

TOWARDS FULLY SYNTHETIC CANCER VACCINES: SYNTHESIS OF TUMOR  
ASSOCIATED CARBOHYDRATE ANTIGENS AND CANCER VACCINE  
CONSTRUCTION AND IMMUNOLOGICAL EVALUATION

by

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ABSTRACT

Cancer is a menacing, worldwide health problem for which there exist no effective therapies. In this thesis, an approach towards the development of novel fully synthetic carbohydrate-based cancer vaccines for the treatment of various human cancers is presented.

Although several vaccines have a proven history in the fight against many serious diseases (such as polio, mumps, and measles), carbohydrate-based cancer vaccines are in their infancy and in the experimental stage. Three main obstacles inhibit the development of a carbohydrate-based cancer vaccine: the availability of pure oligosaccharides, the poor immunogenicity of pure oligosaccharides and T-cell independence of pure oligosaccharides.

In this research, the three obstacles were eliminated through the convergence of three areas of scientific expertise: synthetic chemistry, vaccine formulation and immunology. To assess the cancer vaccine development, the Lewis antigen Le<sup>y</sup> was chosen as the carbohydrate tumor marker. The Lewis antigen Le<sup>y</sup> was chosen because it is overexpressed on the surface of cancer cells and has been previously identified as an important epitope for eliciting antibodies

against colon and liver carcinomas. Large amounts of the tumor-associated Lewis antigen Le<sup>y</sup> were synthesized according to a new, highly efficient strategy and further used in the vaccine construction. Liposome was selected for vaccine formulation. A surface functionalized liposomal carrier system was constructed and optimized using a completely synthetic composition of adjuvants and lipomatrix. The obtained Lewis antigen, Le<sup>y</sup>, and the T-epitope peptide (QYI) were covalently coupled to the structurally-defined liposomal carrier system to afford a glycoconjugate vaccine. The efficacy of the novel therapeutic cancer vaccine was evaluated in an animal model, resulting in the stimulation of the desired immune response. In order to uncover the cross activity among the Lewis antigens, the dimeric tumor associated Lewis antigen Le<sup>y</sup>-Le<sup>x</sup> was also prepared following a similar strategy.

INDEX WORDS: cancer, vaccine, carbohydrate, Lewis antigen, liposome.

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## LIST OF ABBREVIATION

<b>AA</b>	<b>amino acid</b>
<b>Ac</b>	<b>Acetyl</b>
<b>Ac<sub>2</sub>O</b>	<b>Acetic anhydrite</b>
<b>Bn</b>	<b>Benzyl</b>
<b>Boc</b>	<b>benzocycarbonyl</b>
<b>Brs</b>	<b>broad singlet</b>
<b>BSA</b>	<b>bovine serum albumin</b>
<b>dd</b>	<b>double doublet</b>
<b>DBU</b>	<b>1,8-Diazabicyclo[5.4.0]undec-7-ene</b>
<b>DCC</b>	<b>dicyclohexylcarbodiimide</b>
<b>DCE</b>	<b>1,2-Dichloroethane</b>
<b>DCM</b>	<b>Methylene chloride</b>
<b>DIPEA</b>	<b>diisopropylethylamine</b>
<b>DMAP</b>	<b><i>N,N</i>-Dimethylaminopyridine</b>
<b>DMF</b>	<b>dimethylformamide</b>
<b>DMSO</b>	<b>dimethylsulfoxide</b>
<b>Et</b>	<b>Ethyl</b>
<b>EtOH</b>	<b>Ethanol</b>
<b>FAB-MS</b>	<b>Fast atom bombardment mass spectroscopy</b>
<b>Fmoc</b>	<b>fluorenylmethoxycarbonyl</b>

<b>Fuc</b>	<b>Fucoside</b>
<b>Gal</b>	<b>Galactose</b>
<b>gCOSY</b>	<b>gradient Correlation Homonuclear Spectroscopy</b>
<b>gHSQC</b>	<b>gradient Homonuclear Single Quantum Coherence</b>
<b>Glc</b>	<b>Glucose</b>
<b>GlcNAc</b>	<b><i>N</i>-Acetyl glucosamine</b>
<b>HOBt</b>	<b><i>N</i>-hydroxybezotriazole</b>
<b>HPLC</b>	<b>high performance liquid chromatography</b>
<b>Hz</b>	<b>Hertz</b>
<b>IgG</b>	<b>Immunoglobulin G</b>
<b>IgM</b>	<b>Immunoglobolin M</b>
<b>LacNAc</b>	<b><i>N</i>-acetyl lactosamine</b>
<b>Le<sup>x</sup></b>	<b>Lewis x</b>
<b>Le<sup>y</sup></b>	<b>Lewis y</b>
<b>LPS</b>	<b>lipopolysaccharide</b>
<b>m</b>	<b>multiplet</b>
<b>MALDI TOD</b>	<b>MS matrix assisted laser desorption time of flight mass spectrometry</b>
<b>MeOH</b>	<b>methanol</b>
<b>m.p.</b>	<b>Melting point</b>
<b>m/z</b>	<b>Mass to charge ratio</b>
<b>Me</b>	<b>Methyl</b>
<b>Min</b>	<b>Minute</b>

<b>mM</b>	<b>Milimolar</b>
<b>mmol</b>	<b>Milimole</b>
<b>MS</b>	<b>Molecular Sieves</b>
<b>NIS</b>	<b><i>N</i>-iodosuccinimide</b>
<b>NMR</b>	<b>Nuclear Magnetic Resonance</b>
<b>Pal</b>	<b>palmitoyl</b>
<b>Pam<sub>3</sub>Cys</b>	<b><i>N</i>-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteine</b>
<b>Ph</b>	<b>phenyl</b>
<b>ppm</b>	<b>parts per milliom</b>
<b>PyBOP</b>	<b>benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate</b>
<b>q</b>	<b>Quartet</b>
<b>Rf</b>	<b>Retention factor</b>
<b>s</b>	<b>singletamino acid</b>
<b>t</b>	<b>triplet</b>
<b>TEM</b>	<b>transmission electron microscopy</b>
<b>Tf</b>	<b>Trifluoromethanesulfonyl (triflate)</b>
<b>TFA</b>	<b>trifluoroacetic acid</b>
<b>THF</b>	<b>tetrahydrofurane</b>
<b>TLC</b>	<b>Thin layer chromatography</b>
<b>Ts</b>	<b><i>p</i>-Toluenesulfonyl</b>
<b>Z</b>	<b>benzoxycarbony</b>

## **CHAPTER 1:**

### **INTRODUCTION**

Carbohydrates play central roles in a wide range of biological phenomena<sup>1</sup> such as cell-cell adhesion, cell growth, inflammatory processes, fertilization, and bacterial, viral and parasitic infections. Major changes in carbohydrate expression occur during the onset and progression of various diseases such as cancer and rheumatoid arthritis.

Antibodies that bind carbohydrates with high affinity and selectivity could be valuable tools for research, diagnostics, and bio-pharmaceutics, while the stimulation of a strong antibody response towards tumor specific carbohydrate antigens could form the basis of a cancer vaccine. Unfortunately, standard methods used routinely to generate antibodies toward protein and small molecules do not work well in the generation of antibodies toward carbohydrates. Three main obstacles exist for the problem. First, the required unseemly large amount of structurally well-characterized carbohydrate materials is often difficult to obtain from natural sources. Second, tumor associated carbohydrate antigens are auto-antigens and consequently tolerated by the immune system. Therefore their poor immunogenicity presents a major obstacle in the development of effective carbohydrate-based vaccines. Third, tumor associated oligosaccharides, which are themselves T-cell-independent antigens, cannot elicit protective antibodies, and booster injections of them fail to produce an augmented response or promote antibody class switching. As a solution to these problems, the desired carbohydrate antigens can be obtained by chemical and enzymatic synthetic approaches. The available antigens can then be constructed

into a conjugate vaccine, helping to overcome their poor immunogenicity and T-cell-independence.

It has been proven that the coupling of a carbohydrate antigen to a foreign protein (*e.g.* Keyhole Limpet Hemocyanin (KLH), detoxified tetanus toxoid) can overcome the tolerance and the T-cell independent properties. The carbohydrate-protein conjugates can activate the T-cell to produce high levels of carbohydrate specific IgG antibodies and give a booster response after re-exposure.

The efficient linkage of carbohydrates to proteins presents a major problem in the production of conjugate vaccines. For example, such a coupling protocol should minimize structure changes within immunological epitopes of both the saccharide and protein part. An effective coupling procedure would avoid the cleavage of acid, base or oxidation sensitive glycosidic linkages and side-chain functional groups within the protein. Furthermore, saccharide loading must be controlled; too little saccharide incorporation will not give an effective immunological reaction whereas too much may mask protein T-cell epitopes. In addition, the carrier protein may generate high anti-protein responses, and the protein-specific epitope suppression renders the vaccine ineffective. New strategies must be explored for carbohydrate based vaccine development due to these shortcomings, strategies that can properly deliver the antigens to the immune system.

This thesis will combine organic synthetic chemistry, vaccine formulation and immunology to develop novel synthetic carbohydrate based cancer vaccines. In this introduction, cancer vaccine will be introduced and compared to the currently used vaccines; general information and the important roles of cell surface carbohydrates will be presented; the use of cell surface carbohydrates as cancer associated antigens for cancer vaccine development will be

highlighted, and the immunological adjuvants will be reviewed. Liposome will be discussed in greater detail as it was used as an immunological adjuvant for vaccine construction in this thesis.

## **1.1 Background of vaccine development**

When Edward Jenner injected an extract of cowpox lesions into patients to protect against smallpox infection in 1783<sup>2,3</sup>, little could he have known that his crude inoculation would revolutionize the science of disease prevention and control. Another century would pass before it would be firmly established that inoculation with attenuated or inactivated microorganisms can introduce a protective immunological response and that the widespread application of whole-cell vaccines has resulted in the protection against numerous diseases such as diphtheria, polio, measles, rabies, tetanus and whooping cough<sup>4,5</sup>. However, it was discovered that the use of whole-cell vaccines is not useful or safe in all cases. Killed or attenuated cell vaccines may contain the immunizing antigens in too low concentrations. Furthermore, a wide array of non-immunizing agents are introduced, some of which may prove hazardous to humans.<sup>6</sup>

In the past, the risks of whole-pathogen vaccines and limited supply of useful antigens posed barriers to the practical development of vaccines. In the past ten to fifteen years, the advent of the philosophy of vaccine development, genetic engineering progress and the near completion of the human genome project has resulted in the opportunity for new vaccine development.<sup>7</sup> The tremendous advances in genetic engineering and protein sequencing have engendered the subunit vaccine approach, an approach in which the whole-killed or attenuated agents often present in vaccine preparations are replaced with a peptide or protein subunit known to elicit an effective immune response toward a parent organism.<sup>8</sup> The safety profile of subunit vaccines is superior to conventional whole-organism vaccines, because subunit vaccines consist of well-characterized molecules and do not contain the disease-causing agent. Conventional



whole-organism vaccines present the encumbrance of the constant evaluation of the absence of viable infectious agents.<sup>9</sup> Although the new, well-characterized antigens offer advantages in the selection of antigenic epitopes and safety, their poor immunogenicity is a general drawback. Unfortunately, the body's immune system does not respond strongly to these pure antigens, which results in insufficient immune response to exert full protection, therefore these antigens need repeated immunizations to achieve the desired antibody response. This poor immunogenicity of these pure antigens has created an urgent need to identify pharmaceutically acceptable methods capable of initiating a strong immune response. The use of immunological adjuvants is the primary strategy for achieving this goal to improve the immunogenicity of the subunit vaccines.

## **1.2 Cancer vaccine**

Due to its classification as a novel vaccine, the cancer vaccine is quite different from its classical counterparts. A classical vaccine is a substance designed to stimulate an immune response against a specific antigen for the prevention of diseases. Instead of being used to protect against cancer, current cancer vaccines are perceived as a mode of treatment subsequent to the detection of the disease. The goal of the current cancer vaccine is not to prevent cancer, but rather to stimulate an attack of the immune system on existing cancerous cells.<sup>10</sup> Classical vaccines to prevent cancer are not yet possible,<sup>11</sup> as many different types of cancer exist and it is difficult to accurately predict who may or may not develop the disease.

For many years, the treatment of cancer was primarily focused on surgery, chemotherapy, and radiation. However, a vaccine capable of inducing an anticancer immune response has been an enduring vision in medicine.<sup>12</sup> As recent as a few years ago, researchers believed that the immune system constantly patrolled for cancer cells, actively preventing cancer. Therefore,

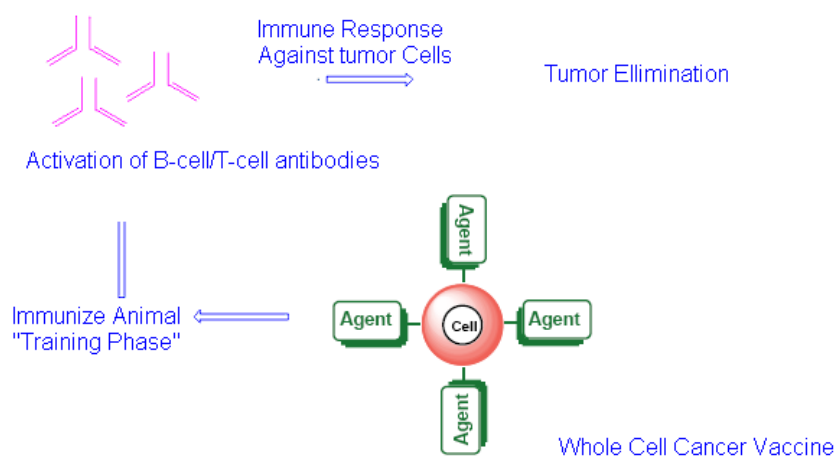
cancer represented a breakdown of the immune system. Today, researchers have begun to realize that the difficulty in the generation of an anti-tumor response leads to cancer development. The immune system is responsible for detecting the difference between healthy cells and bacteria-infected cells, virus-infected cells, or cancerous cells. To remain healthy, the immune system must be able to "tolerate" normal cells and to recognize and attack abnormal cells. The difference between cancer cells and healthy cells are minimal. Therefore, the immune system instinctively tolerates cancer cells rather than attacking them. Although tolerance is essential to keep the normal cells safe from the immune system, tolerance of cancer cells becomes problematic. Cancer vaccines must not only provoke an immune response, but stimulate a response strong enough to overcome the instinct of the immune system to tolerate the cancerous cells. As researchers continue to learn about how the body's natural response to cancer, therapies are being developed that harness the body's natural defense system in the fight against cancer.<sup>13</sup> These therapies, known as immunotherapies, are based on the theory that because tumors possess specific antigens, cancerous cells can be eliminated by stimulating and boosting the body's immune response and activating its natural cancer fighting mechanism.

Fighting an established cancer is a difficult task. Therefore, immunotherapies alone are not effective. The primary tumor can be eliminated by such conventional cancer therapies as surgery, radiation, or chemotherapy. However, following these treatments, cancer vaccines may still be required to provide enhanced protection against tumor reoccurrence and metastasis. Cancer vaccines constitute an emerging type of immunotherapy that remains experimental and in its infancy.<sup>14</sup> At this time, the FDA has not yet approved a single cancer vaccine for use as a standard treatment. Many vaccines are, however, now being tested with response to a variety of

cancer types in clinical trials.<sup>14</sup> As soon as these vaccines are proven effective and obtain FDA-approval, they will be used clinically.

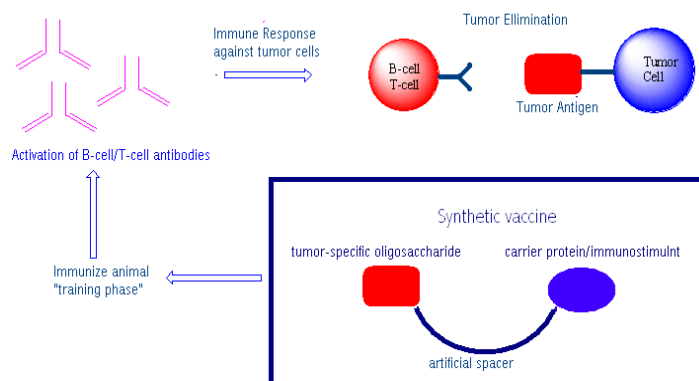
A cancer vaccine can be made either from whole tumor cells or tumor associated antigens. Whole tumor cells display all the potential antigens expressed by the host tumor.<sup>15,16</sup> As such they can easily present the tumor associated antigens to the immune system, functioning as antigen presenting cells. In addition they can be engineered to overexpress tumor antigens or to produce cytokines.<sup>17</sup> To prepare whole cell vaccines, tumor cells are removed from a patient, and grown in the laboratory. The tumor cells are then treated to ensure that 1) they can no longer multiply, and 2) there is nothing present that could infect the patient. When whole tumor cells are injected into a patient, an immune response against the antigens on the tumor cells is generated.

**(Figure 1.1)**



**Figure 1.1** General mechanism of the whole cancer vaccine development

Unlike whole cell vaccines, antigen vaccines are not composed of whole cells, but of tumor associated antigens.<sup>17</sup> The antigen vaccine is based on the theory that tumors possess specific antigens that render cancer cells different from healthy cells.<sup>18,19</sup> These tumor associated antigens are rich on the cancer cell surface. One tumor can have many antigens. Certain antigens are common to all cancers of a particular type, while others are unique to an individual. Tumors of different cancer types often possess identical antigens. Typically, these antigens can be proteins or peptides from the tumor cells or the genetic material coding for those proteins (RNA or DNA). In order to create an antigen vaccine, certain delivery methods are required to aid in the efficient deliver of these antigens to the immune system. These antigens, if delivered successfully could train the immune system to produce opsonizing or cytotoxic antibodies. An effective vaccine must summon antibodies capable of distinguishing between cancer cells and normal cells, so they can recognize and eliminate cancer cells and micro-metastasis from the hosts. **(Figure 1.2)**



**Figure 1.2** General approaches to the antigen cancer vaccine development<sup>20</sup>

With continued progress in the development of antigen cancer vaccines, it is critical to identify the appropriate cancer associated antigens. Aberrant glycosylation on the cancer cell surface provides a unique opportunity for the development of antigen cancer vaccines. The techniques for the structural characterization has identified a number of tumor associated carbohydrate motifs which can be further applied in the antigen cancer vaccine constructions. Due to the promising nature of tumor associated carbohydrate antigen, they will be introduced in greater depth in the following sections. In section 1.2.1, general information pertaining to the cell surface carbohydrate is provided. In section 1.2.2, the application of carbohydrate antigens in cancer vaccine development is summarized.

