A Diagnostic Target Against *Clostridium bolteae,* Towards a Multivalent Vaccine for Autism-Related Gastric Bacteria

by

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Abstract

A VACCINE AND DIGANOSTIC TARGET AGAINST *CLOSTRIDIUM BOLTEAE*

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Constipation and diarrhea are common in autistic patients. Antibiotic treatment against bacteria appears to partially alleviate autistic-related symptoms. The bacterium *Clostridium bolteae* has been shown to be overabundant in the intestinal tract of autistic children suffering from gastric intestinal ailments, and as such is an organism that could potentially aggravate gastrointestinal symptoms. Investigation of the cell-wall polysaccharides of *C. bolteae* was employed in order to evaluate their structure and immunogenicity. Exploration revealed that *C. bolteae* produces a conserved specific capsular polysaccharide comprised of rhamnose and mannose units: [→3)-α-D-Manp-(1→4)-β-D-Rhap-(1→], which is immunogenic in rabbits. This is the first described immunogen of *C. bolteae* and indicates the prospect of using this polysaccharide as a vaccine to reduce or prevent colonization of the intestinal tract in autistic patients, and as a diagnostic marker for rapid detection. This diagnostic target can be used in a multivalent vaccine, which may potentially include *Sutterella* and *Desulfovibrio*.

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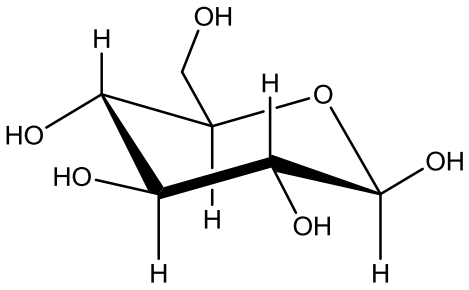
List of Abbreviations

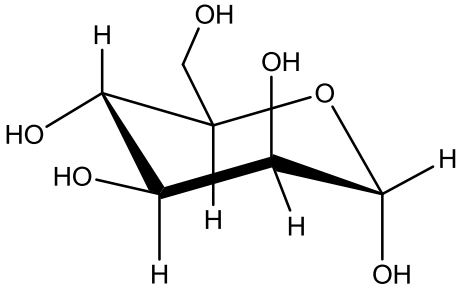
1D	One Dimensional
2D	Two Dimensional
α	Alpha
β	Beta
δ	Chemical Shift
AA	Alditol Acetate
ANS	Autonomic Nervous System
ASD	Autism Spectrum Disorder
BSA	Bovine Serum Albumin
C. bolteae	*Clostridium bolteae*
C. clostridioforme	*Clostridium clostridioforme*
C. difficile	*Clostridium difficile*
C. tetani	*Clostridium tetani*
C. jejuni	*Campylobacter jejuni*
CNS	Central Nervous System
COSY	Correlation Spectroscopy
CPS	Capsular Polysaccharide
DCM	Dichloromethane
dH_2O	Deionized Water
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked Immunosorbant Assay
ENS	Enteric Nervous System
Gal	Galactose
GC-MS	Gas Chromatography Mass Spectrometry

GI	Gastrointestinal
Glc	Glucose
HMBC	Heteronuclear Multiple Bond Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Correlation Spectroscopy
Kdo	Ketodeoxyoctonate
LOS	Lipo-oligosaccharide
LPS	Lipopolysaccharide
Man	Mannose
MWCO	Molecular Weight Cut Off
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser-Effect Spectroscopy
P. aeurginosa	*Pseudomonas aeurginosa*
PMAA	Partially Methylated Alditol Acetate
PPA	Propionic Acid
Rha	Rhamnose
SCFA	Short Chain Fatty Acid
TOCSY	Total Correlation Spectroscopy

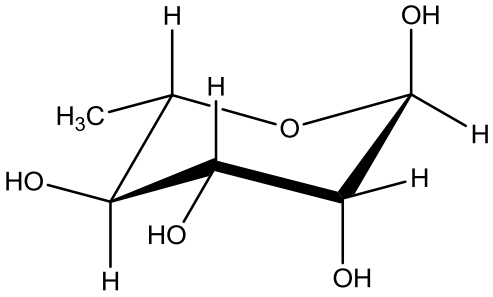
Sugar Structures



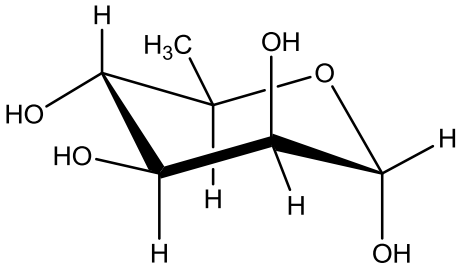
D-Glucose



D-Mannose



L-Rhamnose



D-Rhamnose

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Chapter 1: Introduction

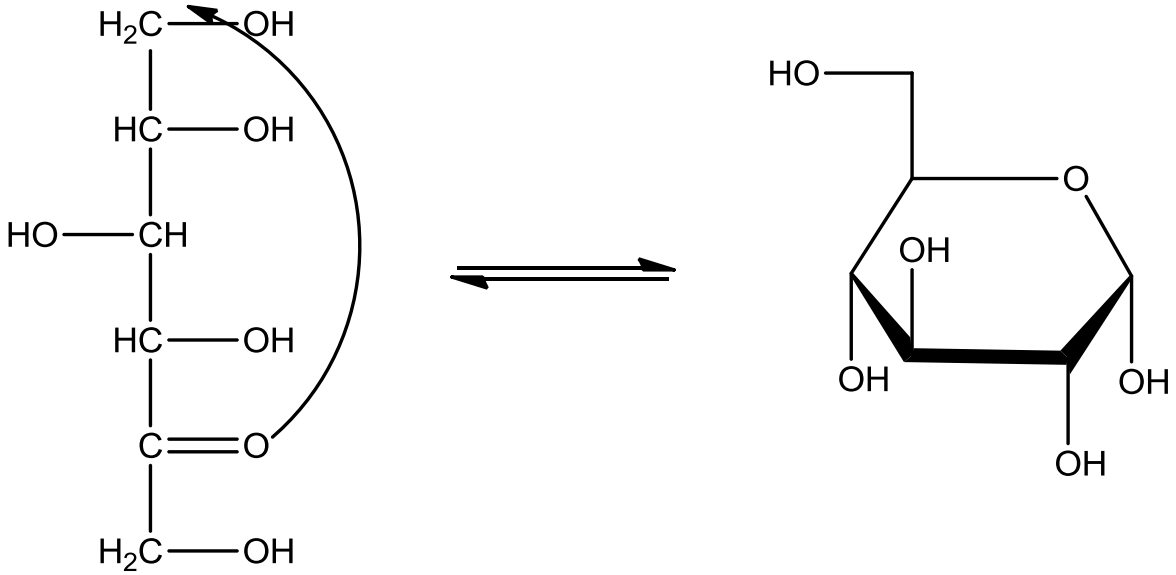
1.1 General Carbohydrates

Traditionally carbohydrates were described based on the overall empirical chemical formula $C_n(H_2O)_n$ [1]. Gradually the naming convention changed and due to carbohydrates demanding a separate chemistry skill set they became their own discipline under the organic chemistry umbrella [1].

The simplest carbohydrate, that which cannot be further hydrolyzed, is referred to as a monosaccharide [1, 2].Two or three residues are named respectively disaccharide and trisaccharide [1]. However, once there are 10 residues the molecule is referred to as a polysaccharide or oligosaccharide, meaning “many” or “a few” respectively [1].

Carbohydrates can be depicted it many different ways. Each representation gives insight to the conformation, spatial orientation, and chirality of the molecule. The Fischer projection displays the carbon chain in the open form [1]. The carbon with the highest oxidation state is normally placed at the top, leaving the asymmetric centre at the bottom of the chain [1]. If the asymmetric centre has the hydroxyl group placed on the right hand side of the chain then the configuration of the monosaccharide would be dextro (D) [1]. Consequently, if the hydroxyl is on the left hand side the assigned configuration would be laevus (L) [1].

a) α-D-Glucose



b) β-D-Glucose

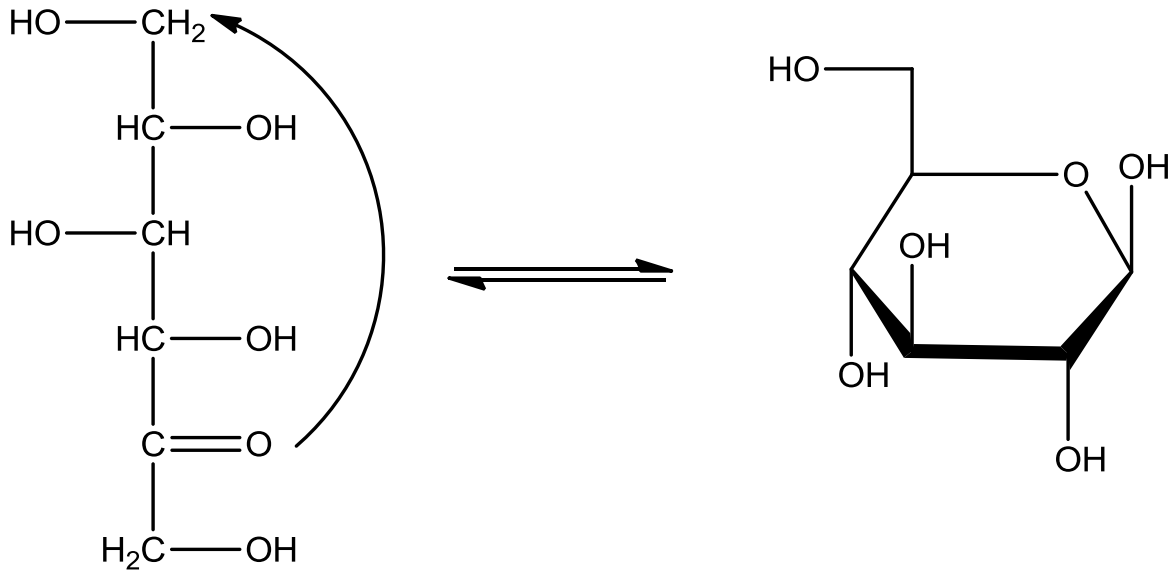


Figure 1: Fischer projections illustrating ring closing into a Haworth projection of the pyranose. A) α-D-glucose ring closure and B) β-D-glucose ring closure.

The Haworth representation displays the sugar in a flat ring form [1], employed most often by biochemists. The flat projection does not provide much information about the ring structure however, which is why chair conformations are usually used. There are two general chair conformations observed for sugars; $^{4}C_{1}$ and $^{1}C_{4}$. Most of the D-form sugars are most stable in the $^{4}C_{1}$ conformation due to the equatorial positions of the bulkier C6 methyl group.

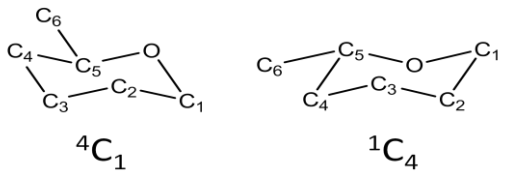


Figure 2: A figure depicting the two chair conformations observed for the pyranose forms of sugars. The $^{4}C_{1}$ configuration is named such due to the C4 being positioned upwards in space, with the C1 positioned downwards. The $^{1}C_{4}$ is the opposite, having the C1 upwards.

Monosaccharides undergo mutarotation and can be found in 4 different orientations. As noted above, monosaccharides are found in the pyranose, 6-membered ring, form with either an α or β anomeric centre. These monosaccharides can also be found in a 5-membered ring, furanose, form. The furanose structure can also possess either an α or β anomeric centre. All 4 of these conformations can co-exist in solution. Certain conformations will be favoured due to increased stability from the anomeric effect, and steric hindrance. Looking at glucose, it can be seen that the α-pyranose form would be most stable. The dipole moment created by the endocyclic oxygen is partially cancelled by the alpha anomer's dipole moment, complying with the anomeric effect [3]. The pyranose conformation also puts the bulkier C6 in an equatorial position which lowers the steric hindrance of the molecule.

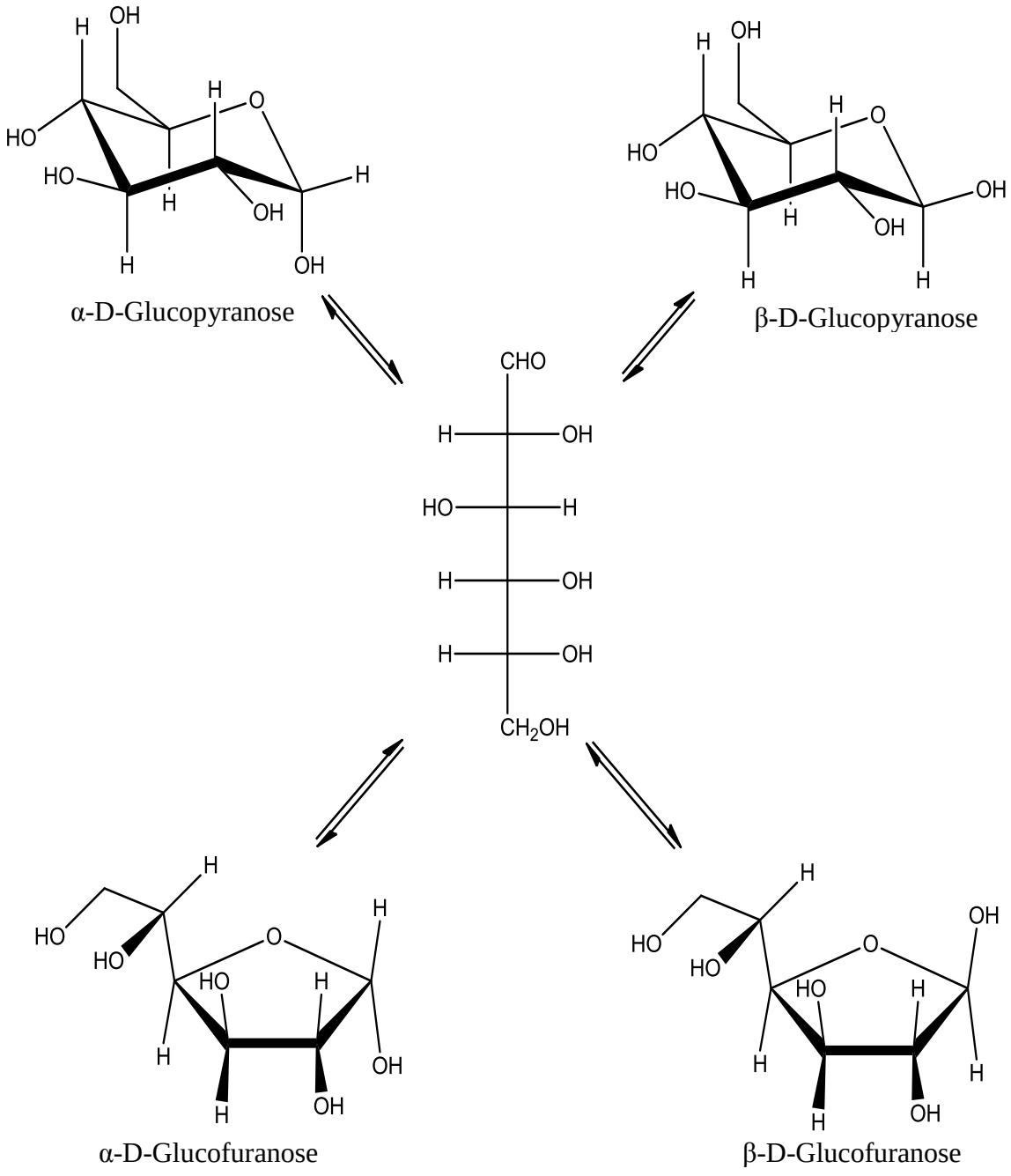


Figure 3: Mutarotation of straight chain glucose to the α and β pyranose forms, and the α and β furanose forms. Where alpha is axial and beta is equatorial.

The polar nature of the anomeric effect would dictate that the α anomer be more stable. As stated above, the cancelling, or partial cancelling, of dipole moments increases the stability of the monosaccharide's conformation [3]. Another reason for increased stability in the α anomer is hyperconjugation [3]. The interaction of the lone pair of electrons in a molecular orbital (n) on the O5 and the unoccupied anti-bonding orbital (σ^*) of C1 [3]. This n → σ^* orbital interaction stabilizes the conformation by allowing for electron density delocalization [3].

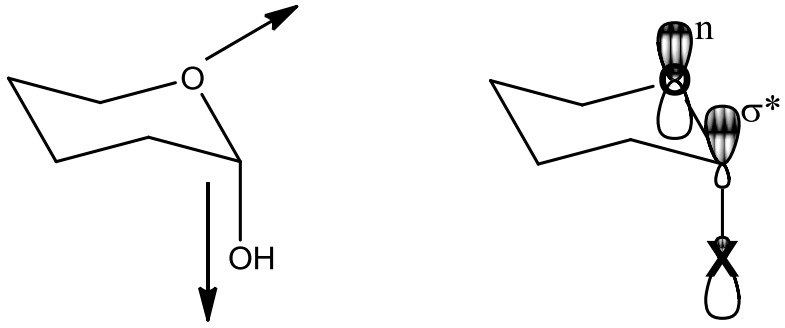


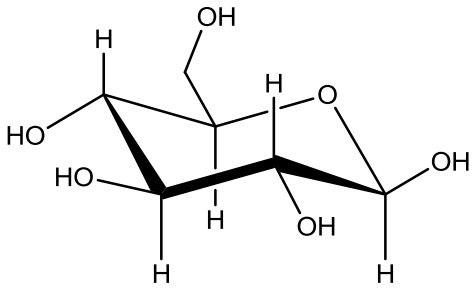
Figure 4: The alpha anomer partially cancels the dipole moment of the endocyclic oxygen and also allows for the n → σ* orbital hyperconjugation which facilitates the delocalization of electron density. This illustrates the stabilizing properties that are the foundation of the anomeric effect.

Some monosaccharides can be present in an oxidized or reduced derivative form [1, 2]. An important group of derivatives have one hydroxyl group (-OH) replaced with a hydrogen (-H) [2]. These sugar derivatives are known as deoxy sugars, and can play important roles in polysaccharides [1, 2]. The most important deoxy sugar is deoxyribose, as it occurs in DNA, other notable deoxy sugars are L-rhamnose (6-deoxy-L-mannose) and L-fucose (6-deoxy-L-galactose) [2].

1.2 Carbohydrates in Nature

Originally, it was thought that carbohydrates were not an important subject matter, as it was believed that they only acted as cellular filters, an energy source, and structural material for plants [2]. Further research uncovered a more interesting depth to carbohydrates, including their ability to couple to lipids and proteins through glycosidic linkages [2]. The resulting glycolipids and glycoproteins have functions in the cell that span a wide range of activities [2].

Glucose (Glu) is a monosaccharide that is found in high abundance in nature. It can be found in the free form; in honey, grapes and raisins [4]. The free Glu residue plays an important role in the blood of animals and humans [4]. It acts as a readily available energy source in the blood and also helps to regulate the osmotic pressure of the blood [4]. Ultimately Glu will become a precursor for glycogen and fat in mammalian bodies [4]. Glu can also be found in a linked form as seen in; cellulose, starch, sucrose and lactose, to name a few [4].



D-Glucose

Figure 5: D-glucose molecule, with the anomeric centre in the β orientation.

Cellulose is the most abundant carbohydrate on the earth, and it holds importance for both commercial and biological use [4, 5]. It is a linear, non-branched, homopolysaccharide composed of (1→4) linked β-D-glucopyranose [5]. The main function for cellulose is its contribution to the rigidity of plant protective cell walls [5].

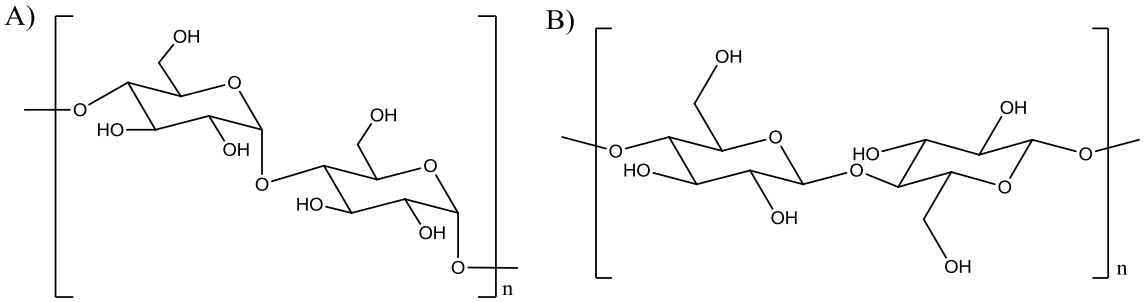


Figure 6: A) The (1→4) linked β-D-glucose repeat of starch. B) The (1→4) linked α-D-glucose repeat of cellulose.

Starch is another carbohydrate with biological importance [4]. Like cellulose it is comprised of repeating units of (1→4) linked D-glucopyranose, however, the glucose residues in this case, are linked via an alpha (α) glycosidic bond [6]. The difference in glycosidic bond orientation results in the function of starch being different from that of cellulose [6]. Starch is more commonly found in the leaves, stem, seeds and roots of the plant, where it stores energy formed through photosynthesis [4].

Polysaccharides can be found in many places in nature, one of which is the surface of bacterial cells. The polysaccharides on the surfaces of bacterial cells are participating in cellular functions and aiding in the adaptations of bacteria to their surrounding environments.

1.3 Gram Negative and Positive Bacteria

A bacterium can be classified by the composition of the cellular membrane that it possesses. The difference in number and thickness of membranous layers results in the ability to differentiate based on a Gram staining process. This counter-stain technique results in either a purple or red dye retained by the bacterial cells. If the first stain, normally crystal violet, is retained by the bacteria then a purple colour is displayed and the sample is deemed to be Gram-positive. Conversely, if the second dye, safranin, needs to be applied for the red to appear, then the bacteria is Gram-negative. Gram-positive bacteria are able to retain the purple dye due to the thick layer of peptidoglycan into which the dye diffuses. When the alcohol wash is applied to the thick peptidoglycan, it dehydrates it and causes the pores of the wall to close around the applied dye. Gram-negative bacteria have a thin layer of peptidoglycan and also have an outer membrane, made up of a lipid-bilayer that is easily penetrated by the alcohol.

