeCN:H₂O solution (4 mL), CAN (100 mg, 0.19 mmol) was added. The reaction mixture was stirred at room temperature for 4 hours before the mixture was poured into H₂O (5 mL) and extracted with EtOAc (10 mL x 3). The organic layer was concentrated and purification by flash chromatography eluting with 2:1 EtOAc-hexanes gave product 35 (29 mg, 78%). Spectroscopy data is in agreement as previously reported.

5-Amino-N-phthalimido-pentanyl

1-O-methylphosphoramidyl-α-D-fructofuranoside 77

\[ \text{C}_{20}\text{H}_{29}\text{N}_2\text{O}_{10}\text{P}: 488.16 \text{ g/mol} \]
Compound 35 (5.0 mg, 6.2 μmol) was dissolved in 0.0033 M methanolic MeONa solution (0.75 mL) and the mixture was stirred for 10 h at room temperature before it was neutralized with acetic acid and concentrated under reduced pressure. Purification by flash chromatography eluting with 9:1 EtOAc-MeOH gave product 77 as a mixture of two diastereomers (2.0 mg, 53%). $^1$H NMR (400 MHz, D$_2$O): δ 7.72 (m, 4H, phthalimido-H); 4.05-3.91 (m, 3H, H-1a, H-1b, H-3); 3.82 (m, 2H, H-4, H-5); 3.70-3.47 (m, 9H, H-6a, H-6b, POCH$_3$, linker-CH$_2$); 1.51 (m, 4H, linker-CH$_2$); 1.30 (m, 2H, linker-CH$_2$). $^{13}$C NMR (100 MHz, D$_2$O): δ 170.9, 134.5, 131.3, 129.4, 123.2 (Ar); 106.7, 106.6, 106.5 (C-2); 83.0, 82.9 (C-5); 80.5, 80.4 (C-3); 76.8, 76.7 (C-4); 62.5, 62.2 (C-1); 61.4, 61.3 (C-6); 61.0, 60.7 (linker); 53.8, 53.7, 53.6, 53.5 (POCH$_3$) 37.7, 28.4, 27.2, 22.7 (linker). $^{31}$P NMR (162 MHz, D$_2$O): δ 14.50, 14.44. HRMS (ESI): Calcd. For C$_{20}$H$_{30}$N$_2$O$_{10}$P: [M+H]$^+$: 489.1638, found: 489.1599.

7.2.8 Experimental procedures for the preparation of MeOPN→GlcNAc-Gal disaccharide

4-Methoxyphenyl 2,6-di-O-allyl-3,4-O-isopropylidene-α-D-galactopyranoside 82

\[
\text{Me} \quad \text{O} \quad \text{OAll} \quad \text{Me} \quad \text{O} \quad \text{AllO} \quad \text{OMP}
\]

C$_{22}$H$_{30}$O$_7$: 406.20 g/mol
A solution of 81 (7.3 g, 22 mmol) dissolved in DMF (80 mL) with allyl bromide (6.8 mL, 79 mmol) was cooled to 0 °C. Sodium hydride, 60% dispersion in mineral oil (2.0 g, 48 mmol) was added and the reaction mixture was stirred for 30 min at 0 °C before warming up to room temperature over 2 hours. The reaction was then quenched with MeOH (2 mL), poured into ice-cold water (200 mL) and extracted with EtOAc (3 × 200 mL). The organic layer was dried over Na₂SO₄ and concentrated. Purification by flash chromatography eluting with 1:6 EtOAc-hexanes gave product 82 (7.2 g, 79%). [α]D²⁵ = +54.8 (c = 0.6, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 7.05-6.72 (m, 4H, MeOC₆H₄); 5.85 (m, 2H, CH₂–CH=CH₂); 5.37 (d, 1H, J = 3.5 Hz, H-1); 5.24-5.06 (m, 2H, CH₂–CH=CH₂); 4.40 (dd, 1H, J₁ = 5.5 Hz, J₂ = 7.7 Hz, H-3); 4.31-3.90 (m, 6H, CH₂–CH=CH₂, H-4, H-5); 3.71 (s, 3H, OCH₃); 3.70-3.56 (m, 3H, H-2, H-6a, H-6b); 1.50, (s, 3H, CH₃); 1.31 (s, 3H, CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 155.1, 151.0 (Ar); 134.8, 134.6 (CH₂–CH=CH₂); 118.3, 117.8, 117.7, 117.6, 116.7, 114.5, 109.3, 105.6 (CH₂–CH=CH₂, Ar); 96.7 (C-1); 76.5 (C-2); 75.8 (C-3); 75.6 (C-4); 73.7, 73.6 (CH₂–CH=CH₂); 69.2 (C-6); 67.1 (C-5); 55.6 (OCH₃); 28.3, 26.4 (CH₃). HRMS (ESI): Calcd. For C₂₂H₃₁O₇: [M+H]⁺: 407.2070, found: 407.2042.
4-Methoxyphenyl 2,6-di-O-allyl-\(\alpha\)-D-galactopyranoside 83