### **1.2.1 Cell surface carbohydrate**

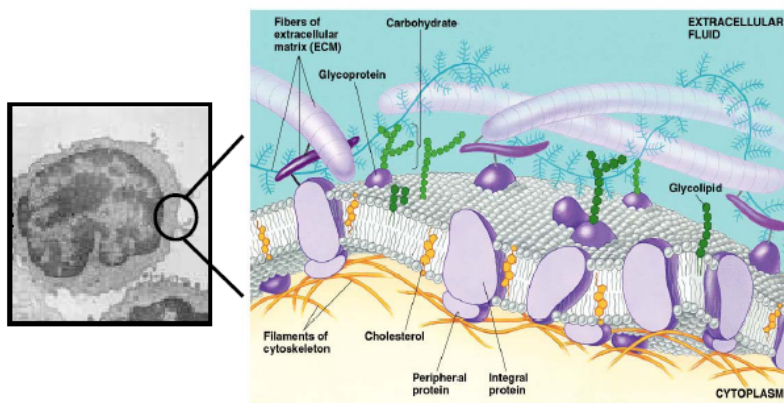
Like DNA and proteins, carbohydrates constitute a significant class of biopolymers. The biological importance of carbohydrates has become increasingly recognized throughout the last two decades. The traditional belief of carbohydrate as nature's energy source (starch and glycogen) and structural material (e.g. cellulose, collagen, proteoglycans, and DNA back bone) has been expanded upon. Today it is an indisputable fact that carbohydrates play increasingly sophisticated and complex roles and perform a variety of functions in mammals. Carbohydrates presented on the cell surface have received particular attention. The surfaces of all cells are decorated with carbohydrates. **(Figure 1.3)** The structures of these carbohydrates differ among cell types and are regulated in development and differentiation. For example, like cells display different patterns depending on their stage of development or environment.

Biologically relevant carbohydrates usually consist of several monosaccharides. Carbohydrates containing up to ten monosaccharides are referred as oligosaccharides, and carbohydrates containing more than ten monosaccharides are referred as polysaccharides. The

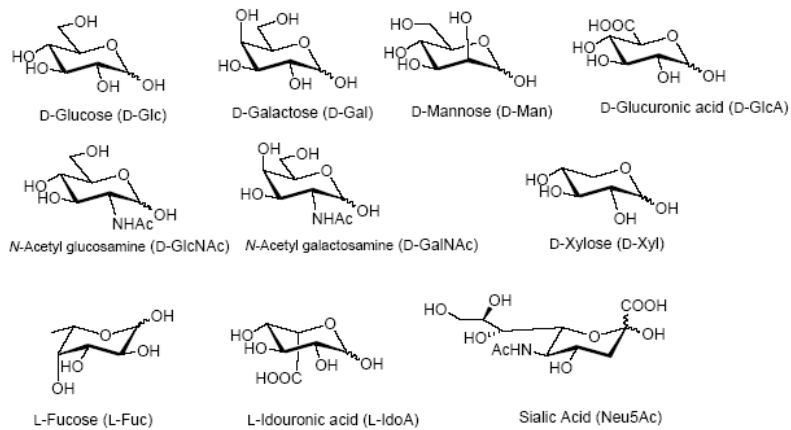
carbohydrate molecule is much more structurally complex than the protein molecule, even though there are only ten different monosaccharides (**Figure 1.4**) present in mammalian systems. The metabolic system combines the ten monosaccharides to form a large number and variety of chemical unique complex branched structures. Compared to the structure of a protein molecule, the structure of carbohydrate molecule is much more complex. For example, while only 24 oligopeptide configurations are possible with four amino acids, more than 100,000 different oligosaccharide configurations are possible with four sugars.

Each cell uses the metabolic pathways,<sup>21</sup> (**Figure 1.5**) to manufacture a unique yet complementary mixture of oligosaccharides. This process consists of two stages. First, the monosaccharides obtained by the cell from dietary sources and recycling and salvage processes are converted into nucleotide-sugar donors. These conversions typically entail the phosphorylation of hydroxyl groups on the monosaccharides under the sequential action of several enzymes. Additionally, the stereocenters of the monosaccharides can be inverted to create related epimers, and one monosaccharide can be transformed into a different sugar. Next, the phosphorylated monosaccharides possessing the desired stereochemical configuration are transferred to the nucleotide-sugar donors. Second, these nucleotide-sugar donors are further assembled so that glycosyltransferases will provide oligosaccharides. The assembly of the monosaccharides into complex carbohydrates is straightforward due to the efficient nucleotide functions of the leaving groups. Each step involves one specific glycosyltransferase, so the exact composition of the final product is determined by allowing (or avoiding) contact with a particular glycosyltransferase. After the assembly, the obtained oligosaccharides are delivered to the surface of mammalian cells, where the diverse oligosaccharide structures exist in their natural

context. Salvage pathways can recycle oligosaccharides on the cell surface to monosaccharides that can be reused by the cell.



**Figure 1.3** Oligosaccharides on the cell surface

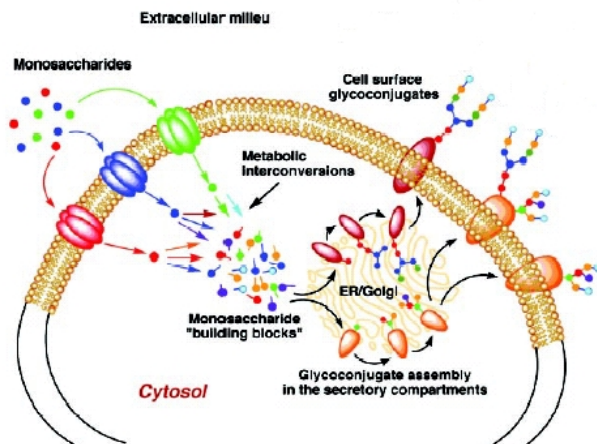


**Figure 1.4** Monosaccharides present in mammals

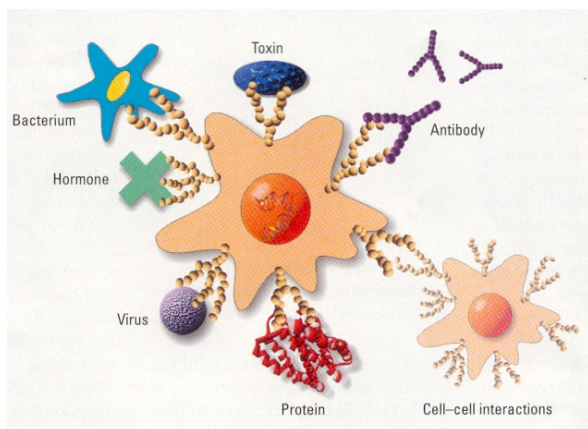
On the cell surface, oligosaccharides are presented as glycoconjugates.<sup>22</sup> Glycoconjugates are compounds which include glycolipids, glycoproteins, and proteoglycans. Glycolipids are oligosaccharides that are covalently bound to a lipid molecule (Sphingosine, Ceramide). The lipid portion of the molecule is non-polar and fits well into the hydrophobic cell membrane as an anchor for these molecules, exposing the oligosaccharides to the extracellular environment. Glycoproteins are oligosaccharides that are covalently bound to proteins, and make up the most the most complex group of glycoconjugates. Two major classes of glycoproteins are: 1) *O*-linked glycoproteins with an oligosaccharide attached to the side chain of serine or threonine, and, 2) *N*-linked glycoproteins with an oligosaccharide attached to the side chain of asparagine. Only the asparagine in the sequence Asn-X-Thr/Ser of the protein is glycosylated (X may be any amino acid, except proline). For *O*-linked glycoproteins, there is no common core structure, while all cell surface *N*-linked oligosaccharides have an identical pentasaccharide core. Proteoglycans comprise the third group of glycoconjugates. Proteoglycans are composed of a protein and a polysaccharide with a molecular weight of up to 30,000. The polysaccharide is a glycosylaminoglycan in which the repeating unit is a disaccharide. These disaccharides typically carry a negative charge. Heparin, a necessary performer in the blood coagulation cascade, is an example of a standard proteoglycan.

The cell surface oligosaccharides are responsible for a broad spectrum of critical biological functions, playing a central role in many types of intercellular communication events. **(Figure 1.6)** Cell surface oligosaccharides play a particular prominent role in the immune system because all cells, foreign or human, are covered with them.





**Figure 1.5** Biosynthesis of cell surface glycoconjugate



**Figure 1.6** Functions of Cell Surface Carbohydrate in mammals.

For example, glycoconjugates on the cell surface serve as points of attachment for pathogens (bacteria, viruses, and toxins), other cells, and molecules (hormones, antibodies, and other carbohydrates). Cell surface oligosaccharides are exploited by bacterial pathogens or viral pathogens for attachment before invasion. The cells of the immune system use carbohydrate-binding proteins to detect subtle differences in sugar structures on the surface of cells in an attempt to recognize foreign pathogens. Since all human cells are covered with carbohydrates, these structures can be used by the cell for communication. For example, cells can communicate with receptors on cognate cells through the multivalent binding of oligosaccharides on glycoproteins. An example of this communication is the attachment of blood-borne leukocytes to endothelial cells at sites of inflammation. Glycolipids, including tumor-associated antigens, present oligosaccharides that can be recognized by antibody molecules. Glycoprotein-based tumor antigens, such as mucin-like molecules on epithelial-derived cancers, also serve as immunogenic epitopes for antibody binding. A number of these interactions are prerequisites for further reactions and events.

The presentation of glycoconjugates on the cell surface is not to be considered a static phenomenon, but as a dynamic system that evolves with the development and differentiation of the cell.<sup>23</sup> The evolution from a healthy cell to a cancer cell is correlated with dramatic changes in the nature and concentration of glycoconjugate presentation. The structural characterization of these altered glycoforms has identified carbohydrate motifs associated with tumor tissue. Some are truly tumor-specific antigens that are not found in normal tissue, while others are present in normal tissue, but overexpressed on tumor cells. Lastly, some carbohydrates are displayed during fetal development, remain dormant into adulthood and eventually arise again during malignant transformation.<sup>24</sup>

Aberrant glycosylation on the cell surface is a hallmark of the malignant phenotype.<sup>25,26</sup> This abnormal glycosylation is an important criterion for the stage, direction and fate of tumor progression. Numerous studies have shown that abnormal glycosylation in primary tumors is strongly correlated with poor survival rates of patients. The close association of glycosylation with malignancy has prompted consideration of tumor-associated oligosaccharides as components of anti-tumor vaccines, with the aim of inducing an antibody response in cancer patients. Monoclonal antibodies against tumor-associated glycoconjugates can react specifically or preferentially with tumor cells. Normal cells have no or very low concentrations of these antigens and as a result the antibodies have no or very low affinities for normal cells.

Cancer vaccines based on carbohydrate-centered tumor antigens have been explored widely. In **section 1.2.2**, information pertaining to this exploration is provided.

### **1.2.2 Carbohydrate-based cancer vaccine**

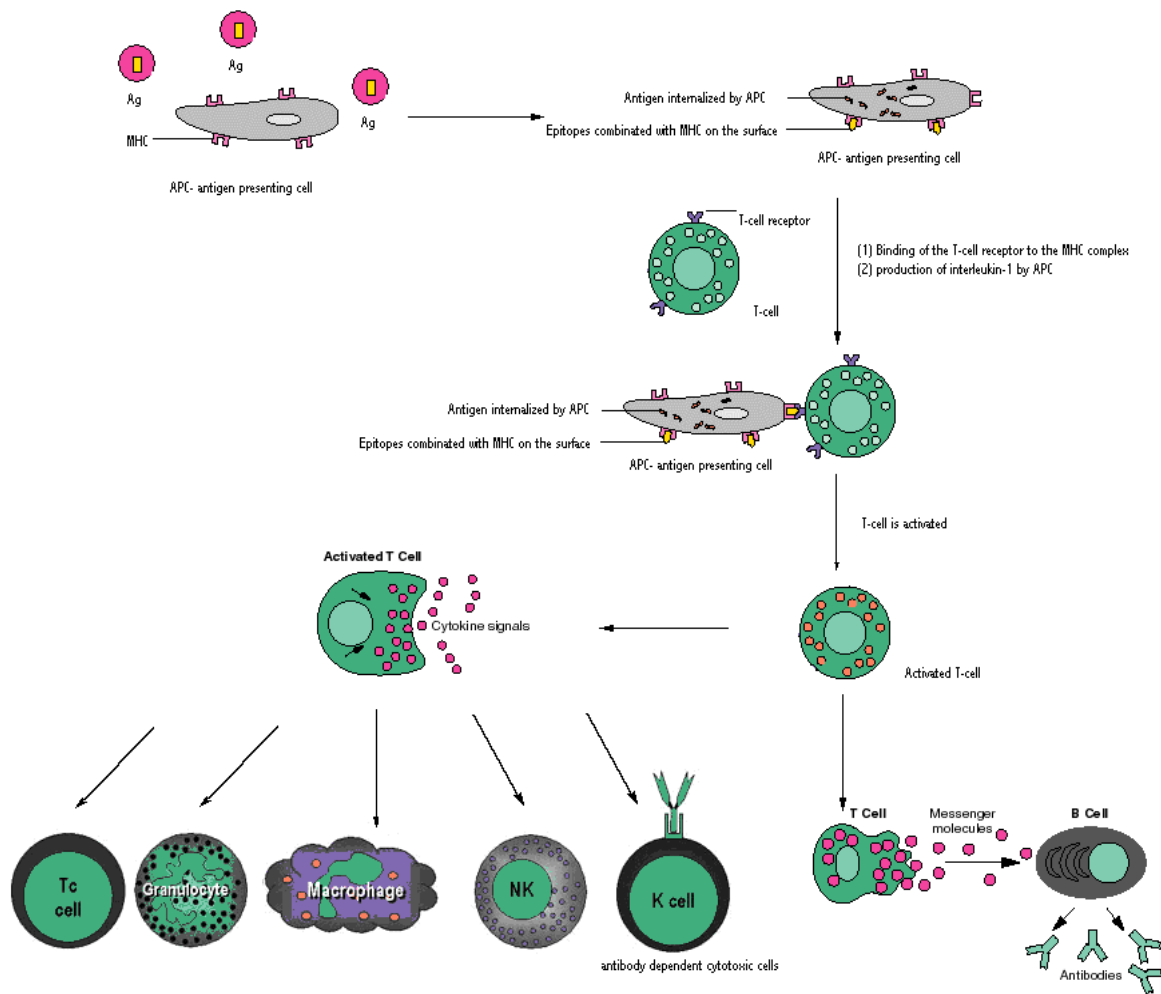
The eventual development of a cancer vaccine is based on the theory that tumor-associated antigens become immunogenic when presented to a properly trained immune system. An immune response directed against tumor-associated antigens results in the induction of antibodies that could eradicate the micro metastasis and circulate tumor cells in the blood stream. Many of the tumor antigens are composed of sugars, because of the aberrant glycosylation of malignant cells. The differential expression of these unusual carbohydrates offers a unique opportunity for the development of cancer vaccines.

However, three major obstacles exist in the development of effective cancer vaccines. Firstl, it is difficult to obtain the necessary carbohydrate antigens from a natural source; however the desired carbohydrate-based cancer antigens can be synthesized using synthetic methodologies. Second, tumor associated oligosaccharides are generally auto-antigens and

consequently are tolerated by the immune system. This tolerance is reinforced due to the presence of antigens shedded from the growing tumor. It is not difficult to understand that the cancer-associated antigens cannot induce an effective immune response when the same antigens on the cancer cells have not done so. Third, pure tumor associated oligosaccharides are T-cell independent antigens. Tumor associated oligosaccharides alone can not activate helper T-cells. Therefore tumor associated oligosaccharides cannot elicit IgG antibodies nor induce immunological memory, thus presenting a major obstacle in the development of effective cancer vaccines.

The production of antibodies by T-cell independent antigens such as oligosaccharides and lipids is based on the cooperative interaction of two types of lymphocytes, the B-cells and T-cells. **(Figure 1.7)** The activation of both T cells and B cells is necessary for an effective immunogenic reaction,<sup>27</sup> because they interact with one another in a complex manner, either directly or through interleukins. The T-helper (Th) cells are the “principal orchestrators” of the immune response because they are needed for the activation of the major effector cells (i.e., cytotoxic T (Tc) cells and antibody-producing B cells) in this response. Antigen presentation is mediated by specialized macrophages (APC). After the internalization of antigen-loaded particles or free antigen, immunorelevant epitopes are presented on the surface of the APC in combination with a major histocompatibility complex (MHC). The T<sub>H</sub> cells are attracted and activated by two signals: the binding of the T-cell antigen receptor to the MHC complex and the production of interleukin-1 by the APC. The activated T<sub>H</sub> cells trigger a complex cascade. They release lymphokines which stimulate B lymphocytes to proliferate and produce specific antibodies after differentiation and activate granulocytes, macrophages, natural killer cells, antibody dependent cytotoxic cells, and cytotoxic T cells. Tumor associated oligosaccharides

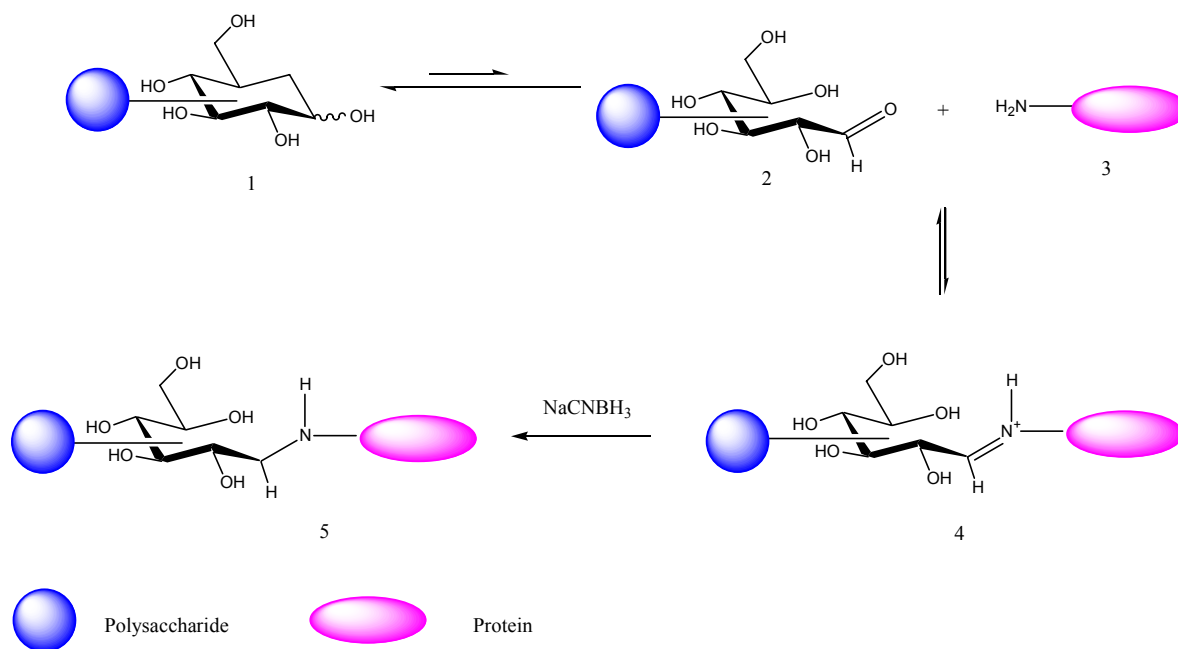
alone can not activate helper T-cells and therefore are unable to elicit IgG antibodies and induce immunological memory, presenting a major obstacle in the development of effective cancer vaccines.