1.4 Polysaccharides of Gram Negative and Positive Bacteria

1.4.1 Lipopolysaccharides

On the surface of Gram-negative bacterial cell walls are lipopolysaccharides (LPSs). These molecules are composed of three distinct regions: the hydrophobic lipid A; the non-repeating core oligosaccharide; and the distal polysaccharide, or O-antigen [7].

The lipid A fatty acid tail, also referred to as endotoxin, has an important function of anchoring the LPS to the bacterial outer membrane. The hydrophobic fatty-acid tails on the lipid A are able to incorporate themselves into the phospholipid bilayer of the outer membrane to avoid the surrounding aqueous environment. The glucosamine-based

phospholipid architecture of lipid A is highly conserved, and often resembles the structure found in *Escherichia coli* [7]. Characteristic features the *E. coli* lipid A architecture are the presence of two phosphate groups and two acyloxyacyl moieties [7]. This translates to the conserved structure being typically composed of a β-d-GlcN-(1→6)-α-d-GlcN disaccharide, which carries two phosphoryl groups [8]. The phosphorylated region of the lipid A is the attachment point for the next region, the core oligosaccharide [8].

The non-repeating core oligosaccharide is the bridge between the lipid A anchor and the O-antigen repeat unit. Similarities in oligosaccharide are often observed between members of the same genus [7]. The core oligosaccharide can be split into two domains; the inner core and the outer core [8]. The outer domain is made up of mainly common sugars such as glucose, N-acetyl glucosamine, galactose, N-acetyl galactosamine; it is more variable than the inner domain [8]. The inner domain often expresses more "unusual" sugars, such as various heptoses and ketodeoxyoctonate (Kdo) [8]. The Kdo residues have been proven previously to be essential to bacterial viability [8]. The O-antigen is invariably linked to the lipid A region via a Kdo residue from the core oligosaccharide.

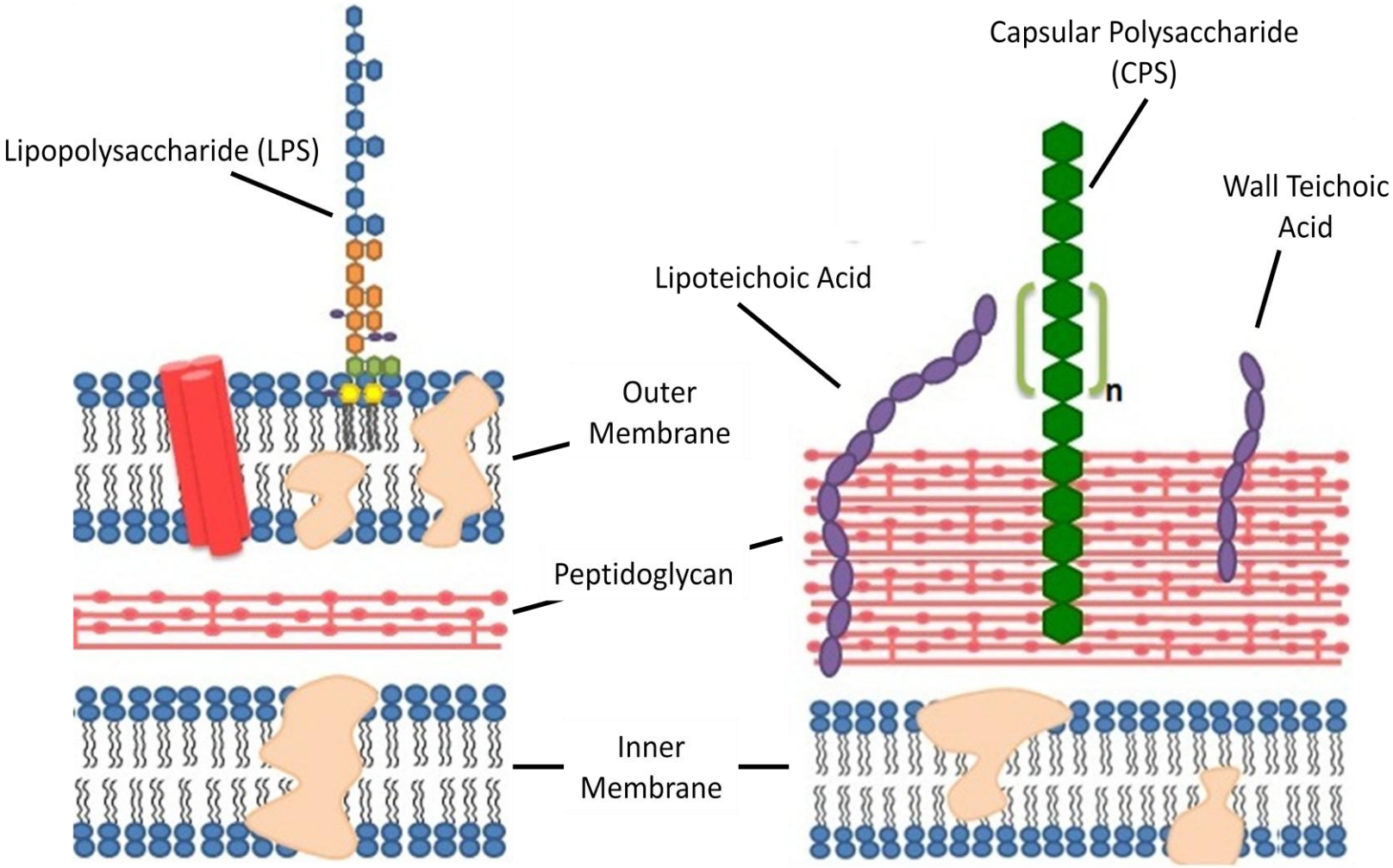


Figure 7**:** The Gram negative (left) and Gram positive (right) cell walls. Gram negative cells have the extra membranous layer, and the Gram positive possess a thick peptidoglycan layer. (Adapted from Brock [6]).

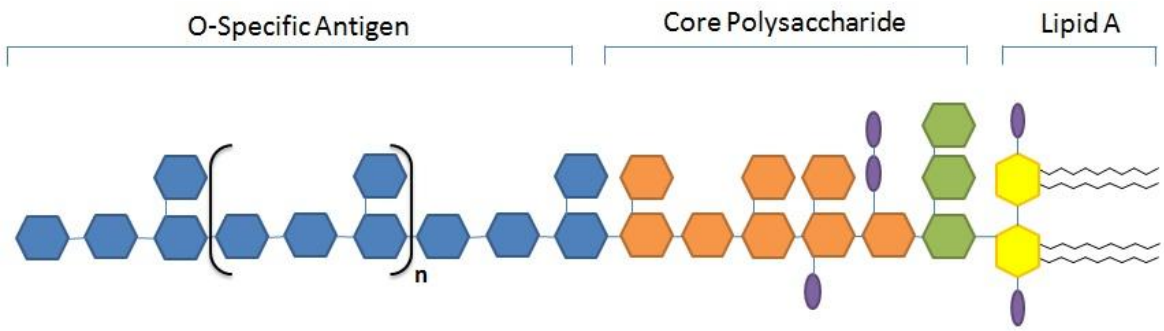


Figure 8: The general structure of the LPS indicating the 3 regions of the polysaccharide structure (Adapted from Brock [6]).

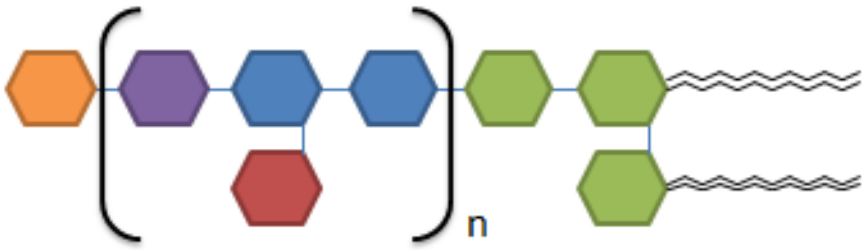


Figure 9: A representation of the general structure of the CPS.

The final region of the LPS is the O-antigen, which is also the most distal region. The O-antigen is placed at the boundary between the cell and the surrounding external environment. It has a structural diversity that is known to include over 60 monosaccharides and approximately 30 non-carbohydrate moieties [7]. A defining feature of the O-antigen is the repeating unit structure, which can have a subunit length of up to fifty [8]. Within a genus, it is possible to observe differences between O-antigen repeating units pertaining to monomer glycosides, stereochemistry, monomer positioning, and O-glycoside linkages [7]. This variable repeating unit may contribute to the organisms' ability to evade the immune system of a host, which makes it an ideal target for vaccine generation.

1.4.2 Capsular Polysaccharides

The capsule of bacteria is thought to have protective properties to aid in the survival of the organism when adverse factors are encountered in a host [9]. The surface of this capsule is riddled with polysaccharide chains, or capsular polysaccharides (CPSs). The CPS has a variable number of repeating units that contain one or more monosaccharide residue [9]. These repeat units are attached to a lipid anchor [9]. The repeat chains also have the possibility of non-sugar moieties being linked to them, such as O-methyl-phosphoramidate, which makes numerous CPS structures possible [9]. The diversity of the CPS is the driving force behind the ability of bacteria to evade the hose immune responses [9]. Under mild conditions the CPS can be easily removed from the surface of the bacteria, which is a trait that none of the other surface polysaccharides display.

1.4.3 Lipo-oligosaccharides

The major glycolipids expressed by mucosal Gram negative bacteria are lipo-oligosaccharides (LOS) [10]. It has also been noted that *Campylobacter jejuni* and *Campylobacter coli* have lipo-oligosaccharides on the cell surface, which is unlike other Gram-negative enteric bacteria [10]. The LOS has a lipid A tail and a core oligosaccharide, but it is missing the O-antigen region that the LPS contains. The oligosaccharide region of the LOS is often limited to only ten saccharide units and these core regions are less conserved than in the LPS structures [10]. These oligosaccharides have been reported to be toxic with potent immunomodulating and immune-stimulating properties [9]. At one time these polysaccharides were thought to be an under-developed LPS. It is now evident that the LOS constructs are distinct structures that have adapted functions and features that allow colonization and pathogenic success of the bacteria expressing them [10].

1.5 Importance of Characterization

The characterization of the polysaccharides isolated from the cell wall surface is important in the generation of a vaccine candidate. It needs to be established that the polysaccharide and protein are both safe to introduce into the human body, and also that the vaccine will be compatible with the scheduled immunizations for infants and young adults.

An example of where human compatibility is concerned is *C. jejuni*, where ganglioside mimicry is a possibility. The lipo-oligosaccharides (LOSs) that can be isolated from *C. jejuni* have similar polysaccharide sequences to the gangliosides of the

brain [9]. There are also CPSs found on the surface of these bacterial strains that are not similar to the gangliosides. If the polysaccharide were isolated without characterization and used for a vaccine, then unwanted LOS may be incorporated into the vaccine candidate. The vaccine would not be specific to *C. jejuni* alone, but would also raise a response to the gangliosides in the brain, which can cause severe health side-effects.

The characterization of the polysaccharide also allows for the proper conjugation technique to be chosen. Without knowledge of the structure and the functional groups present activation and conjugation may not be successful. The more that is known about the polysaccharide the better the chances are that the conjugation will be successful the first time it is performed.

1.2 *Clostridium bolteae*

In 1998, Ellen Bolte hypothesized that *Clostridium tetani* was associated with the symptoms of late onset autism [11]. Since clostridial species produce potent toxins they were a likely candidate in the cause of the irritations in the intestinal tract. Ms. Bolte hypothesized that the disruption of the natural flora in the gut due to increased antimicrobial administration could allow for the colonization of opportunistic bacteria, such as *C. tetani* [11].

This hypothesis generated interest in the diversity of the flora in autistic patients. The GI flora of autistic subjects was analyzed and it was observed that another clostridial species, *Clostridium clostridioforme*, was present in a number of autistic patient cultures, but not in that of controls [12]. Recently it was discovered that *C. clostridioforme* is a mixture of three species of clinical significance: *C. clostridioforme*; *Clostridium bolteae*;

and, *Clostridium hathewayi* [12]. *C. bolteae* had not been previously characterized and the species was named 'bolteae' in honour of Ms. Bolte. The focus of this research will be the bacterial species *C. bolteae* and its capsular polysaccharides.

C. bolteae is a Gram-positive bacterium that is obligately anaerobic and is rod-shaped in appearance [13]. Ideal growth conditions include a temperature of 37°C, which is the average temperature of the human body [13]. These growing conditions attribute to the growth of *C. bolteae* that is observed in the human intestinal tract.

1.2.1 *C. bolteae* Infections

Before *C. bolteae* was classified as its own species, it would have been associated with *C. clostridiforme* cluster infections. This cluster has not been seen to be overly infectious in humans to date. The *C. clostridioforme* cluster is most commonly associated with cases of bacteraemia [12]. Only in one isolated instance *C. bolteae* was found by itself in a patient suffering from intra-abdominal abscess secondary to perforated appendicitis [12].

After the reclassification, the main infection of interest is the association with gastrointestinal irritations in autistic patients as *C. bolteae* has been isolated from stool samples of children with late onset autism [13]. The prevalence of GI irritations in autistic patients is a staggeringly high percentage of 91.4% [14]. The high rate of GI problems, including diarrhea and constipation, sparks an increased interest in *C. bolteae*. These GI problems may have an impact on the behavioural patterns in autism spectrum disorder (ASD) patients due to the production of short chain fatty acids (SCFAs), such as propionic acid (PPA) [15]. PPA is a metabolite of carbohydrate digestion by certain

bacterial families, such as *Clostridia* [15]. The PPA metabolite is known to cause a reduction in gastric motility and also cause an increase in the frequency of contractions in the gut [16]. This puts PPA in the position to interfere with normal peristaltic activity of the GI tract and could lead to inflammation that is reflective of the GI problems reported in patients with ASD [15]. The PPA produced by *C. bolteae* may be one of the many contributing factors that results in GI irritations in ASD patients. Therefore, controlling the production of *C. bolteae* cultures in the GI tract and reducing the concentration of PPA could have extremely positive outcome.

1.2.1.2 Autism Background

Autistic children are more prone to ear infections, and ear infections are often prescribed antimicrobial medications [17]. These medications cause a flushing of the gastrointestinal tract's natural flora. However, *C. bolteae* has shown resistance to some of the medications available, and will therefore be able to continue growth while the competitive bacteria are wiped out [13]. The displayed resistance to antimicrobial agents would allow for the genus to survive and take advantage of the wipe-out of the other natural flora. The lack of competition would then allow proliferation and an increase in overall count of the bacteria in the GI tract. Increased counts, or levels, would then have the ability to contribute to the irritations seen in the GI tract.

Irritations in the gastrointestinal tract can cause severe discomfort and, since the majority of children with autism are nonverbal, their aggressive or negative behavior may be their way of indicating discomfort [18]. Those with autism and GI complications show an increase in occurrence and severity of anxiety, social withdrawal, and irritability [19].

These individuals are also less likely to be receptive to the treatment options provided to them [19].

In 2007, it was reported that the percentage of children with autism, in some areas of the USA, increased to approximately 1% from the previously reported 0.7% (approximate value) in 2002 [17]. This staggering number is accompanied by the $3.2 million lifetime care expense for a person suffering from an autistic spectrum disorder (ASD) [17]. Of these total lifetime costs, $206,337 is spent on average just on the behavioral therapies received [20]. Behavioural therapies are often employed to help with the aggression and severe mood swings displayed by autistic children. Any reduction in costs of these services for these individuals, and their families, can lift a substantial financial burden. Importantly, if it can be shown that specific strains of *C. bolteae* have a definite connection to autism, the impact on those suffering and their families would be substantial. Although vaccination against *C. bolteae* would not be a "cure" for autism it would aid in the decrease of severity of the symptoms in autism and related spectrum disorders.

Improving not only the quality of life for those that are suffering from ASD but also all of those that are in direct contact with them would be an important positive for the research. The up-bringing of a child with disorders that involve developmental and behavioural issues often takes a large toll on the family and care-givers. The stresses of having a child of disability can be handled differently in families, and can sometimes pull apart a family depending of the severity of the need of the child. Mothers and fathers of children with autism reported an increased feeling of family problems [21]. They also reported that they feel a higher rate of pessimism toward their child's future and an

increase in negative behaviour from the child [21]. This is indicative that the impact of an autistic child on a family can be one of high stress and tough adjustments [21]. By reducing the behavioural outbursts of an autistic child, hope of relieving pressures from the care givers is reinforced. Any improvement in their child's life can directly reflect on the stresses and pessimism that the parents carry with them. This can also relieve tension that may have built up between the spouses in the discipline and tolerance that each may want to apply when raising a child that is perceived as "difficult". Relief is not only important for the child, but is also important for the parents that work hard at understanding the disorder, discomfort and road-blocks their child faces every day.

1.2.2 Current Treatment

Treatment options for pathogen causing bacteria often revolve around the use of 'last resort' anti-microbial drugs, such as vancomycin. Vancomycin is commonly used to treat Gram-positive bacterial infections [22]. Vancomycin is not selective for specific Gram-positive bacteria, and therefore will wipe-out most Gram-positive bacterial species, good and bad, in the GI tract. This leaves the GI tract vulnerable to opportunistic bacteria and also can be just a temporary fix. Antimicrobial resistance to vancomycin is on the rise, and due to this, vancomycin therapy is not normally a suggested long-term treatment option. Resistance to vancomycin is observed in coagulase-negative staphylococci, *Staphylococcus aureus*, and enterococci species [22, 23]. Vancomycin has been used to treat infections of *Clostridium difficile* with success [24]. However, reoccurrence rates of *C. difficile* when treated with vancomycin are around 20% [24, 25].

In a trial, vancomycin was used to treat ASD patients suffering from persistent diarrhea and/or constipation [26]. Upon treatment with oral vancomycin improvements were clear in the behaviour of the patients [26]. Follow-up inquiries to the parents of the children involved in the study revealed that after the treatment had been halted, that behavioural deterioration was substantial [26]. These results show that the bacteria associated with the behavioural alterations, as well as the GI discomforts, were suppressed by the vancomycin, but returned upon the end of the treatment program. Children that had a positive response to multiple courses of vancomycin relapsed after each regimen ended, confirming that the response is only temporary [27]. The causative bacteria need to be isolated and treated specifically rather than a general Gram-positive clean sweep of the GI tract. Since CPS is not only unique to the species but also the strain, it would be a good option to target these cellular structures [28]. A polysaccharide-protein conjugate vaccine would have the specificity required to target the harmful bacterial strains.

1.3 History of Immunology

In 1798, Edward Jenner, an English physician, made a giant leap forward in the development of immunity and vaccines [29]. He observed that milkmaids that contracted the mild cowpox disease were then later immune to the much more severe disease of smallpox [29]. He postulated that the giving of pustule fluid from cowpox to those infected with smallpox might, in fact, be protective [29]. He carried out an experiment on an 8 year old boy and discovered that after inoculation with the cowpox pus the boy did not contract smallpox when exposed [29]. Even though this technique spread throughout

Europe to eradicate smallpox outbreaks, the Jenner technique was not used on other diseases for almost a hundred years [29].

Louis Pasteur took the next step towards vaccination when he was growing fowl cholera bacterium [29]. He left culture for a few months before injecting the chickens and surprisingly they grew ill but did not die [29]. Then he injected the same chickens with freshly grown bacterial cultures and they were unexpectedly protected against the disease altogether [29]. The aging of the bacteria seemed to weaken the virulence of the pathogen in question. This unique finding led to the idea of using attenuated strains of bacteria in a vaccine form. Pasteur's experiments laid the groundwork for the beginnings of the study of immunology [29].

Since these dramatic findings in the field of immunology, many diseases have been effectively prevented. Prevention rates between 87.13% and 100% have been seen in a few of these diseases [29]. Prevented diseases include; smallpox, diphtheria, measles, mumps, pertussis, paralytic polio, rubella, tetanus, and invasive hemophilus influenzae [29].

In order to develop vaccines for other diseases, infections and irritations, a lot needs to be understood about the immune system of humans. In section 1.3.1, an outline of the immune system will be established.

1.3.1 The Immune Response

The immunity of humans can be split into two sections, the innate and the adaptive immunity. The innate immunity is the first line of defence when the body in under attack from a pathogen. The response is not pathogen specific and pre-existing

disease protective molecules are involved in the protection. Infections first have to breach existing barriers of the host. These barriers include mucosal membranes and the skin [29]. Acidity of the stomach is another barrier a pathogen may encounter, as well as enzymes in the host that can attack bacteria, such as lysozyme [29].

The adaptive immunity is more specific and is capable of recognizing bacteria that can then be selectively eliminated. In order to achieve this specificity the adaptive immune system has characteristic attributes: antigenic specificity; diversity; immunologic memory; and, self-nonself recognition [29]. Subtle differences in antigens can be detected from the antigenic specificity, which can pinpoint down to a one amino acid difference in proteins [29]. This differs from the broad organism class recognition that is used by the innate immunity. Once the specific antigen has been responded to by the adaptive immunity, an immunologic memory of that antigen is established for secondary attacks [29]. This can allow for the immune system to have a long term memory of many infectious pathogens [29]. The self-nonself recognition is also very important for immunity. If this recognition did not exist, then inappropriate responses to self components could lead to serious disorders or even be fatal [29].

For adaptive immunity to generate an immune response that is effective, two important types of cells must be involved: lymphocytes and antigen-presenting cells (APCs) [29]. The lymphocytes are split into two major populations the B lymphocytes (B cells) and the T lymphocytes (T cells).

The development of the B cells takes place in the stem cells of the bone marrow [30]. These lymphocytes have the ability to make immunoglobins that are specific to all

possible chemical structures, and effectively all infectious microorganisms [30]. The body does not carry all the differentiated B cells at all times, but rather an inventory of all the differentiated cells. These cells can be generated as infections arise in the host [30]. When a naive B cell has an encounter with an antigen that is a match to its membrane-bound antibody, binding of the antigen occurs [29]. Following the binding a rapid division of the B cell occurs, which results in the creation of memory B cells and plasma cells [29]. These resulting daughter cells have important immunological roles as the memory B cells will display the antibody on their outer surface, like the parent B cell did, and the plasma cell will produce an antibody that can be secreted [29]. Memory B cells will survive for longer periods of time and the plasma cells will only survive for a few days in the body [29]. In the plasma cells short life it will produce and secrete hundreds to thousands of antibody molecules [29]. B cells can also act as an APC that presents soluble antigen, toxins, and viruses to naive T cells [30]. Antigen gets internalized by the APCs by phagocytosis or endocytosis, which then display part of the antigen on their membrane bound to a major histocompatibility complex (MHC) class II [29].

