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Synthesis of D-*glycero*-D-*manno*-Heptose 7-Phosphate

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II. ZUSAMMENFASSUNG

D-*glycero*-D-*manno*-Heptopyranose 7-Phosphat ist ein wichtiges Zwischenprodukt in der Biosynthese von Nukleotid aktivierten Heptosen, die als Vorstufen für die Bildung von Lipopolysaccharidstrukturen, Kapselpolysacchariden und anderen glykosylierten Zellwandbestandteilen von Gram negativen Bakterien dienen. Diese Einheiten verschaffen den Mikroorganismen verschiedene funktionale Eigenschaften, indem sie z. B. eine Barriere für hydrophobe Antibiotika bilden oder die Bildung von inflammatorischen Mediatoren im Immunsystem von Säugetieren stimulieren. Da die Antibiotikaresistenz von pathogenen Keimen zunimmt, gewinnt die Untersuchung von Enzymen, die in der Biosynthese von diesen Einheiten beteiligt sind, immer mehr an Bedeutung.

Im Rahmen dieser Arbeit wurde D-*glycero*-D-*manno*-Heptopyranose 7-Phosphat mit einer guten Ausbeute aus Benzyl 5,6-didesoxy-2,3-O-isopropyliden-α-D-*lyxo*-(Z)-hept-5-enofuranosid hergestellt. Phosphitisierung erfolgte mittels Phosphoramidit Methode, mit einer anschließenden *in-situ* Oxidation. Daran wurde eine Osmylierung angeschlossen und lieferte das D-*glycero*-D-*manno*-Derivat (D-D:L-L, 3:1) mit einem guten Ertrag, das leicht von seinem Stereoisomer durch Chromatographie getrennt wurde. Die Hydrierung dieses Zwischenproduktes führte zur simultanen Entfernung der Schutzgruppen und brachte das Zielprodukt mit einer guten Ausbeute hervor. NMR Spektroskopie bestätigte die Struktur der Zwischenprodukte und zuletzt des Endprodukts.

Dieses wurde zur weiteren Studie zu einigen Einrichtungen in Kanada und in den Vereinigten Staaten geschickt. Enzymaktivität- und Enzymkinetikuntersuchungen von Gen-produkten, die eine Rolle in der Biosynthese von Nukleotid aktivierten Heptosen eine Rolle spielen und Röntgenstrukturuntersuchungen der beteiligten Enzyme, profitieren von der Synthese dieses Metabolits, das nicht im Handel angeboten wird.

III. ACKNOWLEDGMENTS

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VI. ABBREVIATIONS

Short	Description	See page
ATP	Adenosine Triphosphate	16
Bn	Benzyl	22
Bz	Benzoyl	22
CAE	Carboxylesterase	32
CE	Capillary Electrophoresis	29
CPS	Capsular Polysaccharide	11
ESI	Electronspray Ionisation	37
GDP	Guanosine diphosphate	16
HR-MAS	High resolution magic angle spinning	28
Kdo	3-Deoxyoct-2-ulosonic acid	10
LPS	Lipopolysaccharide	7
MS	Mass Spectrometry	37
NADP	Nicotinamide adenine dinucleotide phosphate	19
NMR	Nuclear Magnetic Resonance	36
ORF	Open Reading Frame	32
Ph	Phenyl	22
S-layer	Surface-layer	13
SP-D	Surfactant protein D	33
TLC	Thin Layer Chromatography	34

1 Introduction

1.1 The Bacterial Cell Wall

As the outermost boundary of bacterial cells, the cell wall is essential to those microorganisms that produce them. Due to the concentration of dissolved solutes inside the prokaryotic organism, a considerable turgor pressure develops, estimated at two atmospheres in a bacterium like *Escherichia coli*. To withstand these pressures, which is roughly the same as in an automobile tire, bacteria contain cell walls, which also function to give shape and rigidity to the cell. The cell wall has also to fulfil numerous other functions, like the separatory function of the microorganism of its interior from the environment, while protecting from harmful influences and the connecting functions to enable the transport of substances and information from outside to inside and vice versa. These functions can be executed neither with only one cell wall layer, especially in a more complex environment, nor with a cell wall which is constant in all growth phases.[1,2]

Considerable variation in cell wall composition exists among procaryotes; whereas one special group of bacteria lack a cell wall completely, all other bacteria produce them. These bacteria that lacks cell walls survive because their cell membranes contain sterols and other compounds that help with stabilization; in this respect, they are similar to animal cells. [1]

Based upon the chemistry of their cell walls, two major groups of bacteria are recognised: Eubacteria and Archaea. Almost all Eubacteria have a chemical polymer called peptidoglycan in their cell wall structure, while the Archaea do not. Instead of this, they possess a differently composed rigid layer in or on their cell wall, e.g. a pseudomurein or an S-layer.[1]

1.2 The Gram-staining

More than 100 years ago (1884), Gram[a] developed a simple staining method for differentiating all bacteria known at that time into two major groups, that was later named after him. After heat fixation, the bacteria are stained firstly by solutions of carbolgentiana violet and subsequently by Lugol's solution that contains iodine. By that, the dye is retained better by forming an insoluble complex. Both Gram-positive and Gram-negative bacteria appear purple after this treatment. Then the slide is washed with ethanol until no further dye is eluted from the layer. While Gram-positive bacteria keep the stain because of lower cell wall permeability and appear violet, Gram-negative ones are decolourised. Subsequently the bacteria are restained with fuchsine solution. Bacteria appearing dark blue to violet under the microscope are designated Gram-positive (Figure 1), red to pink ones Gram-negative (Figure 2). Reproducible results are only obtained with cells in the exponential growing phase.[3]

Figure 1: Gram positive *cocci*
{http://homepages.wmich.edu/~rossbach/bios312/LabProcedures/Gram%20Stain%20results.html}

[a] H. C. Gram (1853-1938), Danish physician and bacteriologist

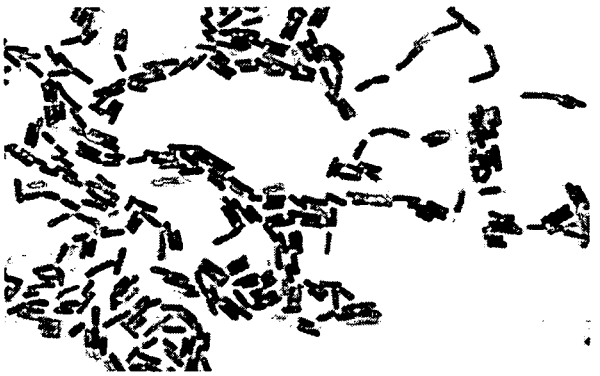

Figure 2: Gram-negative *bacilli*
{http://textbookofbacteriology.net/Bacillus.html}

The difference in stainability depends on the structure of their cell walls. Both types of cell walls contain the peptidoglycan or murein as the rigidity causing component. However, the murein of Gram-positive bacteria is much thicker (up to 40 layers) than that of the Gram-negative. Both groups additionally contain different accessory cell wall components. The composition and structure of Gram-positive cell walls can differ much more from each other than the Gram-negative ones, which follow a more general structural form.[3]

1.3 Peptidoglycan

Each layer that makes up peptidoglycan – also called murein or mureinsacculus – is a thin sheet composed of two sugar derivates, *N-acetylglucosamine* and *N-acetylmuramic acid*, and a small group of amino acids consisting of L-alanine, D-alanine, D-glutamic acid and either L-lysine or diaminopimelic acid (Figure 3 on page 5). The DAP is present in every Gram-negative bacteria and in some Gram-positive, but most of the Gram-positive *cocci* have lysine instead of DAP. A few other Gram-positive bacteria have other amino acids.

While the carbohydrates are connected via β-1,4-glycosidic bonds, all constituents are linked to form a repeating structure, the glycan tetrapeptide (Figure 3). In the basic structure of peptidoglycan, the glycan chains formed by the sugars are connected by peptide cross-links. The glycosidic bonds are very strong, but these chains alone cannot provide enough rigidity in all directions. The full strength of the peptidoglycan structure is realized only when these chains are cross-linked by amino acids. This cross-linking has a characteristically different extent in different bacteria, causing greater rigidity by more complete cross links. In Gram-negative bacteria this occurs by direct peptide linkage of the amino group of the diaminopimelic acid to the carboxyl group of the terminal D-alanine. In the Gram-positive bacteria, the cross-linkage is usually a peptide interbrigde (e.g. in *Staphylococcus aurea* each interbrigde peptide consists of five molecules glycine). Any of the amino acids present in the tetrapeptide and a number of other amino acids (glycine, threonine, serine and aspartic acid) can be found in the interbrigde, whereas certain amino acids such as branched chain amino acids, aromatic amino acids, sulphur containing amino acids and histidine, arginine, and proline are never found there. The kinds and numbers of cross-linking amino acids depend on the organism.[2]

Penicillin prevents the cross linking – it mainly affects Gram-positive bacteria – and therefore meshes in with the formation of the cell wall. It has a bactericidical effect on growing cells, whereas resting cells are escaped.

Lysozyme is a protein that breaks the β-1,4-glycosidic bonds between N-acetylglucosamine and N- acetylmuramic acid in peptidoglycan. Therefore, the cell wall loses strength and due to the osmotic pressure water enters the cell, the cell swells and eventually bursts – a process called lysis. The enzyme is found in animal secretions including tears, saliva and other body fluids and also in egg white and presumably functions as a primary defence against infection by bacteria.[4]

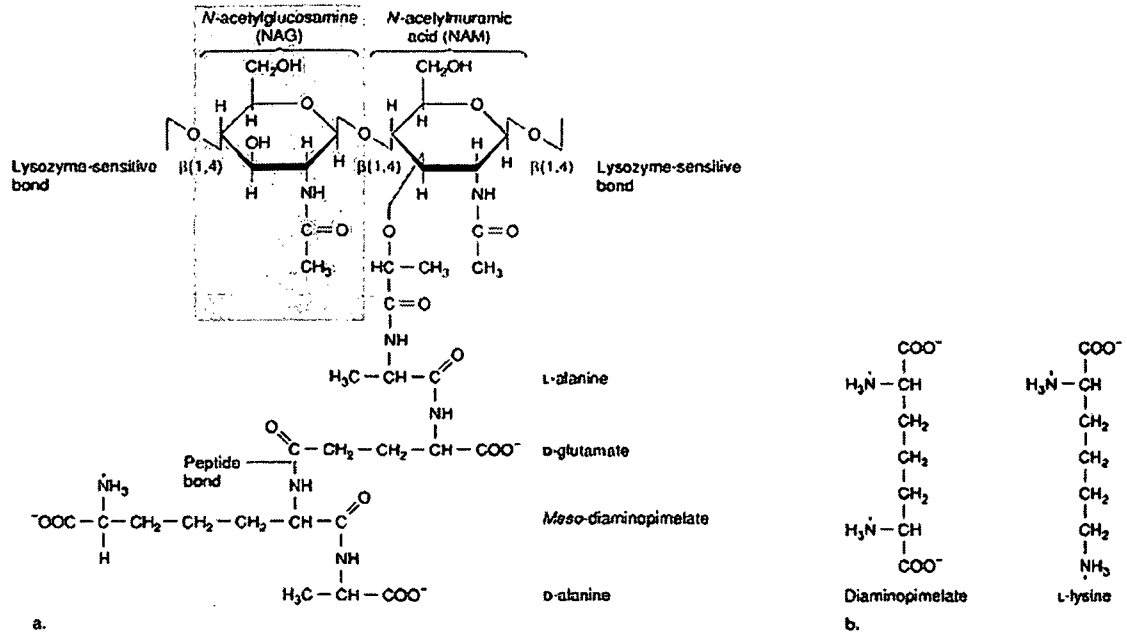

Figure 3: Peptidoglycan composition – structure of NAG and NAM {http://www.rit.edu/~gtfsbi/IntroMicro/images/CHAPTER03/FIGURE03-11.JPG}

Conclusingly it can be stated that although the precise chemistry of peptidoglycan can vary, the structural makeup of peptidoglycan is the same in all forms of the molecule: Glucosamine and muramic acid form the backbone, while the muramic acid molecules are cross-linked with amino acids.[2]

1.4 The Gram-Positive Cell Wall

The major cell wall constituent in the Gram-positive bacteria is the peptidoglycan, making up 30 to 70 percent of the dry weight of the wall depending upon the species. In addition to peptidoglycan, Gram-positive bacteria contain proteins and substantial amounts of polysaccharides (10 – 60%) in their cell walls. With few exceptions they are generally poor in lipids and lack an outer membrane (Figure 4).

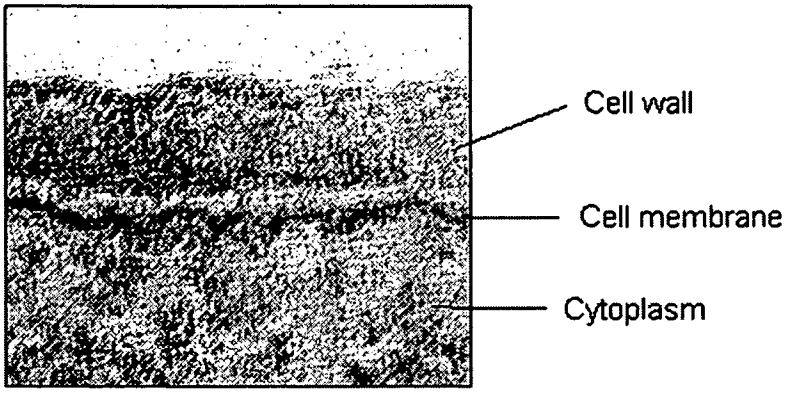

Figure 4: The Gram-positive cell wall [1]

Among the constituents are teichoic, teichuronic acid (Figure 5) and anionic polysaccharides, these are lipoteichoic acid and lipoglycans.[3] The lipid moiety of the lipoteichoic acids is embedded in the cytoplasmic membrane; thus it is not a component of the cell wall. Teichoic acids are polyol phosphate polymers (e.g. polyglycerol phosphate and ribitol phosphate). These are attached covalently to the 6-hydroxy position of muramic acid in the peptidoglycan.