**Figure 1.7:** Interactions of antigens with the immune system.

Since the first obstacle can be overcome by modern synthetic methodology, more attention must be directed directly towards the second and third obstacles. Fortunately, the conjugation of a saccharide to a foreign carrier protein (e.g. Keyhole Limpet hemocyanin (KLH) or detoxified tetanus toxoid) enhances its presentation to the immune system, thereby overcoming the tolerance and the helper T-cell independence properties. In this case, the carrier protein provides T-epitope (peptide fragments of 12-15 amino acids) that can activate T-helper cells. The immune response against tumor associated saccharide antigens can be further improved by including a potent adjuvant. A properly chosen adjuvant may play a significant role in the outcome of immunizations and should favor the induction of both the B-cell response and the T-helper cell response.

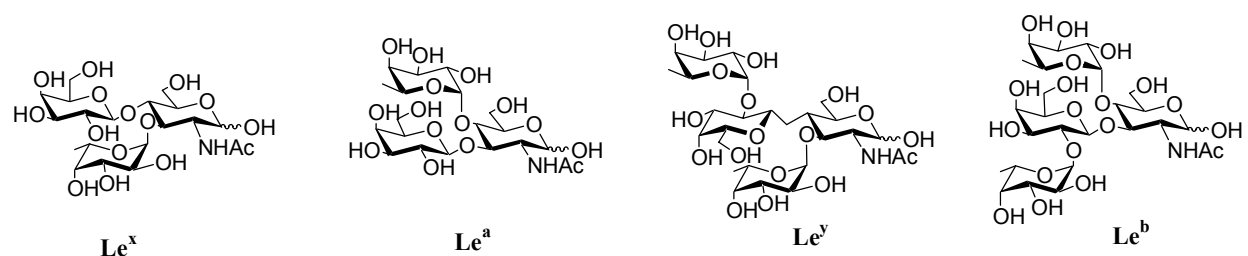
The effective linkage of a carbohydrate to a protein and particularly the minimization of structural changes within immunological epitopes of both the saccharide and protein part constitutes a major problem in the production of conjugate vaccines.<sup>28,29</sup> The tumor associated carbohydrate antigens have a variety of functional groups such as hydroxyl, amino, phosphodiester and carboxyl groups. Any of these functional groups, in principle, can be used to form an effective saccharide-protein coupling. One of the most widely used coupling methods is the so-called reductive amination,<sup>30</sup> which uses the fact that the cyclic form of the reducing terminal sugar is in equilibrium with its acyclic form, having a ketone or aldehyde functionality. These aldehyde functions can form a Schiff's base with free amino groups on the protein (e.g.  $\epsilon$ -amino groups of lysine residues). Reduction of the imine with cyanoborohydride leads to the stable secondary amine and shifts the equilibrium towards the conjugate. (**Figure 1.8**)



**Figure 1.8:** Reductive amination for the coupling of carbohydrate fragments to protein.

Many of the tumor-associated antigens have been made into conjugate vaccines according to this reductive amination strategy. In particular, the Lewis antigen based cancer vaccines elicited more attention.

Lewis antigens are an important family of human tumor associated antigens that are overexpressed on the surface of a variety of tumor cells (breast, prostate, lung, colon, stomach, and ovary cancer).<sup>31-33</sup> Since Lewis antigens are ligands for the endothelial cell-surface receptors (E- and P-selectin) and binding of cancer cells to the endothelium is a necessary step for metastasis, the over-expression of these Lewis antigens promotes tumor-cell invasion and metastasis.<sup>34</sup> Interfering with the differential expression of these tumor-associated antigens provides an exciting opportunity for a possible breakthrough in the development of cancer vaccines.



**Figure 1.9:** Structures of Lewis antigens.

Four different Lewis core structures have been identified as Lewis<sup>a</sup>, Lewis<sup>b</sup>, Lewis<sup>x</sup>, and Lewis<sup>y</sup>. (**Figure 1.9**) Lewis<sup>a</sup> and Lewis<sup>x</sup> antigens are positional isomers. Lewis<sup>a</sup> contains a  $\alpha$ -fucoside at C-4 and a  $\beta$ -galactoside at C-3 of N-acetyl-glucosamine. Lewis<sup>x</sup>, on the other hand, contains a  $\beta$ -galactoside at C-4 and an  $\alpha$ -fucoside at C-3. The tetrasaccharide Lewis<sup>b</sup> has the same core structure as Lewis<sup>a</sup>, but has an additional fucose attached to the galactoside. The tetrasaccharide Lewis<sup>y</sup> has an identical core structure to Lewis<sup>x</sup>, but has an additional fucoside linked to the C-2 of the galactoside. The Lewis<sup>x</sup> and Lewis<sup>a</sup> antigen can be further substituted with a sialic acid moiety at the C-3 position of galactose (SLe<sup>x</sup> and SLe<sup>a</sup>). Lewis antigens also appear as homodimers (e.g. Le<sup>x</sup>-Le<sup>x</sup>) and mixed dimers (e.g. Le<sup>y</sup>-Le<sup>x</sup>), and are attached to lipids and proteins through a lactose moiety.

Expression of sialyl Lewis<sup>x</sup>(SLe<sup>x</sup>) is strongly correlated with the increased metastatic potential of tumor cells and poor patient survival.<sup>35</sup> Recent studies by Ravindranath *et al*<sup>36</sup> have shown that guinea pigs immunized with whole melanoma-cells developed a significant IgG response against SLe<sup>x</sup> and SLe<sup>a</sup>. Melanoma patients immunized with this whole cell vaccine developed significant titers of IgM. Thus it was suggested that these Lewis antigens possess qualities for passive or active immunotherapy in the treatment of melanoma.<sup>13,37</sup> Subsequent



studies demonstrated that the antibody mediates lysis by human complement of breast cancer cells. The antibody could diminish the clonogenic ability of breast cancer.<sup>38,39</sup> A phase one clinical trial with cancer patients demonstrated anti-tumor activity of the antibody. Unfortunately, while whole cancer cell vaccines were able to induce clinically relevant immunological results in particular patients, only IgM antibodies were elicited of low titer.

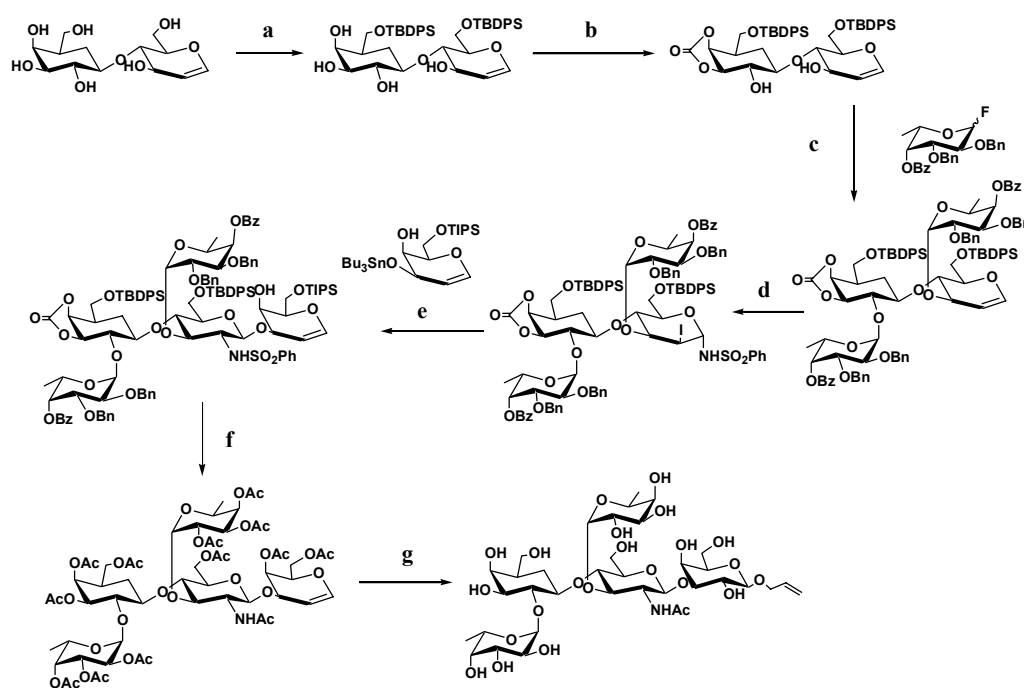
Another attractive Lewis antigen is Le<sup>y</sup>. Le<sup>y</sup> is of particular interest to the scientific community because of its previous identification as an important epitope for eliciting antibodies against colon and liver carcinomas, and as a marker in metastatic prostate cancer.<sup>18</sup> Many formulation methods have been applied to construct Le<sup>y</sup> based cancer vaccines. Of these methods the Le<sup>y</sup> and the carrier protein KLH conjugation is most promising. The immunogenicity of this vaccine has been examined in mice and in a phase one clinical trial.

The Lewis antigen Le<sup>y</sup> is prepared using organic synthetic methods. Organic synthesis offers a highly efficient method to produce large amounts of complex carbohydrates. The advantages of pure, well-characterized synthetic compounds as opposed to naturally extracted compounds include a known reproducible purity, control of quality and supply and scaleable manufacture. Furthermore, organic synthesis permits the incorporation of artificial spacers with a unique reactivity that allows selective conjugation to a carrier protein or immunostimulant.

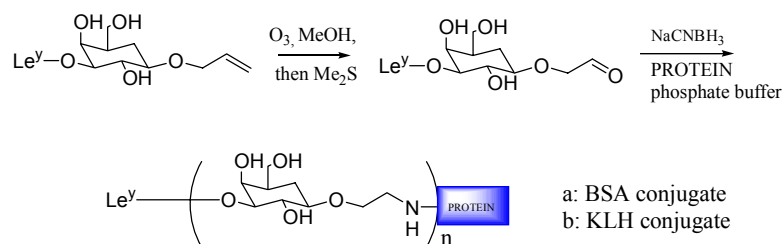
The pentasaccharide as its allyl glycoside containing the Le<sup>y</sup> specificity was prepared<sup>40</sup> by Danishefsky and co-workers as shown in **Figure 1.10**. The double bond of the allyl moiety was ozonolysed to give an aldehyde moiety, which allowed conjugation to the carrier protein KLH by reductive amination. (**Figure 1.11**)

The immunogenicity of the Le<sup>y</sup>-KLH conjugate has been examined in mice and in a phase one clinical trial. Immunization of mice in the presence of the immunological adjuvant

QS21 showed that the conjugate elicited IgG and IgM antibody responses to naturally occurring forms of Le<sup>y</sup> epitopes carried on mucins and glycolipids.<sup>18</sup> The IgM antibody responses were higher in titer than the IgG responses. The antibodies were cytotoxic to a human breast cancer line-expressing Le<sup>y</sup> (MCF-7). It was also found that the conjugation method and nature of the carrier protein affected antibody titers. In particular, the use of BSA as a carrier protein gave poor response. Furthermore, conjugation of Le<sup>y</sup> to KLH by reductive amination gave the most immunogenic conjugates.



**Figure 1.10:** Synthesis of the Lewis<sup>y</sup> allyl glycoside. a) TBDPSCl, imidazole. DMF, 84%; b) Carbonyl diimidazole, THF, 58%; c) AgClO<sub>4</sub>, SnCl<sub>2</sub>, DTBP, Et<sub>2</sub>O, 51%; d) PhSO<sub>2</sub>NH<sub>2</sub>, I(coll)<sub>2</sub>ClO<sub>4</sub>, 99%; e) AgBF<sub>4</sub>, THF, 75%; f) 1. TBAF, THF; 2. Na/NH<sub>3</sub>, MeOH; 3. Ac<sub>2</sub>O, pyridine, 37%; g) 1. DMDO, CH<sub>2</sub>Cl<sub>2</sub>; 2. allyl alcohol, ZnCl<sub>2</sub>, THF; 3. NaOMe, MeOH, 72%; 3 steps.

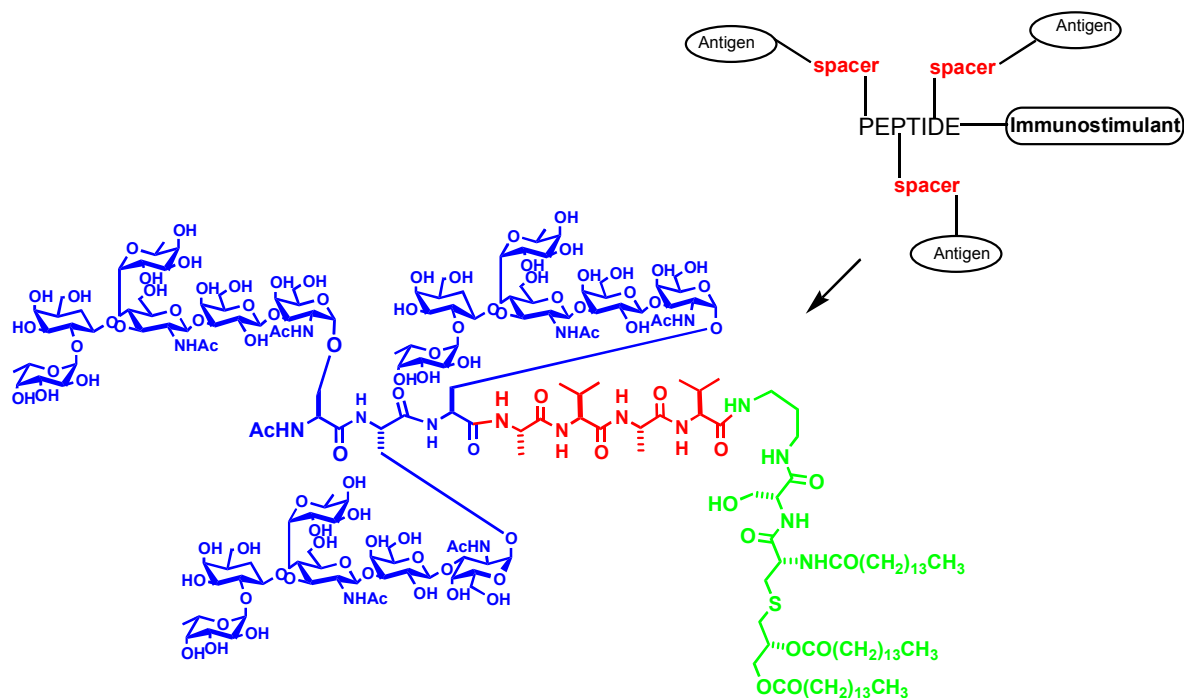


**Figure 1.11:** Conjugation of allyl glycoside  $\text{Le}^y$  to the carrier proteins.

Encouraged by the results from the animal studies, a phase one clinical trial was conducted<sup>41</sup> to test the safety of the vaccine and any antibodies produced in response to it. This trial included twenty-four patients with histologically documented ovarian, fallopian tube or peritoneal cancer. In these studies, the  $\text{Le}^y$ -KLH conjugate obtained by reductive amination was used in combination with the adjuvant QS-21. The results of this study showed that it is possible to induce an antibody response in humans through immunization with the semi-synthetic  $\text{Le}^y$ -KLH conjugate vaccine. The vaccination was well tolerated with no adverse effects related to auto-immunity. Although many of the antibody responses were rather modest, significant antibody titers were attained in a high proportion of the patients. The raised antibodies were capable of reacting with naturally occurring  $\text{Le}^y$  and  $\text{Le}^y$  expressing tumor cells. Unfortunately, the antibodies were mainly of the IgM class with only three patients exhibiting detectable levels of IgG antibodies. This is in sharp contrast to the results from immunizing mice with the same conjugate and adjuvant in which both IgG and IgM antibodies were observed. It appears that the KLH carrier protein cannot induce antibodies of IgG class in the majority of the patients. The above-described results indicate that conjugates of Lewis antigens and a carrier protein show promise as potential cancer vaccines. The inability of the currently used carrier proteins to induce sufficiently strong helper T-cell responses in humans is a major problem.

Alternative carriers for carbohydrate epitopes have been examined. For example, synthetic Le<sup>y</sup> has been coupled to the synthetic immune adjuvant Pam<sub>3</sub>Cys. (**Figure 1.12**)<sup>20,42</sup> This antigen was recognized in the ELISA assay with an antibody elicited against tumor cells displaying the Le<sup>y</sup> ligand. However, this antigen does not contain a helper T-epitope and therefore it is unlikely that it will elicit an IgG antibody response.

The above-described results indicate that the currently used carrier systems produce only low affinity IgM antibodies and little or no levels of desired IgG antibodies in humans. It is clear that new immunological adjuvants need to be explored, adjuvants capable of presenting tumor-associated antigens to the immune system in a more efficient manner. In **section 1.3**, the available immunological adjuvants will be summarized.



**Figure 1.12:** Alternative carrier for Le<sup>y</sup>-based cancer vaccine.

### 1.3 Immunological adjuvants

The term “adjuvant” is a derivative of *adjuvare*, the Latin infinitive meaning “to help”.<sup>43</sup> An adjuvant is a compound administered with antigens, or a compound presenting antigen in a manner that will enhance the immune response toward the antigen.<sup>44</sup> In the context of antibody production, adjuvants possess the ability to influence titer, response duration, isotype, avidity and certain properties of cell-mediated immunity,<sup>45</sup> thus stimulating the rapid and sustained production of high titers of antibodies with high avidity. The use of adjuvants is required for many antigens which otherwise alone are weakly immunogenic.<sup>46</sup>

The mechanism of adjuvant action is complex.<sup>47-49</sup> For simplicity, the adjuvant action may be broken down into three parts. In the first part the adjuvant functions as a depot to promote long-term release of the antigens (for example, mineral compounds, oil-based adjuvants, liposomes, biodegradable polymer microspheres > 10µm). Long-term exposure to the antigen should increase the amount of time the immune system is presented with the antigen for processing as well as the duration of the antibody response. The second part entails the interaction of the adjuvant with immune cells. Adjuvants may act as non-specific mediators of immune cell function by stimulating or modulating immune cells (for example, Freund's complete adjuvant (FCA), muramyl dipeptide (MDP), lipopolysaccharide (LPS), lipid A, monophosphoryl lipid A (MPL), pertussis toxin (PT), cytokines). Additionally, adjuvants may also act as delivery vehicles that may enhance macrophage phagocytosis by targeting antigens to immune competent cells (for example, oil adjuvants, liposomes, biodegradable polymer microspheres <10µm, non-ionic block polymer surfactants).

Adjuvants have been in use as a tool in the augmentation of the immune response to antigens for about 70 years. Ramon was the first to show an increased antitoxin response to

tetanus and diphtheria toxoids together with other compounds such as agar, tapioca, lecithin, starch oil, saponin or even breadcrumbs.<sup>50,51</sup> While for many years the only effective adjuvant available was complete Freund's adjuvant (CFA), this is no longer the case. In recent years, adjuvants have received much attention due to their ability to selectively modulate the immune response to elicit a particular humoral and/or cellular immune response.<sup>52-54</sup> A number of adjuvant formulations have been developed and a few of these have been evaluated in clinical trials. In next section, the adjuvants approved for human use will be summarized.<sup>55-60</sup>

### **Oil emulsion**

Oil emulsions have a long history of use for increasing immune response in animals.<sup>61</sup> In 1916, Le Moignic and Pinoy first found that a suspension of killed *Salmonella typhimurium* in mineral oil increased the immune response.<sup>62</sup> In 1925, Ramon reported starch oil as one of the substances increasing the antitoxic response to diphtheria toxoid. The oil emulsions as adjuvants did not receive much attention until the demonstration of the Complete Freund's Adjuvant (CFA). CFA is a mineral oil adjuvant composed of paraffin oil, killed *mycobacteria* and mannide monooleate. The water-in-oil emulsion without mycobacteria was known as Incomplete Freund's Adjuvant (IFA). Since IFA does not contain the killed mycobacteria, it does not produce the same severe reactions as CFA. Both CFA and IFA have been used in a number of veterinary vaccines. IFA is used for the booster immunizations following the initial injection with antigen-CFA. IFA may be used for initial injection if the antigen is strongly immunogenic.