Like the B cells, T cells arise from bone marrow. Instead of maturation in the bone marrow, the T cells move to the thymus gland to mature [29]. As the T cells mature, they develop into two well defined types of T cells; the T helper (T_H) and T cytoxic (T_C) cells [29]. These two subpopulations are differentiated by either CD4 (T_H) or CD8 (T_C) membrane glycoprotein on the cell surface respectively [29]. T cells are unable to recognize free antigen like the B cells and, as such they require that the antigen be bound to cell membrane proteins MHC classes I and II [29]. One the classes of MHC, class II, is found expressed on the surface of APCs, such as B cells [29].

1.3.2 Vaccine Development

With increasing antibiotic resistance comes the need for vaccinations that are strain-specific. A component of the bacterial structure needs to be targeted to help eliminate the growth without fostering resistance to pharmaceutical agents. Bacterial cell wall polysaccharides are a point of focus as they are the outer-most structure on the cell and protrude into space. Isolation and characterization of these retained structures could lead to the polysaccharide being used as a vaccine candidate.

It is important to consider the immunological response when developing a vaccination against any bacterial, fungal, or foreign agent. The body's ability to respond in a desired manner is the only way that the vaccine can be effective. When looking at a polysaccharide as a target for a vaccine, the inherent immunogenicity of the molecule needs to be analyzed.

1.3.2.1 Polysaccharide Vaccines

Polysaccharides have demonstrated the ability to be used as a vaccine candidate for *Neisseria meningitidis* groups A, C, W-135, and Y, in a quadravalent vaccine [31]. This vaccine is widely used as a preventative vaccine for meningitis and has all but eradicated the bacterial infection [31]. However, not all polysaccharides are successful as a vaccine when administered alone. This lack of success is due to obstacles they face in the immune system.

One of the many obstacles of carbohydrate vaccines is the poor antibody response [28]. While they interact directly with B cells to induce antibody synthesis, interaction with T cells is absent [32]. This inability to give rise to helper T cells limits the immune

response to polysaccharides [29]. Therefore, vaccines based on polysaccharides, usually, only arouse B cells in a type 2 thymus-independent manner [29]. T-independent responses are restricted in that they cannot sustain antibody concentration in young children, specifically those below 18 months [32]. Polysaccharide vaccines are immunogenic in adults and older children, but not without restrictions [32]. Geriatric patients, as well as the immunocompromised, have poor response to polysaccharide vaccines [33].

Dominated by IgM and IgG2 the immunogenic response is relatively short and is not boosted by repeat exposures [32]. Booster immunization fails to promote class switching of the IgM to IgG [34]. IgM is the first immunoglobulin produced in response to an antigen and it is a more efficient activator of complement than IgG, however it is only 5-10% of the total serum composition [29]. IgG2 is somewhat effective at activating complement, not as effective at IgG1 or IgG3, but it has a low affinity to F_c receptors on phagocytic cells [29]. In order to increase the antibody response to the vaccine a carrier may be attached to the polysaccharide.

1.3.2.2 Conjugate Vaccines

When a protein is conjugated to the polysaccharide, CD4+ T cells are generated [28]. This response allows for the generation of high affinity class-switched antibodies, and will ultimately lead to long-lived antibody mediated protection [28].

Commonly used proteins include tetnus toxoid (TT), diphtheria toxoid (DT), mutant diphtheria toxoid (CRM_{197}), and outer membrane proteins [32]. The carrier protein antigenic response is dominated by IgG1 and IgG3 antibodies, and maturation of the affinity can be developed over time [32]. IgG3 is the most effective complement activator

closely followed by IgG1 [29]. Both IgG1 and IgG3 also bind with high affinity to F_c receptors on phagocytic cells [29]. This allows for conjugate vaccines to prime the immune system for memory response [32]. This unique interaction was first noted by Avery and Goebel, in 1929, and has paved the way for conjugate vaccine development [32].

The first commercially available glycoconjugate vaccine formulated for use in humans was for *Haemophilus influenza* type B (Hib) [32]. The conjugate vaccine was soon after introduced into the infant vaccination schedule and was successful in the reduction of invasive Hib disease in children [32]. The success of the conjugate began an acceleration to develop other vaccines for encapsulated bacteria that are the cause of childhood diseases [32]. Other glycoconjugate vaccines that have also shown success commercially are for *Steptococcus pneumonia* (23 serotypes), and *Salmonella typhi*.

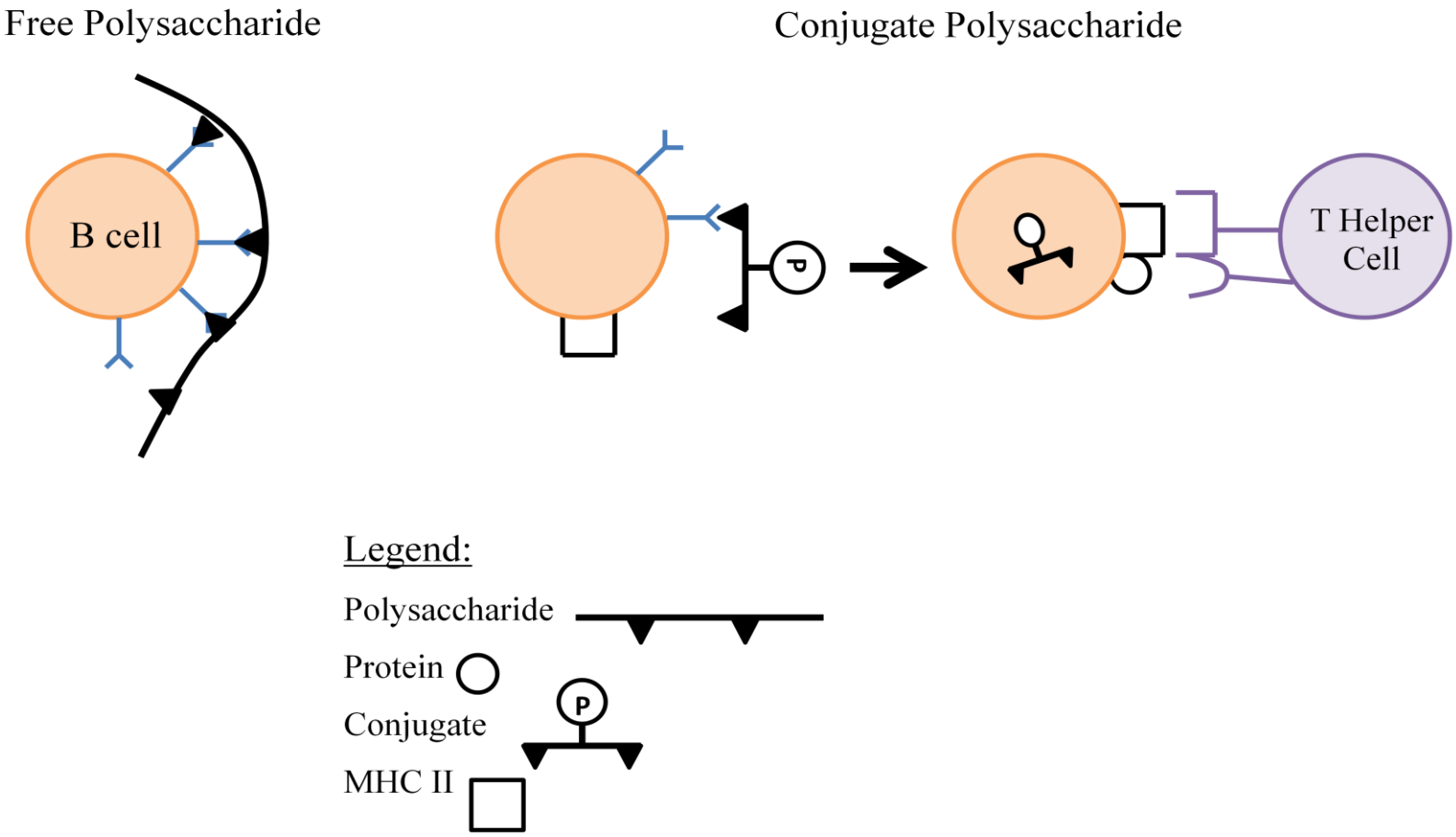


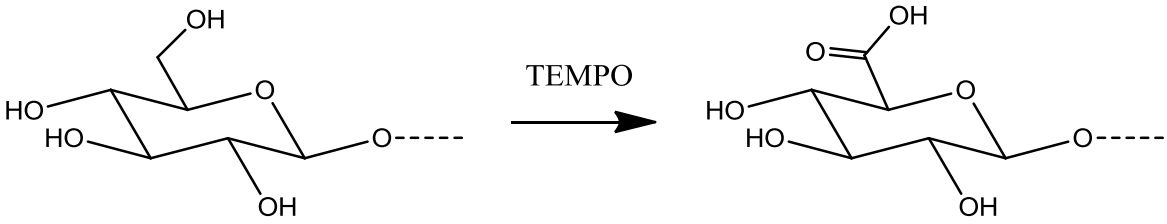
Figure 10: Immunogenic response from both a free polysaccharide, and a protein-conjugated polysaccharide. The polysaccharide bound to the protein elicits a response from the T helper cells which will aid in the development of longer term immunogenic protection. (Adapted from Peeters et al [35])

1.3.2.3 Conjugation Techniques

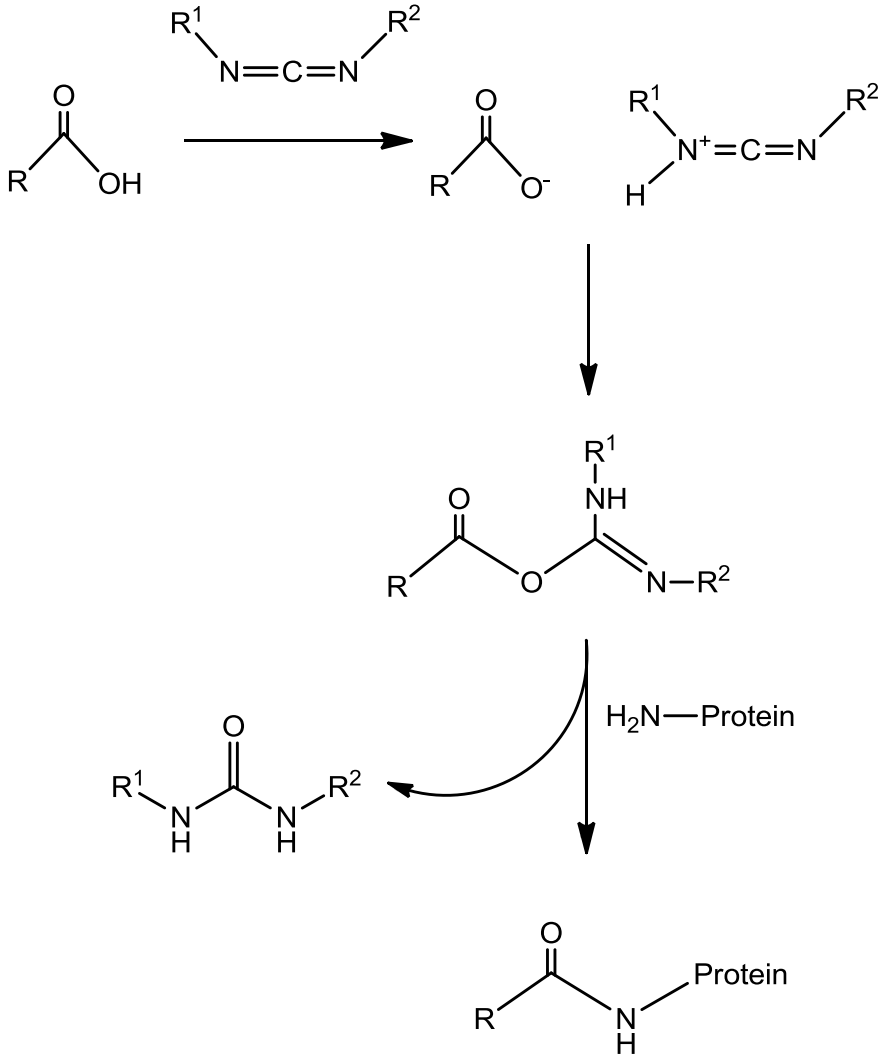
The activation and conjugation technique used for each polysaccharide will need to be chosen to suit the nature of the native structure. Since not all polysaccharides will contain the same functional groups, or the same amount of one specific functional group, the conditions and technique will need to be specifically chosen. Conjugation chemistry usually relies on the modification of a functional group, such as carboxyl, hydroxyl, hemiacetal, disulfide, or amino/imino [34]. Depending on the characterized structure of the polysaccharide a different technique can be used.

If the polysaccharide has an open position that is a primary alcohol, this functional group can be selectively oxidized using 2,2,6,6 – tetramethylpiperidin-1-oxyl (TEMPO) [36]. Previously excessive amounts of TEMPO were used to oxidize a sample, but it has been shown that using a stoichiometric amount of TEMPO to polysaccharide effectively oxidizes 2-3 monosaccharide units of each repeat chain [36]. This oxidation can be followed by conjugation with bovine serum albumin (BSA) or CRM_{197}.

Another way to activate the CPS is by periodate (IO_4^-) oxidation. This oxidation transforms the relatively unreactive hydroxyls of sugar residues into aldehydes that are amine-reactive [37]. Cleavage of carbon-carbon bonds that possess adjacent hydroxyl are the location of the oxidation [37]. Varying concentrations of periodate effect the oxidation and can give some specificity with regard to which of the sugars in the polysaccharide are modified [37].



Scheme 1: TEMPO oxidation generates a carboxylic acid at primary alcohols. Activation of the polysaccharide will allow for conjugation to occur.



Scheme 2: General scheme for coupling with a carboiimide to form an amide bond

Once the polysaccharide has been activated it is ready to be conjugated to a protein carrier. Coupling with EDC (1-ethyl-3(3-dimethylaminopropyl)carbodiimide) is one of the ways to conjugate to a protein [37]. EDC is able to react with carboxylic acids to an active-ester intermediate [37]. When EDC is introduced in the presence of an amine nucleophile an amine bond is generated [37].

Another effective method for introducing the carrier protein is reductive amination. Reductive amination allows for activated molecules containing an aldehyde group to be cross-linked to carrier molecule [37]. The carrier molecule possesses an amine group and this is able to react with the aldehyde groups that form intermediate Schiff bases [37]. The reduction occurs by the use of sodium cyanoborohydride or sodium borohydride which allow for the formation of secondary amine bonds creating a stable conjugate [37].

Chapter 2: Scope of Research

2.1 Information needed for Characterization of Capsular Polysaccharides

There are many different pieces of information needed to complete a characterization of a bacterial capsular polysaccharide. The composition of the monosaccharides must be known, as well as how they link to one another. The enantiomeric configurations must be determined, and also the anomeric configurations of each sugar and resulting linkage. Finally, it must be determined whether or not there are any non-sugar moieties on the polysaccharide, and their locations.

2.2 Aim of Research

This research involves the isolation, purification, and characterization of any capsular polysaccharides that are produced by *C. bolteae*, specifically strains 16351 and 14578. These strains are of interest as they were isolated, by Dr. Sydney Finegold's laboratory, from stool samples of autistic patients [13]. Analytical techniques for characterization of the capsular polysaccharide include; GC-MS and NMR experiments. After characterization, conjugation to a carrier protein was the main objective. The protein-conjugate will then be applied to the creation of a multivalent vaccine for ASD patients.

Chapter 3: Materials and Methods

3.1 Bacterial Growth

The focus of the methodology is the purification and characterization of the CPS. The cells were grown by Dr. Emma Allen-Vercoe's lab at the University of Guelph. The *C. bolteae* cells were grown on fastidious anaerobic agar (Lab 90) supplemented with 5% defibrinated sheep's blood at 37°C in a reduced atmosphere (N_2:CO_2:H_2, 80:10:10) for 36-48 hours. The intact cell mass was then frozen and lyophilized. After a dry intact cell mass was obtained it was purified to obtain the CPS. Analytical detection and quantification techniques were then employed throughout various stages of purification, as well as after completion of purification.

3.2 Extraction and Purification

From the intact cell mass the purification of CPS can begin. This begins with the water/phenol extraction previously described by Westphal et. al., in 1965 [38, 39]. The intact cell pellet is crushed and the sample is added to a round bottom flask, which contains a measured amount of water. The solution is then stirred for one hour in a 75 °C hot bath to suspend and dissolved all of the intact cells [39]. Next, phenol is added to the round bottom flask along with the remaining portion of water. The solution is stirred for 6 – 7 hours in the 75 °C hot bath, and then immediately transferred into ice to cool [39]. The cooling process allows for the water and phenol layers to separate. The aqueous layer is where the CPS will be found, and thus was extracted from the round bottom flask the following day. The volume, of aqueous layer, removed was replaced with fresh deionized water (dH_2O) and the round bottom flask was returned to the hot bath again for 6 – 7 hours. The aqueous layer was removed a total of three times from the round bottom flask.

The aqueous layer still contains trace amounts of phenol. These phenol molecules can be removed using dialysis. SpecrtoPor 1 kDa molecular weight cut-off (MWCO) dialysis bags were prepared and the aqueous layer was placed under running dH_2O overnight. The CPS was retained in the dialysis bag due to the size of the CPS being larger than that of the 1 kD MWCO. The dialyzed aqueous layer was then frozen and lyophilized to obtain the collected CPS.

3.3 Sugar Composition Analysis

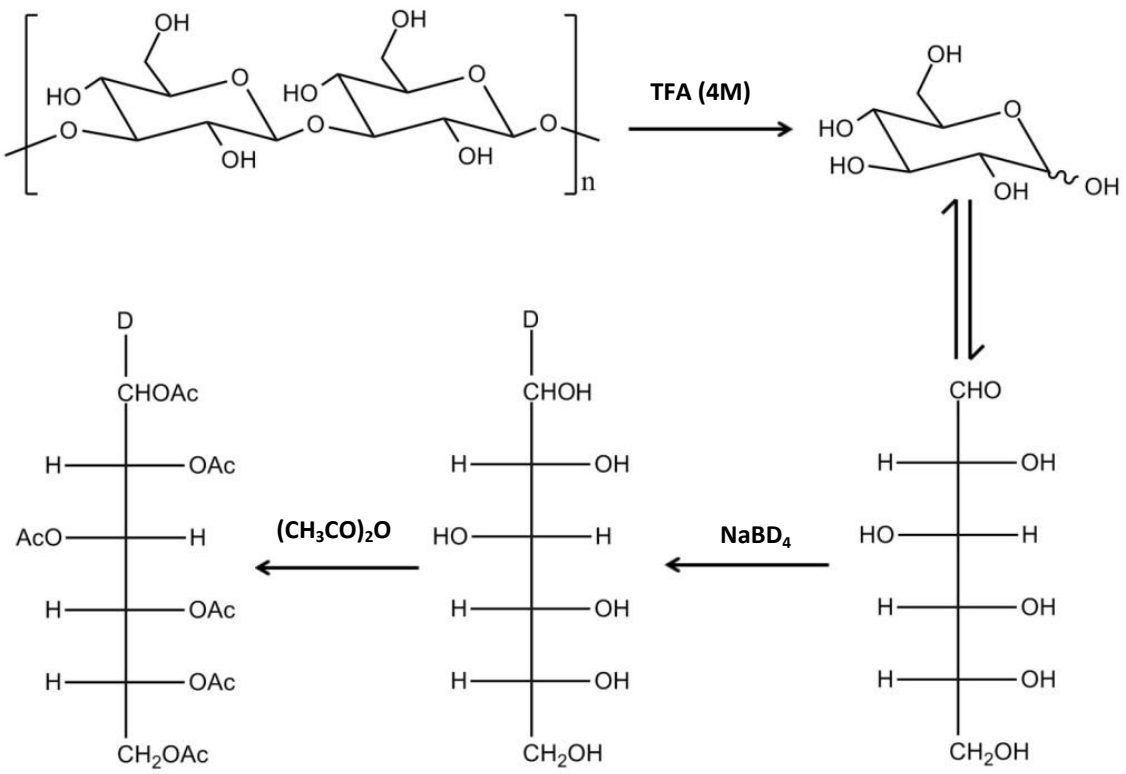
Monosaccharide composition of the isolated CPS is determined by the alditol acetate (AA) experiment. This experiment is composed of 4 major steps, including; hydrolysis, reduction, acetylation and GC-MS analysis.

It is necessary to use the AA preparation for the analysis of carbohydrates by gas-liquid chromatography (GLC), and gas chromatography – mass spectrometry (GC-MS), because carbohydrates are not volatile in their un-acetylated form. Acetylation reduces hydrogen-bonding and an alditol acetate is generated which is sufficiently volatile for GC analysis [40].

The acetylation process was started with hydrolysis, achieved by first adding a strong acid, 4M Trifluoroacetic acid (TFA), and heating at 105°C for 4.5 hours [41]. After, the TFA can be evaporated in air at 40°C. Water was then added along with Sodium borodeuteride ($NaBD_4$) to reduce the monosaccharides. This step is important because it results in the monosaccharides being reduced from aldoses to alditols. Aldoses exist in equilibrium between ring and straight chain structures, whereas, alditols favour

the straight chain form [40]. Sodium borodeuteride also, distinguishes the carbon one (C1) position from the C6 position, by reducing the C1 only [41].

After 24 hours, three drops of pure glacial Acetic acid were added to the solution to catalyze the formation of tetramethyl borate ($B(OCH_3)_4$) [41]. Again evaporation of the aqueous portion of the sample is undertaken. A Methanol (MeOH) and Acetic acid (AcOH) mixture (95:5) was then applied to the sample three times, evaporating to completeness after each addition, to remove all of the $B(OCH_3)_4$ [41]. Acetic anhydride was added to the vial to complete the acetylation of all the free hydroxyl groups [41]. The vial was heated again at 105°C for a duration of 90 minutes, and then the acetic anhydride was evaporated off. The generated alditol acetate was then extracted using dichloromethane (DCM) and a sodium sulfate column [41]. The sodium sulfate column will remove any residual water, acetic anhydride, and basic compounds. The resulting AA sample was then analyzed with GC-MS experimentation.