A solution of 82 (7.2 g, 18 mmol) dissolved in 80% aqueous AcOH (100 mL) was stirred at 80 °C for 1 h. The reaction mixture was then concentrated under reduced pressure. Purification by flash chromatography (1:1 EtOAc-hexanes) gave product 83 (6.5 g, 100%). \([\alpha]_D^{25} = +66.1\) (c = 0.1, CHCl3); \(^1^H\) NMR (400 MHz, CDCl3): \(\delta\) 7.04-6.74 (m, 4H, MeOC\(\text{C}_6\text{H}_4\)); 5.85 (m, 2H, CH\(\text{C}_2\)CH=CH\(\text{C}_2\)); 5.50 (d, 1H, \(J = 3.5\) Hz, H-1); 5.27-5.10 (m, 2H, CH\(\text{C}_2\)CH=CH\(\text{C}_2\)); 4.12-4.05 (m, 5H, CH\(\text{C}_2\)CH=CH\(\text{C}_2\), H-3); 3.97 (m, 2H, H-4, H-5); 3.81 (dd, 1H, \(J_1 = 3.5\) Hz, \(J_2 = 9.5\) Hz, H-2); 3.71 (s, 3H, OCH\(\text{C}_3\)); 3.68 (m, 2H, H-6a, H-6b); 3.05 (s, 1H, 3-OH); 2.62 (s, 1H, 2-OH). \(^{13}\)C NMR (100 MHz, CDCl3): \(\delta\) 155.2, 151.1 (Ar); 134.5, 134.1 (CH\(\text{C}_2\)CH=CH\(\text{C}_2\)); 118.4, 118.2, 117.5, 114.6 (CH\(\text{C}_2\)CH=CH\(\text{C}_2\), Ar); 96.7 (C-1); 76.1 (C-2); 72.5 (C-4); 71.7 (C-3); 70.0 (C-6); 69.9, 69.3 (CH\(\text{C}_2\)CH=CH\(\text{C}_2\)); 68.9 (C-5); 55.7 (OCH\(\text{C}_3\)). HRMS (ESI): Calcd. For C\(\text{C}_{19}\)H\(\text{C}_{27}\)O\(\text{C}_7\): [M+H]^+: 367.1757, found: 367.1738.
4-Methoxyphenyl 4-O-acetyl-2,6-di-O-allyl-α-D-galactopyranoside 80

To a solution of compound 83 (6.2 g, 17 mmol) dissolved in MeCN (60 mL) was added trimethyl orthoacetate (3.3 mL, 25 mmol) and CSA (150 mg). The reaction mixture was stirred for 30 min at room temperature, before the reaction was quenched with Et₃N and concentrated under reduced pressure while maintaining the temperature below 20 °C. The resulting residue was dissolved in 80% aqueous AcOH (60 mL) and stirred for 10 min. The reaction was concentrated under reduced pressure. Purification by flash chromatography eluting with 1:6 EtOAc-hexanes gave product 80 (6.1 g, 88%).

[α]D²⁵ = +51.2 (c = 0.2, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 7.04 -6.74 (m, 4H, MeOC₆H₄); 5.91-5.71 (m, 2H, CH₂–CH=CH₂); 5.49 (d, 1H, J = 3.5 Hz, H-1); 5.45 (t, 1H, J = 2.4 Hz); 5.29-5.09 (m, 2H, CH₂–CH=CH₂); 4.30 (dd, 1H, J₁ = 3.5 Hz, J₂ = 10.0 Hz, H-3); 4.23 (m, 1H, H-5); 4.12-3.82 (m, 4H, CH₂–CH=CH₂); 3.75 (m, 4H, OCH₃, H-2); 3.43 (d, 2H, J = 6.0 Hz, H-6); 2.11 (s, 3H, CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 170.9 (C=O); 155.3, 151.0 (Ar); 134.4, 134.3 (CH₂–CH=CH₂); 118.6, 118.1, 117.3, 114.6 (CH₂–CH=CH₂, Ar); 96.8 (C-1); 76.3 (C-2); 72.3, 71.7 (CH₂–CH=CH₂); 70.5 (C-4); 68.5 (C-6); 68.4 (C-5); 67.9 (C-3); 55.6 (OCH₃); 20.9 (CH₃). HRMS (ESI): Calcd. For C₂₁H₂⁹O₈: [M+H]+: 409.1862, found: 409.1826.
Ethyl-3,4,6-tri-\textit{O}-acetyl-2-deoxy-2-acetamido-1-thio-D-glucopyranoside 85