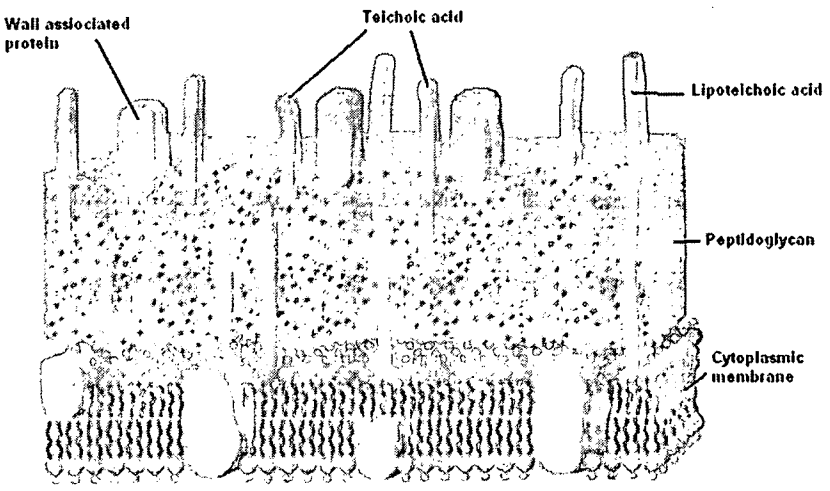

Figure 5: The Gram-positive cell wall (schematically)[2]

Teichuronic acids are polymers of two or more repeating subunits, one of which is always a uronic acid (such as glucuronic acid) or the uronic acid of an amino sugar (such as aminoglucuronic acid).[1]

Besides the polymers teichoic and teichuronic acids, which were termed as 'classical' secondary cell wall polymers (SCWPs) in recent studies of Christina Schäffer and Paul Messner, a different class of SCWPs on Gram-positive cell walls has been identified. These 'non-classical' SCWPs show considerable differences in the chemical make-up, overall structure and charge behaviour. Their function comprises the stable attachment of S-layer proteins (see chapter 1.6) to the cell wall, i.e. 'non-classical' SCWPs as mediators for covalent connection of S-layers to the underlying peptidoglycan meshwork.[5]

1.5 The Gram-Negative Cell Wall

Besides a few layers of peptidoglycan, gram-negative bacteria contain adjacent to the plasma membrane two unique regions: the periplasmic space and an additional cell layer of lipopolysaccharides. This is a second lipid bilayer, which in comparison to the cytoplasmic membrane does not entirely consist of phospholipids, but also incorporates polysaccharides and proteins. These lipids and polysaccharides in the outer layer are closely linked and form specific lipopolysaccharide (LPS) structures, which are also known as the outer membrane (Figure 6).[2]

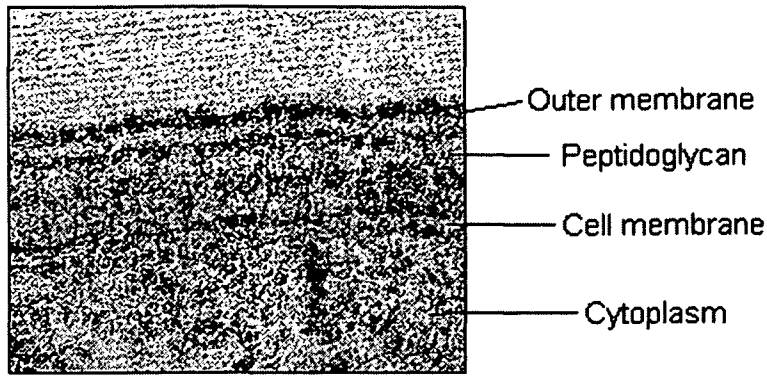

Figure 6: The Gram-negative cell wall[1]

In comparison to the cytoplasmic membrane, the second phospholipid layer of gram-negative bacteria is relatively permeable for small substrate molecules. This is due to channel proteins, also called porins, which are integrated into the outer membrane in great quantity (Figure 7).

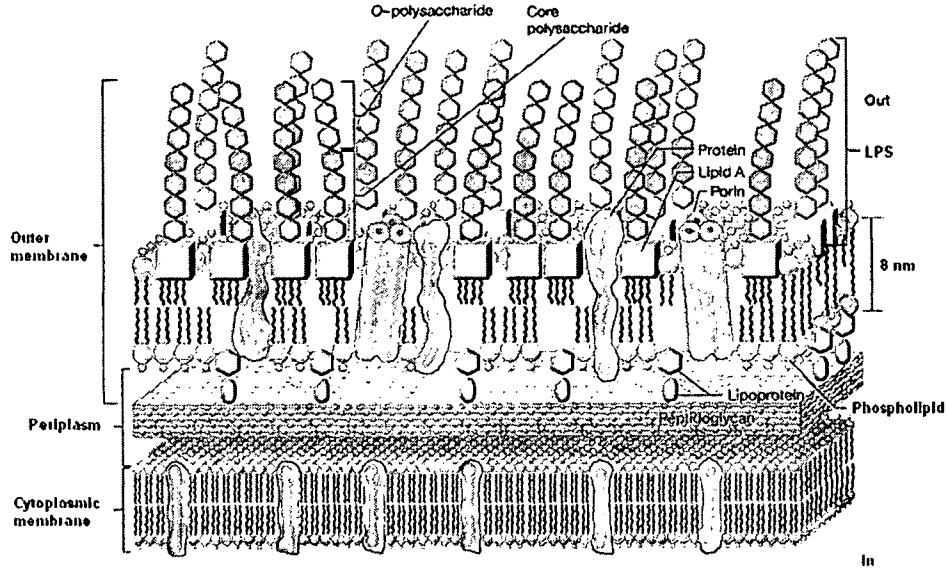

Figure 7: The Gram-negative cell wall (schematically)[2]

1.5.1 Chemistry of Lipopolysaccharides

Despite the complexity, the chemistry of LPSs of various bacteria has already been investigated and noted. In general, these amphiphilic structures comprise three parts: lipid A, the core region and a polysaccharide portion, which may be an O-specific polysaccharide, Enterobacterial Common Antigen or a capsular Polysaccharide (Figure 8).[6]

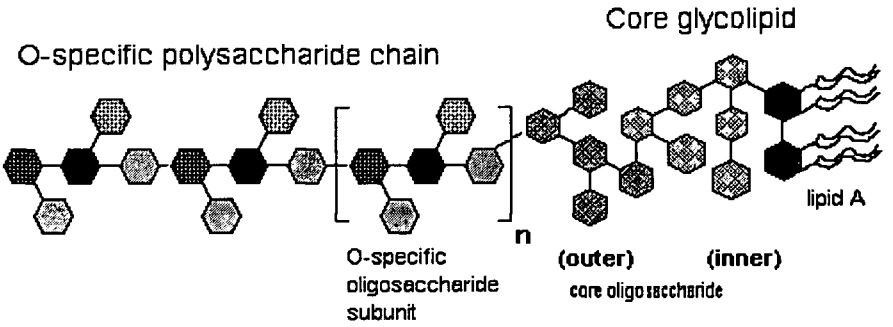

Figure 8: Gram-negative bacterial LPS (Endotoxin[b]) {http://freespace.virgin.net/r.barclay/lpstyl01.gif}

1.5.1.1 Lipid A

The lipid A is essential for the outer membrane stability and the cell viability. It serves as a hydrophobic anchor to the outer membrane. This lipid unit consists of two β-1,6-linked glucosamine residues that are phosphorylated and acetylated with a variable number of fatty acids.[8,9] In lipid A of different microorganisms there is a high diversity of fatty and hydroxy fatty acids. [10] Lipid A is responsible for the endotoxic properties of LPSs by stimulating the production of inflammatory mediators and can potentially lead to a septic shock. While there is a limited number of lipid A structures, they provide regulated modifications in response to environmental stimuli, which results in an altered endotoxicity.[11]

[b] Endotoxin is a toxin that is confined inside the microorganism and is released only when the microorganism is broken down or dies. (http://www.thefreedictionary.com/Endotoxic)
Antonym: Exotoxin is a toxin that is secreted by microorganisms into the surrounding medium.

1.5.2 Core Region

The disaccharide of lipid A is linked to the core region through a 3-deoxyoct-2-ulosonic acid (Kdo).[4] The main function of the core oligosaccharide region is to serve as a barrier to hydrophobic antibiotics.[9] This region can be subdivided into the inner and outer domain. In *Escherichia coli* K-12 and some other enteric bacteria, the inner core generally comprises two residues of Kdo and, depending on the species, two or three residues of L-*glycero*-D-*manno*-heptose (for further description of heptoses see below) – these constituents are largely conserved among gram-negative bacteria. The outer core usually consists of hexoses and hexosamines.[7]

Heptoses are monosaccharides containing seven carbons in a molecule and are widely present in glycoconjugates of few Gram-positive and most Gram-negative bacteria. The nomenclature of heptoses depends on the configuration of hydroxy groups at C-2, C-3, C-4 and C-5 giving D-or L-*manno*, -*gulo*, -*gluco*, -*galacto* etc. and at C-6 leading to D- or L-*glycero* forms. The seven-carbon monosaccharides and their derivates can be found in LPSs and CPSs (chapter 1.5.3) as well as in S-layers (chapter 1.6).[13]

Phosphate groups covalently attached to the heptoses facilitate electrostatic interactions by divalent cations and provide membrane stability and restricted permeability. Hence, *E. coli* mutants lacking heptoses in their LPS structures display much shorter core oligosaccharides and exhibit a wide range of pleiotropic phenotypes due to the reduced stability of the outer membrane. These mutants are hypersensitive to hydrophobic antibiotics, detergents and bile salts. They are also poor recipients for both plasmid conjugation and generalised transduction.[8]

1.5.2.1 O-specific Polysaccharide Chain

The O-specific polysaccharide part is attached to the core region and generally contains Galactose, Glucose, Rhamnose and Mannose (six-carbon sugars) as well as atypical dideoxysugars such as Abequose, Colitose, Paratose and Tyvelose. These sugars are linked to each other in groups of four or five, which are usually branched. In

comparison to the relatively conserved lipid A and core region domains, these form variable, long O-polysaccharide chain.[2,13] This structure constitutes a very effective antigen for the production of antibodies by the mammalian immune system.[4]

1.5.3 Capsular Polysaccharides

Many bacteria secrete specific polysaccharides (sometimes also proteins), that cloak the cell surface and form an additional protective layer. These bacteria are also referred to as polysaccharide encapsulated bacteria. The general denomination for extra-cellular polymeric material is the glycocalyx. Depending on their morphology, capsular polysaccharides (CPSs) are also called capsules or slimes. Whereas capsules are firm, ordered structures and hinder the diffusion of particles to the cell membrane, layers of slime are less structured, do not form a barrier to diffusion and are more difficult to spot under microscope. [4,14]

There is a high structural variety; about two hundred different polysaccharides are produced by *E. coli* alone. CPSs of Gram-negative bacteria contribute to a large extent to the pathogenicity of a broad range of microorganisms.[15]

CPSs have several functions for the bacteria cell. The glycocalyx serves to protect the microorganism or allows it to attach itself to inert surfaces (such as teeth), eukaryotes (e.g. *Streptococcus Pneumoniae* adheres itself to lung cells) or other bacteria (and their glycocalyxes fuse to envelop the colony). CPSs are highly hydrated; thus another function of them is to prevent them from drying out.[14]

1.5.4 Physiologic Action of LPS

When the cell lysis, LPS structures are released from the outer membrane of the cell wall. The LPS binds to the LPS-binding protein in the blood and forms a complex,

which binds to a receptor molecule (CD_{14}) on the surface of macrophages. This reaction is thought to promote the ability of the Toll-like receptor (TLR-4) to release cytokines[c]. These then bind to the cytokine receptors on target cells, initiate inflammation and stimulate both the complement pathway and the coagulation pathway (Figure 9).[7]

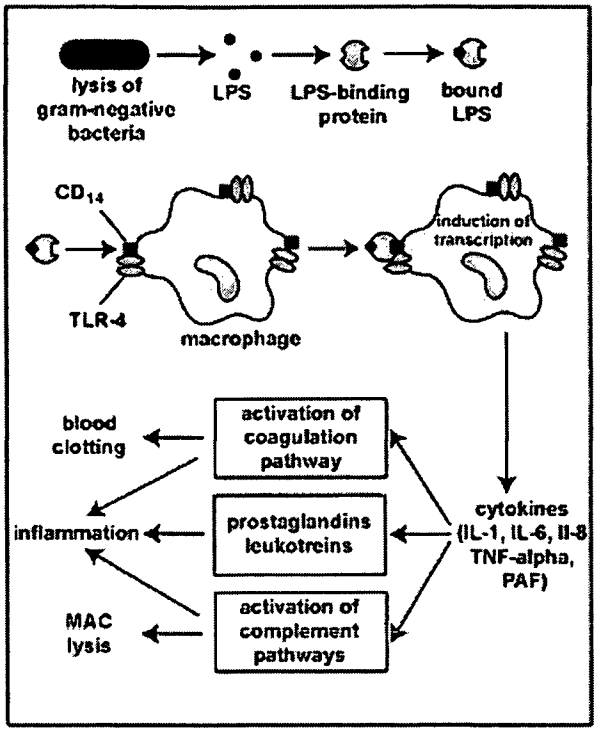

Figure 9: Physiologic action of LPS
(http://faculty.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/lps.html)

1.5.5 Medical Relevance of LPSs and CPSs

Gram-negative bacteria lacking previously mentioned heptoses in their LPS structure exhibit much shorter core oligosaccharides causing reduced stability of the outer membrane. Thus, they are highly sensitive to hydrophobic substances (such as antibiotics), detergents and bile salts. These organisms also show a reduced virulence in animal infection models.[16] In consequence, genes involved in the biosynthesis of

[c] Cytokines are chemical messenger molecules by which immune cells communicate with one another and with other cells. Some cytokines are also used by nerve cells as messenger molecules. (www.ninds.nih.gov/health_and_medical/pubs/sci_report.htm)

heptoses and their products are potentially appealing targets for developing novel antimicrobial compounds.[7] This is of particular importance due to the fact that germs are getting more and more resistant against existing antibiotics.