After the introduction of IFA, various types of emulsions with different oils were evaluated in a search for a stable, non-toxic and effective adjuvant for human vaccines. For example, Montanide ISA<sup>63</sup> (incomplete seppic adjuvant) is a mineral oil adjuvant. Mannide oleate is used as the major surfactant component. The antibody response is generally similar to

that with IFA, while Montanide ISA may have a lessened inflammatory response. Ribi Adjuvant System (RAS)<sup>64</sup> is an oil-in-water emulsion that contains detoxified endotoxin and mycobacterial cell wall components in 2% squalene. RSA is an alternative to CFA, but it has lower viscosity than CFA. The results (titers) of RSA are comparable to those with CFA. The squalene oil is metabolizable, so RSA has lower incidence of toxic reactions. TiterMax<sup>65</sup> is another water-in-oil emulsion, which combines synthetic adjuvant and microparticulate silica with the metabolizable oil squalene. The copolymer is the immunomodulator component. Antigen is bound to the copolymer and presented to the immune system in a highly concentrated form. TiterMax has less toxicity than CFA, but it usually produces the same results as CFA<sup>66</sup>. Syntex Adjuvant Formulation (SAF)<sup>67,68</sup> is an oil-in-water emulsion using a block copolymer as surfactant. A muramyl dipeptide derivative is the immunostimulatory component. All the content is dissolved in squalene, metabolizable oil, so it has less toxic than CFA. SAF may bias the humoral response to IgG2a in the mouse.<sup>69</sup>

### **Mineral compounds**

Aluminum phosphate and aluminum hydroxide are the most frequently used in human vaccines.<sup>70</sup> Calcium phosphate is another mineral salt adjuvant used with routine human vaccines for many years in France. The mechanism of adjuvanticity of mineral compound includes formation of a depot at the site of injection from which antigen is released slowly; stimulation of immune competent cells of the body through activation of complement, induction of eosinophilia and activation of macrophages; and efficient uptake of aluminum absorbed antigen particles by antigen-presenting cells due to their particulate nature and optimal size.<sup>70</sup> Other metal salts, such as cerium nitrate, zinc sulfate, colloidal iron hydroxide and calcium chloride, also increased immune response, but aluminum adjuvants gave the best results. Since this adjuvant is weaker

than emulsion adjuvants, it is best used in combination with strongly immunogenic antigens. Aluminum Salt adjuvants generally show mild inflammatory reactions.

### **Saponin (Quil A, QS-21) and Immunostimulator complex (ISCOMS)**

Saponins are glycosides consisting of a triterpene or a steroid and one or two oligosaccharide sugar moieties. Saponins are well known for their adjuvant activity.<sup>71,72</sup> For example, saponins, which were isolated from the bark of the South American tree *Quillaja saponaria* (Molina) has long been known as an immunostimulator.<sup>73</sup> The crude extracts of plants containing saponin (quillaja saponin) have been used together with vaccines as adjuvant. However, these crude extracts were associated with adverse side-effects when used in vaccines. The reason is the crude saponins are often contaminated with considerable amounts of tannins.<sup>74</sup> The adjuvant active components were partially purified from crude saponins by dialysis, ion exchange and gel filtration chromatographies.<sup>75</sup> This adjuvant active component, known as “Quil A”, showed increased potency on a weight basis and exhibited reduced local reaction when compared to crude extracts. Quil A can be used to construct ISCOM (immunostimulating complexes),<sup>44,76,77</sup> which is non-covalently bound complexes of Quil-A adjuvant, cholesterol and amphipathic antigen. ISCOM has been widely used in veterinary vaccines but Quil A’s hemolytic activity and local reactions make it unsuitable for human vaccines. Quil A has been further separated into more than 21 fractions by high pressure liquid chromatography (HPLC), which revealed a heterogeneous mixture of closely related saponins and led to discovery of QS-21.<sup>73</sup> Q-S21 is a potent adjuvant with reduced or minimal toxicity. Unlike most other immunostimulators, Q-S21 is water-soluble and can be used without emulsion type formulations. In this thesis, QS-21 was used together with liposomal cancer vaccine as immunostimulator.



### **Biodegradable polymer microspheres**

In recent years biodegradable polymer microspheres have received much attention for the development of better vaccine formulations.<sup>78-82</sup> They can function as a vehicle to target antigen to antigen-presenting cells on mucosal surfaces or by parental routes. By entrapping antigens in the microspheres, controlled release vaccines may be prepared to reduce the number of doses for primary immunization or to develop single doses vaccines.<sup>83-85</sup>

The microspheres are made from a variety of polymers such as poly (lactic)/glycolic acid (PLGA), polyphosphazene and polyanhydrides. Lactic/glycolic acid polymers have received particular attention.<sup>79,86</sup> The reason that they have been considered a primary candidate is: first, they have been approved by the FDA for several therapeutic products because of their excellent biodegradability, biocompatibility and a long history of safe use in humans. Secondly the microparticles made from these polymers can provide multiphasic release, which mimic the effect of booster doses required for vaccinations.<sup>87</sup> Third, these polymers can provide the continuous release of antigens over prolonged periods, which may result in the formation of high levels of antibodies similar to those observed after multiple injections. In recent years, a variety of vaccine antigens have been formulated in microspheres. The results of animal studies have shown antibody responses similar to those observed with adjuvants such as aluminum compounds.<sup>88</sup>

### **Liposomes**

Liposomes are lipid membrane particles that can serve as vehicles or delivery systems for vaccine. In the past 20 years, Liposomes have found to act as adjuvants with a number of antigens.<sup>89-94</sup> Antigens from many microorganisms and tumor cells have been incorporated into liposomes. These structures have been characterized and tested in vivo. Antigen presented via

liposomes can introduce humoral as well as cellular immune responses; some of them have been shown to protect against a challenge. Adjuvanticity of liposomes appears due to depot formation at the site of injection and efficient presentation of antigen to macrophages. In this thesis, liposomes were chosen as a carrier system for the development of carbohydrate-based cancer vaccines, in the next section, liposomes will be introduced in more detail.

With so many kinds of adjuvant formulations, selection of an adjuvant is based upon antigen characteristics (size, net charge and the presence or absence of polar groups) as well as the species to be immunized. More work remains to be done and current information cannot be applied across the board to all antigen and adjuvant combinations. Adjuvant selection remains largely empirical. Antigens that are easily purified or available in large quantities may be good choices for starting with the least inflammatory adjuvants for immunization. If antibody response were not suitable, a gradual increase in the inflammatory level of the adjuvant would then be warranted. Antigens which are difficult to come by (e.g., very small quantities are available) may be better choices for combining with the more inflammatory adjuvants such as CFA. In addition, small molecular weight compounds and others known to be weakly immunogenic, may need to be combined with a suitable adjuvant to obtain good antibody titers.

## **1.4 Introduction of Liposome**

### **1.4.1 Liposome Profile**

Liposomes are spherical, colloidal microscopic particles, which were discovered in the mid 1960s by A. Bangham<sup>95,96</sup> and originally as models for the study of biological membrane structure and function. Since then they have gained recognition as a very useful model system in many fundamental studies including topology, membrane biophysics, photophysics and photochemistry, colloid interactions, cell function, signal transduction, and many others. (Table

**1.1)** Especially, they have been used for a large number of applications by pharmaceutical industries<sup>97</sup> such as the solubilizers for difficult-to-dissolve substances, dispersants, sustained release systems, delivery systems for the encapsulated substances, stabilizers, and protective agents and so on. **(Table 1.2)**

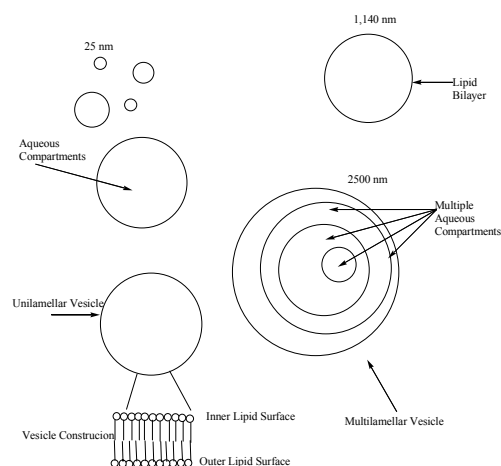
**Table 1.1** Applications of liposomes in the sciences.

<b>Discipline</b>	<b>Application</b>
Mathematics	Topology of two-dimensional surfaces in three-dimensional space governed only by bilayer elasticity
Physics	Aggregation behaviour, fractals, soft and high-strength materials
Biophysics	Permeability, phase transitions in two-dimensions, photophysics
Physical Chemistry	Colloid behaviour in a system of well-defined physical characteristics, inter-and intra-aggregate forces, DLVO
Chemistry	Photochemistry, artificial photosynthesis, catalysis, microcompartmentalization
Biochemistry	Reconstitution of membrane proteins into artificial membranes
Biology	Model biological membranes, cell function, fusion, recognition
Pharmaceutics	Studies of drug action
Medicine	Drug-delivery and medical diagnostics, gene therapy

**Table 1.2** Liposomes in the pharmaceutical industry.

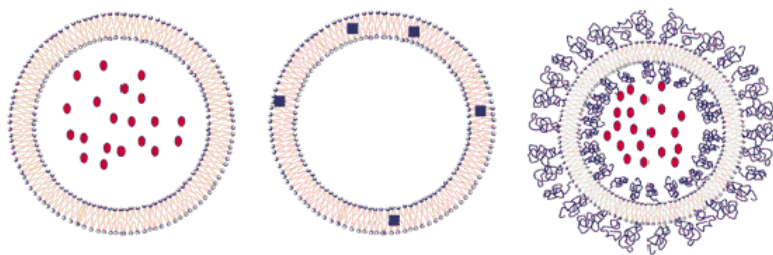
<b>Liposome Utility</b>	<b>Current Applications</b>	<b>Disease States Treated</b>
Solubilization	Amphotericin B, minoxidil	Fungal infections
Site-Avoidance	Amphotericin B-reduced nephrotoxicity, doxorubicin-decreased cardiotoxicity	Fungal infections, cancer
Sustained-Release	Systemic antineoplastic drugs, hormones, corticosteroids, drug depot in the lungs	Cancer, biotherapeutics
Drug protection	Cytosine arabinoside, interleukins	Cancer, etc.
RES Targeting	Immunomodulators, vaccines, antimalarials, macrophage-located diseases	Cancer, MAI, tropical parasites
Specific Targeting	Cells bearing specific antigens	Wide therapeutic applicability
Extravasation	Leaky vasculature of tumours, inflammations, Infections	Cancer, bacterial infections
Accumulation	Prostaglandins	Cardiovascular diseases
Enhanced Penetration Topical vehicles		Dermatology
Drug Depot	Lungs, sub-cutaneous, intra-muscular, ocular	Wide therapeutic applicability

The morphology of a liposome may be classified according to the compartmentalization of aqueous regions between bilayer shells. **(Figure 1.13)** The liposomes whose aqueous regions are segregated by only one bilayer are called unilamellar vesicles (ULV), while liposomes with more than one bilayer surrounding each aqueous compartment are termed multilamellar vesicles (MLV).<sup>98</sup> ULV forms are further classified according to their relative size, although rather crudely. Thus, there can be small unilamellar vesicles (SUV; usually less than 100nm in diameter, with a minimum diameter of about 25nm) and large unilamellar vesicles (LUV, usually greater than 100nm in diameter, with a maximal size of 2500nm). The bilayer structures of MLV, however are not as easily classified due to the existence of an almost infinite number of interconnections for each bilayer. MLVs typically form large, complex honeycomb structures that are difficult to categorize or exactly reproduce. However, MLVs are the simplest to prepare, the most stable, and the easiest to scale up to large production levels.



**Figure 1.13:** The highly varied morphologies of lipid bilayer construction.

Due to their chemical composition, structure, and physical properties, liposomes can serve as highly effective adjuvants in the delivery of vaccine antigens and/or immunostimulators. Liposomes can be made entirely from naturally occurring substances and therefore are nontoxic, biodegradable and non-immunogenic. The most common constituents of a liposome is natural phospholipids and cholesterol, which in aqueous environment spontaneously forms a closed structure by self-assembly.<sup>99</sup> Phospholipids are amphiphilic molecules, which are primarily responsible for bilayer formation. The formed closed bilayer spheres attempt to shield their hydrophobic groups from the aqueous environment while still maintaining contact with the aqueous phase via the hydrophilic head group.<sup>95</sup> Liposomes are a powerful solubilizing system for a wide range of compounds because of their amphiphilic character. The resulting closed sphere may encapsulate aqueous soluble drugs within the central aqueous compartment (**Figure 1.14, left**) or lipid soluble drugs within the bilayer membrane (**Figure 1.14, center**). Alternatively, lipid soluble drugs may be complexed with cyclodextrins and subsequently encapsulated within the liposome aqueous compartment.<sup>100,101</sup> (**Figure 1.14, left**)



**Figure 1.14:** Liposomes as powerful solubilizing systems - (left) A = aqueous soluble drug encapsulated in aqueous compartment; (center) B = a hydrophobic drug in the liposome bilayer; (right) C = hydrophilic polyoxyethylene lipids incorporated into the liposome.

The physical properties of liposomes vary to a large extent. The particles range in rather uniform size from 20 nm to 10  $\mu$ m and can be unilamellar or multilamellar. Their membrane rigidity, mechanical properties, permeability, electric charge density, and pH-sensitivity can be influenced by choosing different phospholipids or by the incorporation of cholesterol, surface bound polymers, or grafted polymers. The membranes can also be functionalized for the attachment of specific ligands.<sup>102,103</sup> In addition to these physicochemical properties, liposomes exhibit many special biological characteristics, including (specific) interactions with biological membranes and various cells.<sup>104</sup> Liposomes interact with the cell surface through two main mechanisms: adsorption and endocytosis. Liposomes can be adsorbed to a cell surface directly (nonspecifically) or through specific interaction with a cell surface receptor. In addition to adsorption and endocytosis, there are two other categories of liposome interaction with the cell surface: fusion of the cell with a vesicle and lipid exchange.<sup>105,106</sup>

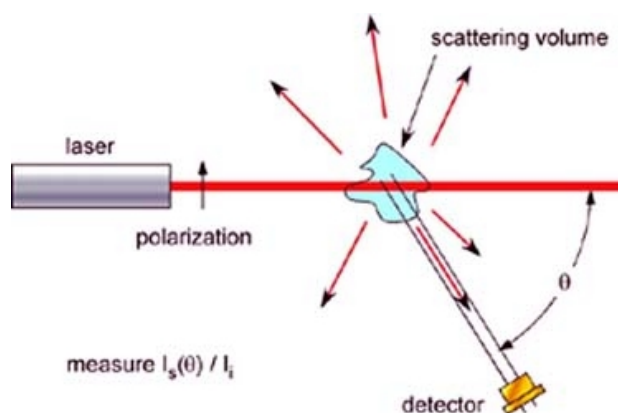
The physical properties introduced above determine the adjuvanticity and type of immune responses elicited. Adjuvancity of liposomes appears to be due to depot formation at the site of injection and efficient presentation of the antigen to macrophages. Both humoral and cell-mediated immune responses have been enhanced by liposomes, which make liposomes act as adjuvants toward a number of antigens.<sup>107</sup>

### **1.4.2 Liposome Characterization**

Liposomes and vesicles have elicited great interest in a number of applications ranging from targeted drug delivery systems to cosmetics. The most important parameters of a liposomal formulation are its size distribution and the absolute characterization of particle sizes. There are several techniques suitable for determining the size of liposome preparations, the most straightforward of which is analysis by dynamic light scattering instrument,<sup>108-110</sup> Coulter N4

plus Dynamic Light Scattering Instrument. This instrument provides for the distribution and mean diameter of the particles and also distinguish as whether the particle population is uniformly distributed around one or more particle sizes (unimodal vs. bimodal).

Dynamic light scattering is a useful, noninvasive probe of macromolecular size and shape. In light scattering, a laser beam is focused on a solution containing the macromolecule of interest, and the scattered light is analyzed. **(Figure 1.15)** A Dynamic Light Scattering Instrument can measure fluctuations in the intensity of the scattered light caused by diffusive motion of the particle.<sup>111</sup> The translational diffusion coefficient (or, equivalently, the hydrodynamic radius) of the particle can be derived from the data. Information regarding the polydispersity of the sample can also be extracted. Particles with diameters in the 3 - 3000 nm range are accessible.

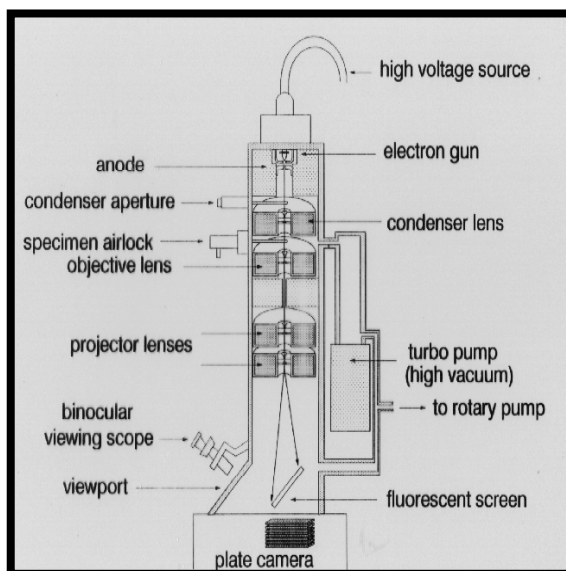


**Figure 1.15:** Mechanism for light scattering

Particle size can also be determined by electron microscopy,<sup>112</sup> a unique technique capable of examining in submicroscopic detail the structure, composition, and properties of the



liposome in a manner in which other equipment is unable. A schematic representation of the principle of a transmission electron microscope is depicted in **Figure 1.16**. To use illumination to magnify the image of an object, the illumination must be able to be deflected from its path. In a light microscope, deflection of the illumination is accomplished through the use of glass lenses. As the light travels into the lens it is bent because it is traveling through a medium with a different refractive index. The bending of the light is referred to as the phenomenon of refraction. The situation in an electron microscope is analogous to a light microscope. Electrons can be emitted by field emission from a tungsten filament, irradiating a thin specimen. A condenser system permits variation of illumination and the target specimen area. An image is obtained using a three to four stage lens system and direct photographic record can be taken. A resolution of 0.2 - 0.5 nm can be achieved.