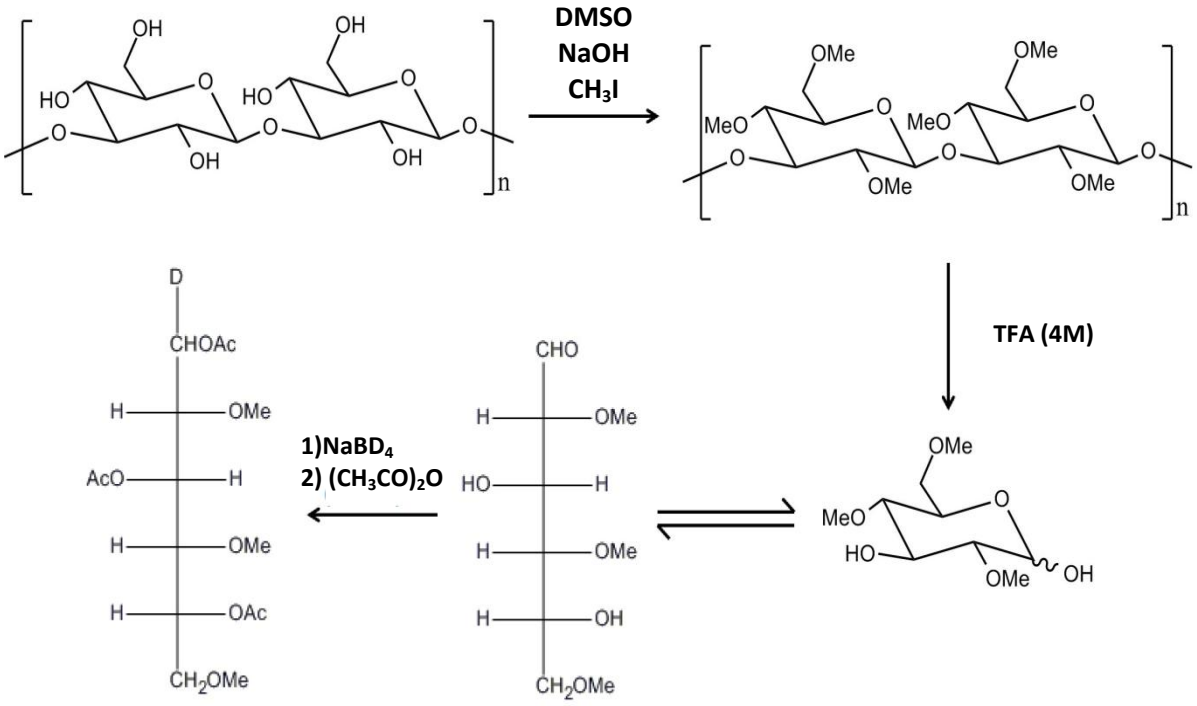
Scheme 3: Alditol acetate (AA) method reaction scheme

3.4 Monosaccharide Linkage Analysis

The determination of the monosaccharide linkages was carried out by using the partially methylated alditol acetate procedure (PMAA). PMAA utilizes similar sample preparation as AA, however, the hydroxyl groups that are not involved in a linkage will be methylated prior to the acetylation process. Methylation of the free hydroxyls will discern the linkage sites from the free sites on each monosaccharide.

Methylation was achieved by first dissolving ≥ 500 µg of sample into dry dimethyl sulfoxide (DMSO), and stirring overnight [41]. The next day, dried powdered Sodium hydroxide was added to the conical vial, acting as a strong base to generate

alkoxide ions [41]. Methyl iodine was then added and stirred into the vial for 3 hours, turning the solution milky white, generating the methylated derivatives [41]. After the mixing time was completed, the methylated sample was extracted by centrifugation using water and DCM. The methylated sample will stay in the organic DCM layer. DCM was then evaporated off and the AA procedure proceeded as normal. Once the PMAA was completed the sample was ready for GLC and/or GC-MS analysis to determine the linkage sites of the monosaccharide.



Scheme 4: Partially methylated alditol acetate (PMAA) method reaction scheme

3.5 Chromatography and Mass Spectrometry

The AA and PMAA samples were then analyzed by GC-MS experiments. A DB-17 column with an internal diameter of 30µm x 0.25µm, and a film thickness of 0.15µm

was used in a ThermoFinigan PolarisQ GC-MS. Ionization was achieved by electron impact (EI), and an ion trap mass analyzer was used for data generation. Helium was used as the carrier gas with a flow rate of 1.1mL/min. Retention times and fragmentation patterns of the monosaccharide derivatives, both AA and PMAA, can be used to determine the identity of the residues. Due to the partitioning between the mobile and stationary phases in the column molecules will be retained and eluted at different times. Retention times of molecules can be used to help identify the sugar residues as well.

Table 1: GC-MS Profile for Alditol Acetate Analysis

	Rate (°C/min)	Initial (°C)	Hold (min)
		37	0.1
Ramp 1	20	160	20
Ramp 2	20	200	22.3
Ramp 3	30	250	30

Table 2: GC-MS Profile for Permethylated Alditol Acetate Analysis

	Rate (°C/min)	Initial (°C)	Hold (min)
		37	0.1
Ramp 1	20	140	30
Ramp 2	20	180	40
Ramp 3	30	230	30

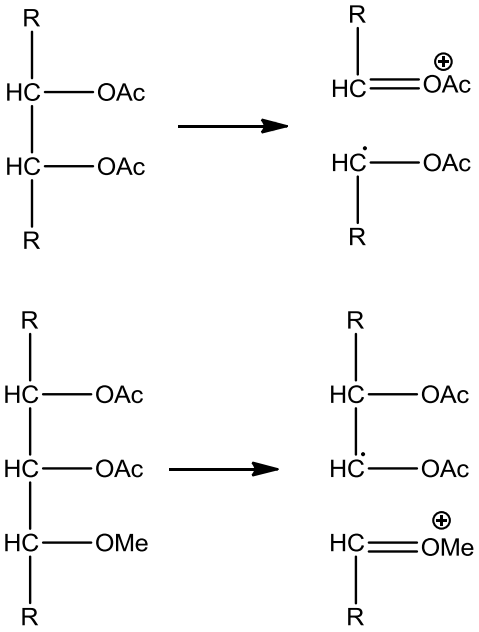
3.5.1 Alditol Acetate Analysis

The fragmentation patterns generated by EI ionization of the AA samples, give insight to the monosaccharide residues that are a part of the CPS. The fragmentation pattern will be characteristic of a sugar structure. The observed primary fragments come

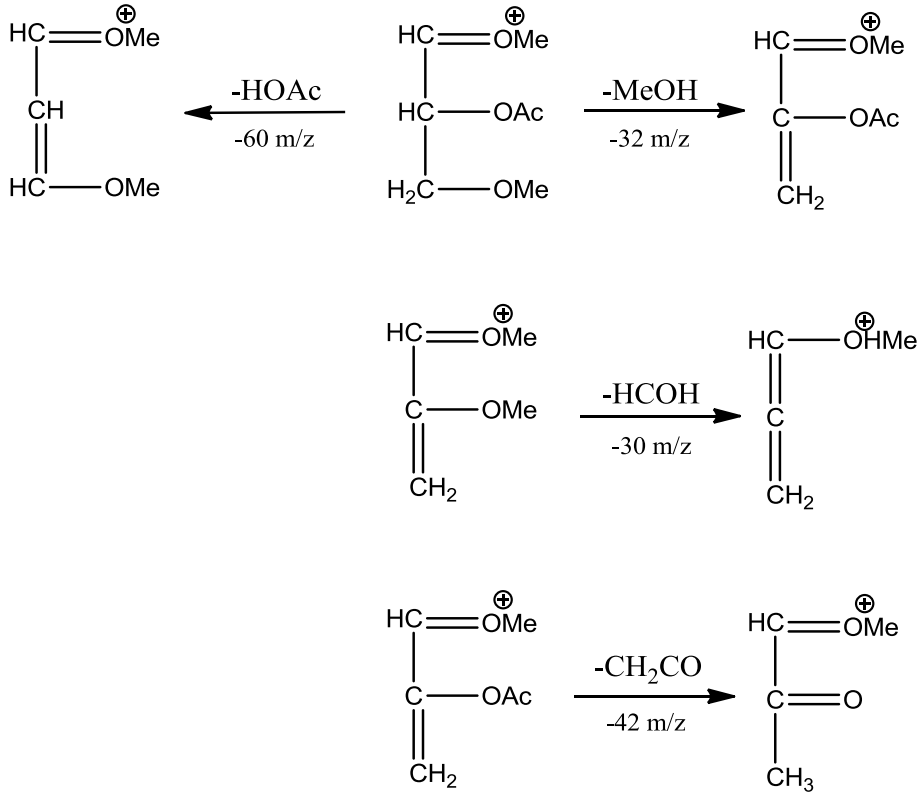
from the generation of radical cations via ionization. Radical cations will decompose by cleavage of carbon-carbon bonds in the sugar backbone, yielding the primary fragments. Since all of the positions of the AA sample are acetoxylated, they have the same probability of occurring. The primary fragments can undergo further fragmentation to secondary fragments, or daughter fragments. For AA, the sources of secondary fragmentation are the loss of acetic acid (-60 m/z) and the loss of ketene (-42 m/z). When cleavage occurs at an α or γ carbon, the loss of acetic acid is preferred.

3.5.2 Partially Methylated Aldiotol Acetate Analysis

The fragmentation patterns generated by EI ionization of the PMAA samples, give insight to the linkages that the monosaccharide residues that are a part of the CPS. The fragmentation pattern will be characteristic of a residue making specific linkages. As in AA, the observed primary fragments come from the generation of radical cations via ionization. Radical cations will decompose by cleavage of carbon-carbon bonds in the sugar backbone, yielding the primary fragments. In the PMAA sample there are both methlated and acetylated positions. The fragmentation will be favoured at the methoxylated carbons, due to an increased stability of the acetoxylated positions. There are an increased number of secondary fragmentation possibilities with the PMAA sample. The loss of methanol (-32 m/z), formaldehyde (-30 m /z), acetic acid (-60 m/z), ketene (-42 m/z), and acetoxyl groups (-59 m/z) are all possible.



Scheme 5: Primary fragmentation of an AA sample cleaves between a C-C bond between acetoxylated carbons. The PMAA primary fragmentation is preferentially at the C-C bond between two methoxylated carbons.



Scheme 6: Secondary fragmentation from AA and PMAA generated by a 70eV ionization source

3.6 Absolute Configuration

Typical GC columns cannot distinguish between enantiomers because both configurations interact similarly with the mobile and stationary phases of the column. Chiral columns can be used for this purpose, however, they are not necessary. A chiral diastereomeric acetate glycoside derivative analysis can also be used, and is used in the Monteiro laboratory.

The chiral diastereomeric acetate glycoside derivative analysis relies on the introduction of chiral centres to the C1 positions, of the sugars, through the addition of (S)-2-butanol and (R)-2-butanol. These diastereometric derivatives can be separated by GC-MS. Standards of the sugars are also prepared and run on the GC-MS to be used for comparison to the S and R derivatives of the CPS sample.

First, 0.5mg of purified CPS are hydrolyzed in 2mL of 2M TFA at 105°C for 2.5 hours. The TFA is then evaporated off under air at 45°C. The hydrolizates are then solvated in 0.2mL of (S)-2-butanol or (R)-2-butanol respectively, along with 0.25mL of pure TFA. This is heated at 100°C for 6 hours to allow for the chiral carbon of the secondary alcohol to become linked to the glycosidic oxygen, thereby differentiating between the D and L isomers. Evaporation of the sample occurs under air at 45°C overnight. Peracetylation of the derivatives is achieved using 0.3mL of 1:1 pyridene-acetic anhydride at 100°C for 1 hour. Following a final evaporation, the sample is extracted using DCM and a sodium sulphate column.

Each sugar will produce 4 peaks that represent the α and β anomers of both of the cyclic forms (pyranose and furanose). The GC profile must be inspected closely to

identify retention times of each peak. The sample GC trace is compared to the GC trace of a standard. If the sample trace matches the standard trace it can mean one of two things; the sample is the same as the standard and would have the same R or S and D or L chiral identifiers, or the sample is the enantiomer of the standard and would therefore have the opposite chiral identifiers.

3.7 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) can also be used to determine the sugar structure in the CPS. There are 1D and 2D experiments that allow for the sequencing and structure of the polysaccharide to be determined. The 1D – proton experiments were carried out on a Bruker AMX 400 MHz spectrometer at 273K. All other 1D and 2D experiments were carried out on a Bruker AMX 600 MHz spectrometer at 273K, equipped with a cryoprobe. All data was analyzed using Bruker Topspin 2.1 software. The samples were prepared using 3 exchanges with deuterated water (D_2O). Freezing and lyophilisation occurred between each exchange. The deuterated sample was dissolved in 600µL of D_2O for introduction to the NMR analysis tube. 3-trimethylsilyl-tetradeutro sodium propionate (TSP) is used as the proton standard, with a δ_H 0ppm.

1D proton NMR (^{1}H-NMR) is the first experiment used. It is targeting the hydrogen atoms present at each carbon position within a given sugar (ex. in a pyranose C1 – C6) [42, 43]. Proton NMR also indicates the number of anomerics present in the polysaccharide. The anomerics will book end the water peak around δ 4.4 – 5.5, with the alpha-anomer resonating downfield of the beta-anomer [44]. The number of anomeric protons observed will give insight to the number of monosaccharides in the polysaccharide structure. Another characteristic region is δ 1.6 – 1.9 where the deoxy

broad doublets are observed [45]. The ring protons will be found in the region of δ 3.0 – 4.3. The overlap in the ring region can allow for ambiguity, and therefore other 1D and 2D techniques must be utilized.

Correlation spectroscopy (COSY) is a 2D-NMR technique that illustrates the correlation of protons to one-another [42, 43]. By observing the cross-peaks that arise, the assignment of each proton will become easier. These cross-peaks are generated by the coherence transfer between protons. Correlation, however, is limited to protons within 2 to 3 bonds from one-another [42]. A proton can generate a singlet, doublet, triplet, quartet, doublet-of-doublets, or multiplet all depending on the number of protons in the immediate vicinity [42]. Starting from an identifiable proton, such as the anomeric or 6-deoxy, the ring region can be assigned step-wise [45]. Not all protons will be able to be assigned this way, and therefore more NMR experiments can be carried out.

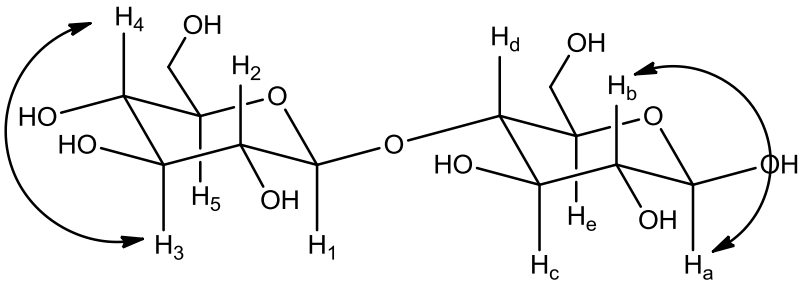


Figure 11: The ^{1}H-^{1}H COSY experiment shows correlations between protons that are adjacent to one another. For example; H_a to H_b and H_3 to H_4, in their respective ring systems.

Heteronuclear single-quantum correlation spectroscopy (HSQC) is a 2D-NMR technique that depicts the overlapping signals from 2 different nuclei that are one bond length apart [43, 46]. This overlapping allows for the accurate determination of H1 and

H6/H6’ by the known field areas for C1 and C6 in a sugar ring structure, which are δ_C 100 and δ_C 20 respectively [42]. This is the clearest way to determine the number of sugars in the polysaccharide sample.

Heteronuclear multiple bond correlation (HMBC) is another 2D-NMR technique that looks at correlations between 2 different nuclei. This technique differs from HSQC in that it can look across 2, 3 and sometimes 4 bonds between the nuclei. HMBC is often used to assign the linkages of monosaccharides as it can see across the glycosidic linkage between monosaccharides.

Total correlation spectroscopy (TOCSY) is a 1D-NMR technique that allows for all homonuclear correlations in a spin system to be determined. It is often helpful for solving the ambiguities in the ring region of the spectrum [47]. By irradiating a proton resonance, it can be seen which of the other protons are seen by the original irradiated centre. In some cases, the TOSCY spectra can be used to finalize the assignments made by the COSY and HSQC.

Nuclear Overhauser-Effect spectroscopy (NOESY) is a 1D-NMR technique that facilitates a means of obtaining conformational details of compounds in solution. A requirement of the technique is that two protons must be separated by 2-5Å. Even two axial protons can see each other as long as they are separated by the appropriate distance [46]. The peaks observed through NOESY are the cross-peaks of protons within the specified proximity, and can be protons from two attached molecules seeing one another. This allows for the assignment of linkages between two sugars. NOESY can also aid in the determination of anomeric configuration of the sugar residues [46]. From the cross-

peaks a better understanding of both linkage and orientation of molecules can be achieved [46].

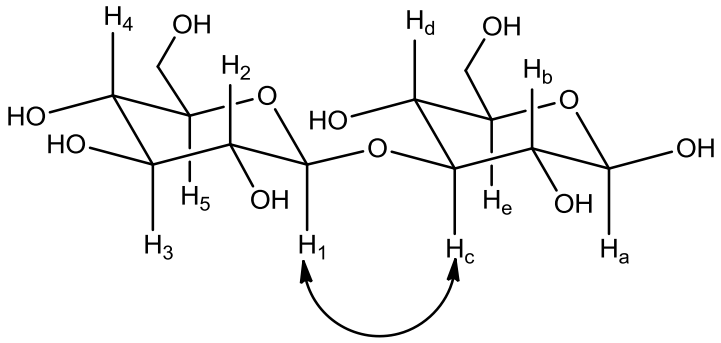


Figure 12: The ^{1}H-^{1}H 1D-NOESY shows the nuclear Overhauser effect connectivity between protons that are 2-5Å apart in distance. This experiment can uncover which protons are involved in linkages, for example the H_1 to H_c in the disaccharide depicted.

3.8 Immunogenicity Studies

An immunoblot study can be done to determine the immunogenicity of the CPS in mammals. New Zealand rabbits will be immunized 4 times, every 10 days, with 200µg of pure CPS combined with Complete Freund's Adjuvant. Serum can be extracted from the rabbits after 35 days. Purified *C. bolteae* CPS solution, 1mg/mL, can be applied onto nitrocellulose membrane in a 2µL volume. The membrane can then be blocked with 5% milk powder (w/v) in a Tris-buffered saline solution containing 0.05% Tween20 (v/v). The nitrocellulose membrane will then be incubated for 2 hours with the isolated rabbit serum at a 1:2000 fold dilution. Next, the membrane was incubated with a horseradish peroxidise-coupled goat-anti- rabbit IgG antibody at a 1:2000 dilution for 1 hour. Finally the membrane can be washed and incubated with TMB precipitating stain (SkyTek). Imaging of the stained membrane is performed with the BioRad XR+ imager.

Chapter 4: Results and Discussions

4.1 Sugar Compositional Analysis of Strain 16351

On the purified CPS a sugar composition analysis was carried out, via the alditol acetate (AA) procedure. Observed in the GC-MS data were four monosaccharide peaks at characteristic relative retention times. Retention times of 26.34, 27.47, 36.51 and 37.44 minutes were evident. Fragmentation patterns of the each peak, coupled with the relative retention times, allowed for the identification of each of the peaks. Respectively the peaks were found to be rhamnose (Rha), ribose (Rib), mannose (Man) and galactose (Gal).

The Rib component observed in the AA results is attributed to residual bacterial RNA. The Gal component can also be attributed to the fact that most bacterial cells will give rise to both Glu and Gal as a large part of the cell mass. The remaining two peaks, of Rha and Man, had to be looked at in greater detail to confirm the assignments.