![Chemical structure of 85](image)

To a mixture of compound 84 (13 g, 33 mmol) and ethanethiol (3.6 mL, 50 mmol) dissolved in CH$_2$Cl$_2$ (260 mL), tin (IV) chloride (4.3 mL, 36 mmol) was introduced at room temperature. Reaction mixture was refluxed for 24 hours at 40 $^\circ$C before quenching with NaHCO$_3$ (300 mL). The reaction mixture was then extracted with CH$_2$Cl$_2$ (200 mL x 3) and the organic layer was washed with brine (300 mL). The organic layer was then concentrated under reduced pressure. Purification by flash chromatography eluting with 1:2.5 EtOAc-hexanes gave product 85 as a $\alpha/\beta$ mixture (7.5 g, 58%).

85A: [$\alpha$]$_{D}^{25}$ = -6.2 (c = 0.2, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 5.74 (d, 1H, $J = 8.8$ Hz, NH); 5.38 (d, 1H, $J = 5.4$ Hz, H-1); 5.05 (m, 2H, H-3, H-4); 4.47 (m, 1H, H-2); 4.33 (m, 1H, H-5); 4.23 (m, 1H, H-6a); 4.04 (m, 1H, H-6b); 2.66-2.51 (m, 2H, -SCH$_2$CH$_3$); 2.07-1.92 (4s, 12H, Ac), 1.24 (t, 3H, -SCH$_2$CH$_3$). HRMS (ESI): Calcd. For C$_{16}$H$_{26}$NO$_8$S: [M+H]$^+$: 392.1379, found: 392.1351.

85B: [$\alpha$]$_{D}^{25}$ = +17.2 (c = 0.1, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 5.50 (d, 1H, $J = 9.4$ Hz, NH); 5.17-5.05 (m, 2H, H-3, H-4); 4.57 (d, 1H, $J = 10.3$ Hz, H-1); 4.20 (m, 1H, H-6);
4.13-4.02 (m, 2H, H-6b, H-2); 3.66 (m, 1H, H-5); 2.77-2.61 (m, 2H, -SCH₂CH₃); 
2.10-1.92 (4s, 12H, Ac), 1.26-1.20 (t, 3H, -SCH₂CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 
171.2, 170.8, 170.1 169.3 (C=O); 84.4 (C-1); 75.9 (C-5); 73.9 (C-3); 68.4 (C-4); 62.3 
(C-6); 53.3 (C-2); 24.2 (SCH₂CH₃); 20.9, 20.8, 20.7, 20.6 (OAc); 14.8 (SCH₂CH₃). 

**Ethyl-2-deoxy-2-acetamido-1-thio-D-glucopyranoside 86**

![Chemical structure of ethyl-2-deoxy-2-acetamido-1-thio-D-glucopyranoside 86]

C₁₀H₁₉NO₅S: 265.10 g/mol

Compound 85 (5.0 g, 13 mmol) was dissolved in 0.05 M methanolic MeONa solution (90 mL) and the mixture was stirred for 2 h at room temperature before it was neutralized with acetic acid (0.26 mL) and concentrated under reduced pressure. Purification by flash chromatography eluting with 3:1 EtOAc-hexanes gave product 86 (3.2 g, 95%);

**86A**: [α]D²⁵ = +33.5 (c = 0.1, H₂O); ¹H NMR (400 MHz, D₂O): δ 5.34 (d, 1H, J = 5.3 Hz, H-1). 4.02-3.89 (m, 2H, H-2, H-5); 3.80-3.65 (m, 2H, H-6a, H-6b); 3.55 (m, 1H, H-3); 3.36 (m, 1H, H-4); 2.60-2.45 (m, 2H, -SCH₂CH₃); 1.93 (s, 3H, Ac); 1.17-1.08 (t, 3H, -SCH₂CH₃). ¹³C NMR (100 MHz, D₂O): δ 174.3 (C=O); 83.0 (C-1); 72.4 (C-5); 70.9
(C-3); 70.2 (C-4); 60.4 (C-6); 53.8 (C-2); 24.6 (SCH₂CH₃); 21.7 (OAc); 14.1 (SCH₂CH₃).

HRMS (ESI): Calcd. For C₁₀H₂₀NO₅S: [M+H]⁺: 266.1062, found: 266.1043.