**Figure 1.16:** Schematic ray path for a transmission electron microscope.

## CHAPTER 2

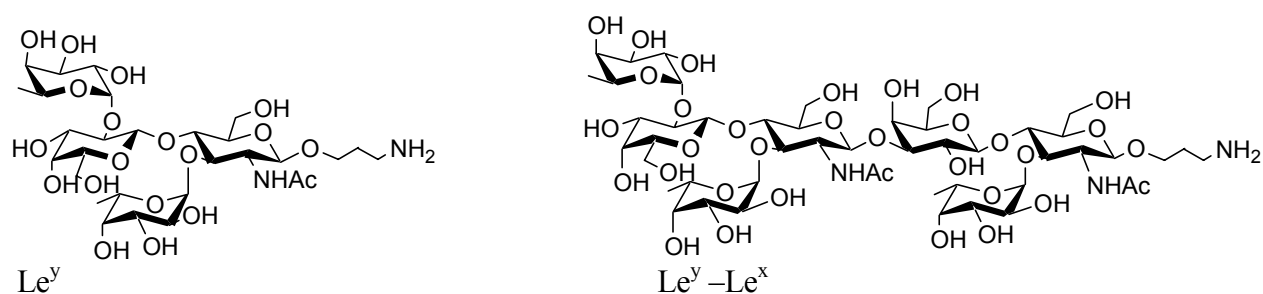
### RESEARCH OBJECTIVES

The project presented in the thesis is based on the fact that cancer cells are very often characterized by the expression of aberrant cell surface glycoconjugates.<sup>32,113</sup> The differential expression of the tumor-associated antigens offers a unique opportunity for the development of anti-cancer vaccines. Advances in tumor cell immunology have led to the identification of tumor associated antigens that represent a diverse array of oligosaccharide structures. Lewis antigens are an important family of oligosaccharides that are over-expressed on cancer cells.<sup>20,31</sup> In this project, the Lewis antigen Lewis<sup>y</sup> (Le<sup>y</sup>), which has been identified as an important epitope for eliciting antibodies against colon and liver carcinomas,<sup>20,114,115</sup> was chosen as a cancer marker used for vaccine development. A liposomal carrier system was selected as an immunological adjuvant.

This project is divided into two broad components. The primary objective of this project focuses on new strategies and methods for the assembly of the spacer equipped Lewis antigens. A large quantity of Le<sup>y</sup> was synthesized. The dimeric Lewis antigen Le<sup>y</sup>-Le<sup>x</sup> was also synthesized using a similar strategy (**Scheme 2.1**). The cross reactivity of the particular Le<sup>y</sup> antibody with respect to other similar Lewis antigen was studied for the prudent use of Le<sup>y</sup> antigen. The second objective of the project aimed to conjugate the antigens to an appropriate carrier substance to increase its immunogenicity as well as boost T-cell activity against antigens. In this thesis, particular liposomal di-epitope constructs were designed and constructed, allowing for the presentation of B and T<sub>H</sub> epitopes as structurally separate entities on the same vesicles.

## 2.1 A highly efficient strategy for the synthesis of lewis antigens

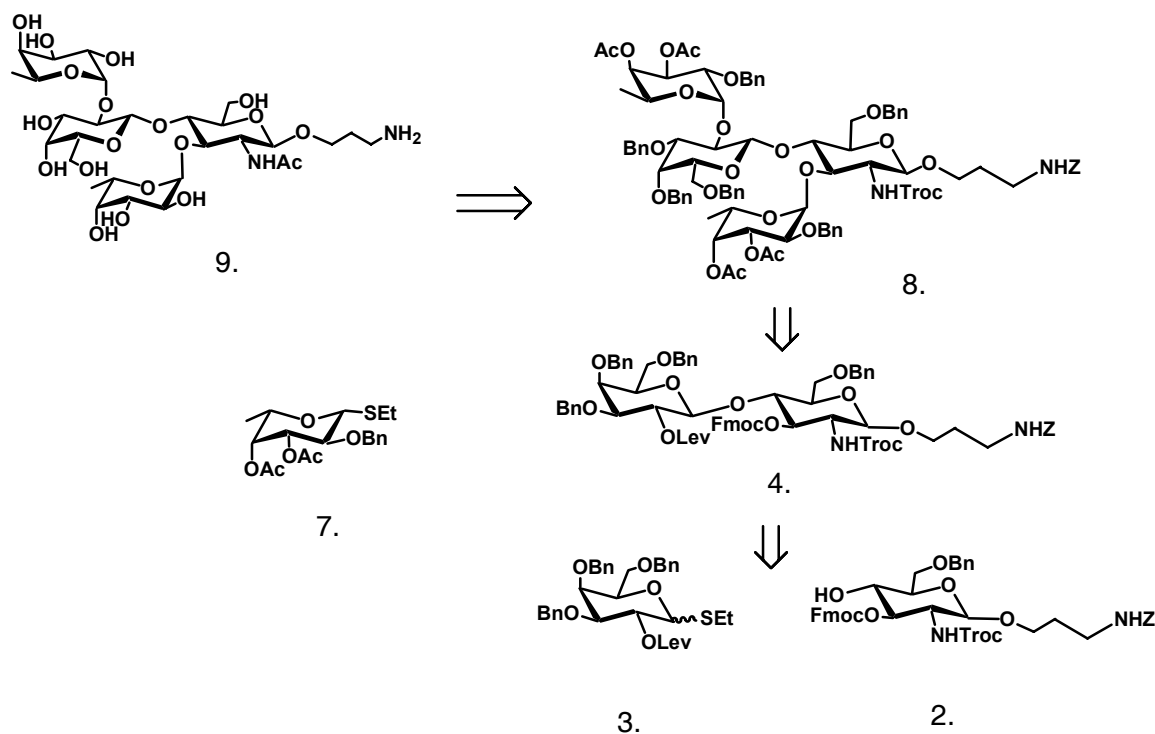
A major obstacle in the development of fully synthetic carbohydrate-based cancer vaccines is that substantial quantities of the tumor-associated oligosaccharide are required. The first major project of this thesis is the preparation of a Lewis<sup>y</sup> tetrasaccharide and a Lewis<sup>y</sup>-Lewis<sup>x</sup> heptasaccharide modified by an artificial aminopropyl spacer. (**scheme 2.1**)



**Scheme 2.1** Target Lewis antigens Le<sup>y</sup> and Le<sup>y</sup>-Le<sup>x</sup>

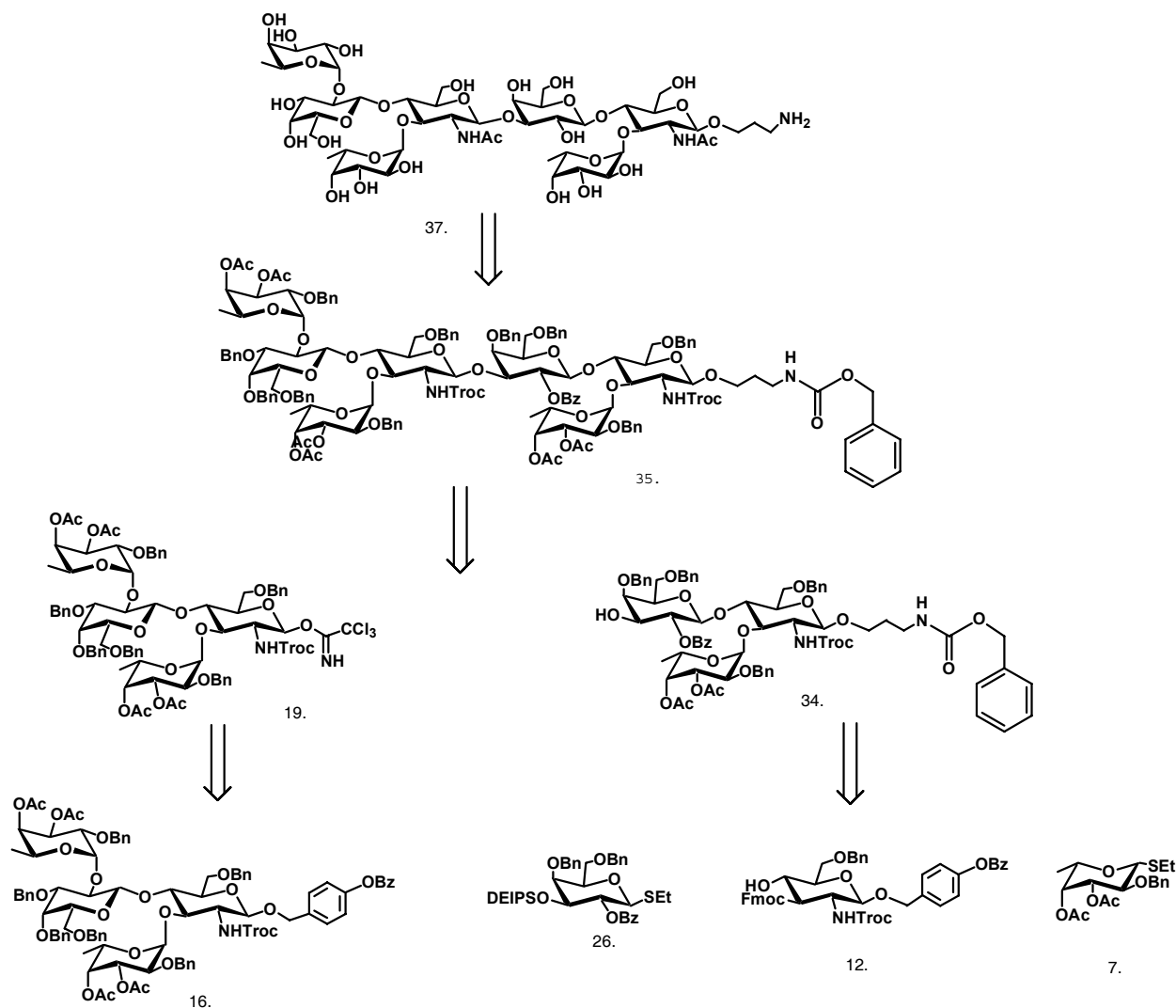
The Lewis<sup>y</sup> oligosaccharide has a Galα(1-4)GlcNAc core structure with two fucosides at C-3 of the GlcNAc and C-2 of the Gal moiety. In the preparation of the Lewis antigen Le<sup>y</sup>, the compound was broken down into three building blocks (**Scheme 2.2**). The levulinoyl ester (Lev) and 9-fluorenylmethoxycarbonate (Fmoc) were selected as an ideal set of orthogonal hydroxyl protecting groups. The Fmoc group can be deprotected by β-elimination using triethylamine in DCM.<sup>116</sup> (**Scheme 2.4**) Such a mild condition does not affect the Lev group. The Lev group can be removed by treatment with hydrazine acetate,<sup>117</sup> which cyclizes with the ketone moiety of Lev to give a free hydroxyl group. (**Scheme 2.5**) Since this reaction follows a different mechanism, the Fmoc is not cleaved. A trichloroethyloxycarbonyl (Troc) was employed as an amino

protecting group, which ensures high glycosyl accepting properties of the C-3 hydroxyl of the glucosamine unit<sup>118-120</sup> and offers efficient neighboring group participation to give stereo selective formation of  $\beta$ -glycoside. **(Figure 2.6)** In addition, the deprotection conditions for the set of the orthogonal groups did not affect the Troc protecting group. An artificial aminopropyl spacer was introduced that will facilitate the attachment of Lewis<sup>y</sup> to the desirable carrier system via its anomeric center. The new linker is stable with regard to the Lewis acidic conditions used in glycosylations and compatible with both the the base sensitive amino protecting group trichloroethyloxycarbonyl (Troc) and the set of orthogonal groups. As for the fucosyl donor, the two acetyl groups are essential to stabilize the fucoside, while the benzyl group ensures the formation of  $\alpha$ -glycosides because they will not perform non-neighboring group participation.



**Scheme 2.2** Retro-synthesis of Lewis antigen Le<sup>y</sup>

In the preparation of the Lewis antigen Le<sup>y</sup>-Le<sup>x</sup>, the compound was broken down into a Le<sup>y</sup> glycosyl donor **19** and a Le<sup>x</sup> glycosyl acceptor **34**. (Scheme 2.3)



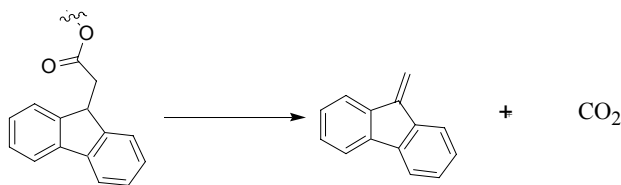
**Scheme 2.3** Retro-synthesis of Lewis antigen Le<sup>y</sup>-Le<sup>x</sup>

The strategy of the synthesis of the Le<sup>y</sup> glycosyl donor **19** is almost identical to the strategy for the preparation of the Le<sup>y</sup>, except for the introduction of a new linker to the anomeric

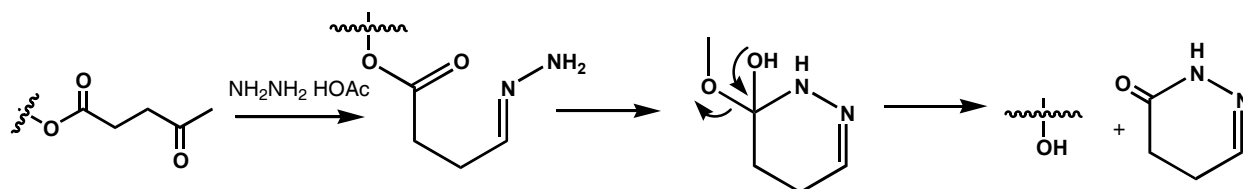
center of the GlcNAc in the Lewis<sup>y</sup> tetrasaccharide. Instead of using the aminopropyl spacer, a novel phenolic ester linker was used, (**scheme 2.7**) functioning as a temporary anomeric protecting group. This ester linker is stable towards the Lewis acidic conditions used in glycosylation and perfectly compatible with Troc and the set of orthogonal protecting groups. The resulting Lewis<sup>y</sup> tetrasaccharide **16** can be changed into a glycosyl donor after linker cleavage and activation of the anomeric center. The phenolic ester linker can be deprotected by treatment with hydrogen peroxide/Et<sub>3</sub>N, followed by an oxidative removal of the *p*-hydroxyl benzyl moiety with 3,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The obtained lactol can be easily converted into a glycosyl donor for linkage with the Le<sup>x</sup> acceptor. (**scheme 2.7**)

The Le<sup>x</sup> oligosaccharide has an identical Gal $\alpha$ (1-4)GlcNAc core structure to that of Le<sup>y</sup>, but differs in its fucoside substitution pattern. Lewis<sup>x</sup> has only one fucoside at the C-3 of the GlcNAc whereas Lewis<sup>y</sup> has two fucosides at both the C-3 of the GlcNAc and C-2 of the Gal moiety. Since the C-1 of GlcNAc in Le<sup>y</sup> and C-3 of Gal in Le<sup>x</sup> is linked together to form the target molecule Le<sup>y</sup>-Le<sup>x</sup>, a few changes were made to the synthesis of the Le<sup>x</sup> acceptor, particularly in the Gal building block preparation. The 9-fluorenylmethoxycarbonate (Fmoc) and diethylisopropylsilyl (DEIPS) were selected as a set orthogonal hydroxyl protecting groups. The Fmoc group can be deprotected by  $\beta$ -elimination using triethylamine in DCM,<sup>116</sup> (**Scheme 2.4**) which does not affect the DEIPS group. The DEIPS could be removed by treatment of TBAF buffered with acetic acid. This treatment did not affect the Fmoc protecting group. Due to the advantage that *N*-trichloroethoxycarbonyl (Troc) group and the artificial aminopropyl spacer can provide, they were also applied in the new synthetic strategy development. The DEIPS protecting group must be introduced to the C-3 of a Gal in Le<sup>x</sup>, which can be achieved through the preparation of Gal building block. Starting from Ethyl -1-thiol- $\beta$ -D-galactopyranoside, 1, 2-

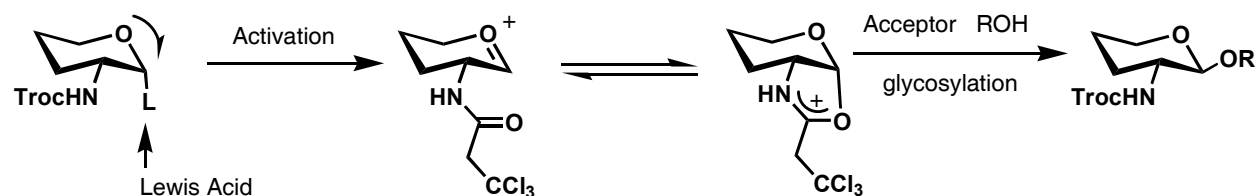
diacetal can protect the C-2 and C-3 selectively. After benzylation of the C-4 and C-6 hydroxyl groups, 1, 2-diacetal was easily cleaved and left C-2 and C-3 hydroxyl groups for the addition of DEIPS. Since the C-3 hydroxyl of Gal is much more active compared to that of C-2, DEIPS can be selectively introduced to the desired C-3 position. Initially, protection of the free hydroxyl on C-2 was attempted by the levulinoyl ester (Lev). However this levulinoyl ester (Lev) reduced the reactivity of the Lewis<sup>x</sup> acceptor, which proved to cause difficulty at later stage in the synthesis. Instead of using a levulinoyl ester (Lev), a benzoate (Bz) was used to protect C-2 of Gal. Coupling of the available glycosyl donor and acceptor gave the target Lewis<sup>y</sup>-Lewis<sup>x</sup> heptsaccharide. The synthesis of all the target compounds will be described in Chapter 3.