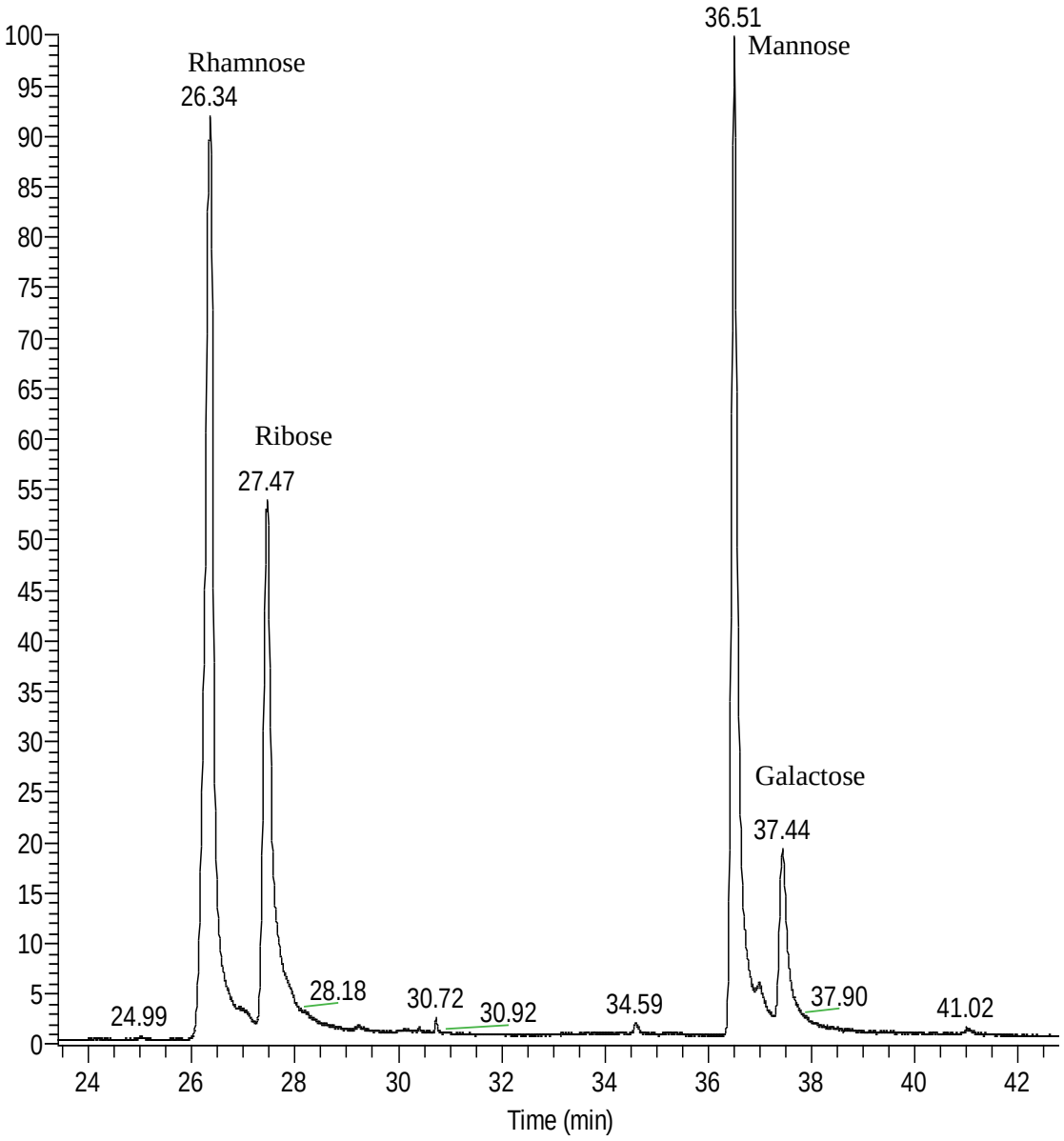


Figure 13: GC elution profile of CPS of *C. bolteae*

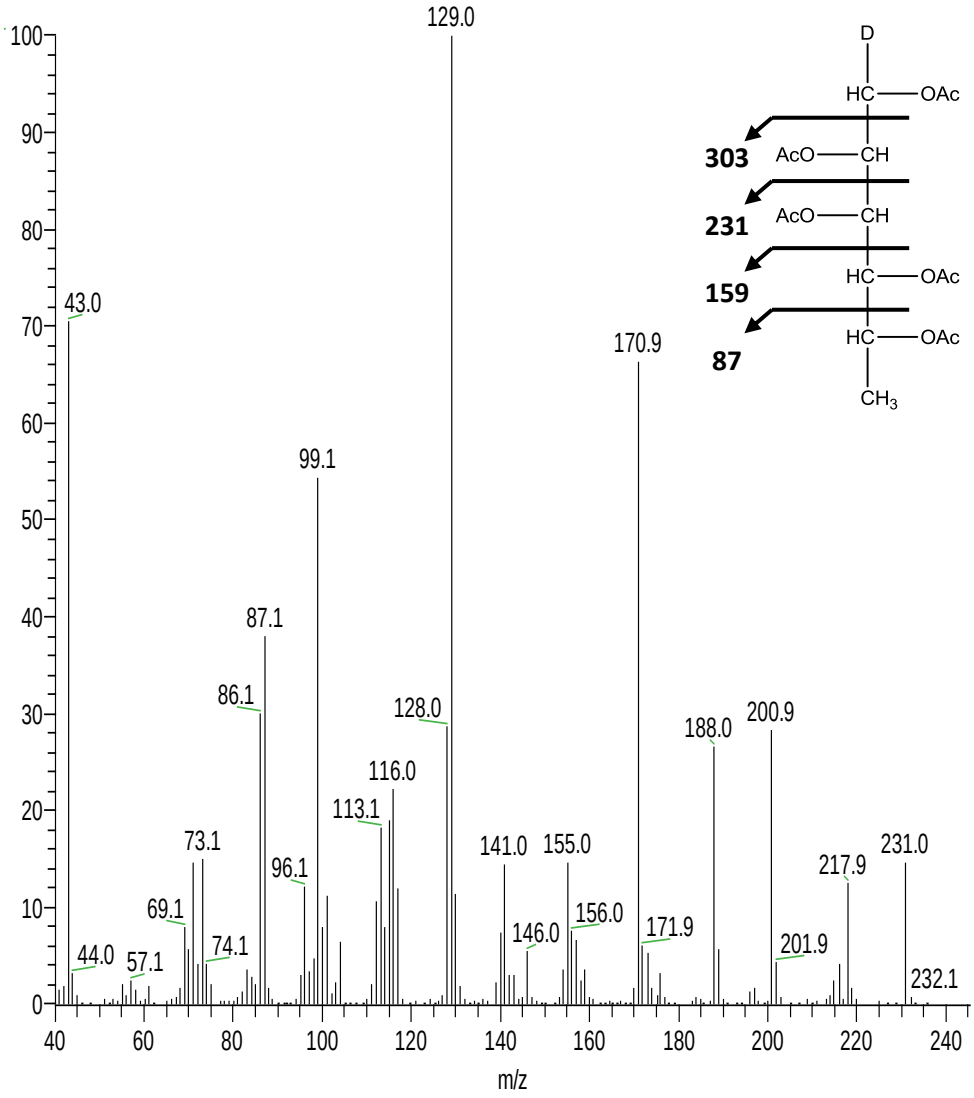


Figure 14: Secondary fragmentation by GC-MS of the Rhamnose residue.

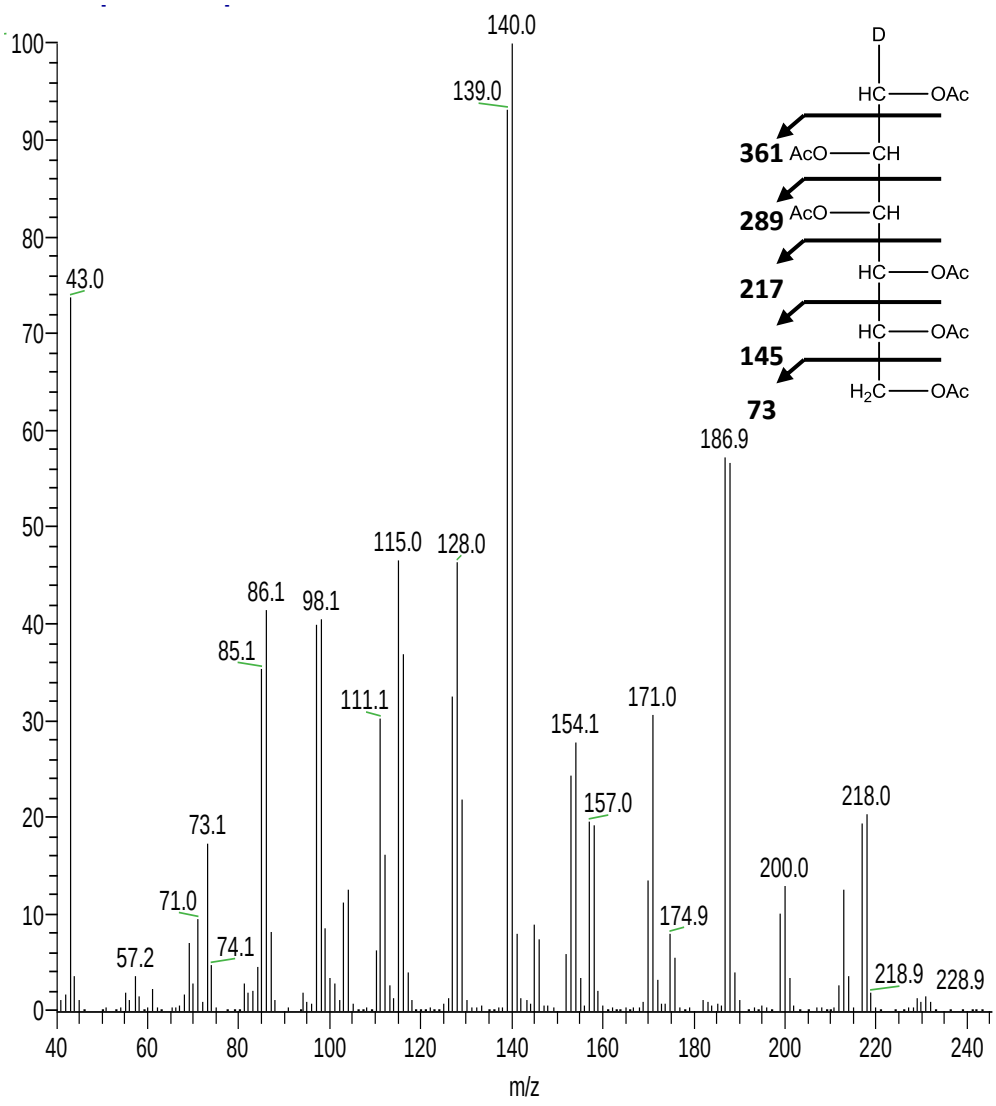


Figure 15: Secondary fragmentation by GC-MS of the Mannose residue.

The fragmentation of the Rha residue contained the characteristic intensities of 87, 99, 129, 171, and 201 m/z (Table 3). This fragmentation pattern differs from monosaccharides, such as Glu, Gal and Man, because of the 6-deoxy. The C6 position in a 6-deoxy will not be able to be acetylated giving the fragments different m/z values than an un-substituted hexose.

Table 3: High intensity fragments from the peak located at a retention time of 26.26min. The fragment loss of 60 is attributed to the loss of a ketene, and fragment loss of 42 is attributed to the loss of acetic acid.

Peak (m/z)	Primary Fragment	Fragment Loss
87	87	N/A
99	159 303	-60 -60, -60, -42, -42
129	231	-60, -42
171	231	-60
201	303	-60, -42

These fragmentations could have been attributed to any other 6-deoxy-heptose. In order to confirm that the residue in question was Rha, the relative retention time was compared to retention times found in literature and previously determined in the lab. The retention of 26.26 min was close to the value seen of 26.10 min for Rha residues.

The fragmentation of the Man residue also showed characteristics intensities of a hexose. These characteristic intensities were; 43, 139, 140, 187, 188, 259, and 260 m/z (Table 4). Since all hexoses would have the same characteristic fragmentation the relative retention time had to be used to determine that this was in fact Man. The retention time of 36.47 min was very close to the 36.40 min seen in laboratory experimentation.

Table 4: High intensity fragments from the peak located at a retention time of 36.40min. The fragment loss of 60 is attributed to the loss of an acetic acid, and fragment loss of 42 is attributed to the loss of ketene.

Peak (m/z)	Primary Fragment	Fragment Loss
139	361	-60, -60, -60, -42
140	362	-60, -60, -60, -42
187	289	-60, -42
188	290	-60, -42
259	361	-60, -42
260	362	-60, -42

Once the composition of monosaccharides in the CPS was determined the arrangement of these monosaccharides was of interest. To determine the arrangement of the residues, the linkage analysis is used.

4.2 Sugar Linkage Analysis

Purified CPS was subjected to PMAA and this allowed for the linkages to be determined. GC-MS analysis showed major intensities at 31.57min, 40.26min and 41.74min. These retention times, respectively, would be indicative of 4-substituted Rha, a 2-substitded Glc and a 3-substituted Man. Since Glc was not observed in the AA of the purified crude sample it could be attributed to a contaminant, and this would be confirmed by NMR experiments.

The fragmentation of the peak at 31.57min was analyzed further to confirm that the peak could be attributed to the 4-subsituted Rha residue. Observation of the major fragmentation peaks uncovered that the assignment was correct. Notable fragments include; 101, 118, 143, 203, and 247 m/z (Table 5). These fragments are typical of a 6-deoxy hexose that has a linkage at the 4 position.

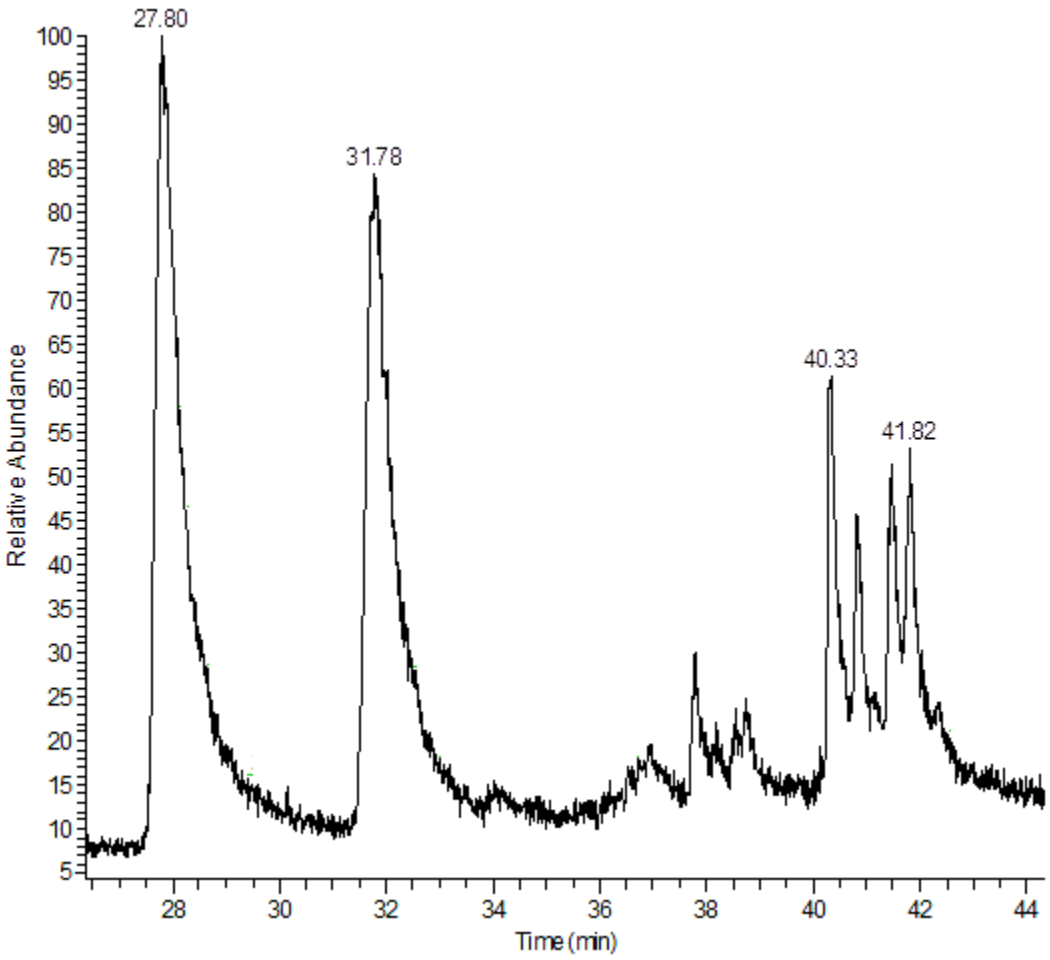


Figure 16: GC elution profile of the PMAA prepared CPS.

Table 5: High intensity fragments from the peak located at a retention time of 31.57min. The fragment loss of 60 is attributed to the loss of a ketene, and fragment loss of 42 is attributed to the loss of acetic acid.

Peak (m/z)	Primary Fragment	Fragment Loss
101	203	-60, -42
118	N/A	N/A
143	203	-60
203	N/A	N/A
247	N/A	N/A

The intensity at 41.74 min could also be confirmed to be a 3-subsituted hexose. Characteristic fragmentation was observed for this residue. Peaks at: 45, 129, 161, 202, 234, and 277 m/z (Table 6), were evidence of a correct assignment. To determine that the hexose was Man, a relative retention time collected in the lab was compared to the observed retention time. The retentions were comparable and based on the AA GC/MS results the residue was assigned to be a 3-substitued Man.

Table 6: High intensity fragments from the peak located at a retention time of 41.74min. The fragment loss of 32 is attributed to the loss of a methanol group.

Peak (m/z)	Primary Fragment	Fragment Loss
45	N/A	N/A
129	161	-32
161	N/A	N/A
202	234	-32
234	N/A	N/A
277	N/A	N/A

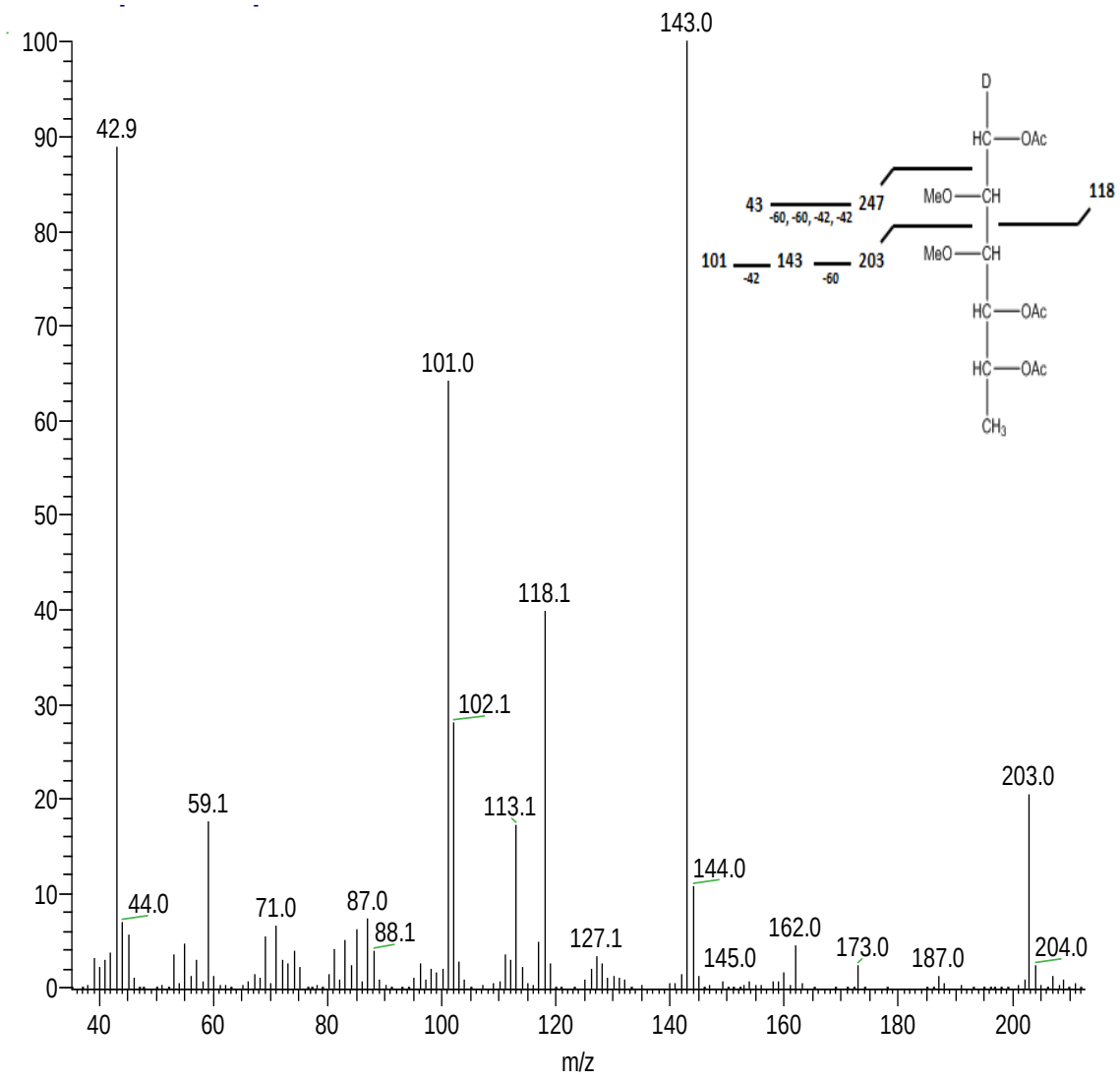


Figure 17: Secondary fragmentation of the intensity at 31.57min by GC-MS.

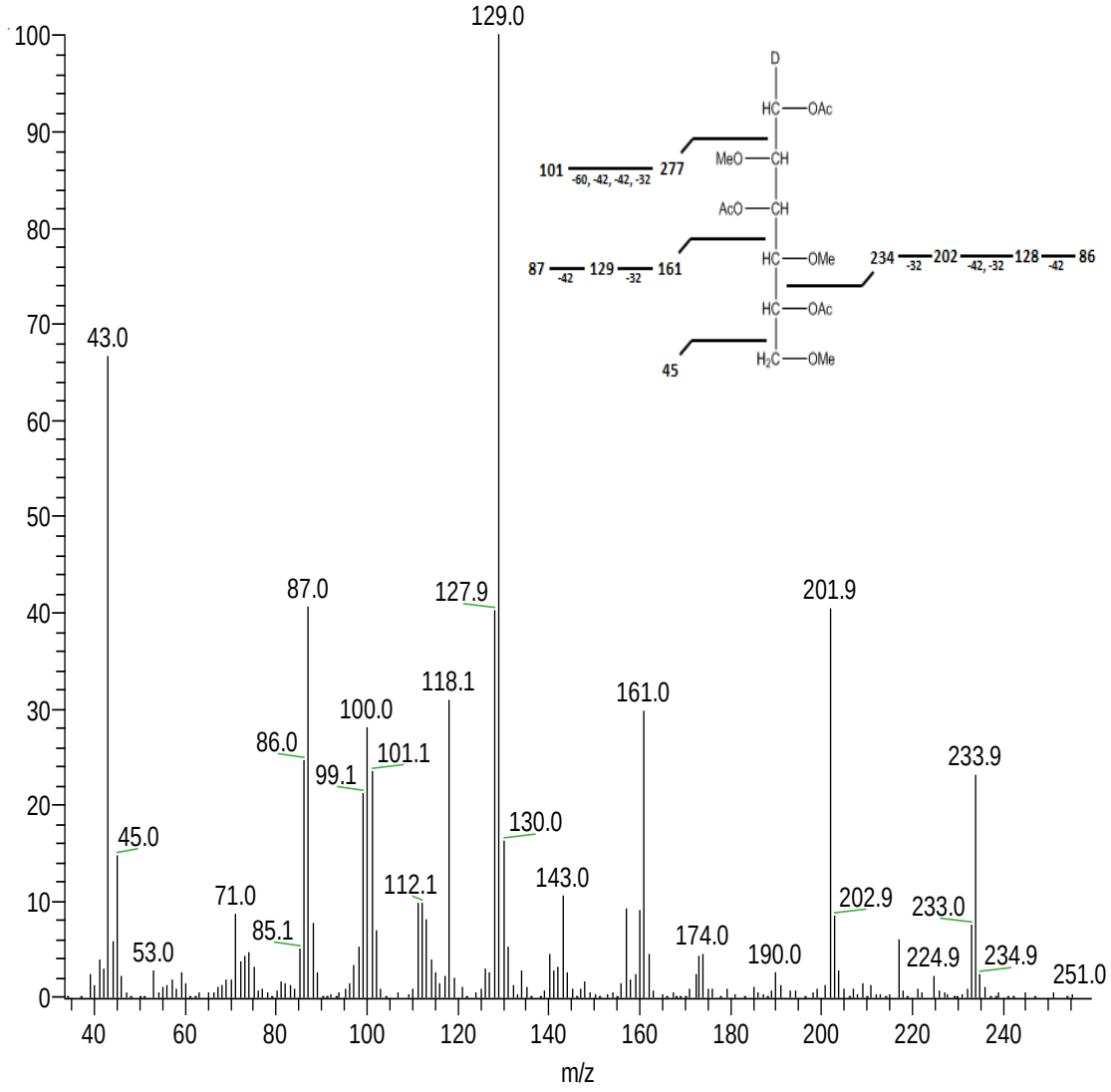


Figure 18: Secondary fragmentation of the intensity at 41.74min by GC-MS.