86B: [α]D²⁵ = +19.1 (c = 0.1, H₂O); ¹H NMR (400 MHz, D₂O): δ 4.52 (d, 1H, J = 10.5 Hz, H-1). 3.80 (m, 1H, H-6a); 3.68-3.51 (m, 2H, H-2, H-6b); 3.44-3.33 (m, 3H, H-3, H-4, H-5); 2.61 (m, 2H, -SCH₂CH₃); 1.93 (s, 3H, Ac); 1.13 (t, 3H, -SCH₂CH₃). ¹³C NMR (100 MHz, D₂O): δ 174.4 (C=O); 83.9 (C-1); 79.8 (C-5); 75.1 (C-3); 69.7 (C-4); 61.0 (C-6); 54.7 (C-2); 24.4 (SCH₂CH₃); 22.1 (OAc); 14.2 (SCH₂CH₃). HRMS (ESI): Calcd. For C₁₀H₂₀NO₅S: [M+H]⁺: 266.1062, found: 266.1043.

Ethyl-3,4,6-tri-O-allyl-2-deoxy-2-acetamido-1-thio-D-glucopyranoside 79

A solution of compound 86 (2.89 g, 10.9 mmol) dissolved in DMF (60 mL) with allyl bromide (4.74 mL, 54.8 mmol) was cooled to 0 °C. Sodium hydride, 60% dispersion in mineral oil (1.75 g, 43.8 mmol) was added and the reaction mixture was slowly warmed up to room temperature over 2.5 hours. The reaction was then quenched with MeOH (2 mL), poured into H₂O (90 mL) and extracted with EtOAc (3 × 100 mL). The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure.
Purification by flash chromatography eluting with 1:1 EtOAc-hexanes gave compound 79 as a $\alpha/\beta$ mixture (2.8 g, 68%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 5.95-5.75 (m, 3H, CH$_2$–CH=CH$_2$); 5.48 (d, 1H, NH); 5.32 (d, 0.3H, $J = 5.1$ Hz, H-1$\alpha$); 5.28-5.08 (m, 6H, CH$_2$–CH=CH$_2$); 4.74 (d, 0.7H, $J = 10.2$ Hz, H-1$\beta$); 4.39-392 (m, 7 H, CH$_2$–CH=CH$_2$, H-2); 3.80-3.65 (m, 1H); 3.65-3.56 (m, 1H), 3.55-3.46 (m, 1H, H-3), 3.44-3.32 (m, 1H); 2.71-2.50 (m, 2H, -SCH$_2$CH$_3$); 1.94 (s, 3H, OAc); 1.30-1.18 (m, 3H, -SCH$_2$CH$_3$). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 170.2 (C=O, $\alpha$); 169.7 (C=O, $\beta$); 135.0-134.5 (CH$_2$–CH=CH$_2$); 117.4-116.9 (CH$_2$–CH=CH$_2$); (CH$_2$–CH=CH$_2$); 25.6, SCH$_2$CH$_3$, $\beta$); 24.3 SCH$_2$CH$_3$, $\alpha$); 23.7(OAc, $\alpha$); 23.5 (OAc, $\beta$); 15.2 (SCH$_2$CH$_3$, $\beta$); 14.9 (SCH$_2$CH$_3$, $\alpha$). HRMS (ESI): Calcd. For C$_{19}$H$_{32}$NO$_5$S: [M+H]$^+$: 386.2001, found: 386.1988.

4-Methoxyphenyl 3,4,6-tri-O-allyl-2-deoxy-2-acetamido-1-thio-β-D-glucopyranosyl-(1→3)-4-O-acetyl-2,6-di-O-allyl-α-D-galactopyranoside 87