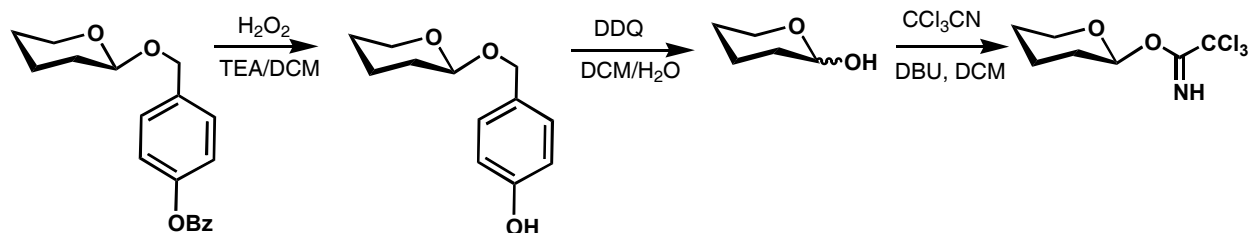
**Scheme 2.4** Mechanism of Fmoc Cleavage



**Scheme 2.5** Mechanism of Lev Cleavage



**Scheme 2.6** Common Mechanism for  $\beta$ - glycosidation



**Scheme 2.7** Common Mechanism for transition from phenolic ester to glycosyl donor

## 2.2 Construction of carbohydrate-based liposomal vaccine

The second major project presented in this thesis is the construction of cancer vaccines. While the availability of the tumor-associated Lewis antigens offers a unique opportunity for the developments of cancer vaccine, several obstacles remain. These tumor associated oligosaccharides are auto-antigens and consequently tolerated by the immune system. Furthermore, the inability of saccharides to activate helper T-lymphocytes diminishes their usefulness for vaccine development.<sup>121,122</sup> The classical solution in the attempt to increase the immunogenicity of oligosaccharide antigens consists in their coupling to carrier proteins.<sup>123-125</sup> The carrier proteins provide their own  $T_H$  epitopes. It is well known that combining certain proteins with carbohydrate antigens can drive an immune response producing IgG antibodies. However, the shortcomings to this strategy do exist and are discussed in **Chapter 1**. Additionally,

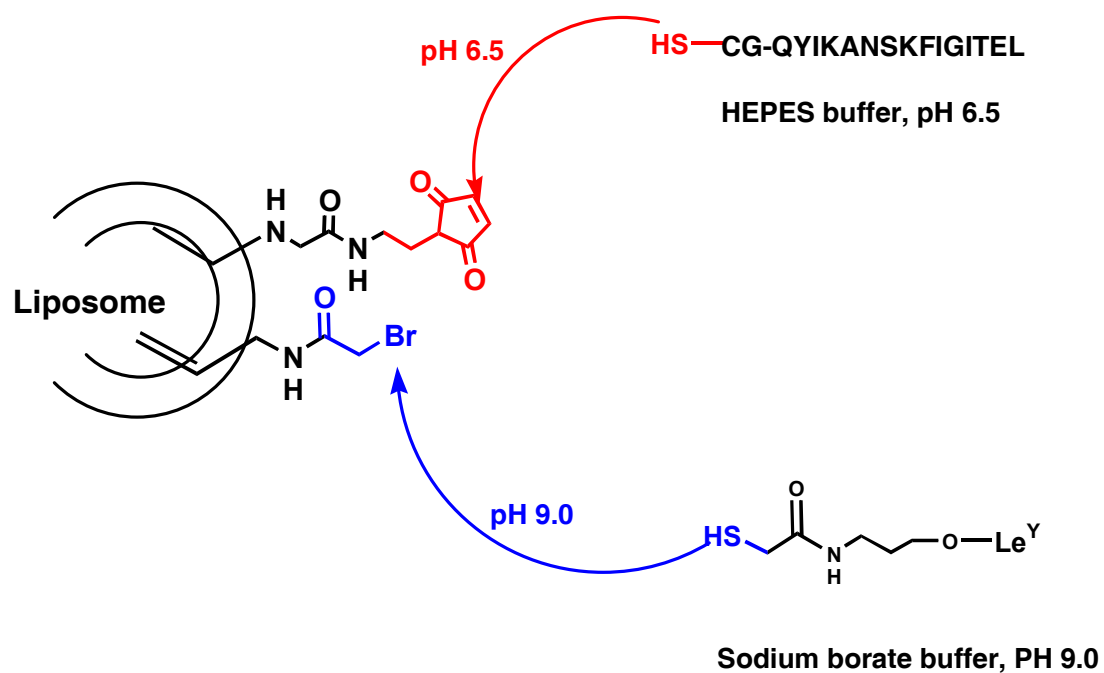


the conjugation steps are usually poorly controlled from a chemical perspective, thus the advantage of using structure defined T<sub>H</sub> epitopes is somewhat offset. The synthetic peptide sequence QYIKANSKFIGITEL (QYI) functionalized with a thiol at the N terminal was introduced. This peptide is a “universal” Th epitope from tetanus toxin (residues 830-844) and is characterized by its promiscuous recognition by T-helper cells. This peptide can activate T-cells and help oligosaccharide antigens to overcome T-cell independence.<sup>126</sup>

In the research of vectors for the construction of vaccines, liposomes are of great interest. These highly versatile phospholipid vesicles are characterized by a low toxicity and a low intrinsic immunogenicity, making them ideal delivery systems for vaccine development. In the past twenty years since liposomes were discovered to possess an adjuvant potential, studies have shown that variation in lipid composition, liposomal size, and antigen location (e.g., whether it is adsorbed or covalently coupled to the liposome surface or encapsulated in liposomal aqueous compartments) can significantly influence immunological response. Optimization of these variables by various techniques may ensure the efficacy, stability, homogeneity, and safety of a liposomal vaccine.

In this study, di-epitope constructs were assembled from typical liposome content like cholesterol and phospholipids. Some special lipopeptide, Pam<sub>3</sub>Cys, was also included, which plays a role as built-in adjuvant in the liposome formulation. This strategy is based on the different reactivity of maleimide and bromoacetyl functions towards thiol groups. Following the procedure of Boeckler *et.al.*,<sup>127,128</sup> liposomes with different thiol-reactive functionalities (maleimide and bromoacetyl) on their surface were prepared (**Scheme 2.8**). These groups exhibit a marked difference in reactivity under different pH conditions, *i.e.* the maleimide reacts rapidly with a sulfhydryl compound at pH 6.5, whereas the bromoacetyl requires a slightly

higher pH 8-9 for an efficiently reaction with a thiol compound. For the conjugation to the thiol-reactive anchors, both the oligosaccharide and the peptide were prepared with a thiol-containing linker. A diepitope construct carrying the B-epitope Le<sup>y</sup> tetrasaccharide and the Th-epitope peptide QYIKANSKFIGITEL (QYI) was prepared. The details regarding vaccine construction will be discussed in **Chapter 4**.



**Scheme 2.8** Design of diepitope liposomal constructue.

## **CHAPTER 3:**

### **SYNTHESIS OF LEWIS ANTIGENS**

The difficulty in obtaining reasonable quantities of well-defined complex carbohydrate from natural sources is well known. Furthermore, when relatively short carbohydrate fragments are linked to a carrier protein, it is quite possible that a vital recognition element may be destroyed during this process, thus leading to a decrease or complete loss of immunogenicity. In this thesis, a highly efficient strategy for the synthesis of complex Lewis antigens has been developed. The advantages of synthetic compounds over their naturally extracted counterparts include reproducible and known purity, control of quality and supply as well as scaleable manufacture. Furthermore, organic synthesis may incorporate functionality with a unique reactivity allowing selective conjugation to a carrier.

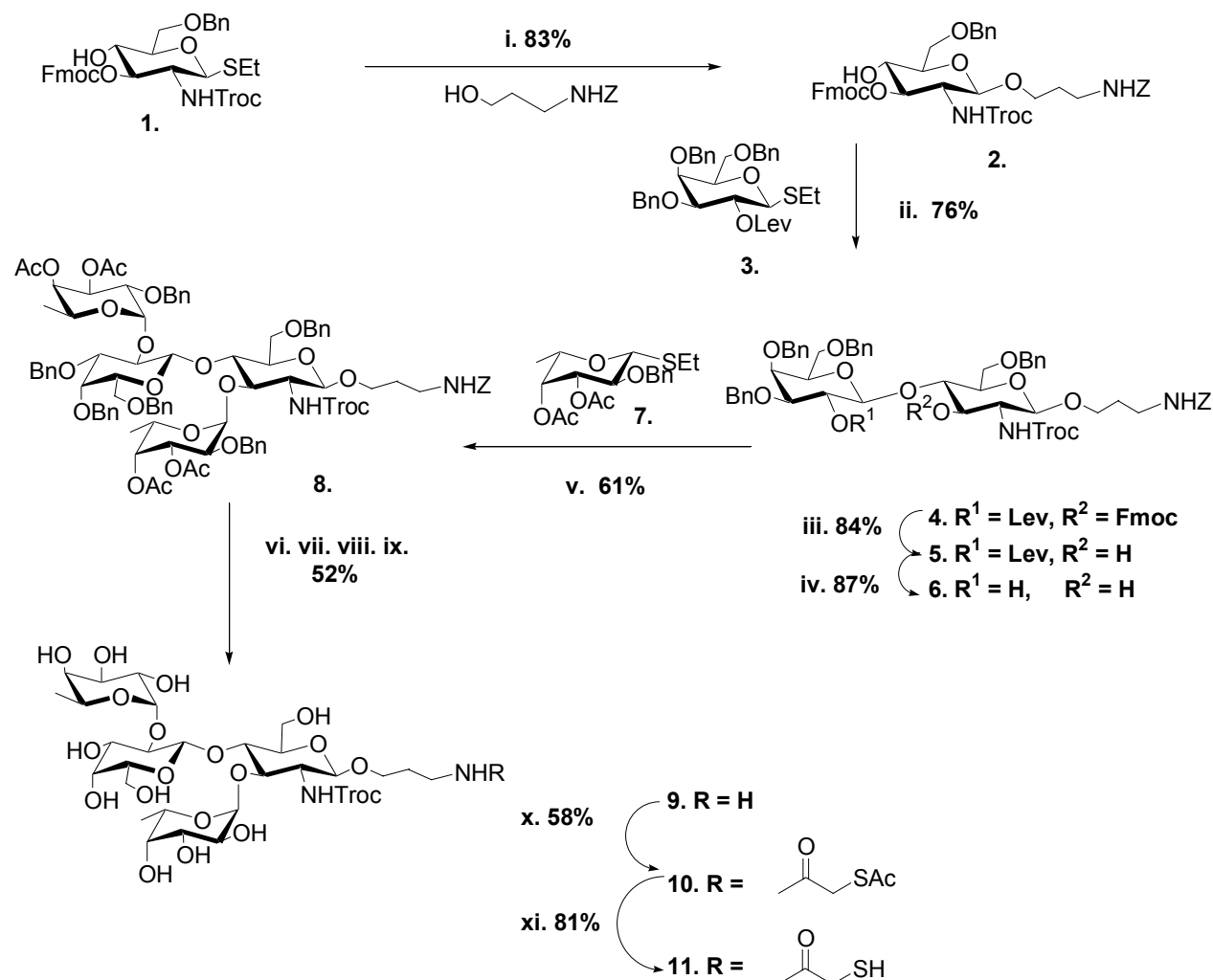
A large number of synthetic approaches have been reported in literature for the synthesis of the Lewis antigens.<sup>18,40,129-131</sup> In this chapter, a novel highly efficient synthetic methodology for the preparation of the Lewis antigens with an artificial aminopropyl spacer will be presented. The methodology is based on the use of only several monosaccharide building blocks, which will be achieved through the use of a set of orthogonal protecting groups, a base sensitive amino protecting group (Troc), and a novel phenolic ester protecting group. In section **3.1.**, the synthesis of Lewis antigen Lewis<sup>y</sup> is described followed by the synthesis of Lewis<sup>y</sup>-Lewis<sup>x</sup> in section **3.2.** The synthesis of Lewis<sup>y</sup>-Lewis<sup>x</sup> includes the synthesis of Lewis<sup>y</sup> donor and Lewis<sup>x</sup> acceptor and their coupling. Since a Gal building block is an essential component for the Lewis<sup>x</sup> acceptor preparation, the synthesis of this building block will be highlighted.

### 3.1 Synthesis of Lewis<sup>y</sup>

As part of a program to develop a fully synthetic anti-cancer vaccine, we required substantial quantities of the tumor-associated antigen Le<sup>y</sup>. The target compound, Le<sup>y</sup> derivative **9** (**Scheme 3.1**) was selected as it has an artificial aminopropyl spacer at the anomeric center for selective conjugation to a carrier system.

An efficient solution phase synthesis for **9** was developed which employs the building blocks **1**,<sup>132</sup> **3**,<sup>133</sup> and **7**.<sup>134</sup> Thus, coupling of **1** with 3-[(*N*-benzyloxycarbonyl)amino]propanol<sup>135</sup> in the presence of NIS/TMSOTf gave, after purification by silica gel column chromatography, **2** in an excellent yield of 83%. The compound **2** was immediately used in the next NIS/TMSOTf-mediated glycosylation with galactosyl donor **3** to give disaccharide **4** in a yield of 76%. The levulinoyl (Lev) protecting group of **4** was cleaved by a treatment of hydrazine buffered with acetic acid. The resulting compound **5** was subjected to Et<sub>3</sub>N in DCM to remove the 9-fluorenylmethoxycarbonate (Fmoc) to give diol **6**. Coupling of **6** with 3.8 equivalents of fucosyl donor **7** resulted in a clean and stereoselective glycosylation at C-3 and C-2' hydroxyls and the fully protected tetrasaccharide **8** was isolated in a yield of 61%. The fucosylation proceeded with complete  $\alpha$ -selectivity as confirmed by <sup>1</sup>J<sub>H,H</sub>-couplings (*J* = 3.5 Hz). Deprotection of **8** could easily be accomplished by a four-step procedure involving removal of the trichloroethyloxycarbonyl (Troc) group by reaction with nanosize activated Zn followed by acetylation of the resulting amine with acetic anhydride and pyridine. Next, the acetyl esters were saponified by treatment with NaOMe in methanol. Finally, the benzyl ethers and the benzyloxycarbonyl moiety were removed by catalytic hydrogenation over Pd/C in a mixture of ethanol and acetic acid to give, after purification by P2 Bio-gel size exclusion column chromatography, target compound **9**. The amino functionality of **9** was derivatized with an acetyl

thioacetic acid moiety by reaction with *S*-acetylthioglycolic acid pentafluorophenyl ester (SAMA-OPfp) to give **10**. Derivative **10** may be employed for glycosylation of proteins that are modified by electrophilic or nucleophilic moieties, respectively.



**Scheme 3.1:** Synthesis of Lewis<sup>y</sup> i: NIS, TMSOTf, DCM, 0 °C; ii: NIS, TMSOTf, DCM, 0 °C; iii: Et<sub>3</sub>N, DCM; iv: NH<sub>2</sub>NH<sub>2</sub>-HOAc, MeOH, DCM; v: NIS, TESOTf, DCM, 0 °C; vi: Zn, HOAc; vii: Ac<sub>2</sub>O, pyridine; viii: NaOMe, MeOH; ix: Pt/C, EtOH, HOAc; x: SAMA-OPfp, DIPEA, DMF; xi: 7% NH<sub>3</sub> (g)/DMF.

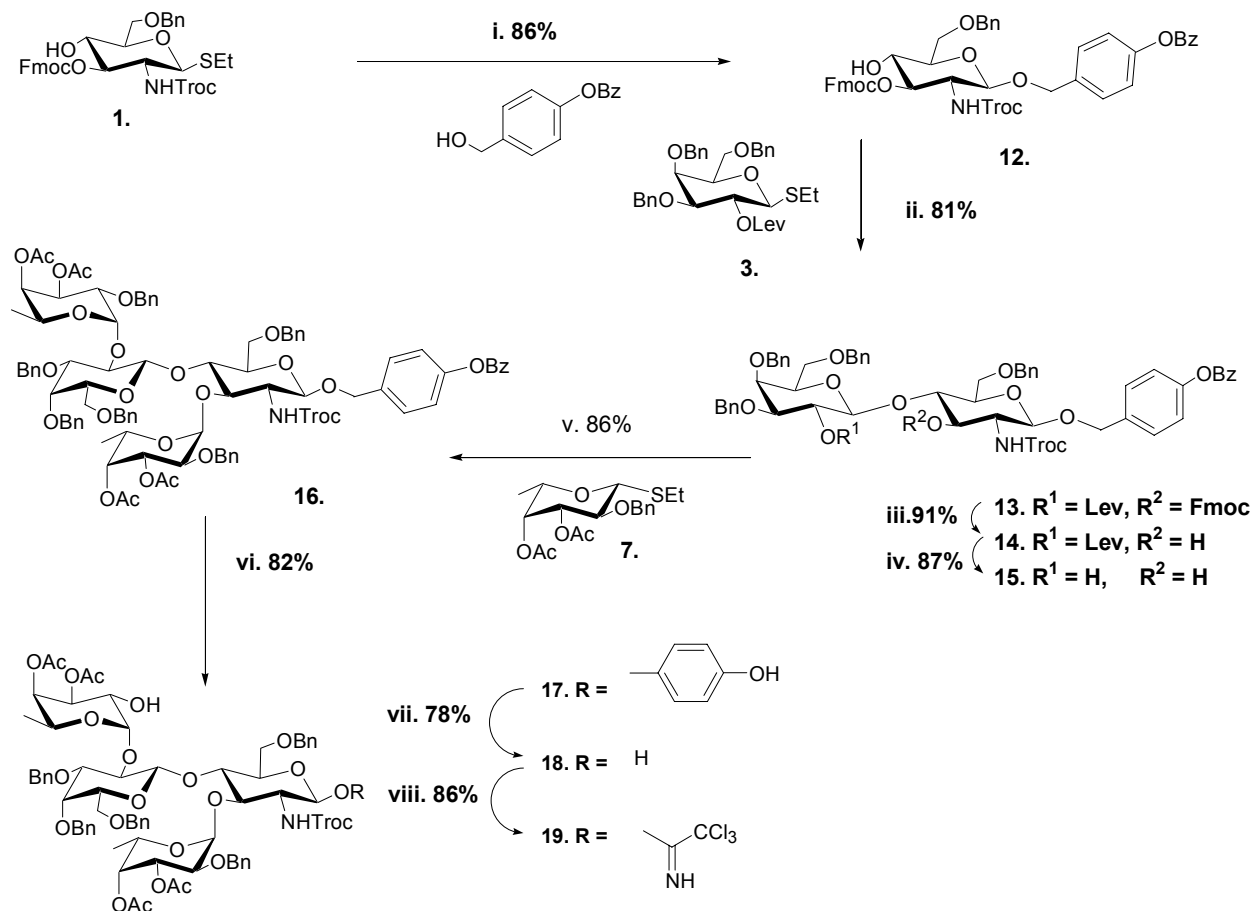
### 3.2 Synthesis of Lewis<sup>y</sup>-Lewis<sup>x</sup>

The synthesis of Lewis<sup>y</sup>-Lewis<sup>x</sup> consisted of the creation of a Lewis<sup>y</sup> donor and a Lewis<sup>x</sup> acceptor. The Lewis<sup>y</sup> donor construction followed an almost identical strategy as that of the Lewis<sup>y</sup> antigen assembly, except that a phenolic ester was included as a temporary protecting group on the anomeric center of GlcNAc in the Le<sup>y</sup> donor. This new linker allows for the successful synthesis of the Le<sup>y</sup> donor. However, there are a few changes in the synthesis of the Lewis<sup>x</sup> acceptor, which appeared during the galactose building block preparation. The DEIPS group was used to protect the C-3 hydroxyl of the galactose in the Le<sup>x</sup> acceptor, since this silyl protecting group can be deprotected selectively to release the free C-3 hydroxyl group for further coupling.

#### 3.2.1 Synthesis of Lewis<sup>y</sup> donor

A Coupling of the glucosamine acceptor **1** with the phenolic ester linker in the presence of *N*-iodosuccinimide/trimethylsilyl triflate (NIS/TMSOTf)<sup>136-138</sup> gave the formation of the main building block **12**. The key disaccharide **13** was synthesized in good yield (81%) in a *N*-iodosuccinimide/trimethylsilyl triflate (NIS/TMSOTf) promoted galactosylation of C-3 hydroxyl of glucosamine acceptor **12** (Scheme 3.2). Removal of the Lev group of **13** using triethylamine in dichloromethane (DCM) to give **14** and subsequent treatment with hydrazine acetate to remove the Fmoc group gave the 3,2'-diol derivative **15**. Fucosylation of **15** to afford the fully protected tetrasaccharide **16** was achieved in 86% yield by activating thioglycoside **7** in the presence of *N*-iodosuccinimide/trimethylsilyl triflate. The anomeric protecting group (phenolic ester) was cleaved by treatment with hydrogen peroxide in the presence of triethylamine. The so formed *p*-hydroxybenzyl derivative **17** was further treated with DDQ to completely remove the temporary protecting group from the anomeric position, which gave hemiacetal **18**. For the

further coupling with Lewis<sup>x</sup> acceptor, hemiacetal **18** was converted into the corresponding trichloroacetimidate donor **19** using standard conditions.