O-specific polysaccharides of gram-negative bacteria are important in the serological classification of enteric bacilli[d]. The O-specific chains determine the specificity of the O antigens, which are the immunodominant part of the lipopolysaccharide molecule in the intact bacterial cell.[17] However Gupta *et al.* suggest that the native O-specific polysaccharide is not immunogenic[e], most probably because of its comparatively low M_r. Bearing this in mind and due to the toxicity of LPSs, both are not suitable native vaccines and need to be conjugated for an efficient immunisation.[18]

Glycocalyxes are also immunogenic for mammals, thus mixtures of capsular polysaccharides, either conjugated[f] or native are used as vaccines. It is standard medical practice to recommend vaccination to people (e.g. persons with sickle cell anaemia) who are more susceptible to pathogens.[19]

1.6 S-Layer

The Surface-Layer is mostly found in bacteria and archaea and consists of a monomolecular paracrystalline coating made of glycoproteins or proteins.[4] As a part of the cell envelope, this two dimensional structure is built via self-assembly and encloses the whole cell surface. S-layer proteins are hardly conserved and can vary noticeably even between related species. Depending on species, the S-layer has a thickness between 5 and 25 nm and exhibits identical centre-to-centre spacing of approximately 2.5 to 35 nm.[20]

[d] Enteric Bacilli are microorganisms, especially the gram-negative rods, found in the intestinal tract of humans and animals (http://www.answers.com/topic/enteric-bacilli)
[e] Capable of inducing an immune response; antigenic (http://www.thefreedictionary.com/immunogenic)
[f] Conjugated vaccines are vaccines made from capsular polysaccharides bound to proteins of known immunogenicity, such as tetanus toxoid. (http://www.sabin.org/vaccine_science_GlossaryB_D.htm)

The biological function of S-layer is not fully understood. For many bacteria, the S-layer is the outermost interaction zone with their environment. Its function is thought to differ considerably from species to species. In some archaea it is the only cell wall component and therefore is important for mechanical stabilisation. Additional functions of S-layers are protection against phagocytosis, resistance against low pH, stabilisation of the cell membrane or adhesion sites (especially for glycosylated S-layers).[2]

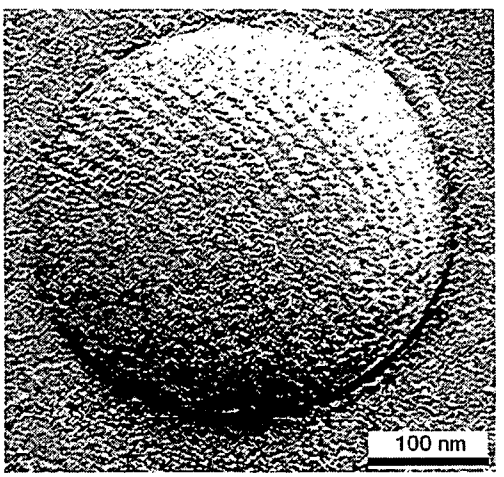

Figure 10: Freeze etching preparation of a liposome completely covered by an S-layer (http://www.nano.boku.ac.at/1145.html?&L=1)

1.7 The Biosynthesis of Core Region Heptoses

First investigations concerning the biosynthesis of nucleotide-activated heptoses were made by Eidels and Osborn (1971, 1974). They established that the synthesis of L-*glycero*-D-*manno*-heptoses utilises sedoheptulose-7-phosphate, an intermediate in the non-oxidative portion of the pentose phosphate pathway. According to their studies, Eidels and Osborn suggested a four-step biosynthesis pathway implying the conversion of the sedoheptulose-7-phosphate into D-*glycero*-D-*manno*-heptose 7-phosphate by a phosphatase isomerase, the formation of D-*glycero*-D-*manno*-heptose 1-phosphate by a mutase reaction, then transfer of a nucleotide via phophodiester linkage and concluding

the epimerisation of D-*glycero*-D-*manno*-heptose residue of the sugar nucleotide to L-*glycero*-D-*manno*-heptose (Figure 11).

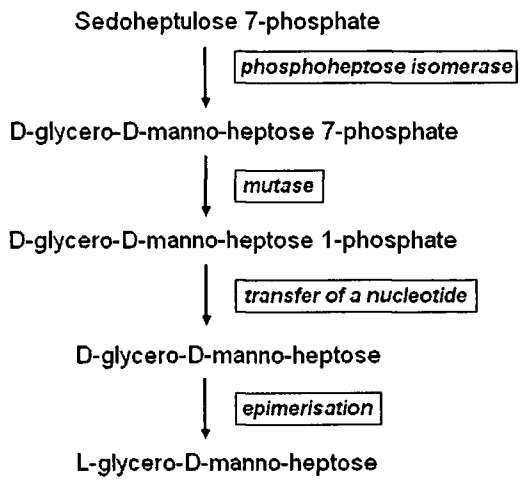

Figure 11: Biosynthesis pathway proposed by Eidels and Osborn (1971, 1974)

At a later date, Kontrohr and Kocsis (1984) figured out that ADP is the nucleotide sugar residue attached to *glycero-manno*-heptose in *Shigella sonnei* and *Salmonella enterica.* However, recent studies in the analysis of biosynthesis pathways of core region heptoses brought more insight.

Essentially, the biosynthesis starts with the isomerisation of D-sedoheptulose-7-phosphate giving D-*glycero*-D-*manno*-heptose 7-phosphate (Figure 13) by phosphoheptose isomerase GmhA. The *gmhA* gene is highly conserved in many Gram-negative bacteria as well as in some Gram-positive, such as *Clostridium acetobutylicum* or *Mycobacterium tuberculosis.*[16]

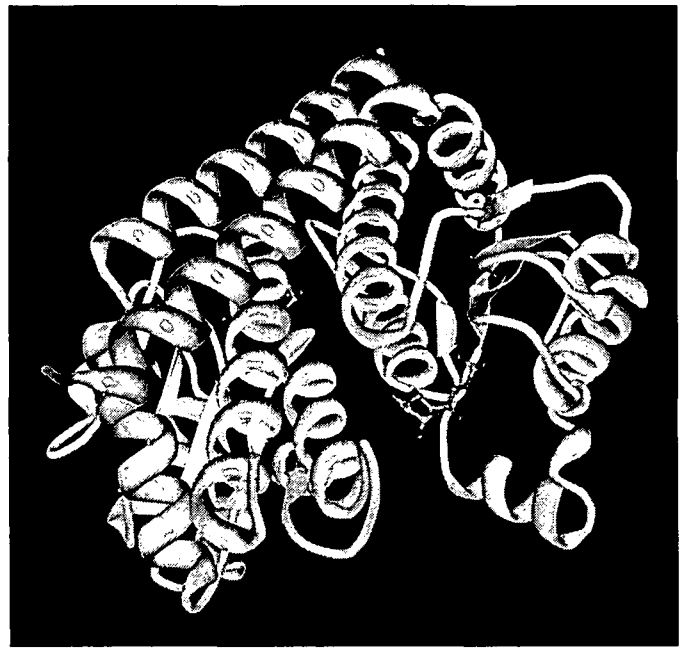

Figure 12: Phosphoheptose isomerase in complex with reaction product D-*glycero*-D-*manno*-heptopyranose 7-phosphate [21]

The product of the isomerase reaction serves as a reactant for the formation of two different nucleotide-activated metabolites, the ADP-L-*glycero*-β-D-*manno*-heptose or GDP-D-*glycero*-α-D-*manno*-heptose. These two separate pathways were termed as L-β-D-heptose and the D-α-D-heptose pathway by M. Valvano, P. Messner and P. Kosma and can be distinguished based on the specificity of the kinase and nucleotidylyltransferase steps (Figure 13).

The following step involves a C-1 phosphorylation of the D-D-heptose 7-phosphate by either HddA (for the D-α-D configuration) or HldE (formerly RfaE) (for the D-β-D configuration). HldE is a bifunctional protein with two clearly distinguishable functional domains. The N-terminal or HldE1 domain corresponds to a polypeptide related to the ribokinase family and the C-terminal or HldE2 domain belongs to the cytidylyltransferase superfamily.[16] Studies of McArthur *et al.* provide genetic and biochemical evidence that HldE heptokinase functions as a dimer.[8] The HldE1 domain converts *glycero*-β-*manno*-heptose 7-phosphate in the presence of ATP into *glycero*-*manno*-heptose 1,7-bisphosphate. Morrison and Tanner analysed whether alternate

substrates would be phosphorylated by the kinase activity of HldE by incubating it with mannose and phosphate buffer. Both substrates could bind in the active site of the enzyme, mimicking the natural substrate D-D-heptose 7-phosphate.[22] For the D-α-D-heptose pathway, HddA is responsible for the conversion of D-α-D-heptose 7-phosphate into the 1,7-bisphosphate. It is specific for the α-anomer of the D-D-heptose.[16]

The removal of the C-7 phosphate group is the next step and common in both (in D-D- and L-D-heptose) pathways and is carried out by the phosphatase GmhB. Kneidinger *et al.* constructed a deletion in the *gmhB* gene, which resulted in the formation of an altered LPS core. This mutation confirmed that this enzyme is required for the production of nucleotide activated D-D-heptose.[23]

The enzyme involved in the activation of the D-D-heptose 1-phosphate by GDP is the HddC for the D-D-heptose pathway, which is a guanidylyltransferase. ADP is attached to the D-β-D-heptose by the adenylyltransferase domain of the bifunctional HldE in the L-D-heptose pathway (see below for further details of epimerisation in this pathway). The GDP-activated heptose is used as a substrate by specific glycosyltransferases for the assembly of bacterial glycoproteins as well as polysaccharide capsules. Precursors for the 6-deoxy-heptose sugars found in CPS and flagellin glycoproteins in *Campylobacter* strains may be derived from GDP-D-*glycero*-α-D-*manno*-heptose. Some *Burkholderia* strains express polysaccharide capsules containing D-D-heptose that are important for the virulence of these microorganisms.[16]

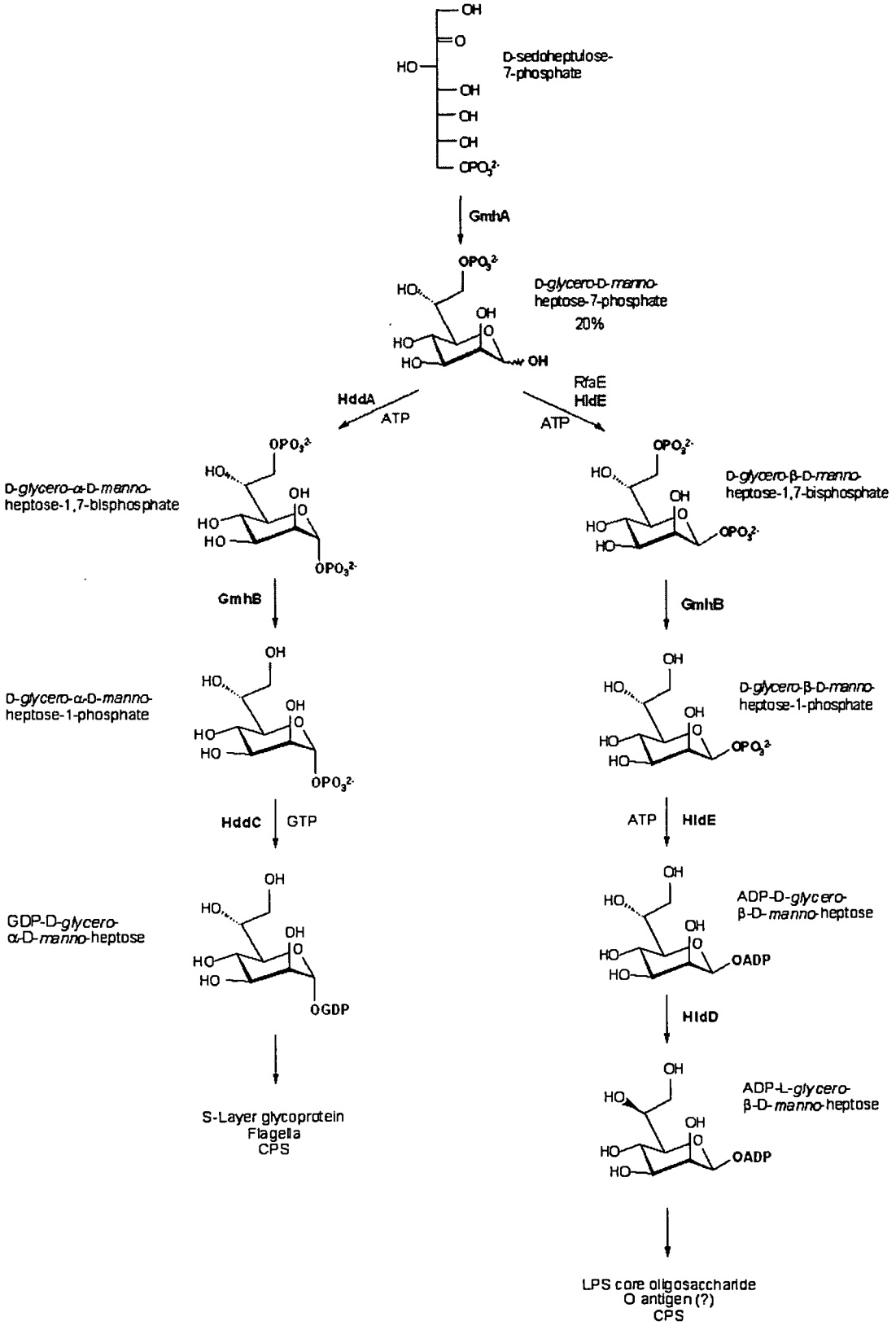

Figure 13: Biosynthesis pathway of NDP-*glycero*-D-*manno*-heptoses

The epimerisation of ADP-D-*glycero*-β-D-*manno*-heptose to the L-D-configuration is accomplished by the HldD. The enzyme ADP-D-*glycero*-β-D-*manno*-heptose 6-epimerase has been well characterised. Current research of Tanner et al. shows that the mechanism of the epimerisation appears to involve a transient oxidation/ reduction step employing a tightly bound $NADP^+$. There were at least four quite distinct mechanisms that could be postulated for the epimerisation reaction (Figure 14). These were path A) a scheme of direct oxidation/ reduction step at C-6, B) oxidation of C-4 (dehydration/ rehydration), C) oxidation of C-4 (retroaldol aldol) and D) oxidation at C-7 employing a deprotonation/ reprotonation reaction.