Acceptor 80 (54 mg, 0.133 mmol) and donor 79 (103 mg, 0.268 mmol) were dissolved in anhydrous CH$_2$Cl$_2$ (2 mL) with molecular sieves (4 Å). NIS (66 mg, 0.293 mmol) was introduced and the mixture was cooled to 0 °C. TfOH (11 μL, 0.120 mmol)
was added drop-wise and the reaction mixture was stirred for 48 hours at room temperature. The reaction was then neutralized with Et₃N (16 μL) and concentrated. Purification with flash chromatography (1:1 EtOAc-hexanes) gave disaccharide 87 (53 mg, 54%). \([\alpha]_D^{25} = +39.2\) (c = 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃): \(\delta\) 7.04 - 6.74 (m, 4H, MeOC₆H₄); 5.92 - 5.78 (m, 5H, CH₂–CH=CH₂); 5.51 (m, 3H, NH, H-1Gal, H-4Gal); 5.38 - 5.10 (m, 10H, CH₂–CH=CH₂, H-4GlcNAc); 3.89 (m, 3H, H-3Gal, H-3GlcNAc, H-5GlcNAc); 3.80 (s, 3H, OCH₃); 3.71 - 3.42 (m, 7H, H-2Gal, H-2GlcNAc, H-5Gal, H-6aGal, H-6bGal, H-6aGlcNAc, H-6bGlcNAc); 2.14 (s, 3H, OAc); 1.99 (s, 3H, NHAc). ¹³C NMR (100 MHz, CDCl₃): \(\delta\) 170.2, 169.9 (C=O); 155.3, 151.1 (Ar); 135.1, 135.0, 134.9, 134.7, 134.4, 134.3 (CH₂–CH=CH₂); 119.0, 118.9, 117.6, 117.3, 117.1, 117.0, 116.9, 116.6, 114.5 (Ar, CH₂–CH=CH₂); 101.4 (C-1GlcNAc); 97.4 (C-1Gal); 81.3 (C-2GlcNAc); 77.6 (C-3GlcNAc); 75.5, 75.4, 75.0, 73.4, 73.1, 72.4, 72.3, 71.8, 71.0, 69.2 (C-2Gal, C-3 Gal, C-4Gal, C-5Gal, C-4GlcNAc, C-5GlcNAc, CH₂–CH=CH₂); 69.0 (C-6GlcNAc); 68.9 (C-6Gal); 55.6 (OCH₃); 23.7, 21.0 (OAc). HRMS (ESI): Calcd. For C₃₈H₆₄NO₁₃: [M+H]⁺: 732.3595, found: 732.3553.
4-Methoxyphenyl
3,4,6-tri-O-allyl-2-deoxy-2-acetamido-1-thio-β-D-glucopyranosyl-(1→3)-2,6-di-O-allyl-α-D-galactopyranoside 88

![Chemical structure](attachment:chemical_structure.png)

C_{36}H_{51}NO_{12}: 689.34 g/mol

Compound 87 (325 mg, 0.445 mmol) was dissolved in 0.25 M methanolic MeONa solution (5 mL) and the mixture was stirred for 14 h at room temperature before it was neutralized with acetic acid and concentrated under reduced pressure. Purification by flash chromatography eluting with 2:1 EtOAc-Hexanes gave product 88 (215 mg, 70%).

\[[\alpha]_D^{25}\] = +49.1 (c = 0.05, CHCl₃); ^1H NMR (400 MHz, CDCl₃): δ 7.05-6.75 (m, 4H, MeOC₆H₄); 5.85 (m, 5H, CH₂–CH=CH₂); 5.50 (d, 1H, J = 7.8 Hz, NH); 5.39 (d, 1H, J = 3.7 Hz, H-1Gal); 5.25-5.08 (m, 10H, CH₂–CH=CH₂); 4.85 (d, 1H, J = 7.6 Hz, H-1GlcNAc); 4.29-3.90 (m, 13H, CH₂–CH=CH₂); 3.87 (dd, 1H, J₁ = 3.7 Hz, J₂ = 10.1 Hz, H-2Gal); 3.74 (s, 3H, OCH₃); 3.69-3.58 (m, 6H); 3.44 (m, 2H); 1.96 (s, 3H, NAc). ^13C NMR (100 MHz, CDCl₃): δ 170.3 (C=O); 155.1, 151.0 (Ar); 134.9, 134.5 (CH₂–CH=CH₂); 118.8, 117.3, 116.9, 114.5 (Ar, CH₂–CH=CH₂); 101.4 (C-1GlcNAc); 97.4 (C-1Gal); 81.1 (C-2GlcNAc); 79.2 (C-3GlcNAc); 77.8 (C-5Gal); 74.7 (C-5GlcNAc); 74.6 (C-2Gal); 73.6 (C-4GlcNAc); 73.5, 72.4, 72.2, 72.0 (C-4Gal, C-3Gal, CH₂–CH=CH₂); 69.0 (C-6GlcNAc); 68.8
(C-6Gal); 56.2 (OCH₃); 23.8 (OAc). HRMS (ESI): Calcd. For C₃₆H₅₂NO₁₂: [M+H]⁺: 690.3490, found: 690.3447.
Chapter 8

References


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Epilogue
The scientific breakthroughs described in this document represent the first attempts in the creation of synthetic carbohydrate vaccines against the gastrointestinal pathogens, *C. difficile* and *C. jejuni*. The synthesis of the pentasaccharide repeating block of *C. difficile* PS-I cell-surface polysaccharide was finalized in this work and conjugated to subunit of *C. difficile* exotoxin B (ToxB) to yield a dual antigen *C. difficile* vaccine. The synthetic PS-I pentasaccharide was immunodetected by circulating IgG antibodies present in horse sera, which indicated that the synthetic pentasaccharide possessed structural regions that may be capable of generating antibodies against the *C. difficile* native cell-surface PS-I polysaccharide.