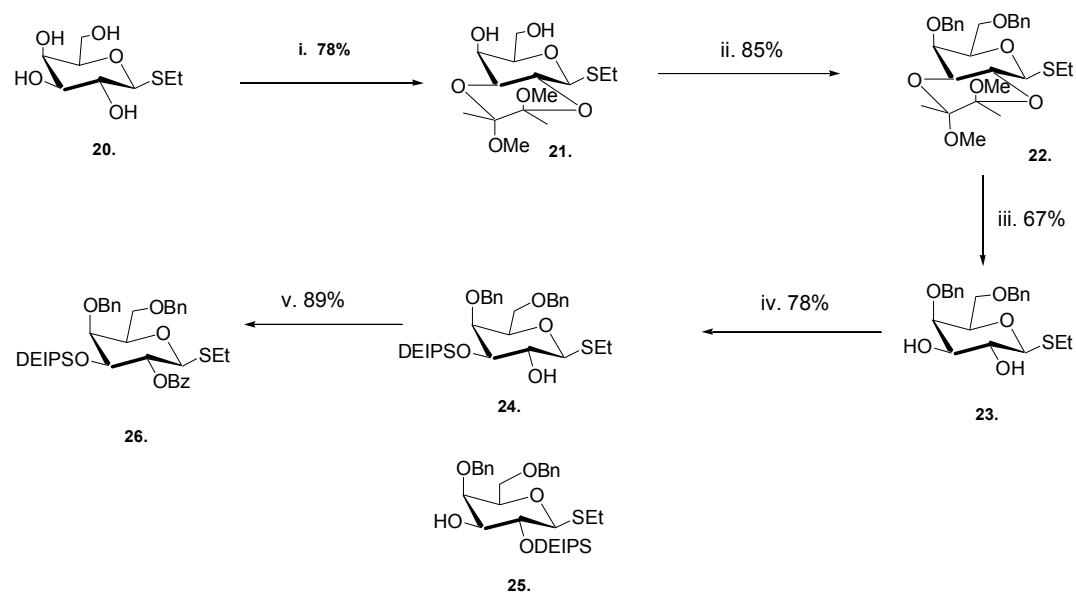


**Figure 3.2:** Synthesis of Lewis<sup>y</sup> glycosyl donor i: NIS, TMSOTf, DCM, 0 °C; ii: NIS, TMSOTf, DCM, 0 °C; iii: Et<sub>3</sub>N, DCM; iv: NH<sub>2</sub>NH<sub>2</sub>-HOAc, MeOH, DCM; v: NIS, TESOTf, DCM, 0 °C; vi: H<sub>2</sub>O<sub>2</sub>, Et<sub>3</sub>N, THF; vii: DDQ, DCM/H<sub>2</sub>O, 19/1 v/v; viii: CCl<sub>3</sub>CN, DBU, DCM.

### 3.2.2 Synthesis of galactose building block

In the synthesis of this glucose building block **26**, a new protecting group, 1,2-diacetals, was introduced. While 1,2-diacetals have been explored since 1938,<sup>139</sup> their specific

application in organic synthesis has been recognized only recently. This 1,2-diacetal is a critical component in the construction of the desired galactose building block for the Lewis<sup>x</sup> acceptor preparation.



**Scheme 3.3:** Synthesis of glucose building block i: butane-2,3-dione, HC(OCH<sub>3</sub>)<sub>3</sub>, CSA, MeOH, reflux, 78%; ii: NaH, BnBr, DMF, 85%; iii: TFA/H<sub>2</sub>O 9/1, 2min, 67%; iv: DIEPSCI, imidazole, THF, 78%; v: BzCl, TEA, DMAP, DCM, 89%.

The tetraol Ethyl 1-thiol-β-D-galactopyranoside **20** is protected by butane diacetal (BDA) in high yield on large scale (78% at 65.7 mmol) and produces an analytically pure product **21** without a need for further purification. By using standard conditions, di-O-benylation of diacetal **21** gave **22** in a good yield of 85%. The BDA moiety of **22** was deprotected very easily (5 min) with trifluoroacetic acid (TFA)/water (90%) to give diol **23** in excellent yield (67%). Selective silylation of diol **23** by reaction with diethylisopropyl chloride in the presence of imidazole gave the desired alcohol **24** in 78% yield. The selectivity was expected due to the



much greater reactivity of the hydroxyl of C-3 compared to the C-2 hydroxyl. The undesired isomer **25** (21%) was deprotected and recycled. Benzoylation of the alcohol **24** gave the galactose building block **26** in a yield of 89%.

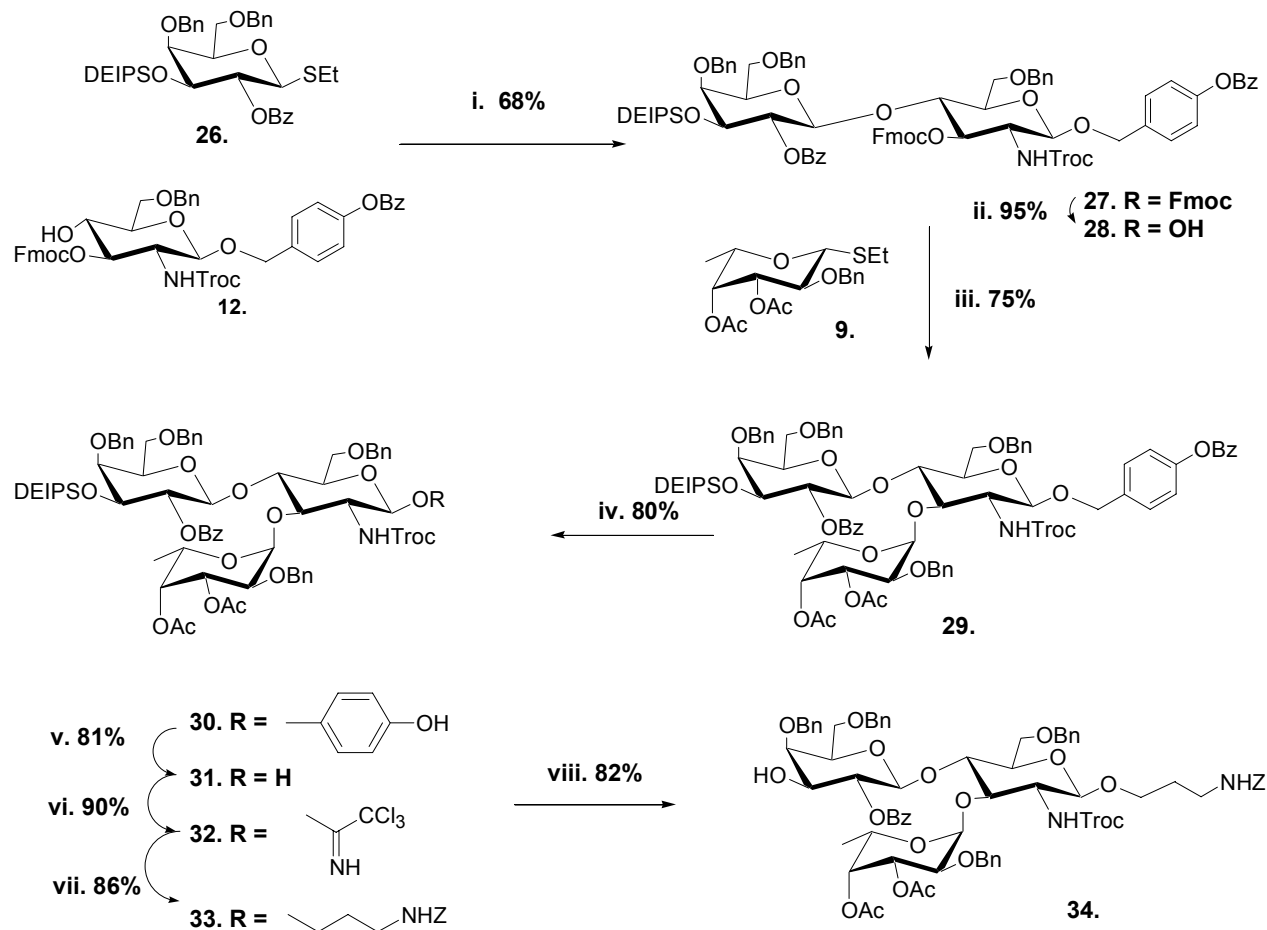
### 3.2.3 Synthesis of Lewis<sup>x</sup> acceptor

The significant disaccharide **27** was obtained by NIS/TMSOTf mediated coupling of thioglycoside **26** with the glucosamine acceptor **12**, which is modified with the new phenolic ester protecting group. Treatment of **27** with the non-nucleophilic base Et<sub>3</sub>N in dichloromethane removed the Fmoc group and revealed the C-3 hydroxyl of the glucosamine unit to give **28**. The revealed hydroxyl of **28** was glycosylated with fucosyl donor **9** in the presence of NIS/TESOTf to give the protected Le<sup>x</sup> core trisaccharide **29** in good yield (75%). The phenolic ester linker of **29** could be completely cleaved by a two step procedure including H<sub>2</sub>O<sub>2</sub>/EtN<sub>3</sub> and DDQ in H<sub>2</sub>O/DCM (1/19), which gave the hemiacetal **31**. Using standard condition, the hemiacetal **31** was converted into the trichloroacetimidate **32**, which was then attached to the aminopropanol spacer to give the fully protected Le<sup>x</sup> trisaccharide **33** in an excellent yield at 86%. In order to continue with further coupling, the hydroxyl group of C-3 of galactoside in this trisaccharide protected by DEIPS need be released. The DEIPS group of Le<sup>x</sup> trisaccharide **33** could be readily removed by a treatment with TBAF/AcOH/THF to give compound **34** in a yield of 82% without affecting all other protecting groups present in the compound **33**. The obtained trisaccharide **34** can be used as a glycosyl acceptor for the preparation of Le<sup>y</sup>-Le<sup>x</sup> heptsaccharide.

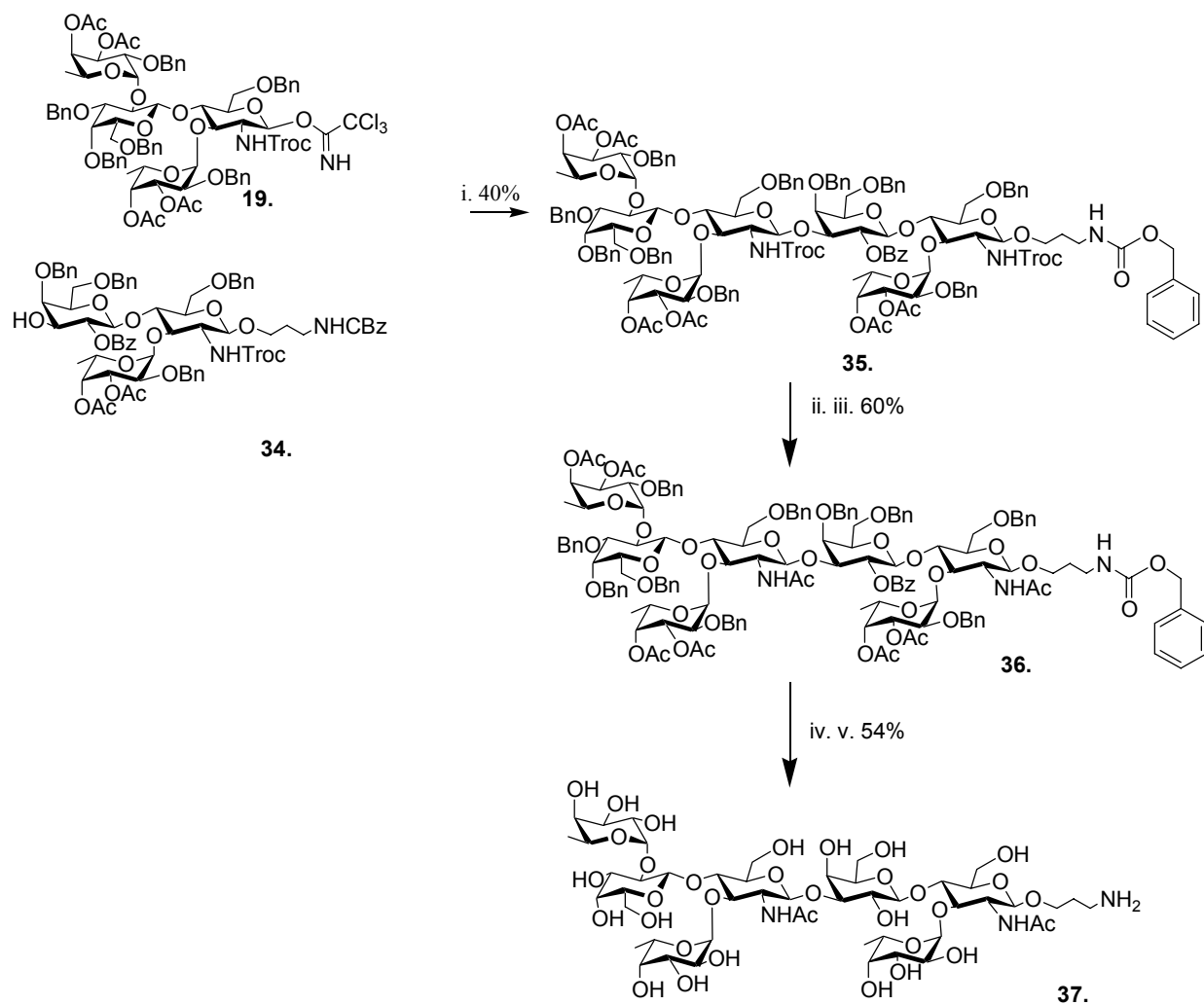
### 3.2.4 Synthesis of Lewis<sup>y</sup>-Lewis<sup>x</sup>

Glycosyl donor **19** was coupled with the spacer modified Lewis<sup>x</sup> acceptor **34** in the presence of TBSOTf at -20°C with a reasonable yield (49%). The Lewis<sup>y</sup>-Lewis<sup>x</sup> heptsaccharide was then deprotected in a sequence of four steps. First, the *N*-Troc group was converted into

acetamido functionality. The *N*-Troc group was removed by a treatment with zinc in acetic acid. Standard acetylation gave the *N*-acetylated tetrasaccharide. Alkaline removal of the *O*-acetyl groups followed by hydrogenolysis over Pd/C in a mixture of ethanol and HOAc gave the deprotected 3-aminopropyl tetrasaccharide **37** in an overall yield of 32%.



**Scheme 3.4:** Synthesis of Lewis<sup>x</sup> acceptor i: NIS, TMSOTf, DCM, 0 °C; ii: Et<sub>3</sub>N, DCM; iii: NIS, TESOTf, DCM, 0 °C; iv: H<sub>2</sub>O<sub>2</sub>, Et<sub>3</sub>N, THF; v. DDQ, DCM/H<sub>2</sub>O 19/1 v/v; vi: CCl<sub>3</sub>CN, DBU, DCM; vii: BF<sub>3</sub>-Et<sub>2</sub>O, DCM, 0 °C; viii. TBAF/HOAc 1/1 v/v, THF.



**Scheme 3.5:** Synthesis of Lewis<sup>y</sup> i: TBSOTf, DCM, -30 °C; ii: Zn, HOAc; iii. Ac<sub>2</sub>O, pyridine; iv: Pd/C, EtOH, HOAc; v: MeOH/NH<sub>2</sub>NH<sub>2</sub> H<sub>2</sub>O 4/1.

## CHAPTER 4:

### CONSRUCTION OF CANCER VACCINE

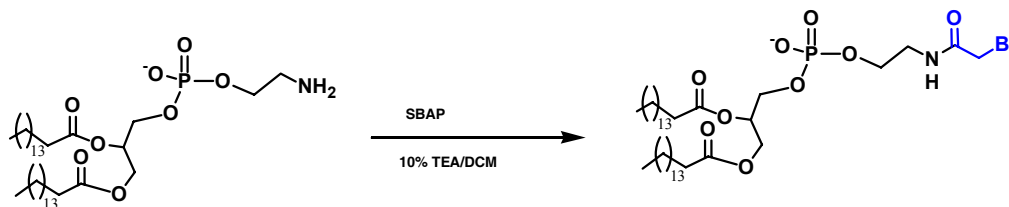
Liposomes are considered prime candidates in the improvement of antigen immunogenicity. Animal immunization studies by numerous laboratories have shown that liposomes promote humoral and cell-mediated immunity towards a wide spectrum of bacterial, protozoan and viral antigens as well as tumour cell antigens, venoms and allergens. The immunoadjuvant function of liposomes is supplemented by their ability to act as a carrier for co-entrapped B and T-cell epitopes, thus eliminating the need for a carrier protein. Recently, a designed liposomal diepitope constructs were developed by Schuber *et al.*,<sup>127,128</sup> which allow for the physical combination of B and T<sub>H</sub> epitopes as structurally separate entities within the same vesicle. The immunoadjuvant efficiency was tested by several peptide antigens which induced highly anamnestic and long-lasting immune responses, showing that this strategy can be successful. These results indicate that liposomal diepitope constructs could be attractive vesicles in the development of vaccines with other antigens. In this thesis, a liposomal construct was used for the preparation of a carbohydrate-based cancer vaccine.

The following chapter will discuss the use of liposomes to induce a desired immune response to the cancer associate Lewis antigen Le<sup>y</sup>. In this chapter, section **4.1** is dedicated to the synthesis of lipid components for liposomal vaccine formulation, while section **4.2** explores the method of liposome preparation. In section **4.3** the bioconjugation of the B-epitope and T-epitope to functionalized liposomes is presented. The final section (**4.4**) concentrates on the characterization of the liposomal vaccines.

#### 4.1. Synthesis of liposome lipid composition

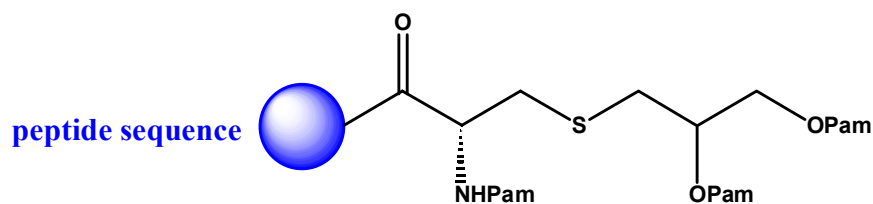
A unique trait of liposomes lies in the composition of their membranes which are composed of cholesterol and phospholipids. The membrane structure, composition and proportion of the membranes are almost identical to host cell membranes. Thus, the liposomes can be avidly phagocytosed by macrophages and the other cells of the reticuloendothelial system, making them excellent adjuvants for many purified antigens. Additionally, liposomal constructs are appealing in the development of carbohydrate-based cancer vaccines because they incorporate the minimal vaccination elements and give access to chemically defined formulations. However, these advantages can only be achieved when the correct lipid composition is chosen. In this project, the following five contents were chosen for liposome construction:

Lipo peptide *S*-[2,3-bis (palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteinylalanyl-glycine (Pam<sub>3</sub>CAG), derivatives of di-palmitoylphosphatidylethanolamine-bromoacetyl (DPPE-BrAc), Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Cholesterol (Chol). Three contents are commercially available (Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Cholesterol (Chol)), so only DPPE-BrAc and Pam<sub>3</sub>CAG need be synthesized.