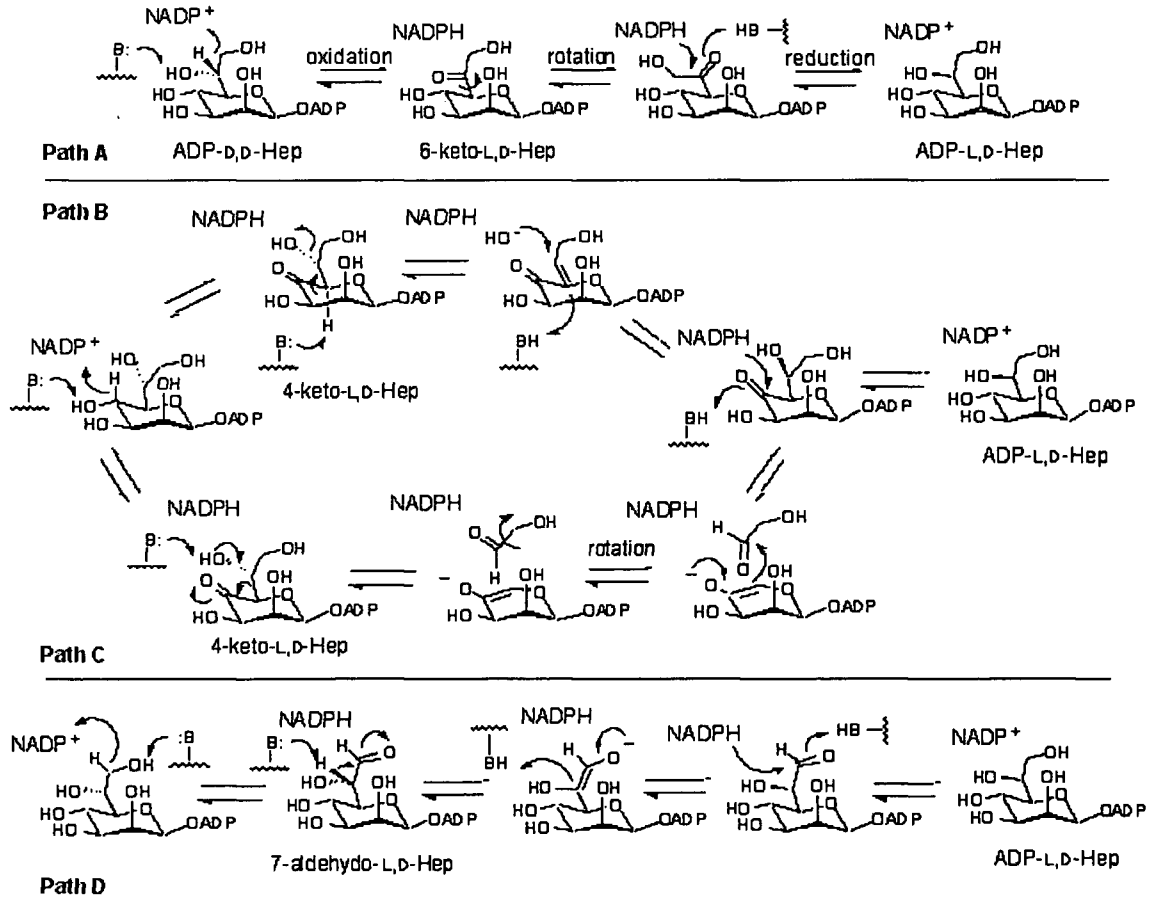

Figure 14: Mechanism of ADP-L-D-heptose 6-epimerase reaction (B_1 and B_2 are active site residues) (http://www.chem.ubc.ca/faculty/tanner/current/currentresearch.html)

In order to distinguish these pathways, experiments with isotopically labelled samples were prepared. These were substrates of ADP-D,D-heptose that contained ^{18}O and ^{2}H isotopes at C-7'' and C-6'' respectively. This experiment gave proof of mechanism A.[24,25]

The product of the epimerisation step in the L-β-D-heptose pathway is also involved in the LPS core biosynthesis as well as other capsular polysaccharides. The enzymes that occur in D-α-D pathway are involved in the biosynthesis of components of S-layers, e.g. in *A. thermoaerophilus*.[16]

1.8 Objectives of Master Thesis

The biosynthesis of nucleotide-activated heptoses of the D-*glycero*-D-*manno*- and L-*glycero*-D-*manno*-configurations has only been recently fully elucidated. However there are still gaps in the full explanation of the heptose biosynthetic pathway (e.g. lacking structural data of enzymes complexed to respective substrates). Hence, the chemical synthesis of the corresponding intermediates is still needed for a full functional and structural characterisation of the involved enzymes.

The aim of this research project was to synthesize D-*glycero*-α,β-D-*manno*-heptose 7-phosphate (see Figure 15). It is an important precursor in the biosynthetic pathway of LPS core region heptose as well as CPS and S-layer glycans.[25,13] In Figure 13 it becomes apparent that this metabolite occurs in early phases of the synthesis. This adds significant impact on investigations of starting steps for a series of bacterial membrane saccharides.

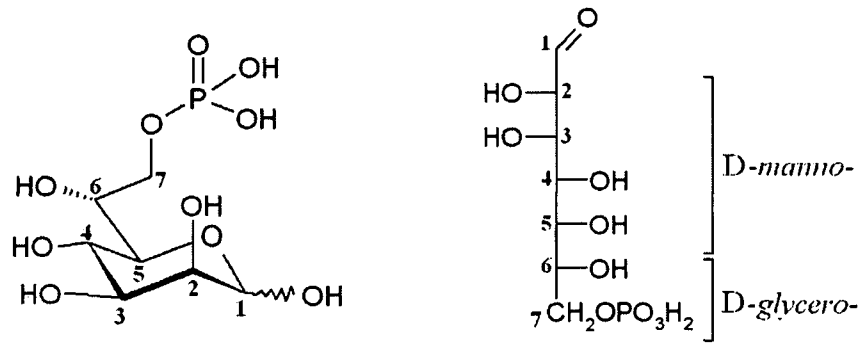

Figure 15: Structure of D-*glycero*-α,β-D-*manno*-heptose 7-phosphate

2 Results and Discussion

The initial synthesis way of the D-*glycero*-D-*manno*-heptose 7-phosphate followed the reaction steps of "The synthesis of some seven-carbon sugars..." by Brimacombe *et al.*[26]

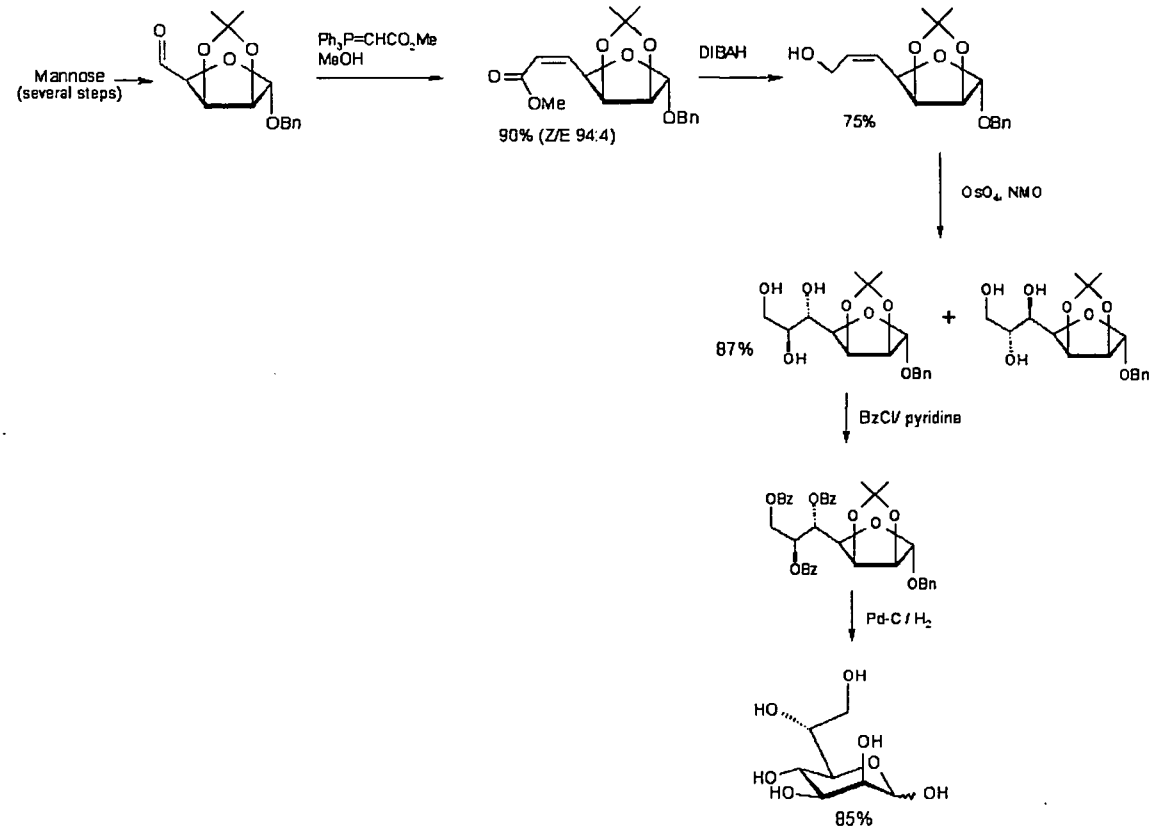

Figure 16: Synthesis of D-glycero-D-manno-heptose by J. Brimacombe, A.K. Kabir 1986, Graziani 2005

2.1 Synthesis of Benzyl-2,3-*O*-isopropylidene-α-D-*lyxo*-hept-5-enofuranoside

The synthesis of unsaturated *Z*-configured heptofuranoside **3**, which was the key-intermediate of our target-synthesis, was performed through a two step synthesis (Figure 17). These steps were preferred since the starting compound, the pentadialdose

1, was already available in our laboratory and these reactions were well known in the working group of Prof. Kosma.[12]

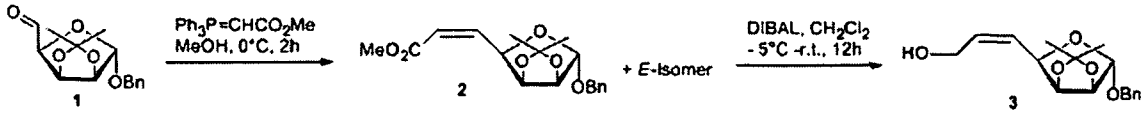

Figure 17: Reaction scheme: synthesis steps for **3**

2.1.1 The Wittig Reaction

Wittig reactions are exceedingly important as they provide the formation of a C-C-double bond (see Figure 18). In contrast to elimination reactions, these double bonds yield in most cases unambiguous alkenes (only one isomer). Generally, it is a chemical reaction of an aldehyde or ketone with a triphenyl phosphonium ylide.[27]

For the synthesis of the methyl-*Z*-heptofuranuronate **2**, the Wittig reaction between **1** and (methoxycarbonylmethylene)triphenylphosphorane in methanol at 0 °C was performed and gave 85% isolated product. The *Z*-configuration of the alkene was assigned on the basis of the vicinal coupling constant between H-5 and H-6. This value was smaller for the *cis* configuration ($J_{5,6} \approx 12$ Hz) in comparison to that of the *trans* isomer ($J_{5,6} \approx 16$ Hz).[12] The *E*-isomer formed in 4% yield was separated by column chromatography.

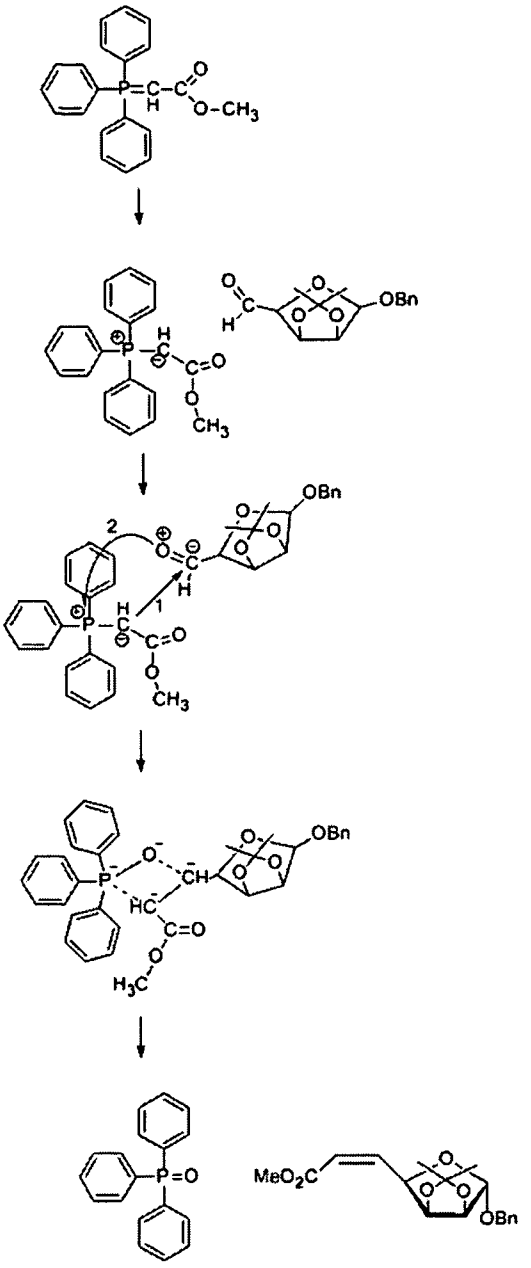

Figure 18: A scheme of the Wittig reaction mechanism

2.1.2 The Reduction of the Carboxygroup

The reduction of **2** was accomplished by treatment with diisobutylaluminium hydride at -5 °C and took about 3 h for the completion of the reaction. Finally, **3** was obtained in a yield of 67% by subsequent chromatography on silica gel.

2.2 The Phosphorylation of the Heptofuranoside

Whereas Zamojski published the synthesis of all monophosphates of the L-glycero- D-manno-heptose, the C-7 phosphorylation of D-*glycero*-D-*manno*-heptose had never been reported before. Recently Mayer and Tanner implemented the chemical synthesis of a mixture of D-D- and L-D-heptose 7-phosphate through a four step procedure from 6-keto-heptopyranose. These reagents were employed for an isotope crossover experiment to investigate the hydride transfer steps in the reaction catalysed by ADP-L-*glycero*-D-*manno*-heptose 6-epimerase (also known as HldD, formerly RfaD).[25]

For the accomplishment of the phosphorylation, several reagents could be considered in order to obtain the heptenofuranoside 7-phosphate.

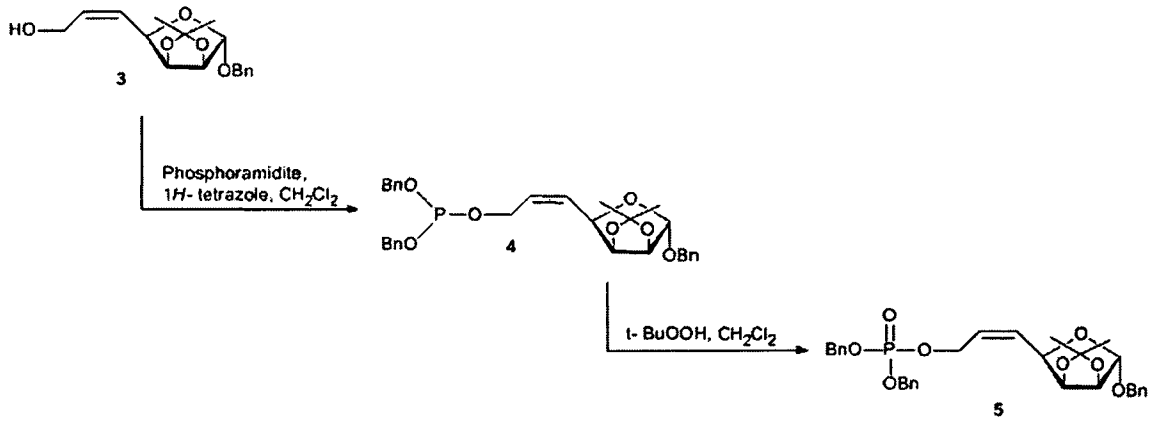

Figure 19: Reaction scheme of phosphorylation

As a first attempt the highly efficient and well established phosphoramidite method was employed for the phosphitylation of **3**. Thus bis(benzyloxy)-*N,N*-diisopropyl-aminophosphine with 1*H*-tetrazole as a catalyst in dichloromethane was used to produce the intermediate **4** under inert atmosphere. TLC showed full conversion of the reactant after 2h. Subsequent treatment with t-BuOOH promoted the oxidation of **4** and the phosphotriester **5** was isolated in 65% overall yield. The presence of the phosphate

group was firmly established on the basis of the NMR-spectra, which showed a 31P NMR signal at 0.05 ppm.