4.3 Nuclear Magnetic Resonance (NMR)

4.3.1 Proton

The 1D ^{1}H NMR spectra gives insight into the proton configuration of the purified CPS. This information coupled with the GC-MS data collected from both AA and PMAA samples will ultimately give rise to the composition and configuration of the CPS.

Different chemical shift regions in the 1D ^{1}H spectra are characteristic of specific proton resonances. Due to the sample being analyzed in deuterated water (D_2O), there is a significant resonance contribution at δ_H 4.800 ppm referred to as the HOD. Often this is a broad intensity that can overpower any resonances that are of a similar shift. The anomeric resonances, for example, can be found in a similar shift as the HOD, with the α configuration being between δ_H 4.90 – 5.30, and the β configuration at δ_H 4.30 – 4.90. Temperature variation during the collection of 1D ^{1}H NMR can be used to shift the HOD further upfield to reveal any hidden anomeric resonances. In the region of δ_H 3.00 – 4.20 there will be resonances found that are attributed to the ring protons of the monosaccharides. The peaks found here are often overlapping one another and hard to distinguish and assign from the 1D ^{1}H alone. This overlap and uncertainty is due to the fact that the ring protons are all methylene. Other substitutions on monosaccharides can also be observed, such as a sharp singlet at δ_H 2.00 -2.20 for N-acetyl-glucosamine and a broad doublet between δ_H 1.10 - 1.30 for 6-deoxy-hexose residues (NMR 1992).

The 1D ^{1}H NMR spectra showed that there are two anomeric resonances at δ_H 4.90 and δ_H 5.30. The δ_H 4.90 resonance was determined to be a β configuration due to it being shifted upfield in the spectra. The δ_H 5.30 anomeric resonance is attributed to an α

configuration. Multiple resonances in the ring region were also observed but unable to be assigned from only the information provided from the proton scan. Further upfield at δ_H 1.34 a broad doublet was evident. This doublet is in the region where methyl (CH_3) proton resonances are often observed. This spectral data is consistent with of one of the two ring systems being a 6-deoxy-monosaccharide. The Rha residue, seen in the AA analysis, is a 6-deoxy-mannose, and would therefore account for the δ_H 1.34 doublet.

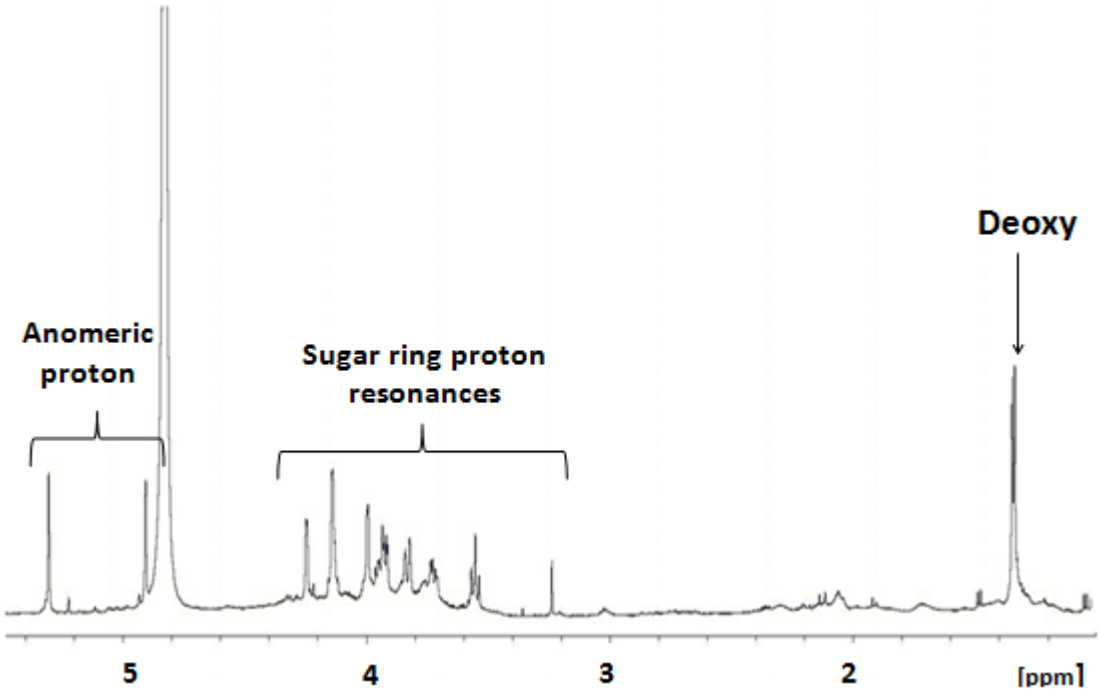


Figure 19: Preliminary ^{1}H-1D NMR spectrum. Known characteristic regions for monosaccharide chemical shifts are labelled.

4.3.2 COSY

The 2D ^{1}H-^{1}H spectra allows for the assignment of the protons in the observed ring systems of the isolated CPS. Based on the 1D ^{1}H spectra there are two anomeric resonances at δ_H 5.30 and δ_H 4.90. These resonances are used to determine the other ring protons for the two monosaccharides in the CPS. The α anomeric, at 5.30 ppm, will be henceforth referred to as the A system, and the β anomeric, at 4.90 ppm, will be the B system.

Looking first at anomeric resonance A1, there is a cross peak seen with the resonance at δ_H 4.24. This cross-peak gives the relationship between the anomeric proton and the proton on carbon two of system A. This resulted in the proton resonances of A1-A5 being assigned as follows: δ_H 5.30, δ_H 4.24, δ_H 4.13, δ_H 3.72 and δ_H 3.82 ppm. The assignment of both of the A6/6$^{'}$ protons could not be made based on the data collected from the COSY alone. System A did not have any relation to the deoxy resonance, resulting in A being assigned as the Man residue.

The COSY also provided information for the B system. Starting with the anomeric resonance B1, the proton resonances for B2, B3, B4, and B6 could be assigned. Starting from the anomeric resonance the chemical shifts for, B1-4 and B6, respectively were; δ_H 4.90, δ_H 3.99, δ_H 3.92, δ_H 3.55, and δ_H 1.34 ppm. System B, since it contained the peak at δ_H 1.34, can be assigned as the 6-deoxy-hexose, Rha residue. The B6 resonance has a cross-peak with B3, this leads to the assumption that B5 may in fact be at a similar chemical shift as B3. Further NMR analysis will be needed to confirm that B5 is in fact at the assumed chemical shift.

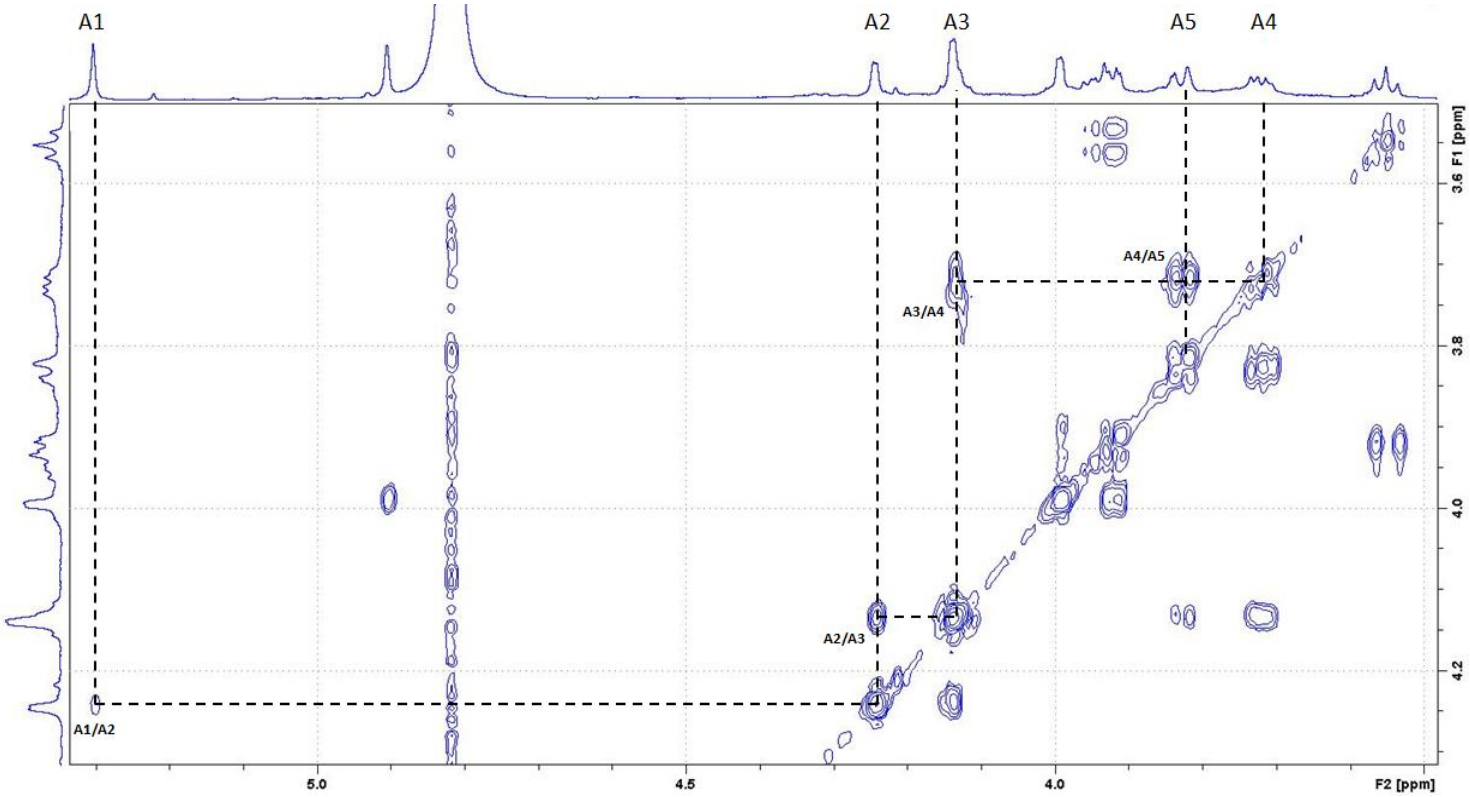


Figure 20: ^{1}H-^{1}H COSY of the ring system A. Proton resonances of A1-A5 assigned as follows: δ_H 5.30, δ_H 4.24, δ_H 4.13, δ_H 3.72 and δ_H 3.82

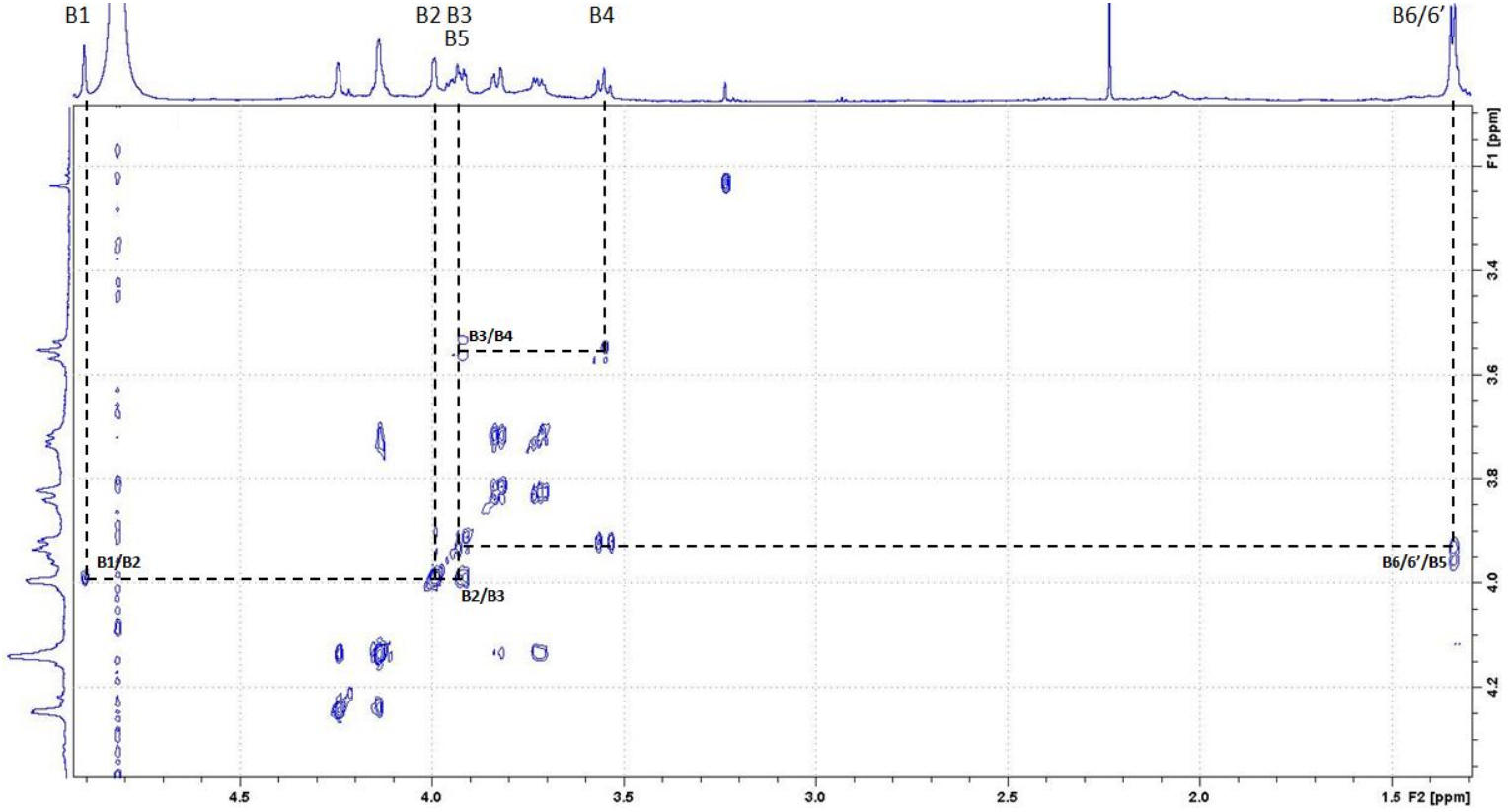


Figure 21: ^{1}H-^{1}H COSY of ring system B. Proton chemical shifts for, B1-4 and B6, respectively were; δ_H 4.90, δ_H 3.99, δ_H 3.92, δ_H 3.55, and δ_H 1.34

4.3.3 TOCSY

Since not all the proton resonances could be assigned from the 1D – ^{1}H alone, further 1D – NMR experiments were required. A TOCSY experiment will provide information as to which protons see each other in the contained ring system.

The irradiation of the A1 resonance showed correlation between the peaks already previously assigned as A2, A3, A4, and A5 (Figure 22). Further confirmation of the assignments being correct for the proton systems came from irradiating both A2 and A3, respectively seeing A1, A3, A4, A5, and A1, A2, A4, A5 (Figure 23, Figure 24). The A6/6' were still unable to be assigned from the secondary 1D experiment.

The irradiation of the B1 resonance also allowed for the confirmation of the ring proton assignments. The anomeric B1 resonance showed correlation between the peaks previously found to be B2, B3, B4, and B6 (Figure 25). The peaks that were assigned both B2 and B4 also confirmed the previous assignment in the B system. The deoxy B6 resonance gave correlation peaks for the previously assigned B1, B2, B3 and B4 resonances (Figure 26). These TOCSY results confirmed that both ring system protons were properly assigned to the Man and Rha residues.

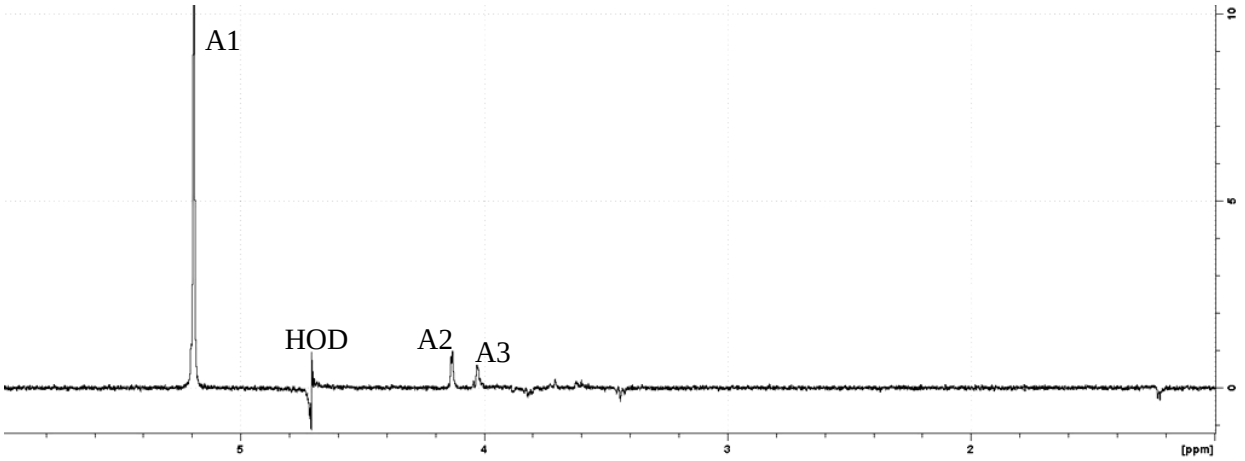


Figure 22: TOCSY depicting the irradiation of the Man anomeric (A1). The irradiation shows that A1 sees A2 and A3 in the Man ring structure.

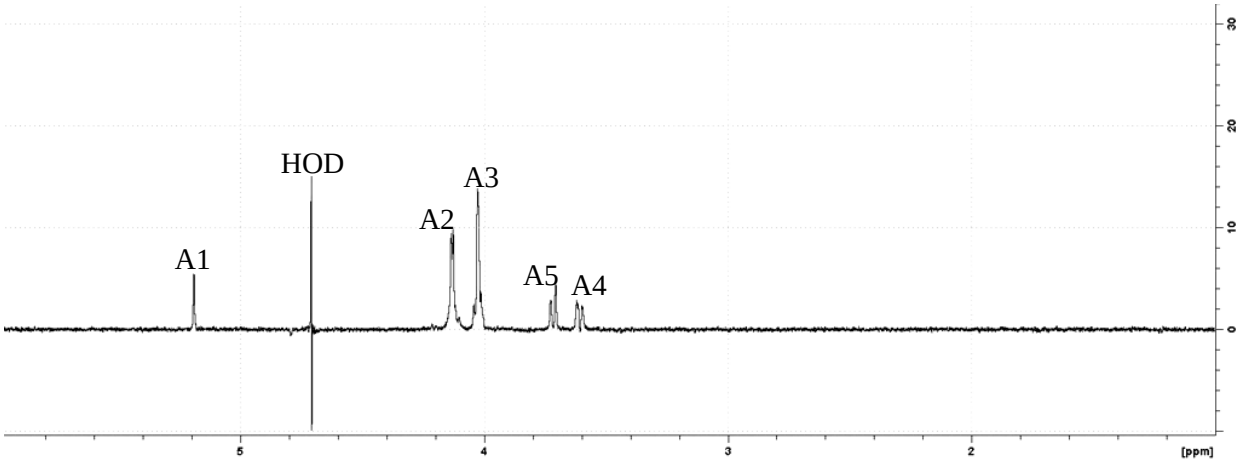


Figure 23: TOCSY depicting the irradiation of the Man proton at position 2 (A2). The irradiation shows that A2 sees A1, A3, A4 and A5 in the Man ring structure.

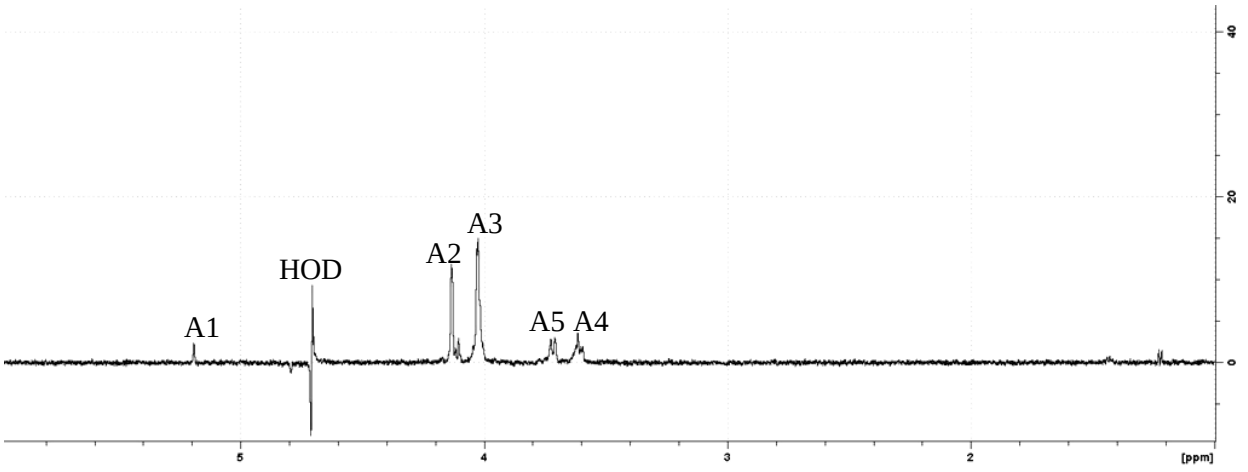


Figure 24: TOCSY depicting the irradiation of the Man proton at position 3 (A3). The irradiation shows that A3 sees A1, A2, A4 and A5 in the Man ring structure.