The main body of this thesis described the synthesis of *O*-methyl-phosphoramidate (MeOPN)-glycan epitopes that are present in *C. jejuni* capsule polysaccharides. Here, a two-step-one-pot MeOPN introduction strategy was developed, which offered a relatively inexpensive and straight-forward path in the generation of MeOPN-saccharide antigens. The synthesis of MeOPN→3-Fru was a great challenge due to unexpected side-reactions, migrations and degradations of the protecting groups. This epitope was synthesized using alternative MeOPN introduction methods. The synthesis of MeOPN→3-Fru revealed important insight into the unique reactivity of D-frucofuranoside, protecting group manipulation and mechanism of MeOPN introduction. Immunodetection studies on the MeOPN-glycan epitopes, using *C. jejuni* whole-cell and capsule conjugate antisera, helped to understand the role that MeOPN plays in the immunogenicity of native *C. jejuni* capsule polysaccharide. In particular, a glycoconjugate based on the
MeOPN→6-D-Gal epitope was shown to raise bactericidal antibodies that reacted with the cell-surface of *C. jejuni* cells. These findings represent a notable advancement in the development of well-defined carbohydrate vaccines that in time may replace the presently available conjugate vaccines based on native polysaccharides.
Spectral data associated with PS-I pentasaccharide

7: $^1$H NMR (D$_2$O, 600 MHz)

[Graph of 1H NMR spectrum]

7: $^1$H NMR (D$_2$O, 600 MHz)

[Graph of 1H NMR spectrum]
Spectral data associated with MeOPN→6-Gal

14: $^1$H NMR (CDCl$_3$, 400 MHz)

14: $^{13}$C NMR (CDCl$_3$, 100 MHz)
15: $^1$H NMR (CDCl$_3$, 400 MHz)

15: $^{13}$C NMR (CDCl$_3$, 100 MHz)
16: $^1$H NMR (CDCl$_3$, 400 MHz)

16: $^{13}$C NMR (CDCl$_3$, 100 MHz)
17: $^1$H NMR (CDCl$_3$, 400 MHz)

17: $^{13}$C NMR (CDCl$_3$, 100 MHz)
17: $^{31}$P NMR (CDCl$_3$, 162 MHz)

18: $^1$H NMR (CDCl$_3$, 400 MHz)
$^{13}$C NMR (CDCl$_3$, 100 MHz)

$^{31}$P NMR (CDCl$_3$, 162 MHz)
9: $^1$H NMR (D$_2$O, 400 MHz)

9: $^{13}$C NMR (D$_2$O, 100 MHz)
9. $^{31}$P NMR (D$_2$O, 162 MHz)

9. $^1$H-$^{31}$P NMR (D$_2$O, 400 MHz)
Spectral data associated with MeOPN→2-Gal

**20:** $^1$H NMR (CDCl$_3$, 400 MHz)

![H NMR spectrum](image)

**20: $^{13}$C NMR (CDCl$_3$, 100 MHz)

![C NMR spectrum](image)
21: $^1$H NMR (CDCl$_3$, 400 MHz)

21: $^{13}$C NMR (CDCl$_3$, 100 MHz)
22: $^1$H NMR (CDCl$_3$, 400 MHz)

22: $^{13}$C NMR (CDCl$_3$, 100 MHz)
23: $^1$H NMR (CDCl$_3$, 400 MHz)

23: $^{13}$C NMR (CDCl$_3$, 100 MHz)
24: $^1$H NMR (CDCl$_3$, 400 MHz)

24: $^{13}$C NMR (CDCl$_3$, 100 MHz)
24: $^{31}$P NMR (CDCl$_3$, 162 MHz)

25: $^1$H NMR (CDCl$_3$, 400 MHz)
25: $^{31}$P NMR (CDCl$_3$, 162 MHz)

10: $^1$H NMR (D$_2$O, 400 MHz)
10: $^{13}$C NMR (D$_2$O, 100 MHz)

10: $^{31}$P NMR (D$_2$O, 162 MHz)
$^{10*}$: $^1\text{H NMR (D}_2\text{O, 400 MHz)}$

$^{10*}$: $^{13}\text{C NMR (D}_2\text{O, 100 MHz)}$
10*: $^{31}$P NMR (D$_2$O, 162 MHz)