2.3 Oxidation of the Double-bond

The cleavage of the C-C double bond was implemented by a catalytic osmylation step with osmiumtetroxide and NMMO (see Figure 20). This gave rise to the formation of two isomers, the L-*glycero*-L-*gulo*-heptofuranoside 7-phosphate (**6a**) and D-*glycero*-D-*manno*- (**6b**) in a ratio of 1:3. The osmylation yielded 71% of the mixture.

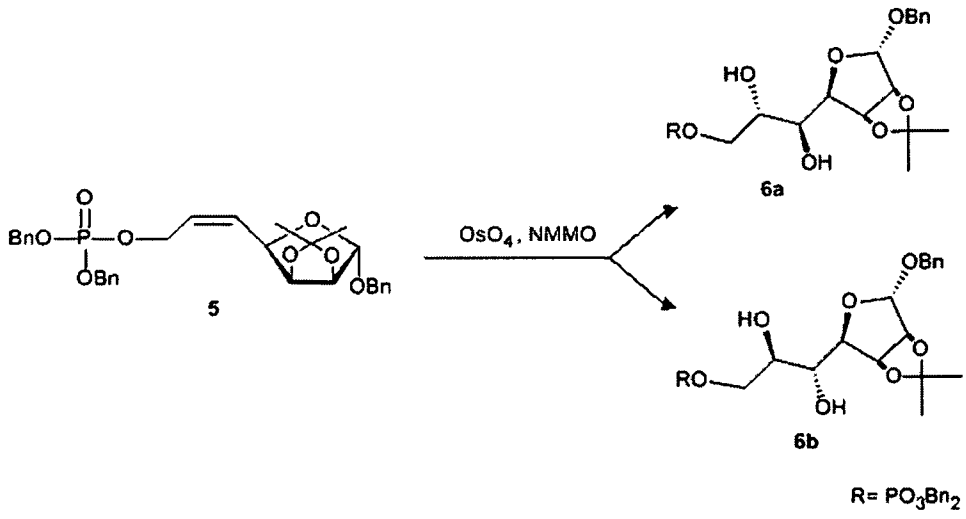

Figure 20: Reaction scheme of the osmylation

The acquired isomers were easily separated by gel chromatography with hexane/ ethyl acetate (2:3) as eluent.

The D-D isomer was successfully crystallised whereas the L-L isomer remained as a syrup. The assignment of the configuration of **6a** and **6b** was based on the vicinal coupling constant of H-4 and H-5. This value was smaller for the L-L-isomer ($J_{4,5} \approx 2.5$ Hz) than for the D-D-isomer ($J_{4,5} \approx 7.0$ Hz).

2.4 Hydrogenation and Acid Cleavage

The next step after the osmylation was the elimination of the benzyl protecting groups and the removal of the isopropylidene group to allow the rearrangement of the heptofuranose to a heptopyranose.

The protecting groups were removed by catalytic hydrogenation at atmospheric pressure in the presence of 10% Pd/C as a catalyst for 12h (Figure 21).

After the hydrogenation, the compound would need an acid treatment in order to form the heptopyranose configuration. However, the pH of the solution was 1 before addition of sulphuric acid, *de facto* it was presumed that the phosphate group rearranged the heptofuranose form thus acidic treatment was not necessary any more. The NMR spectrum of 7 confirmed this assumption. The pH was then adjusted to 5.5 by adding triethylamine and the product was obtained in 81% yield.

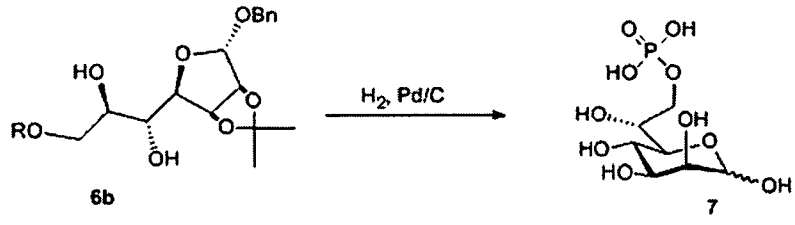

Figure 21: Reaction scheme of hydrogenation

The last purification step was achieved by elution on a PD-10 column with water. The lyophilisation of the end product gave an amorphous material. Integration of the ^{1}H NMR signals gave evidence of the presence of a mono triethylammonium salt. ^{31}P NMR showed one clear peak at 0.92 ppm for the phosphate residue.

Finally two fractions, the acidic fraction at pH 1 and the triethylammonium salt at pH 5, of the end product was obtained.

The structural assignments of the target compound were confirmed by NMR- and MS-data (see Appendices). The 1H NMR-spectrum of 7 indicated the presence of β- and α-heptopyranose forms in a ratio of 1:1.54.

The obtained NMR spectra of the final product were compared to the spectra of the native compound. These were previously recorded from samples, which were isolated from cell lysates of *Aneurinibacillus thermoaerophilus*, during biosynthesis pathway analysis of nucleotide-activated heptoses by Prof. P. Messner and Prof. P. Kosma.[28] Signals of both probes corresponded to each other.

The final product obtained from this research project was sent to several institutes based in Canada and the US. Studies of enzyme activity and kinetics as well as X-ray experiments had been awaiting the synthesis of *glycero-manno*-heptose 7-phosphate, which is not available from commercial sources.

2.5 Further Heptose experiments

High Resolution Magic Angle Spinning (HR-MAS) NMR was applied to identify genes involved in the biosynthesis of nucleotide-activated heptoses by Reid *et al.* They screened *Campylobacter jejuni* NCTC 11168 and putative heptose mutants Δ*gmhA2*, Δ Cj1427, ΔCj1428 and ΔCj1430 by HR-MAS NMR for the loss of heptose. During this study, all four mutants showed the lack of the heptose anomeric ^{1}H signal at δ 5.70 ppm (Figure 22).

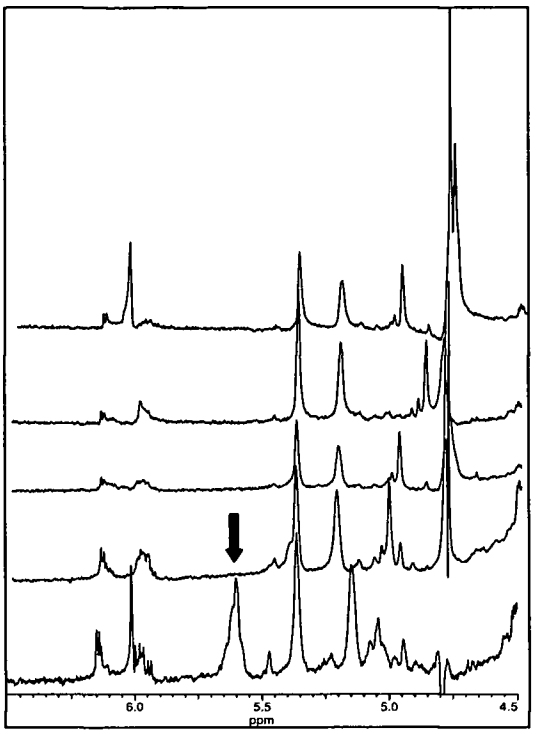

Figure 22: HR-MAS NMR spectrum of *C. jejuni* strain (bottom graph) and heptose lacking mutants (top four)[31]

Selective methods for the detection of sugar phosphates[29] and nucleotide-activated heptoses[30] using CE-ESMS and precursor ion scanning have been developed. Soo *et al.* implemented selective analysis strategies for nucleotide-activated sugars. They demonstrated the application of this MS methods for the identification of intracellular pools of sugar nucleotides of wild-type and isogenic[g] mutants from the bacterial pathogen *Campylobacter jejuni*. These techniques were also applied in a metabolomics study of heptose biosynthesis pathway in the same species by Hui *et al.*[29]

Reid *et al.* performed metabolomics studies of cell lysates from *Campylobacter jejuni* 11168 and the mutants of Δ*gmhA2*, Δ Cj1427, ΔCj1428 and ΔCj1430 in order to investigate the accumulation of sugar-phosphates and sugar-nucleotides relevant to the

[g] Isogenic means genetically identical (except for sex) - coming from the same individual or from the same inbred strain (http://www.biochem.northwestern.edu/holmgren/Glossary/Definitions/Def-I/isogenic.html)

biosynthesis of heptose implying CE-ESMS and precursor ion scanning. These experiments verified the accumulation of heptose intermediates.[31]

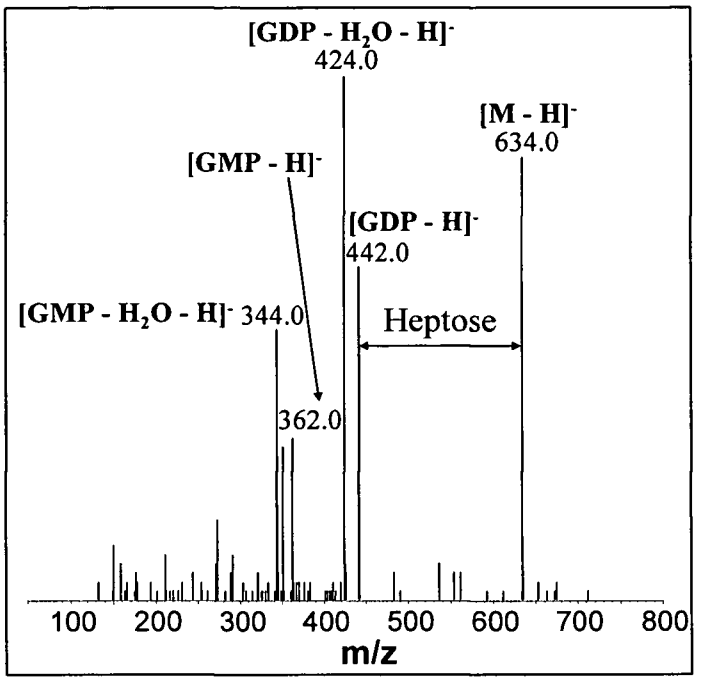

Figure 23: CE-ESMS/MS spectra of two mutant lysates of *C. jejuni* showing the presence of GDP-heptose (m/z 634)[31]

3 Conclusion

3.1 Outlook of the Master Thesis

The aim of this project was to synthesize D-*glycero*-D-*manno*-heptose 7-phosphate **(7)** chemically. A way of producing this molecule has not been published before and hence it was not available from any sources. Several studies for the elucidation of core polysaccharide, CPS and S-layer pathways have been awaiting this product – as a substrate or related compounds to the native substrate – for both enzymatic and structural analysis.[8,32]

The production of the activated heptose sugar was implemented successfully. The synthesis was performed conveniently through a six-step procedure, including a Wittig reaction with (methoxycarbonylmethylidene)triphenyphosphorane, phosphorylation using phosphoramidite and dihydroxylation via osmiumtetroxide. Further reactions that were used during the accomplishment of the work were reduction, oxidation and hydrogenation steps (Figure 24). Protecting groups remained until the last step (hydrogenation) was performed. In this stage, deacetalisation through an acid treatment was not necessary, because under the acidic nature of the compound itself (due to the phosphate group), the respective hydroxyl groups were deprotected autonomously.

This master thesis has been published: Güzlek, H.; Graziani, A.; Kosma, P. A short synthesis of D-*glycero*-D-*manno*-heptose 7-phosphate. *Carbohydr. Res.*, **2005**, 340, 2808-11.

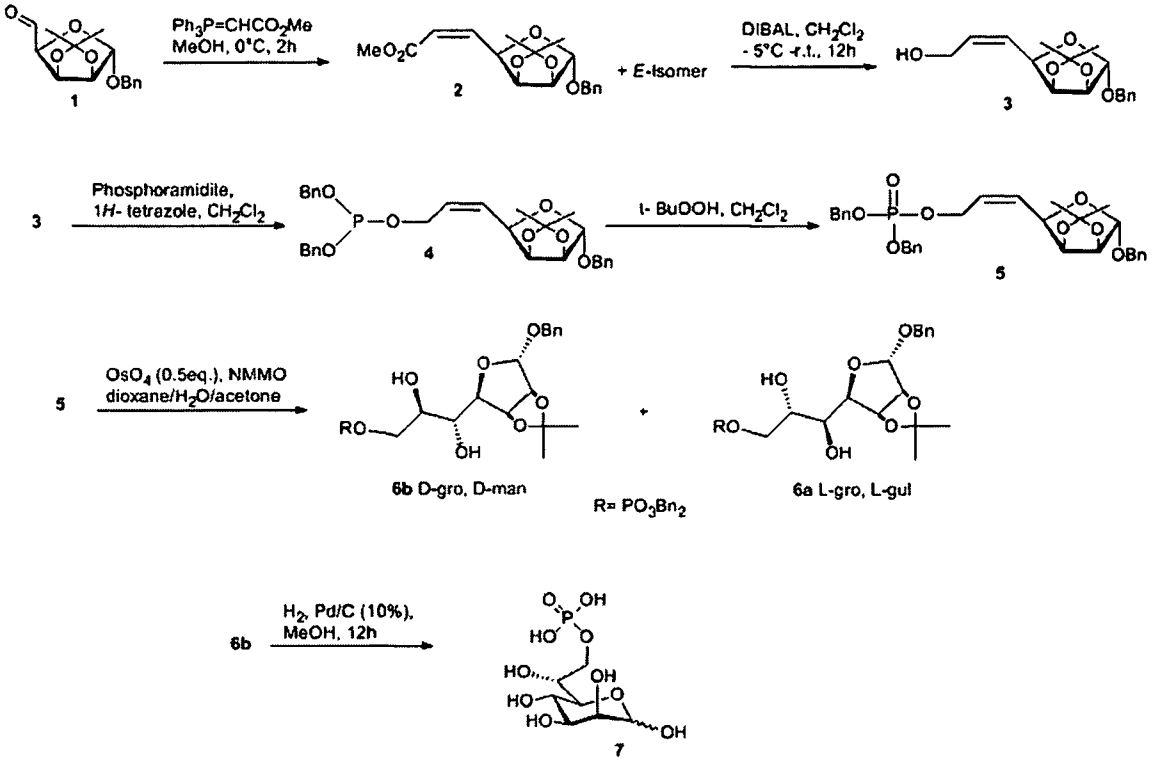

Figure 24: Reactions performed

3.2 Biological Studies involving D-*glycero*-D-*manno*-Heptose 7-Phosphate

As mentioned before, several chemoenzymatic and enzyme kinetics studies and experiments involving structural determination of enzyme-substrate complexes have been awaiting the synthesis of the D-D-heptose 7-phospate.