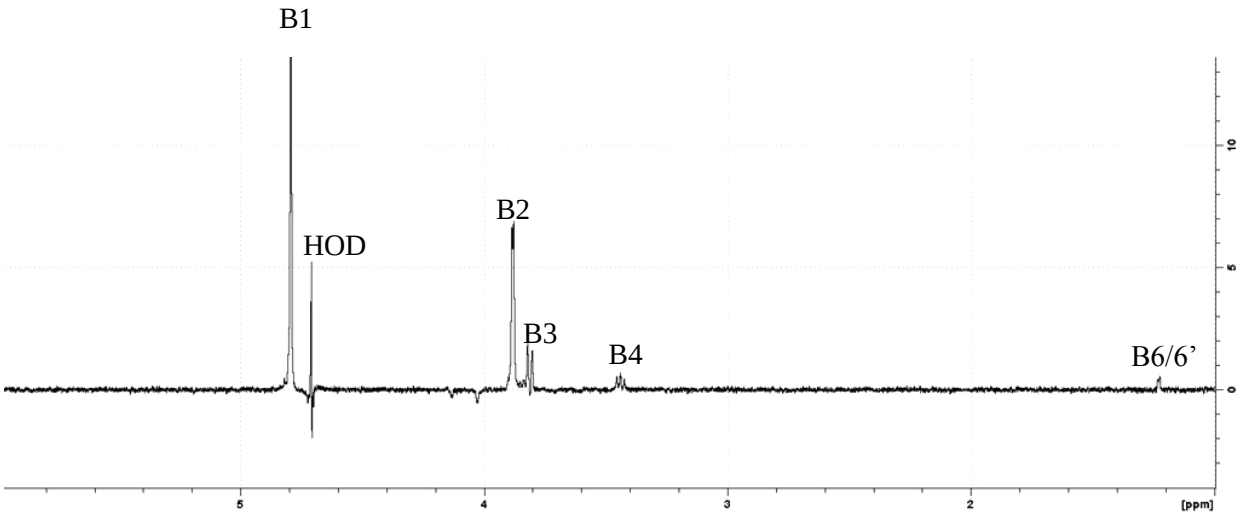


Figure 25: TOCSY depicting the irradiation of the Rha anomeric proton (B1). The irradiation shows that B1 sees B2, B3, B4 and B6/6’ in the Rha ring structure.

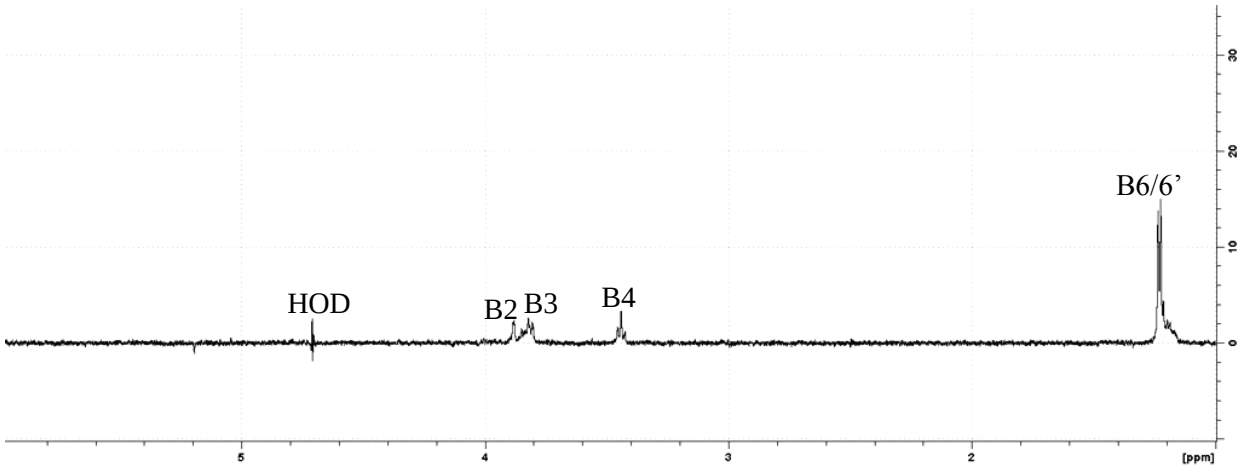


Figure 26: TOCSY depicting the irradiation of the Rha deoxy protons (B6/6’). The irradiation shows that B6/6’ sees B2, B3, and B4 in the Rha ring structure.

4.3.4 HSQC

The ^{1}H-^{13}C HSCQ allows for the assignment of the carbon resonances for the monosaccharide ring structures. It is helpful for this experiment to know characteristic chemical shifts for carbon in the ring. Anomeric carbons are typically at δ_C 90-112 [45], and 6-deoxy carbons are typically seen at approximately δ_C 20. Knowing these ranges, combined with knowing the proton assignments from the COSY, the carbons can be assigned.

The proton resonances of the Man residue (A system) displayed corresponding carbon chemical shifts at δ_C 111.1, δ_C 76.8, δ_C 80.1, δ_C 72.4, δ_C 65.3, and δ_C 65.2 ppm (Figure 27). Two carbon resonances were observed underneath the A4 proton shift at δ_C 65.2 and δ_C 72.4. Another observation in the carbon shifts was that the A5 had two cross peaks at δ_C 65.3. These two unique cross peaks are notable as they can be attributed to the A6/6’ resonances previously unassigned. Since A6 and A6’ are attached to the same

carbon they would correspond to a carbon with the same chemical shift, which is why the cross peaks at δ 3.82/65.3 and δ 3.17/65.2 correspond to A6/6'. These carbon-proton cross peaks allow for the completion of the assignment of the Man ring system, Table 7.

The Rha residue (system B) also had defined carbon chemical shifts that could be assigned. These resonances were located at δ_C 103.6, δ_C 73.2, δ_C 72.8, δ_C 82.1, δ_C 70.6, and δ_C 19.8 ppm. The β-anomeric (B1) correlated to the δ_C 103.6, ^{13}C resonance. Another expected correlation was between the B6/6' resonance and the ^{13}C shift of δ_C 19.8, characteristic of $-CH_3$ chemical shifts. The ring protons all had associated carbons between δ_C 70 and δ_C 82. Under the previously characterized B3 resonance there are two observed carbon-proton cross-peaks. One of the carbon-proton cross-peaks is likely due to the B5 proton being at a similar chemical shift to B3. This will need to be confirmed by further NMR experiments. The complete ring assignment was made, and is found in Table 7.

Table 7: ^{1}H-^{13}C HSQC proton and carbon assignments for the disaccharide unit.

Residue	**H_1** **C_1**	**H_2** **C_2**	**H_3** **C_3**	**H_4** **C_4**	**H_5** **C_5**	**$H_{6/6'}$** **$C_{6/6'}$**
α – Man	5.30 111.1	4.24 76.8	4.13 80.1	3.72 72.4	3.82 65.3	3.82/3.71 65.3/65.2
β – Rha	4.90 103.6	3.99 73.2	3.92 72.8	3.55 82.1	3.93 70.6	1.34 19.8

Note: all chemical shift reported are in ppm.

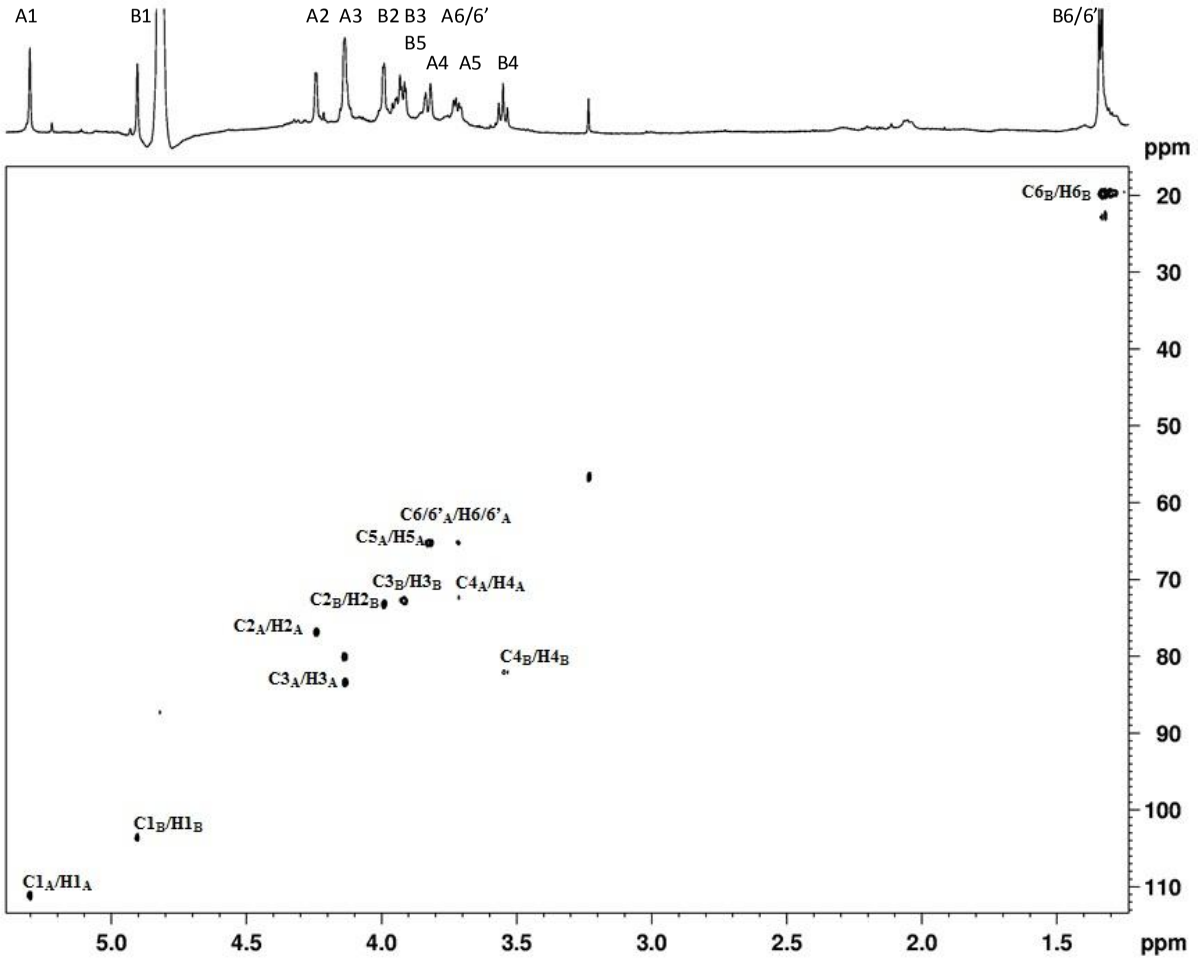


Figure 27: ^{1}H-^{13}C HQSC spectra showing the correlations between carbons and their respective protons. Where A denotes the Man residue, and B denotes the Rha reside.

4.3.5 HMBC

The MS data collected from the PMAA preparation of the disaccharide showed 4-subsituted Rha and 3-substitued Man. The results collected from the 2D-HMBC experiment solidify the assignment of these linkages. Since the HMBC can see 2-4 bonds between heteronuclear correlations any linkage between two monosaccharides, as well as the carbon-proton bonds in a ring system, can be observed. The carbon shifts of interest would be the anomeric resonances previously observed at δ_C 111.1 and δ_C 103.6, for Man and Rha respectively.

Looking at Figure 28, there are evident cross-peaks at δ 3.55/111.1 and δ 4.13/103.6. HSQC results determined δ 111.1 to be the Man anomeric carbon, and 1D-^{1}H results gave the δ 3.55 chemical shift the assignment of H4 of Rha. The cross-peak is therefore attributed to H_B4/C_A1 (Figure 28) The same deductive logic can be applied to the cross-peak at δ 4.13/103.6, giving it the overall assignment of H_A3/C_B1 (Figure 28).

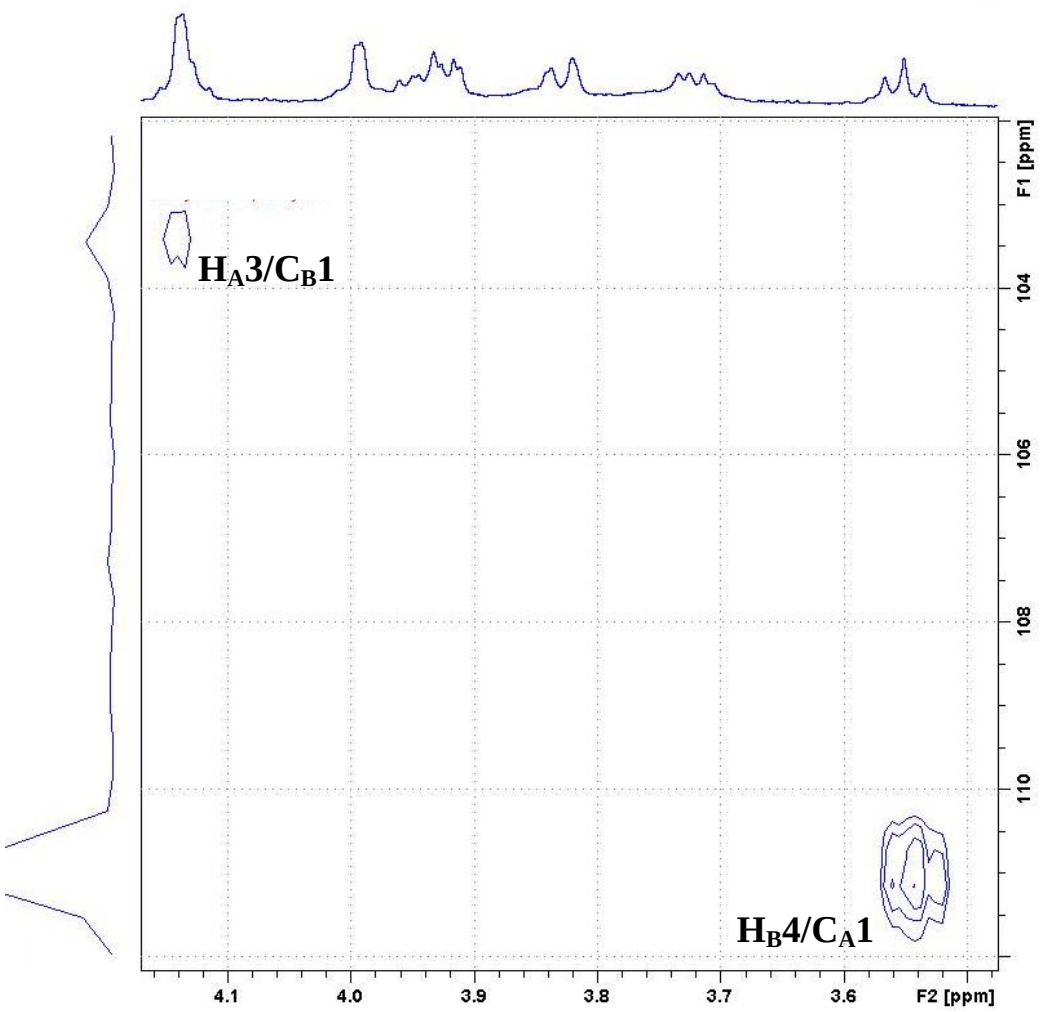


Figure 28: ^{1}H-^{13}C HMBC spectra showing the correlation between the anomeric carbon and the proton involved in the linkage site. Where A denotes the Man residue, and B denotes the Rha reside.

4.3.6 NOESY

The 1D-NOESY experiment was the final confirmation of the configuration of the monosaccharides in the disaccharide repeat unit. Each of the peaks identified from the proton experiment were irradiated and the nuclear-Overhauser effects were observed.

Starting with the Man residue, the anomeric proton A1 was irradiated. The resulting spectral data revealed that A1 has strong inter-NOE connections with A2, B4, and B6/6'. Weaker connections are observed with A3 and B5 as well. The previous conclusion that Rha was 4-substitued is solidified here with the strong interaction between A1 of Man and B4 of Rha.

The Rha residue proton resonances were also irradiated. The anomeric proton B1 displayed strong inter-NOE connectivity to A3 and B2. The connectivity to A3 confirms the assignment of the 3-substituted Man linking to the Rha residue. The B6/6' residue, when irradiated, showed strong inter-NOE connectivity to A1, A2, A3, B4, and the peak assigned B3 previously. The connectivity to the B3 peak confirms that the second carbon-proton cross-peak seen underneath the B3 chemical shift, seen in the HSQC, can be attributed to the B5 proton. Also, the interesting connectivity between A1, A2, A3 gives insight as to the absolute configuration of the Rha. Only a specific orientation of the Rha in the D-isomer would give rise to the inter-NOE connectivity observed. However, this speculation needs to be confirmed by chiral glycosides.

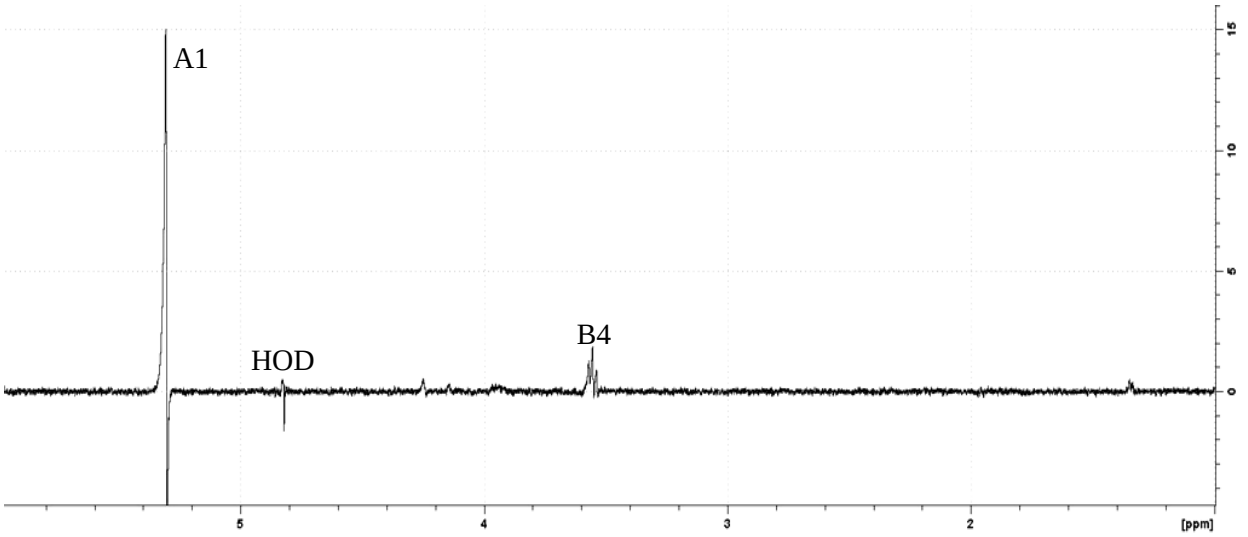


Figure 29: NOESY depicting the irradiation of the mannose anomeric proton (A1). The irradiation shows that A1 sees the A2 resonance as well as the rhamnose proton 4 (B4) and a small connectivity to B6/6', confirming the glycosidic linkage of Man(1→4)Rha.

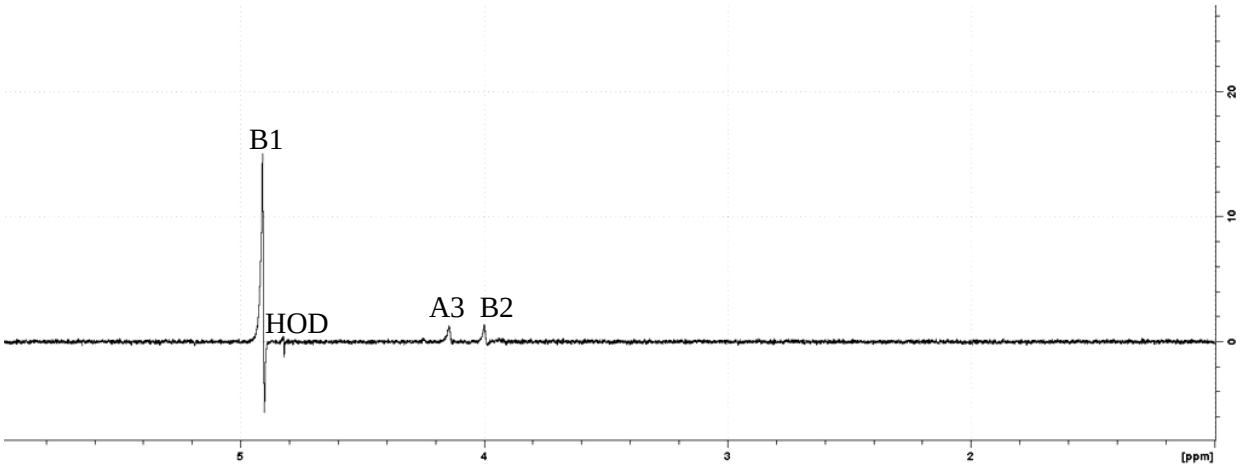


Figure 30: NOESY depicting the irradiation of the rhamnose anomeric proton (B1). The irradiation shows that B1 sees the B2 resonance as well as the mannose proton 3 (A3), confirming the glycosidic linkage of Rha(1→3)Man.

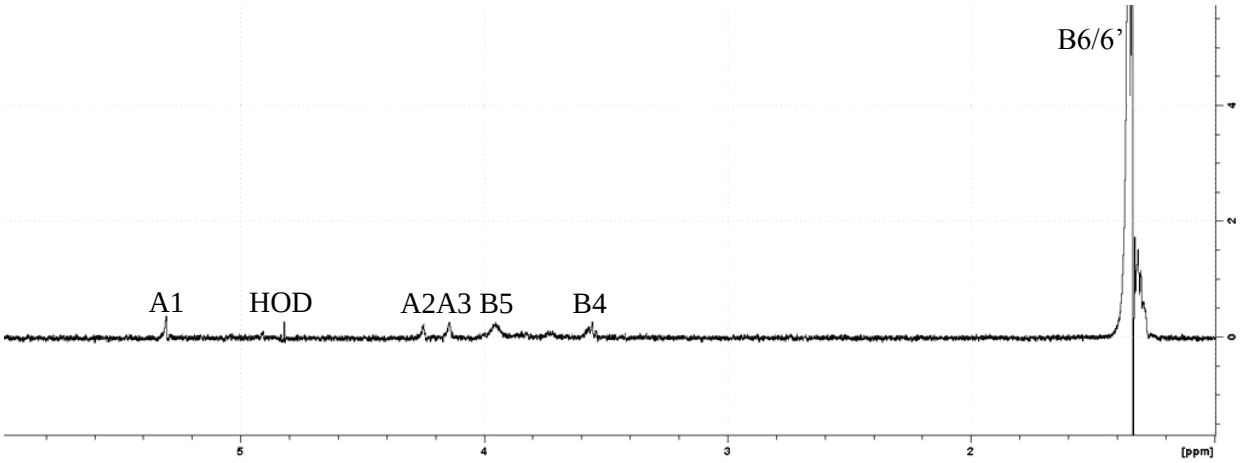


Figure 31: NOESY depicting the irradiation of the rhamnose 6-deoxy protons (B6/6'). The irradiation shows that B6/6' sees the B4 and B5 resonances as well as the mannose anomeric proton (A1), A2 and A3. This irradiation helps to confirm that the rhamnose is in the D form.