10*: $^1$H-$^{31}$P HMBC NMR (D$_2$O, 400 MHz)
Spectral data associated with MeOPN→4-Gal

27: $^1$H NMR (CDCl$_3$, 400 MHz)

27: $^{13}$C NMR (CDCl$_3$, 100 MHz)
28: $^1$H NMR (CDCl$_3$, 400 MHz)

28: $^{13}$C NMR (CDCl$_3$, 100 MHz)
28: $^{31}$P NMR (CDCl$_3$, 162 MHz)

29: $^1$H NMR (CDCl$_3$, 400 MHz)
29: $^{31}$P NMR (D$_2$O, 162 MHz)

30: $^{31}$P NMR (D$_2$O, 162 MHz)
11: $^1$H NMR (CDCl$_3$, 400 MHz)

11: $^{31}$P NMR (D$_2$O, 162 MHz)
11: $^{13}$C NMR (CDCl$_3$, 100 MHz)

11: $^1$H-$^{31}$P HMBC NMR (D$_2$O, 400 MHz)
Spectral data associated with MeOPN→3-Fruc

32: $^1$H NMR (CDCl₃, 400 MHz)

32: $^{13}$C NMR (CDCl₃, 100 MHz)
33: $^1$H NMR (CDCl$_3$, 400 MHz)

33: $^{13}$C NMR (CDCl$_3$, 100 MHz)
34: $^1$H NMR (CDCl$_3$, 400 MHz)
35: $^1$H NMR (CDCl$_3$, 400 MHz)

35: $^{13}$C NMR (CDCl$_3$, 100 MHz)
35: $^{31}$P NMR (D$_2$O, 162 MHz)

36: $^1$H NMR (CDCl$_3$, 400 MHz)
36: $^{13}$C NMR (CDCl$_3$, 100 MHz)

![C NMR spectrum]

36: $^{31}$P NMR (D$_2$O, 162 MHz)

![P NMR spectrum]
37: $^1$H NMR (CDCl$_3$, 400 MHz)

37: $^{31}$P NMR (D$_2$O, 162 MHz)
38: $^1$H NMR (CDCl$_3$, 400 MHz)

38: $^{13}$C NMR (CDCl$_3$, 100 MHz)
39: $^1$H NMR (CDCl$_3$, 400 MHz)

39: $^{13}$C NMR (CDCl$_3$, 100 MHz)
43: $^1$H NMR (CDCl$_3$, 400 MHz)

43: $^{13}$C NMR (CDCl$_3$, 100 MHz)
44: $^1$H NMR (CDCl$_3$, 400 MHz)

44: $^{13}$C NMR (CDCl$_3$, 100 MHz)
45: $^1$H NMR (CDCl$_3$, 400 MHz)

45: $^{13}$C NMR (CDCl$_3$, 100 MHz)
49: $^1$H NMR (CDCl$_3$, 400 MHz)

49: $^{13}$C NMR (CDCl$_3$, 100 MHz)
50: $^1$H NMR (CDCl$_3$, 400 MHz)

50: $^{13}$C NMR (CDCl$_3$, 100 MHz)
52: $^1$H NMR (CDCl$_3$, 400 MHz)

52: $^{13}$C NMR (CDCl$_3$, 100 MHz)
53: $^1$H NMR (CDCl$_3$, 400 MHz)

54: $^1$H NMR (CDCl$_3$, 400 MHz)
54: $^{13}$C NMR (CDCl$_3$, 100 MHz)

55: $^1$H NMR (CDCl$_3$, 400 MHz)
55: $^{13}$C NMR (CDCl$_3$, 100 MHz)

56: $^1$H NMR (CDCl$_3$, 400 MHz)
56: $^{13}$C NMR (CDCl$_3$, 100 MHz)

56: $^{31}$P NMR (CDCl$_3$, 162 MHz)
56: $^1$H-$^{31}$P HMBC NMR (CDCl$_3$, 400 MHz)

57: $^1$H NMR (CDCl$_3$, 400 MHz)
$\text{57}: ^{13}\text{C NMR (CDCl}_3, \text{ 100 MHz)}$

$\text{57}: ^{31}\text{P NMR (D}_2\text{O, 162 MHz)}$
Spectral data associated with MeOPN→6-Gal conjugate

58A: $^1$H NMR (CDCl$_3$, 400 MHz)

58A: $^{13}$C NMR (CDCl$_3$, 100 MHz)
58B: $^1$H NMR (CDCl$_3$, 400 MHz)

58B: $^{13}$C NMR (CDCl$_3$, 100 MHz)
59: $^1$H NMR (CDCl$_3$, 400 MHz)