Finally, the end product was utilised in biochemical assays to determine the kinetics and mutated enzyme forms of HldE to unequivocally probe the function of mutated residues by M. Valvano *et. al.*

Another investigation, in which the heptose 7-phosphate was employed, was to test it as a substrate for an ORF phosphatase from *E. coli* denoted CAE. Although it was established that the substrate for this protein was the D-*glycero*-D-*manno*-heptose 1,7-

bisphosphate, the related heptose was employed as the next best possibility. Furthermore, the product was used for cocrystallisation with its enzyme.

In addition, the compound has been used as a potential ligand for human lung surfactant protein D, a component of the innate immune system. It could be shown that the 7-phosphate inhibits binding to SP-D.[33]

4 Experimental Part

4.1 Materials and Methods

4.1.1 Chromatography

"Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction."[34]

4.1.1.1 Thin Layer Chromatography

Thin layer chromatography (TLC) is a widely-used separation and differentiation technique for chemical compounds. The TLC plate consists of a sheet (e.g. of glass, metal or plastic), which is coated with a solid absorbent such as silica or alumina. After a small amount of the mixture containing the analytes is spotted near the bottom of a plate, it is then placed in a shallow pool in the development chamber. As the eluent – the mobile phase – slowly rises up the TLC plate by capillary action, the analytes will be carried differently (depending on their solubility in the mobile phase and the strength of their adsorption to the stationary phase) farther up.[35]

In course of the project, analytical TLC was performed using silica gel 60 F_{254} HPTLC plates with 2.5 cm concentration zone (Merck). Spots were detected by treatment with anisaldehyde – H_2SO_4 or by UV-absorption.

4.1.1.2 Column Chromatography

Column Chromatography is a preparative separation method, in which the stationary phase is within a tube. In classical column chromatography the glass tube with a tap at the bottom is filled with stationary phase. After a solution of analytes has been applied

on the top of the stationary phase, the eluent is passed through the column. The components are retained differently by the stationary phase and thus separate from each other. Fractions are collected and tubes are checked by TLC to determine further processing.[35]

During the accomplishment of this work, column chromatography was performed on silica gel 60 (230 – 400 mesh) by Merck.

4.1.2 Optical Rotation

A polarimeter (Figure 25) in its simplest form consists of a monochromatic light source, a fixed polariser (e.g. Nicol prism), a sample tube, an analyser (rotatable polariser) and a detector. When the analyser is rotated 90° to the polariser (starting condition), no light reaches the detector.[36]

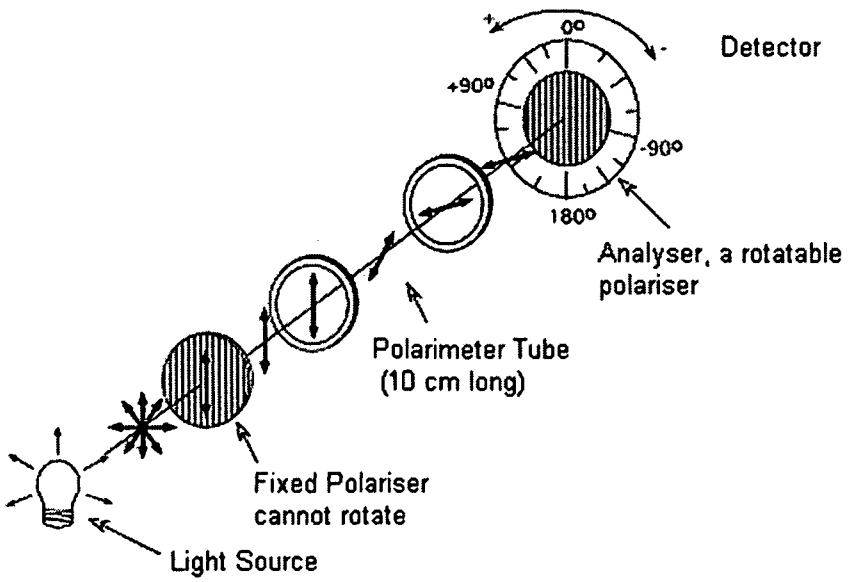

Figure 25: Polarimeter – schematically (http://www.creation-science-prophecy.com/amino/polarimeter.gif)

After the light passes through the polariser, it consists of light waves in which the electromagnetic oscillation occurs in a single plane. It is then sent through a sample cell (tube with flat glass ends containing a solution of the analyte), where the plane of polarised light is changed by the optically active substance. The angle that the analyser has to be rotated in order to get the minimum detector signal is the optical rotation, [α]. This rotation angle depends on the length of the sample tube, the wavelength (λ) of the light, temperature (T), the concentration (c) of the analyte and the specific rotation ($[\alpha]^T{}_\lambda$) of the substance, according this equation: $[\alpha] = [\alpha]^T{}_\lambda * c * l/100$

Optical rotations of compounds in this thesis were measured with a Perkin–Elmer 243 B polarimeter at 20 °C and a wavelength of 589 nm. The concentrations are given in g/ 100 mL.

4.1.3 Nuclear Magnetic Resonance

4.1.3.1 NMR Techniques

A spinning charged nucleus (such as ^1H, ^2H or D, ^{13}C, ^{15}N, ^{19}F, ^{17}O, ^{31}P) generates a magnetic field. In the course of an NMR experiment, these isotopes generate signals, which can be detected by the NMR spectrometer and displayed as a plot of signal frequency versus intensity.[37,38]

4.1.3.2 Application of NMR Spectroscopy for this Project

In the course of this research project, NMR spectra were recorded at 297 K in D_2O and $CDCl_3$ with a Bruker DPX 300 spectrometer equipped with an inverse QNP-probehead and Z-gradients using standard Bruker NMR software. ^1H were recorded at 300.13 MHz, ^{13}C at 75.47 MHz and ^{31}P at 121.50 MHz. ^1H NMR spectra were referenced to tetramethylsilane or 2,2-dimethyl-2-silapentane-5-sulfonic acid. ^{13}C NMR spectra were referenced to chloroform for solutions in $CDCl_3$ (δ 77.00) or dioxane (δ 67.40) for

solutions in D_2O. ^{31}P NMR spectra were referenced externally to 85% aq. H_3PO_4 (δ 0.0). The NMR spectra in this work were recorded by Dr. Andrea Graziani and Dr. Andreas Hofinger.

4.1.4 Mass Spectrometry

4.1.4.1 Principles of Mass Spectrometry

Mass Spectrometry (MS) is an analytical tool used for characterisation of a molecule by measuring the mass-to-charge ratio of its ions. It is a powerful technique used to identify unknown compounds, to quantify known compounds, and to elucidate the structure and chemical properties of molecules.[39]

A typical MS comprises three parts: an ion source, a mass analyser and a detector system. In the first phase of the MS experiment, the compounds are evaporised and ionised into electrically charged particles. There are different ionisation methods. The most common ionisation techniques are EI (electron impact), ESI (electron spray ionisation), laser desorption and MALDI (matrix assisted laser desorption ionisation). The ions are then accelerated by impressing a voltage and focussed to a beam. In the mass analyser, they are deflected in a magnetic field by their mass-to-charge ratio (Figure 26). Typical mass analysis instrumentations are sector, time-of-flight, quadrupole, fourier transform ion cyclotron resonance etc. Finally, the detector records the charge induced or current produced when an ion passes by or hits the surface of the detector. Typical detectors used in MS experiments are electron multipliers; other detectors like Faraday cups and ion-to-photon detectors are also implied.[40,41]

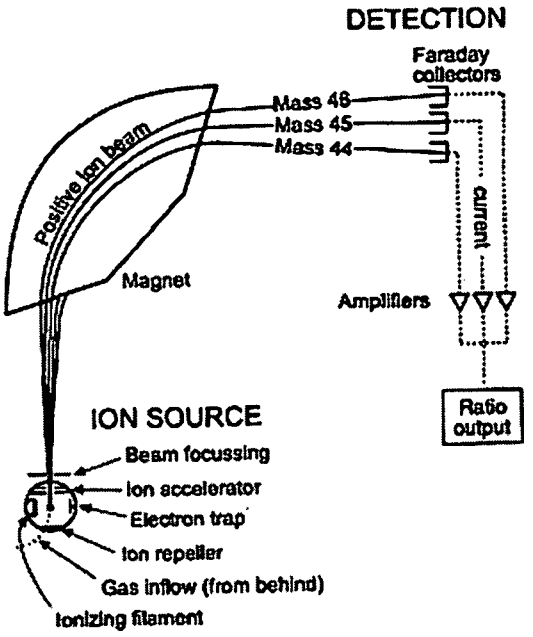

Figure 26: Schematics of MS (http://en.wikipedia.org/wiki/Image:Mass_spectrometer_schematics.png)

4.1.4.2 Application of MS for this project

During the research project, Waters Micromass Q-TOF Ultima Global instrument was used to obtain ESI-MS data of the final compound. The measurement was accomplished by Dr. Daniel Kolarich.

4.2 Synthetic Procedures

4.2.1 Synthesis of Methyl-[benzyl-(*Z*)-5,6-dideoxy-2,3-*O*-isopropylidene-α-D-*lyxo*-hept-5-enofuranosid]-uronate (2)

The syrup of **1** (2.3 g, 8.36 mmol) and (methoxycarbonylmethylidene)triphenyl-phosphorane (3.08 g, 9.2 mmol) was dissolved in dry methanol (40 mL) was and injected through a septum. The solution was stirred at 0 °C for three hours. After the

completion of the reaction that had been monitored with TLC, the mixture was concentrated. The residue was purified by silica gel chromatography (4:1, n-hexane/diethyl ether) and gave the colourless oil of **2** (2.15 g, 85%) as the major product; a small amount (80 mg) of its *E*-isomer (Methyl-[benzyl-(*E*)-5,6-dideoxy-2,3-*O*-isopropylidene-α-D-*lyxo*-hept-5-enofuranosid]-uronate) was also obtained. Data for **2**: R_f 0.60

4.2.2 Synthesis of Benzyl-(*Z*)-5,6-dideoxy-2,3-*O*-isopropylidene-α-D-*lyxo*-hept-5-enofuranoside (3)

The oil of **2** (2.15 mg, 6.43 mmol) was cooled (-5 °C) and stirred under Argon-atmosphere in anhydrous dichloromethane (27 mL). A solution of diisobutylaluminium hydride in 1 M dichloromethane (32 mL) was added to the reaction mixture, which was then stirred at 0 °C for three hours.

When the reaction ended, the mixture was diluted with dichloromethane and the excess of the reagent was destroyed with saturated, aqueous ammonium chloride. The insoluble material was filtered off and the filtrate was washed with water first and then with brine. $MgSO_4$ was added to the organic phase to remove the remaining water. It was then filtered off and the liquid remainder was concentrated. Column chromatography of the residue on silica gel (7:3, toluene/ ethyl acetate) gave **3** as a colourless oil (1.26 g, 67%).

^{1}H NMR ($CDCl_3$): δ 7.36-7.29 (m, 5H, Ph), 6.00-5.90 (m, 1H, H-6), 5.74-5.81 (m, 1H, H-5), 5.10 (s, 1H, H-1), 4.80 (dd, 1H, H-4), 4.71-4.65 (m, 2H, H-3, H-2), 4.5 (ABq, 2H, $^2J_{A,B}$=12.0 Hz, CH_2Ph), 4.30-4.14 (m, 2H, H-7a,b), 1.60 (s, 1H, OH), 1.48, 1.30 [2s, 6H, $C(CH_3)_2$].

4.2.3 Benzyl-(Z)-5,6-dideoxy-2,3-O-isopropylidene-α-D-*lyxo*-hept-5-enofuranosyl-7-dibenzylphosphite (4) and Benzyl 7-*O*-[bis(benzyloxy)phosphoryl]-5,6-dideoxy-2,3-*O*-isopropylidene-α-D-*lyxo*-(*Z*)-hept-5-enofuranoside (5)

Compound **3** (680 mg, 2.22 mmol) and bis(benzyloxy)-*N,N*-diisopropylaminophosphine (1.87 mL, 5.55 mmol) were dried by repeated evaporation with dry toluene (4 x 10 mL) and then under reduced pressure for 5 h. Then CH_2Cl_2 (6 mL) was added to the sample. The flask was charged with a solution of 1*H*-tetrazole (467 mg, 6.66 mmol) in dry acetonitrile (3 mL) and stirred at room temperature for 2 h under Argon. Monitoring of the reaction by TLC showed the formation of phosphite trieesters **4** (7:3, toluene-EtOAc). The reaction mixture was cooled to 0 °C and a solution of *t*-BuOOH (617 μL, 3.33 mmol, 80% solution in di-*tert*-butyl peroxide) was gradually added. The solution was stirred for 15 h at room temperature and the solvent was evaporated using a stream of Argon. The residue was dissolved in 2:1 diethyl ether-EtOAc (50 mL) and washed sequentially with satd $NaHCO_3$-water (until pH 9 was reached) and brine. The organic phase was dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography (1:1, toluene-diethyl ether) to give **5** as a colourless syrup (815 mg, 65%).

$[\alpha]^{20}_D$ +26 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.36- 7.25 (m, 15 H, Ph), 5.85 (dd, 1 H, $J_{6,5}$ 10.8, $J_{5,4}$ 1,3 Hz, H-5), 5.79 (ddd, 1 H, $J_{6,7a} = J_{6,7b}$ 2.3 Hz, H-6), 5.05 (s, 1 H, H-1), 5.09-4.94 (m, 4 H, CH_2Ph), 4.71- 4.59 (m, 5 H, H-4, H-7a, H-7b, H-2, H-3), 4.65 and 4.47 (AB system, 2 H, $J_{A,B}$ 11.9 Hz, CH_2Ph), 1.43 and 1.27 [s, each 3 H, $C(CH_3)_2$]; ^{13}C NMR ($CDCl_3$): δ 137.22 (Ph), 135.83 and 135.74 (C-5, C-6), 128.71-127.42 (Ph), 112.56 [$C(CH_3)_2$], 105.32 (C-1), 85.29 (C-3), 81.35 (C-2), 75.61 (C-4), 69.31, 69.24 and 69.05 (CH_2Ph), 63.55 ($J_{C,P}$ 5.3 Hz, C-7), 26.04 and 24.77 [$C(CH_3)_2$]; ^{31}P NMR ($CDCl_3$): δ 0.05. *Anal.* Calcd for $C_{31}H_{35}O_8P$: C, 65.72; H, 6.23. Found: C, 65.47; H, 6.60.