4.4 Absolute Configuration

Chirality of the monosaccharides is important to determine as it can affect both the efficacy and binding properties of the polysaccharide. Since Man and Rha can be found in either the D or L-enantiomer the chirality of each monosaccharide needed to be checked.

The most commonly observed conformation of most hexapyranose is the D form. Man residues are almost always in this form. The 6-dexoy-hexapyranose residues, including Rha, are most commonly found in the L form. From the chiral glycoside data it was observed that the Man was in fact the D-enantiomer, as was to be expected. Unexpectedly, however, it was seen that the Rha residue was also in the D conformation.

Looking at literature it was seen that other bacteria also are found to produce this rare monosaccharide conformation. One example is the A band lipopolysaccharide (LPS) of *Pseudomonas aeurginosa,* which is composed of a D-Rha repeat unit [48]. This A band

D-rhamnan production is attributed to the conversion of the GDP-D-Man nucleotide precursor to GDP-D-Rha [49].

For further evidence that the Rha is in fact in the D-enantiomer form the 1D-NOESY data was revisited. The irradiation of B6/6' showed inter-NOE connectivity to both A2 and A3 (figure 31). Also, seen in the 1D-NOESY are the inter-NOE interactions between both B3 and B5 with the A1 resonance (figure 29). These inter-NOE relationships point to the disaccharide having the D-enantiomer of Rha (Figure 32).

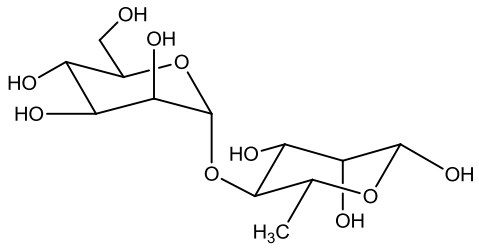


Figure 32: Disaccharide repeat unit of *C. bolteae*. The Rha residue is in the D conformation and rotated 180° in order to give the NOE-interconnectivities observed.

4.5 Strain 14578

After the characterization of the first strain, 16351, a second human isolate strain, 14578, was purified. The purified sample was analyzed by GC-MS and NMR. Resulting spectra from the second strain were identical to the data that was collected from 16351. Looking at the fact that both strains produced the same spectral data it was determined that strain 14578 has the same disaccharide on the cell surface as that of 16351. The data from the immunogenicity study was collected from samples that were from both strains, as both would require the same conjugate-vaccine. This is an important finding in that only one vaccine would be needed to be generated for prevention of two different human isolate strains.

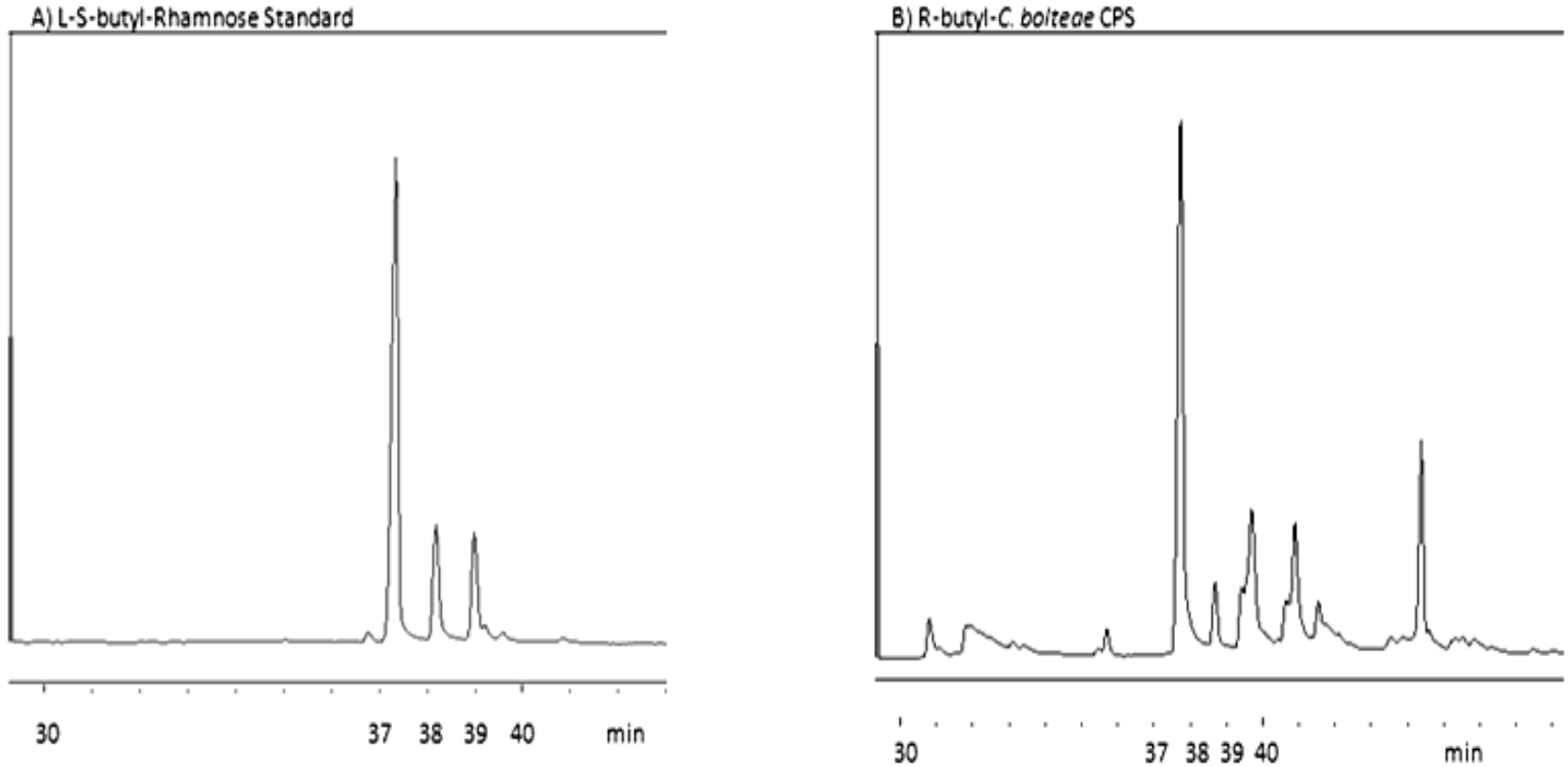


Figure 33: Chiral glycoside analysis of *C. bolteae* CPS. A) The L-S-butyl-Rhamnose standard, having major peaks at approximately 37, 38, and 39 minutes. B) The R-butyl-*C. bolteae* CPS also has major peaks at approximately 37, 38, and 39 minutes, and with similar intensities. Therefore, it was confirmed that the *C. bolteae* CPS contains a D-Rha residue.

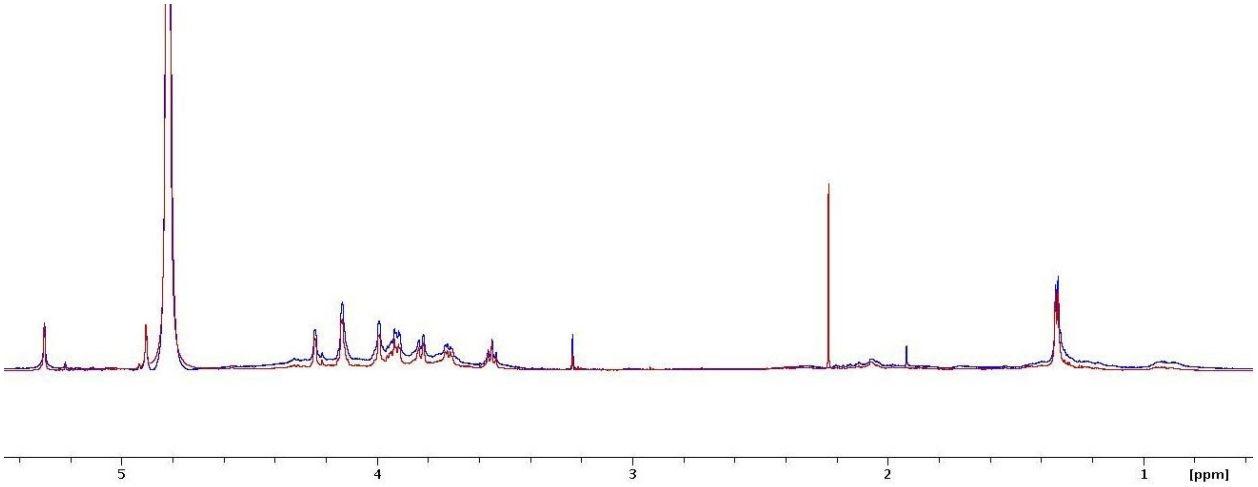


Figure 34: 1D-^{1}H overlay of strain 16351 and 14578.

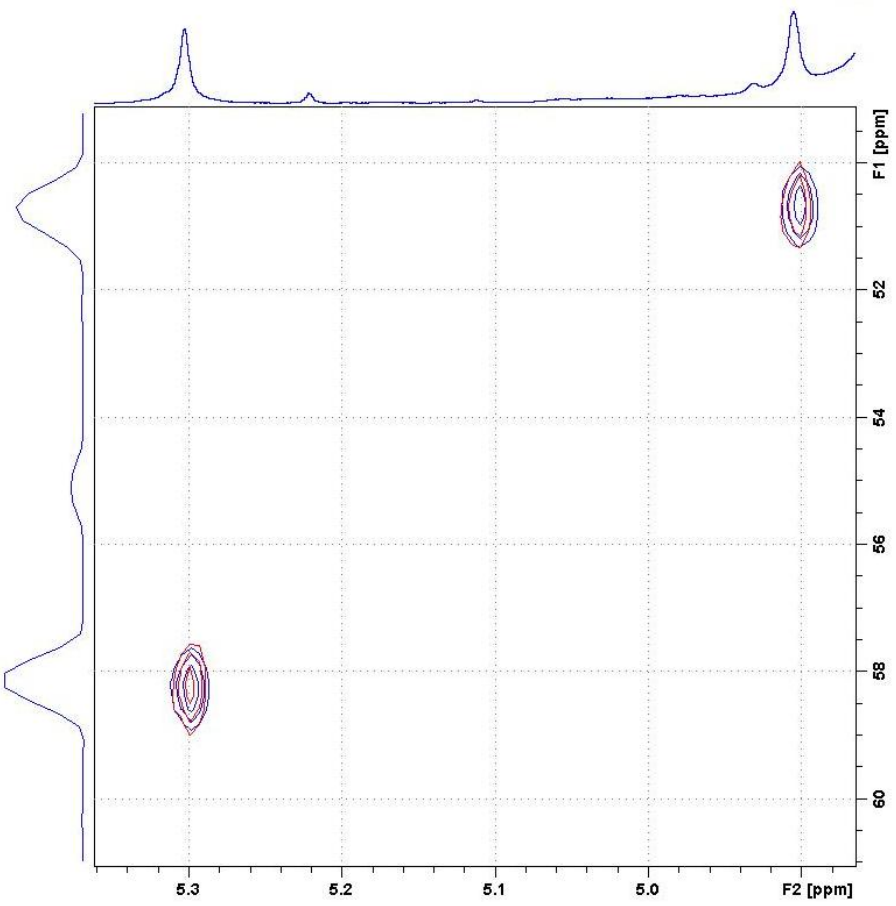


Figure 35: Overlay of the anomeric protons of 16351 (red) with 14578 (blue) from the 1H-13C HSCQ experiment.

4.6 Immunogenicity Study

Immunogenicity of the TEMPO oxidized CPS was observed through immunoblot analysis. The immunoblot was carried out by Stellar Biotechnologies Inc, in California. Rabbit serum was used to measure potential interactions of the CPS with antibodies. A total of five concentrations of CPS were utilized in the immunoblot. It was observed that even at a 1:1000 dilution (i.e. 2.0ng) the CPS exhibited a strong interaction with the antibodies in the rabbit serum (Figure 35). A *C. difficile* CPS was also blotted against the antibodies and did not show any interaction. This shows that the immunogenic interaction was specific to *C. bolteae*. Therefore, the isolated CPS proves to be a sufficiently immunogenic target for vaccine development against *C. bolteae*.

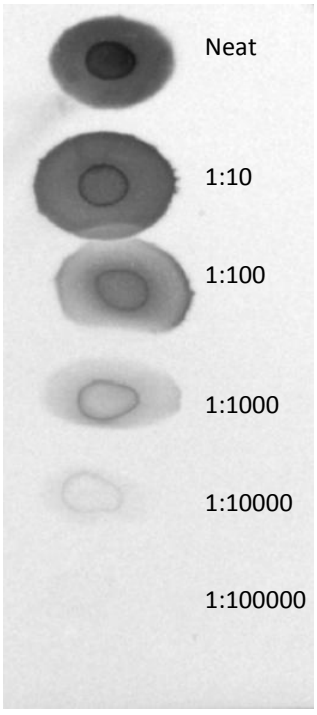


Figure 36: Immunoblot depicting the neat concentration of *C. bolteae* native CPS, as well as 5 dilutions. Immunogenic properties to rabbit serum are observed up to the 1:10000 dilution of the CPS.

Chapter 5: Concluding Remarks

5.1 Conclusions

Copious amounts of evidence were gathered to determine the structure of the CPS produced by *C. bolteae*, strains 16351 and 14578. Extraction and purification techniques isolated the CPS of strain 16351 from the cellular mass. GC-MS analysis was utilized to determine the monosaccharide residue composition, as well as the linkages of the residues. Anomeric configuration and chirality were also determined by GC-MS. Analysis by NMR confirmed the monosaccharide composition and linkages and allowed for full assignment of each proton and carbon in the CPS structure.

The results from the AA analysis, by GC-MS, show that the monosaccharides involved in the CPS are Rha and Man. Chiral glycosides were used to determine that the configuration of both residues was D. Based on the PMAA analysis results the linkages of the sugars were →4)Rha(1→ and →3)Man(1→.

NMR results confirmed the information provided from the GC-MS experiments. The 1D-^{1}H depicted the presence of two anomeric resonances, corresponding to Rha and Man. The chemical shifts of these anomeric resonances showed that Rha is in the β configuration, and Man in the α. The COSY and TOCSY experiments allowed for the assignment of the ring protons, and the HSQC gave the carbon assignments. HMBC and NOESY showed that the inter-residue linkages between both H3 of Man and H1 of Rha, and H4 of Rha and H1 of Man.

These results combined to give the repeat structure [→3)α-D-mannopyranose(1→4)β-L-rhamnopyranose(1→]$_{n}$, shown in Figure 36. GC-MS

integration gave the ratio of terminal Man to 3-substitued Man to be 7:1. The ratio of Rha to Man gives insight to the number of repeats in the polysaccharide chain. An immunogenicity study was performed and illustrated that the isolated CPS is in fact immunogenic in rabbits. Further immunological tests can be carried out to confirm this observation, and broaden the understanding of the immunogenicity of the CPS.

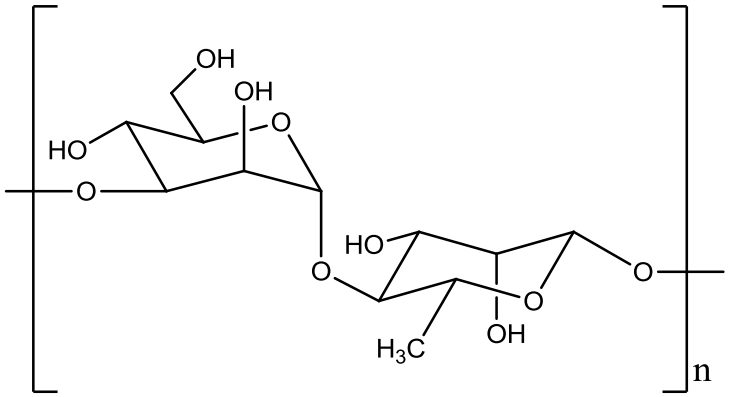


Figure 37: The disaccharide repeat unit isolated from *C. bolteae* strain 16351 [50].

Strain 14578 was also extracted, via water/phenol, and analyzed by GC-MS and NMR. The results from the strain were identical to those gathered from 16351. Both strains were assigned the same CPS disaccharide. Therefore, one vaccine candidate could be generated for both strain 16351 and 14578.

5.2 Future Work

5.2.1 Native Capsular Polysaccharide Conjugation

With the structure of the CPS known, conjugation can take place. The repeat unit of Man-Rha only has one residue with a primary alcohol group. Therefore, to activate the CPS for conjugation, TEMPO oxidation will be used. Oxidation of the C6 position of the

Man residue will yield a carboxylic acid at the position. A protein will need to be chosen to conjugate to the activated CPS. Depending on the protein and stability of the TEMPO oxidized CPS different conjugation techniques can be chosen from, such as reductive amination, and EDC coupling.

The conjugation can then be confirmed by gel electrophoresis, specifically SDS PAGE. If the conjugation is successful the conjugate can be looked at via ELISA for immunogenicity, using the collected rabbit sera.

5.2.2 Synthetic Disaccharide Conjugate

The discovered polysaccharide being a disaccharide allows for the option of synthesis. A synthetic product will be valuable if the vaccine is to be produced by a private sector company.

When generating the synthetic scheme all of the potential difficulties have to be considered. Since the Rha residue of the disaccharide is in the D-configuration the price of the molecule can be quite expensive. Another potential difficulty will be the formation of a beta linkage on the Rha residue. Due to the anomeric effect the Rha will be more likely to generate an alpha linkage at the anomeric center. This problem can be overcome by have the disaccharide synthesized in a Man – Rha order, where the glycosidic linkage is in the favoured alpha configuration.

The proposed synthesis is a total of 12 reactions starting from D-Man and D-Rha. The D-Man will be used as the donor and the D-Rha will be used for the acceptor. By having the Man as the donor, the glycosidic linkage can be alpha between Man and Rha and more of the native structure can be conserved.

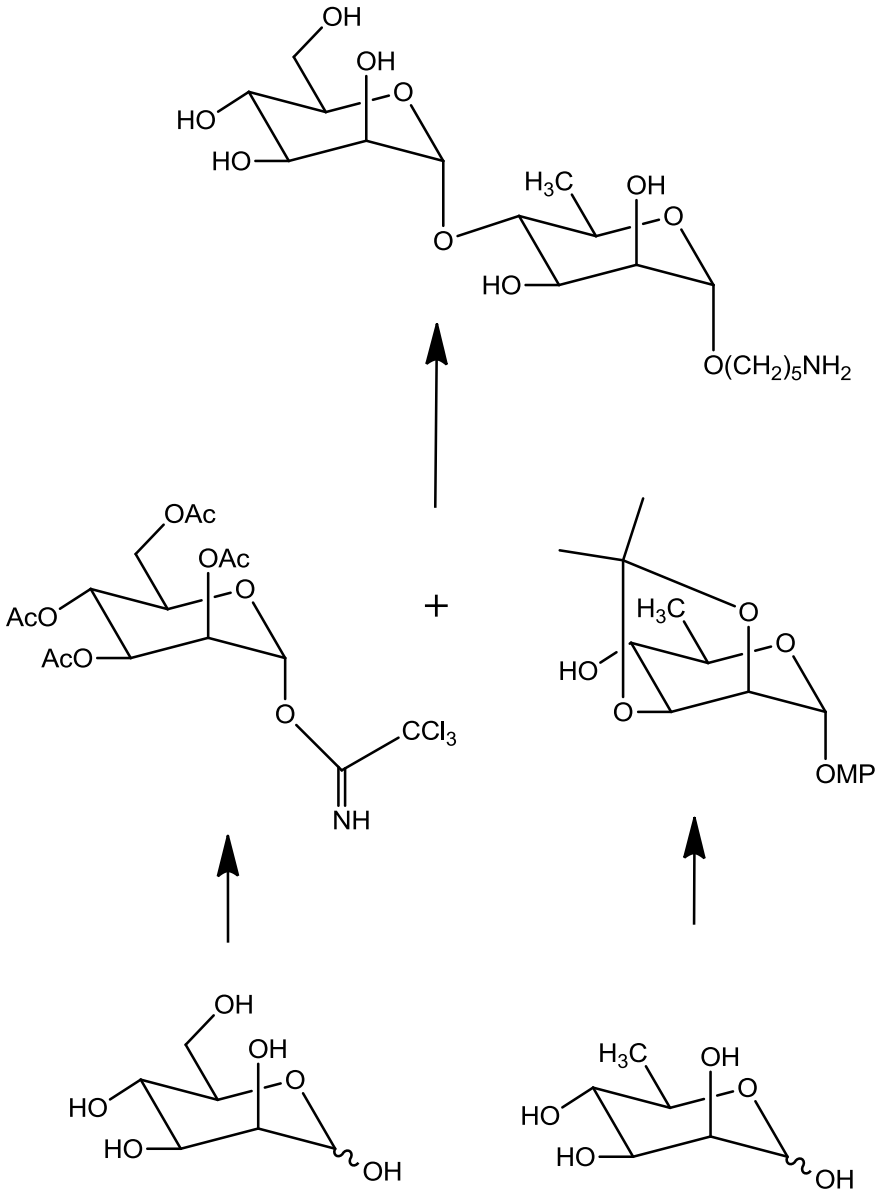
Once the Man donor and the Rha acceptor have been synthesized glycosylation can take place. The resulting disaccharide can then have a linker attached at the C1 position of the Rha residue. This linker will aid in the addition of a protein for conjugate vaccine generation. This 5-carbon linker has been used previously, in the Monteiro research group, for generation of synthetic conjugates [51].

The synthetic disaccharide, with the linker, can then be analyzed for immunogenicity. The immunogenic response can be compared to that of the native polysaccharide to determine the usefulness as a vaccine option. If the synthetic disaccharide raises an adequate response the scheme can be looked at for modifications to find optimal yield conditions.

5.2.3 Multivalent Vaccine

The investigation into the GI microflora of autistic children uncovered that not only *C. bolteae* was present at elevated level, but also some species from the Gram negative genera of *Desulfovibrio* and *Sutterella* [52]. While there is no causative link, yet established between these genera and autism, focus has recently shifted to include them in research investigations [52].

Prevalent strains of these genera such as; *Desulfovibrio piger, Desulfovibrio fairfieldensis, Sutterella wadsworthensis,* and *Sutterella stercoricans,* will be investigated to purify and characterize cell wall polysaccharides [53]. The polysaccharides, once characterized, can all be worked into making a multivalent vaccine [53].



Scheme 7: Retrosynthetic scheme of the disaccharide repeat unit of *C. bolteae* using D-Man and D-Rha as the starting compounds.

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