59: $^{13}$C NMR (CDCl$_3$, 100 MHz)
60: $^1$H NMR (CDCl$_3$, 400 MHz)

60: $^{13}$C NMR (CDCl$_3$, 100 MHz)
61: $^1$H NMR (CDCl$_3$, 400 MHz)

61: $^{13}$C NMR (CDCl$_3$, 100 MHz)
62: $^1$H NMR (CDCl$_3$, 400 MHz)

62: $^{13}$C NMR (CDCl$_3$, 100 MHz)
63: $^1$H NMR (CDCl$_3$, 400 MHz)

63: $^{13}$C NMR (CDCl$_3$, 100 MHz)
63: $^{31}$P NMR (CDCl$_3$, 162 MHz)

64: $^1$H NMR (CDCl$_3$, 400 MHz)

64: $^{13}$C NMR (CDCl$_3$, 100 MHz)
65: $^1$H NMR (CDCl$_3$, 400 MHz)

65: $^{13}$C NMR (CDCl$_3$, 100 MHz)
65: $^{31}$P NMR (D$_2$O, 162 MHz)

66: $^1$H NMR (CDCl$_3$, 400 MHz)
66: $^{13}$C NMR (CDCl$_3$, 100 MHz)

66: $^{31}$P NMR (D$_2$O, 162 MHz)
67: $^1$H NMR (CDCl$_3$, 400 MHz)

67: $^{13}$C NMR (CDCl$_3$, 100 MHz)
67: $^{31}$P NMR (D$_2$O, 162 MHz)

68: $^1$H NMR (CDCl$_3$, 400 MHz)
68: $^{13}$C NMR (CDCl$_3$, 100 MHz)

68: $^{31}$P NMR (D$_2$O, 162 MHz)
68: $^1\text{H}$$-^{31}\text{P}$ HMBC NMR (CDCl$_3$, 400 MHz)
Spectral data associated with MeOPN→6-Gal and MeOPN→3-Fruc in chapter 5

71: $^1$H NMR (CDCl$_3$, 400 MHz)

71: $^{13}$C NMR (CDCl$_3$, 100 MHz)
71: $^{31}$P NMR (D$_2$O, 162 MHz)

73: $^1$H NMR (CDCl$_3$, 400 MHz)
73: $^{13}$C NMR (CDCl$_3$, 100 MHz)

73: $^{31}$P NMR (D$_2$O, 162 MHz)
76: $^1$H NMR (CDCl$_3$, 400 MHz)

76: $^{13}$C NMR (CDCl$_3$, 100 MHz)
76: $^{31}$P NMR (D$_2$O, 162 MHz)

77: $^1$H NMR (CDCl$_3$, 400 MHz)
77: $^{13}$C NMR (CDCl$_3$, 100 MHz)

77: $^{31}$P NMR (D$_2$O, 162 MHz)
Spectral data associated with the preparation of GlcNAc-β-(1→3)-Gal disaccharide

**82: **\(^1\)H NMR (CDCl\(_3\), 400 MHz)

![\(^1\)H NMR spectrum of GlcNAc-β-(1→3)-Gal disaccharide]

**82: **\(^{13}\)C NMR (CDCl\(_3\), 100 MHz)

![\(^{13}\)C NMR spectrum of GlcNAc-β-(1→3)-Gal disaccharide]
83: $^1$H NMR (CDCl$_3$, 400 MHz)

83: $^{13}$C NMR (CDCl$_3$, 100 MHz)
80: $^1$H NMR (CDCl$_3$, 400 MHz)

80: $^{13}$C NMR (CDCl$_3$, 100 MHz)
85A: $^1$H NMR (CDCl$_3$, 400 MHz)

85B: $^1$H NMR (CDCl$_3$, 400 MHz)
85B: $^{13}$C NMR (CDCl$_3$, 100 MHz)

86A: $^1$H NMR (CDCl$_3$, 400 MHz)
86A: $^{13}$C NMR (CDCl$_3$, 100 MHz)

86B: $^1$H NMR (CDCl$_3$, 400 MHz)
86B: $^{13}$C NMR (CDCl$_3$, 100 MHz)

79: $^1$H NMR (CDCl$_3$, 400 MHz)
**79**: $^{13}$C NMR (CDCl$_3$, 100 MHz)

![C NMR spectrum](image)

**87**: $^1$H NMR (CDCl$_3$, 400 MHz)

![H NMR spectrum](image)
87: $^{13}$C NMR (CDCl$_3$, 100 MHz)

88: $^1$H NMR (CDCl$_3$, 400 MHz)
$^{13}$C NMR (CDCl$_3$, 100 MHz)