4.2.4 Synthesis of Benzyl 7-*O*-[bis(benzyloxy)phosphoryl]- 2,3-*O*-isopropylidene-L-*glycero*-β-L-*gulo*-heptofuranoside (6a) and benzyl 7-*O*-[bis(benzyloxy)phosphoryl]-2,3-*O*-isopropylidene-D-*glycero*-α-D-*manno*-heptofuranoside (6b)

A solution of **5** (815 mg, 1.44 mmol) and NMMO (388.7 mg, 2.88 mmol) in 2:1 dioxane-water (10 mL) and acetone (3 mL) was stirred at r.t. Then osmium tetraoxide (1.5 mL, 0.12 mmol; 2% in water) was transferred into the flask and the mixture was stirred for 5 h. The solution was then diluted with chloroform (150 mL). After treatment with ice-cold 5 M hydrochloric acid (6 mL) the mixture was vigorously shaken with 45% aqueous $Na_2S_2O_5$ (9 mL) and water. The organic phase was dried ($MgSO_4$) and concentrated to a syrupy residue (580 mg, 71%). D-*Glycero*-α-D-*manno* heptofuranoside **6b** and the L-*glycero*-β-L-*gulo* diastereoisomer **6a** were separated by chromatography on silica gel (2:3 *n*-hexane-EtOAc) to give 445 mg of **6b** as colourless crystals.

Mp 82-83° (hexane-EtOAc), R_f 0.62; $[\alpha]^{20}_D$ +40 (*c* 0.9, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.37- 7.26 (m, 15 H, Ph), 5.12 (s, 1 H, H-1), 5.10- 5.04 (m, 4 H, $POCH_2Ph$), 4.91 (dd, 1 H, $^3J_{3,2}$ 6.0, $^3J_{3,4}$ 3.8 Hz, H-3), 4.70 (s, 2H, CH_2Ph), 4.63 (d, 1 H, H-2), 4.61 (dd, 1 H, $J_{7a,7b}$ 11.8, $^3J_{7a,6}$ 5.0 Hz, H-7a), 4.45 (m, 1 H, H-7b), 4.16 (dd, 1 H, $^3J_{4,5}$ 7.0 Hz, H-4), 4.01 (t, 1 H, $^3J_{5,6}$ 7.0 Hz, H-5), 3.92 (m, 1 H, $^3J_{6,7b}$ 3.0 Hz, H-6), 3.78 (br. s, OH), 1.44 and 1.30 [2 s, 6 H, $C(CH_3)_2$]; ^{13}C NMR ($CDCl_3$): δ 137.09 (Cquart. Ph), 128.65-126.99 (Ph), 112.70 [$C(CH_3)_2$], 105.46 (C-1), 84.73 (C-2), 80.44 (C-3), 79.03 (C-4), 72.87 (d, $J_{C6,P}$ 4.9 Hz, C-6), 69.75 (d, $POCH_2Ph$), 69.35, 69.25 and 69.17 (C-5, C-7, $POCH_2Ph$), 65.36 (CH_2Ph), 25.92 and 24.45 [$C(CH_3)_2$]; ^{31}P NMR ($CDCl_3$): δ 1.14. *Anal.* Calcd. for $C_{31}H_{37}O_{10}P$: C, 61.99; H, 6.21. Found: C, 61.46; H, 6.26.

Further elution furnished **6a** as a syrup (115 mg), R_f 0.57; $[\alpha]^{20}_D$ +43 (*c* 1.3, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.36-7.26 (m, 15 H, Ph), 5.16 (s, 1 H, H-1), 5.11-5.04 (dd, 4 H, $POCH_2Ph$), 4.82 (dd, 1 H, $^3J_{3,2}$ 5.9, $^3J_{3,4}$ 3.6 Hz, H-3), 4.66 (d, 1 H, H-2), 4.67 and 4.49 (AB-system, 2 H, $J_{A,B}$ 11.7 Hz, CH_2Ph), 4.33 (dd, 1 H, $J_{7a,7b}$ 11.0, $^3J_{7a,6}$ 2.6 Hz, H-7a),

4.28 (dd, 1 H, $^3J_{4,5}$ 2.5 Hz, H-4), 4.23 (dd, 1 H, $^3J_{7b,6}$ 5.0 Hz, H-7b), 3.99 (dd, 1 H, H-5), 3.89 (m, 1 H, H-6), 2.60 (br. s, OH), 1.47 and 1.30 [2 s, 6 H, $C(CH_3)_2$]; ^{13}C NMR ($CDCl_3$): δ 137.42, 135.84, 135.72 (Cquart. Ph), 128.76- 128.02 (Ph), 112.91 [$C(CH_3)_2$], 104.88 (C-1), 85.54 (C-2) , 81.87 (C-3), 76.65 (C-4), 70.96 (d, $J_{C6,P}$ 4.9 Hz, C-6), 69.85, 69.82, 69.78 and 69.73 (C-5, C-7, $POCH_2Ph$), 69.23 (CH_2Ph), 25.77 and 24.09 [$C(CH_3)_2$]; ^{31}P NMR ($CDCl_3$): δ 1.36. *Anal.* Calcd. for $C_{31}H_{37}O_{10}P$: C, 61.99; H, 6.21. Found: C, 61.48; H, 6.37.

4.2.5 Synthesis of D-*Glycero*-D-*manno*-heptopyranose 7-phosphate (triethylammonium salt) (7)

A solution of **6b** (125 mg, 0.21 mmol) in dry MeOH (5.5 mL) was stirred at room temperature in the presence of 10% Pd/C (45 mg) for 12 h under hydrogen at atmospheric pressure. After completion of the reaction, the catalyst was removed by filtration through Celite and washed with MeOH. The filtrate was concentrated, redissolved in water (4 mL; the compound was acidic: pH 1) and stirred for 5 h at room temperature. Then the pH was adjusted to 5.5 by adding triethyl amine (200 µL) and the solution was lyophilized to give **7** as amorphous material. Final purification was achieved by elution on a PD-10 column (PD-10 desalting column containing Sephadex^{TM} G-25) with water as eluent to furnish 66 mg (81%) of **7**.

$[\alpha]^{20}_D$ +6.5 → +9.7 (*c* 0.85, H_2O); R_f 0.36 (10:10:3 MeOH-$CHCl_3$-H_2O); ^1H NMR (D_2O): δ 5.16 (d, 1H, $^3J_{1,2}$= 1.69 Hz, H-1α), 4.87 (d, 1H, H-1β), 4.22- 4.01 (m, 3H, H-6β, H-2α), 4.01- 3.51 (m, 9H, H-2β, H-6α, H-7a, H-7b, H-3, H-4, H-5α), 3.50- 3.38 (dd, 1H, H-5β); ^{13}C NMR (D_2O): δ 94.85, 94.77 (C-1α, C-1β), 76.79 (C-5α), 74.06 (C-5β), 72.90 (C-3), 71.84- 71.29 (C-6), 68.34 (C-7), 68.15, 68.05 (C-4α, C-4β), 66.50, 66.43, 66.30, 66.23 (C-2α, C-2β); ^{31}P NMR (D_2O): δ 1.49; ESI-MS: 291.0508 $[MH]^+$; calcd. for $C_7H_{15}O_{10}P$: 291.0476.

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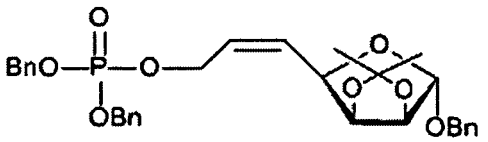
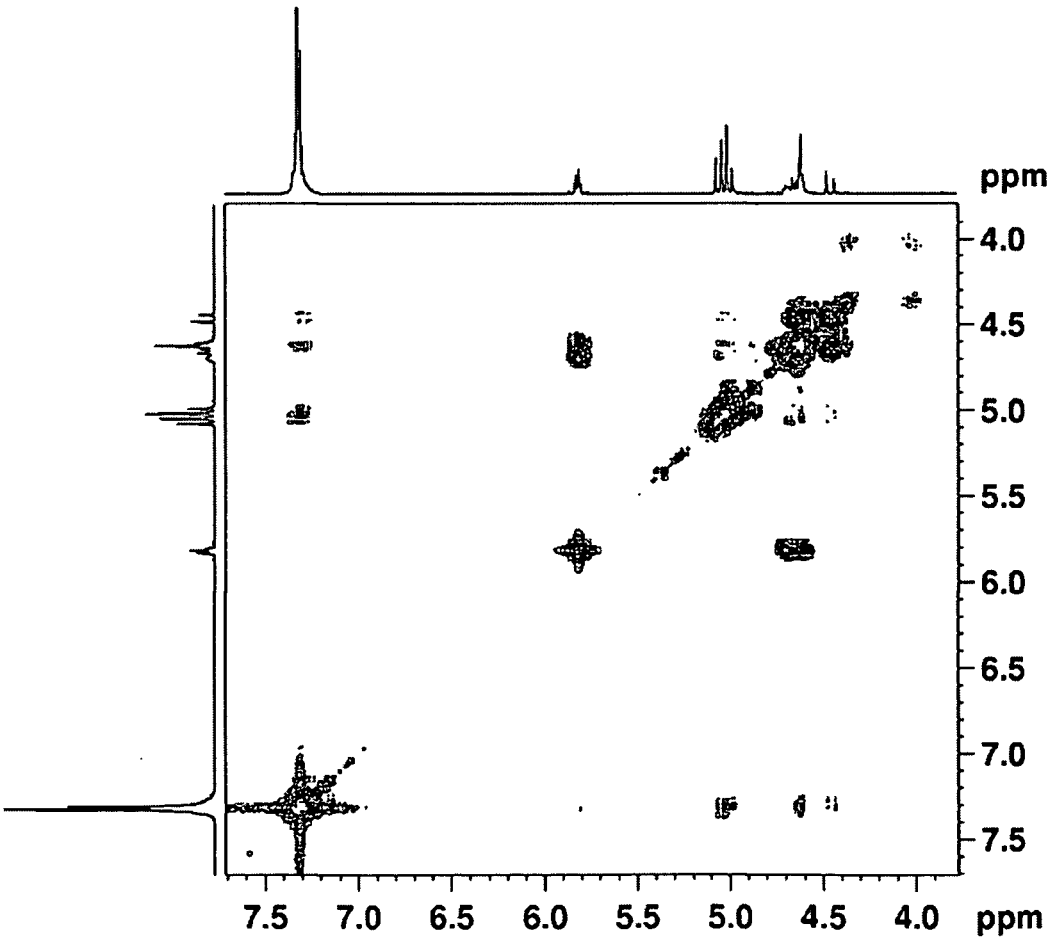
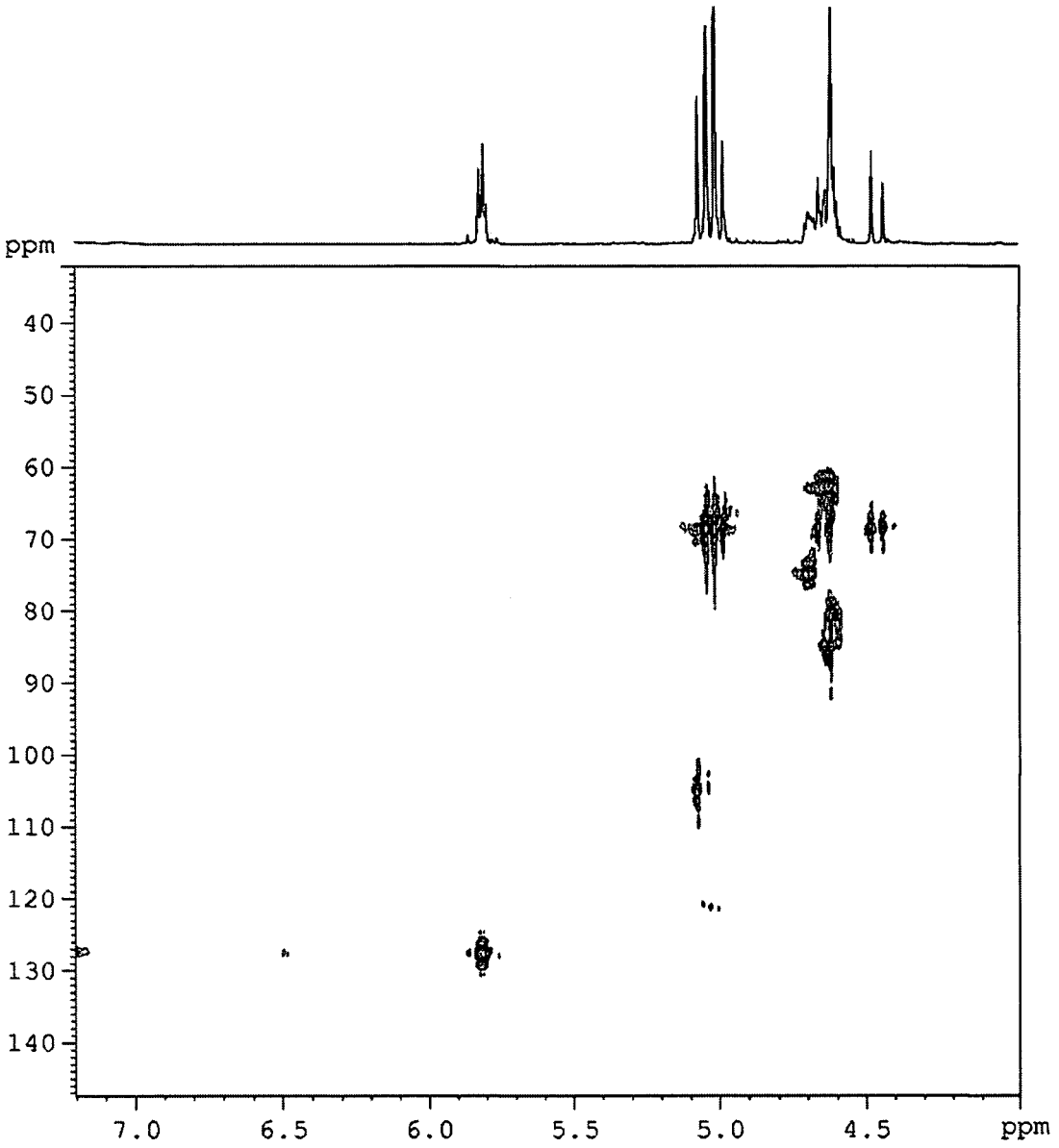
6 Appendices

6.1 NMR Spectra

6.1.1 Benzyl 7-*O*-[bis(benzyloxy)phosphoryl]-5,6-dideoxy-2,3-*O*-isopropylidene-α-D-*lyxo*-(*Z*)-hept-5-enofuranoside (5)



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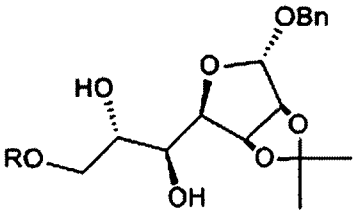
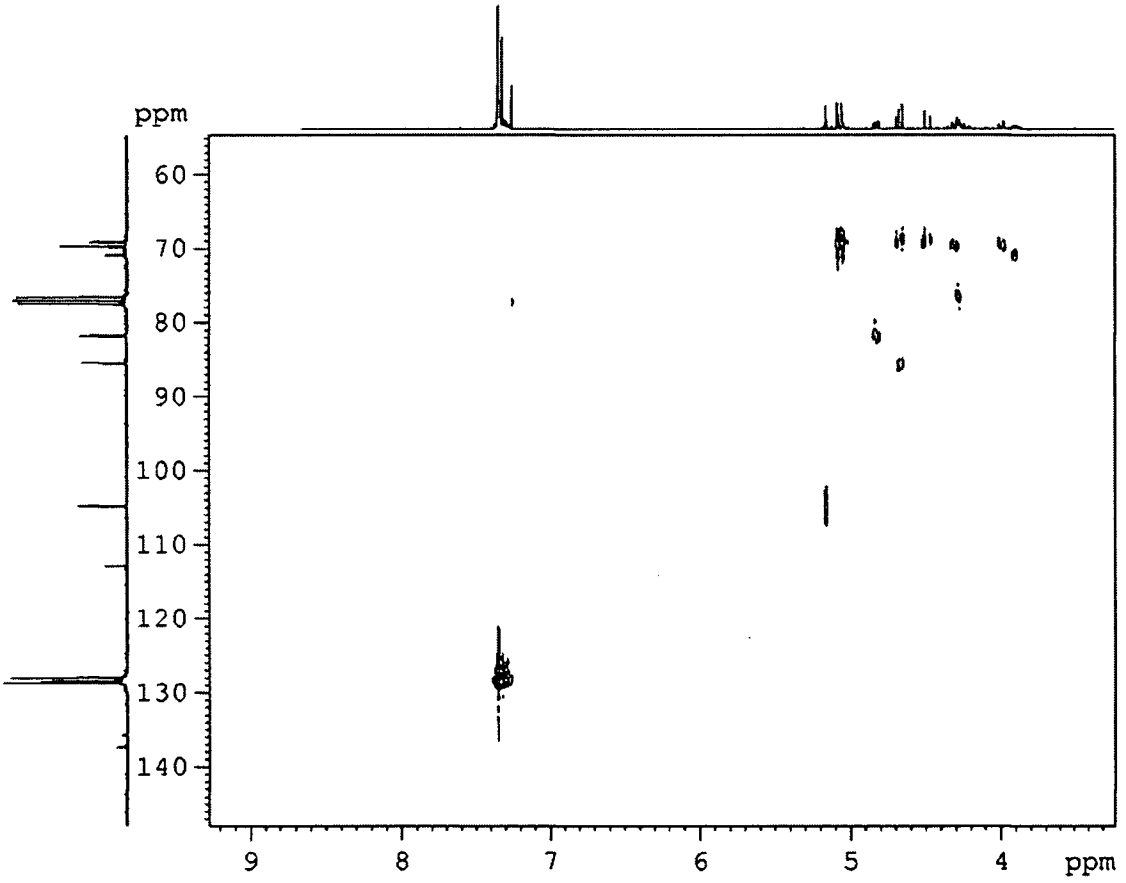
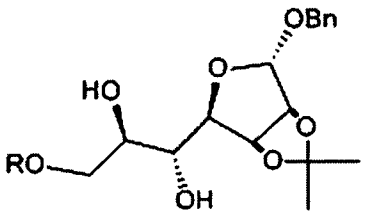
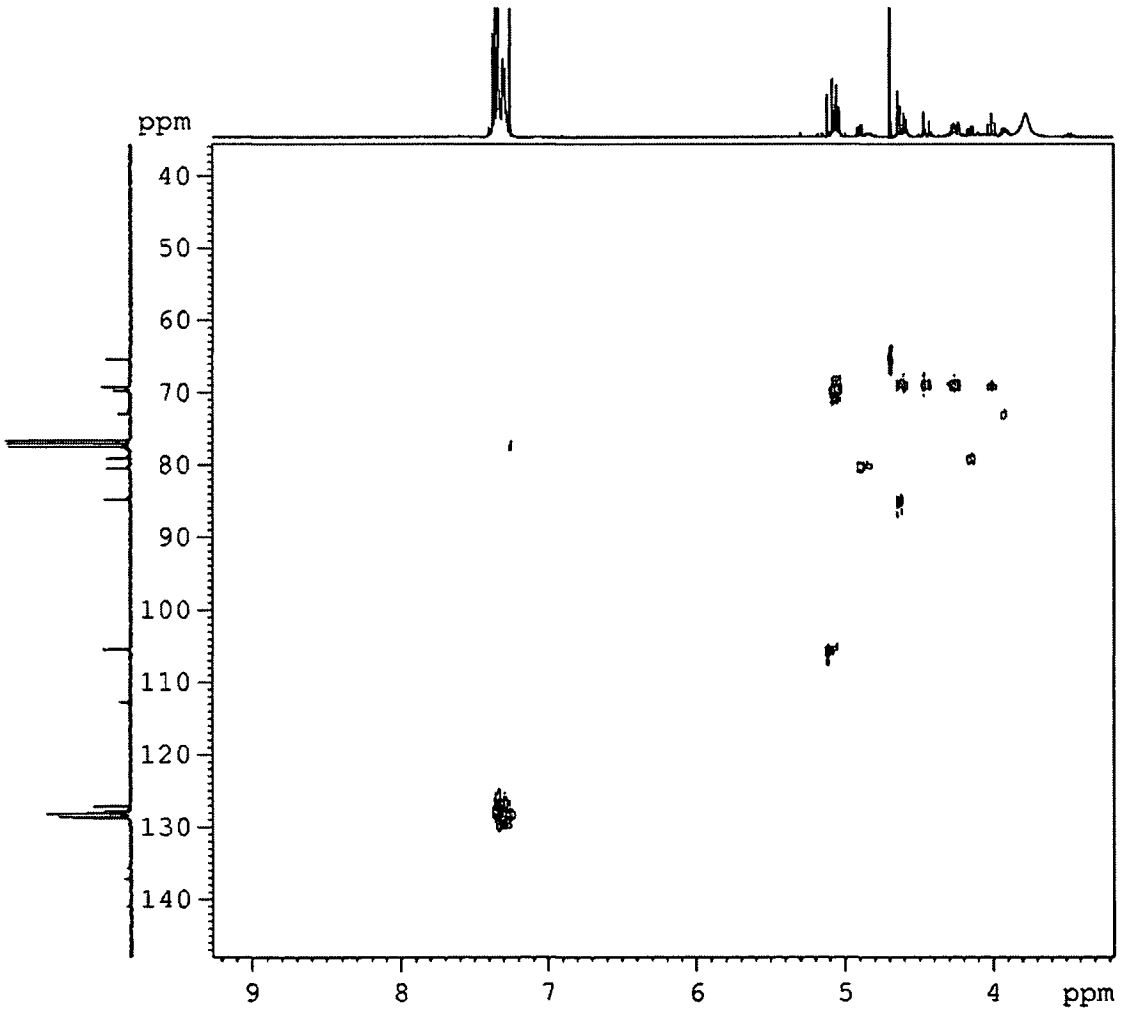
6.1.2 Benzyl 7-*O*-[bis(benzyloxy)phosphoryl]- 2,3-*O*-isopropylidene-L-*glycero*-β-L-*gulo*-heptofuranoside (6a)



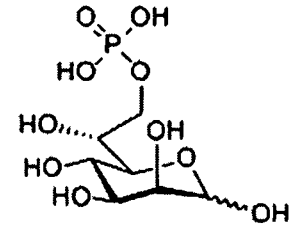


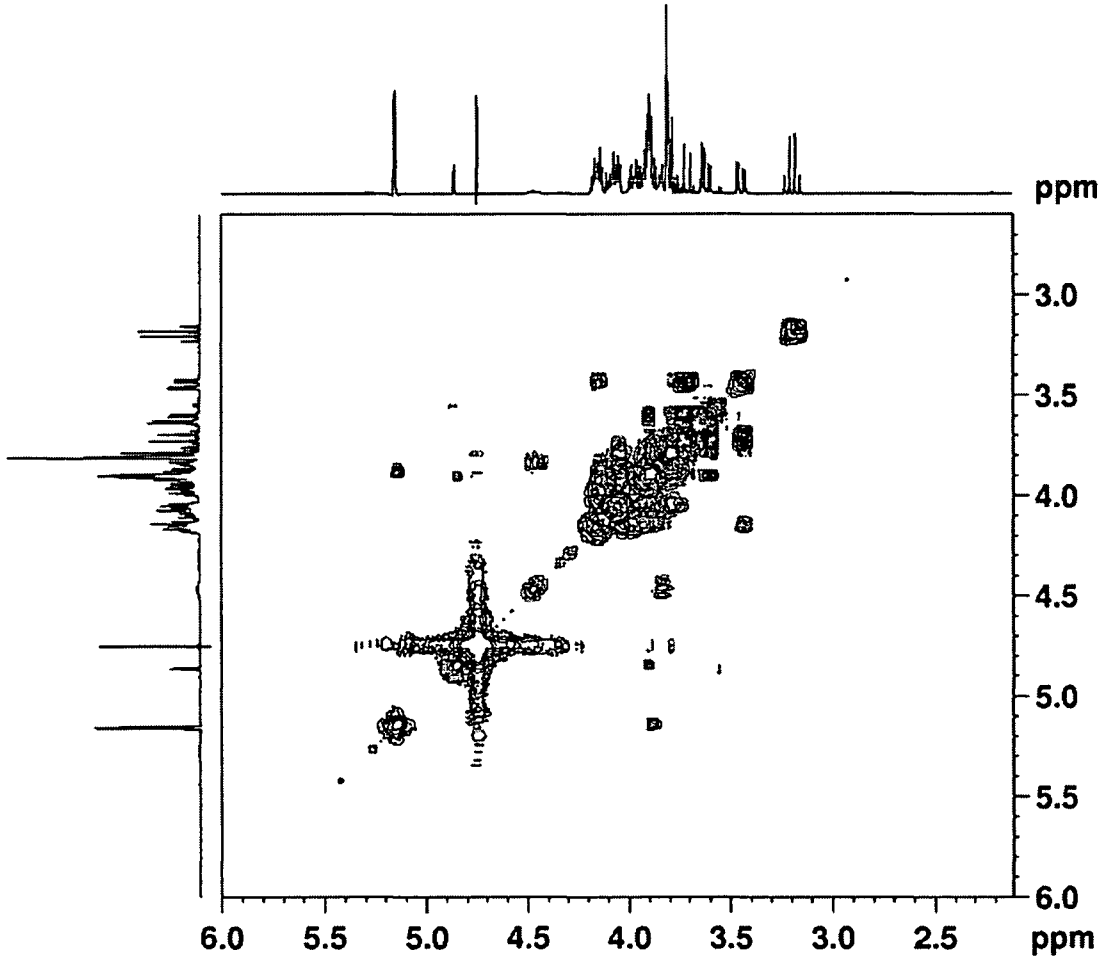
6.1.3 Benzyl 7-*O*-[bis(benzyloxy)phosphoryl]-2,3-*O*-isopropylidene-D-*glycero*-α-D-*manno*-heptofuranoside (6b)

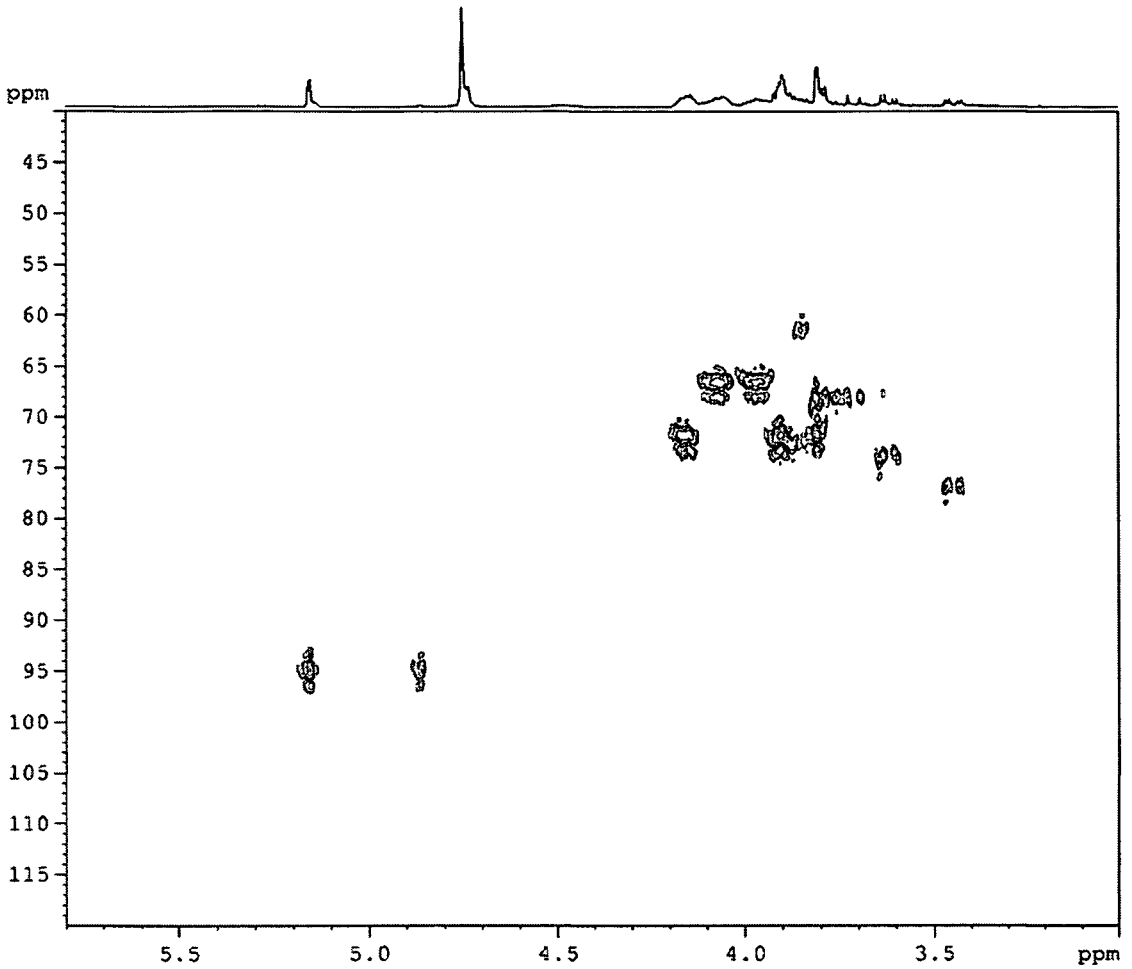




6.1.4 D-*Glycero*-D-*manno*-heptopyranose 7-phosphate (triethylammonium salt) (7)



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