**Scheme 4.1:** Synthesis of di-palmitoylphosphatidylethanolamine-bromoacetyl (DPPE-BrAc).

Preparation of DPPE-BrAc is very straightforward.<sup>140</sup> Di-palmitoylphosphatidylethanolamine was bought from Sigma-Aldrich Co. and was activated by *N*-acylation. Bromoacetyl groups were introduced by treatment with succinimidyl 3-(bromoacetamido) propionate (SBAP) in 10% TEA/DCM solution. (**Scheme 4.1**)

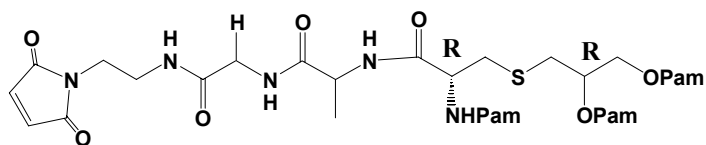


**Scheme 4.2:** Basic structure of lipopeptide containing Pam<sub>3</sub>Cys.

Pam<sub>3</sub>CAG is the derivative of Pam<sub>3</sub>Cys. (**Scheme 4.2**) Pam<sub>3</sub>Cys is a type of lipopeptide found from the N-terminal sequence of the principal lipoprotein of *Escherichia coli*. Jung and co-workers<sup>141</sup> have extensively investigated the molecular requirements of Pam<sub>3</sub>Cys derivatives for optimal immunological activities and found that:

a) The lipopeptide Pam<sub>3</sub>Cys-Ser, which contains an additional polar serine moiety, is much more mitogenic than Pam<sub>3</sub>Cys, so it is the preferred compound for use in vaccines. b) The peptide sequence can be varied (length, sequence) without loss of biological activity, but *R*-configuration in the glycery moiety has a higher activity than the corresponding *S*-diastereoisomers. c) The length of the fatty acid chain has only a marginal influence on the biological activity, but three acyl chains yield an optimum biological activity.

According to these rules, Pam<sub>3</sub>CAG has been developed and used as an adjuvant successfully for a long time. To perform the project in this thesis, the thiol-reactive derivative of Pam<sub>3</sub>CAG, PamCysAlaGlyMal, (**Scheme 4.3**) was synthesized.<sup>142</sup> This lipopeptide can introduce the maleimide moiety to the surface of the target liposomal constructs. Bromoacetyl, the other thio-reactive moiety, was incorporated into the same vesicle by use the thiol-reactive phospholipids derivative, di-palmitoylphosphatidylethanolamine-bromoacetyl.



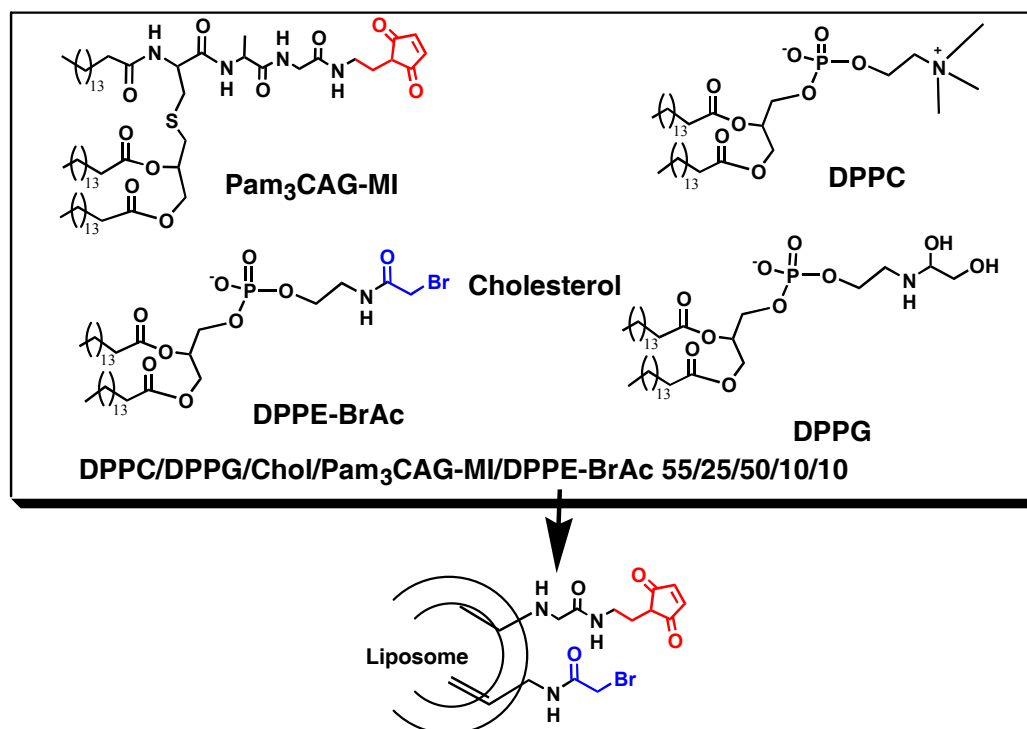
**Scheme 4.3:** The thiol-reactive lipopeptide Pam<sub>3</sub>CysAlaGlyMal.

Lipid building block Pam<sub>3</sub>Cys was synthesized following the method described by Wiesmüller *et al.*<sup>141</sup> The commercially available FmocGly (O<sup>t</sup>Bu)-Wang resin was chosen for the lipopeptide synthesis. The reason to choose wang resin is because of its linker that can afford a carboxylic acid at the peptide C-terminal after resin cleavage, which makes later modification possible.

## 4.2. Liposome preparation

Liposomes were prepared from egg phosphatidylcholine (PC), phosphatidylglycerol (PG), cholesterol, di-palmitoylphosphatidylethanolamine-bromoacetyl (DPPE-BrAc), and S-[2,3-bis(palmitoyloxy)-(2-RS)-2propyl]-N-palmitoyl-cysteinyl-alanyl-glyciny-maleimide (Pam<sub>3</sub>CAG-Mal) (55/25/50/10/10 molar ratio) according to the reversed-phase evaporation

method. Briefly, this involves preparing the lipids for hydration, hydration with agitation followed by sizing to a homogeneous size distribution. Thus, the lipids were dissolved in DCM to assure homogeneous mixing. The solvent was then removed to produce a thin lipid film, which was hydrated by suspending it in 10 mM Hepes buffer, pH 6.5. The vesicle suspension was vortexed on a shaker, to achieve aging, and the large multilamellar vesicle (LMV) suspension was extruded through polycarbonate membranes to obtain 100 nm small unilamellar vesicles (SUV). Photon correlation spectroscopy technique and transmission electron microscopy (TEM) confirmed the uniform size distribution and mean diameter of the vesicles. (Scheme 4.4).

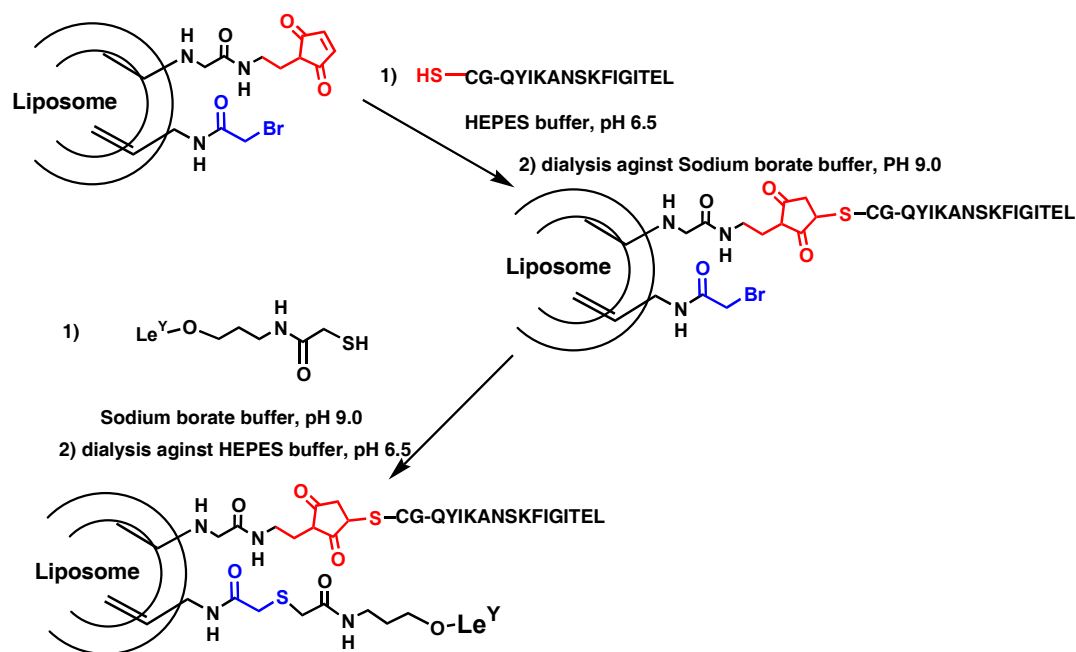


**Scheme 4.4** Liposome Preparation



### 4.3. Conjugation of T- and B-epitope to the liposomes

The thiol modified T- and B-epitopes were chemoselectively conjugated to the freshly prepared liposomes in a two step fashion. The cysteine modified T-epitope peptide QYI was conjugated to the maleimide moieties at pH 6.5 using a twofold excess with regard to Pam<sub>3</sub>CAG-Mal (**Scheme 4.5**). Un-conjugated peptide was removed by dialysis against sodium borate buffer pH 9.0. The conjugation of the B-epitope Le<sup>y</sup> tetrasaccharide was then performed by adding a twofold excess (with regard to DPPE-bromoacetyl) to the pH 9.0 liposome solution. Finally, any un-reacted maleimide- or bromoacetyl-groups were quenched by addition of 2-mercaptoethanol (ME). The diepitope liposomal constructs were purified by extensive dialysis. For comparison, a blank liposome suspension was prepared by conjugating 2-mercaptoethanol to freshly prepared liposomes at pH 9.0.



**Scheme 4.5:** Mechanism of the conjugation of T- and B-epitope to the liposomes

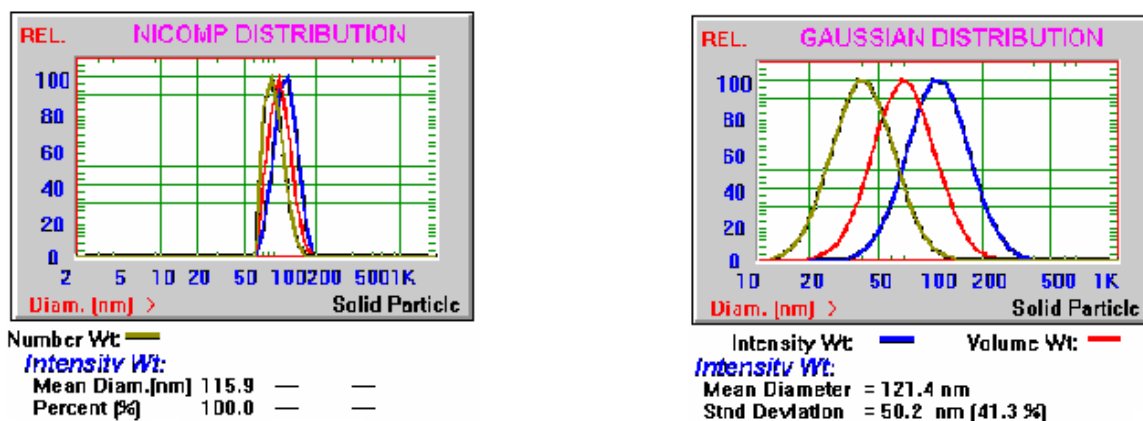
## 4.4. Characterization of the liposomal vaccines

### 4.4.1. Quantitation of peptide and carbohydrate conjugation

The amount of conjugated peptide CG-QYI was quantified using Lowry protein concentration test.<sup>143</sup> The incorporation of Le<sup>y</sup> oligosaccharide to the liposomes was determined by Dubois's phenol-sulfuric acid total carbohydrate assay.<sup>144</sup> Typical yield of conjugation of the T-epitope peptide was 65-70% and 70-80% for the Le<sup>y</sup> oligosaccharide.

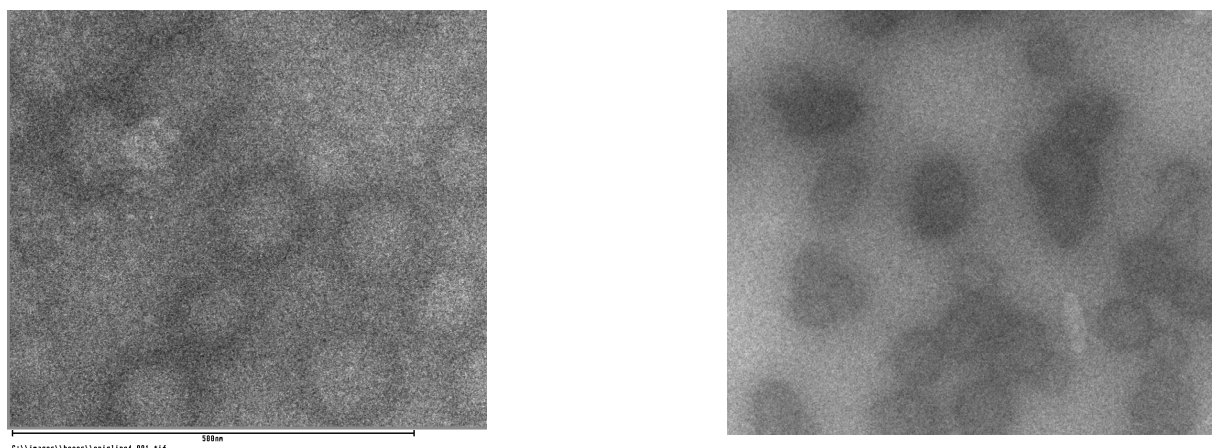
### 4.4.2. Analysis of liposomal size

The particle size distribution of the liposomal formulations was tested by dynamic light scattering (N4 Plus, Coulter Electronics, Miami, FL, USA). The liposome suspension was injected into the dispersion unit of the machine, which contains stirrer and stirres in order to reduce the interparticle aggregation. The laser obscuration range was maintained between 15% and 20%. The average volume-mean particle size was measured. The average particle size of the prepared liposome formulations is given in **Scheme 4.6**.



**Scheme 4.6:** The particle size distribution before (left) and after (right) conjugation.

An electron transparent support film (collodion film) covered grid is prepared to deposit the sample. A thin suspension of the original liposome solution is placed on the film covered grid and all but a tiny excess is removed with a small piece of filter paper. The remainder is allowed to dry completely to the film (30 min). After the complete drying of the specimen, a thin layer of negative stain (uranyl acetate) is similarly applied, removed and allowed to dry. Once the stain is completely dry the grid may be examined using the TEM. The actual liposome sizes before and after conjugation were recorded, which is shown in **Scheme 4.7**.



**Scheme 4.7:** The actual particle size tested by TEM before (left) and after (right) conjugation.

## CHAPTER 5:

### CONCLUSIONS

In **Chapter 3**, the synthesis of the two Lewis antigens was presented. A highly efficient strategy has been developed for the synthesis of Lewis antigen  $\text{Le}^y$  and  $\text{Le}^y\text{-Le}^x$  with the same artificial aminopropyl spacer, which facilitates the attachment of Lewis antigens to the desired carrier system via its anomeric center. The strategy is based on the use of only three monosaccharide building blocks for the preparation of the target compounds by the use of a set of orthogonal protecting groups. Lewis antigens,  $\text{Le}^y$ ,  $\text{Le}^y$  donor and  $\text{Le}^x$  acceptor have been successfully made by following this new strategy.  $\text{Le}^y$  donor and  $\text{Le}^x$  acceptor were further coupled to synthesize the dimeric  $\text{Le}^y\text{-Le}^x$  structure.

The developed methodology should allow the preparation of a variety of other monomeric and dimeric Lewis antigens like  $\text{Le}^a\text{-spacer}$ ,  $\text{Le}^b\text{-spacer}$ ,  $\text{Le}^x\text{-Lac-spacer}$ ,  $\text{Le}^y\text{-Lac-spacer}$ ,  $\text{Le}^a\text{-Lac-spacer}$ ,  $\text{Le}^b\text{-Lac-spacer}$ ,  $\text{Le}^x\text{-Le}^x$ ,  $\text{Le}^a\text{-Le}^a$ ,  $\text{Le}^a\text{-Le}^x$ ,  $\text{Le}^b\text{-Le}^a$ ,  $\text{Le}^x\text{-Le}^a$ , et.c.

In **Chapter 4**, a liposomal carrier system with two different functionalities were constructed and optimized. Then  $\text{Le}^y$  B-epitope and “universal” helper T-epitope with the sequence QYIKANSKFIGITEL were conjugated selectively to the available liposomes to make the desired di-epitope glycoconjugate vaccines. The efficiency of the liposomal vaccines had been tested on the model animal and verified the desired humoral immune responses to the target antigen. The liposomal vaccine technology may be equally applicable to all the other Lewis antigens.

## CHAPTER 6:

### EXPERIMENTAL SECTION

**General:** Succinimidyl 3-(bromoacetamido) propionate (SBAP), sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC), 2-iminothiolane (Traut's reagent), keyhole limpet hemocyanin (KLH), maleimide activated mariculture KLH (mcKLH-MI) and bovine serum albumin (BSA-MI) were purchased from Pierce Endogen, Rockford, IL. BSA was purchased from Sigma. NIS was purchased from Fluka and recrystallized from dioxane/ $\text{CCl}_4$ . All other chemicals were purchased from Aldrich, Acros, and Fluka and used without further purification. Molecular sieves were activated at  $145^\circ\text{C}$  for 10 h. All solvents employed were of reagent grade and dried by refluxing over appropriate drying agents. All the reactions were performed under anhydrous conditions and monitored by TLC using Kieselgel 60  $\text{F}_{254}$  (Merk) plates, with detection by UV light (254 nm) and/or by charring with 8% sulfuric acid in ethanol. Column chromatography was performed on silica gel (Merk, mesh 70-230). Size exclusion column chromatography was performed on Sephadex LH-20 or Sephadex G10 gel (Pharmacia Biotech AB, Uppsala, Sweden) and dichloromethane/methanol (1/1, v/v) was used as eluent.. Extracts were concentrated under reduced pressure at  $\leq 40^\circ\text{C}$  (water bath).  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded on a Varian Inova300 spectrometer and a Varian Inova500 spectrometer equipped with Sun workstations.  $^1\text{H}$  spectra recorded in  $\text{CDCl}_3$  were referenced to residue  $\text{CHCl}_3$  at 7.26 ppm or TMS, and  $^{13}\text{C}$  spectra to the central peak of  $\text{CDCl}_3$  at 77.0 ppm. Assignments were made using standard 1D and gCOSY, gHSQC and TOCSY 2D experiments. Negative ion matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass

spectra were recorded using an HP-MALDI instrument using gentisic acid as a matrix. Centrifugal filter devices were purchased from Millipore Inc. The immunoadjuvant QS-21 was a gift from Antigenics Inc., Lexington MA. ELISA plates Immulon II Hb was purchased from Fisher Scientific Inc.

**3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2[[*(2,2,2*-trichloroethoxy) carbonyl]amino]-3-*O*-(9-fluorenylmethoxycarbonyl)- $\beta$ -D-glucopyranoside (2):** A mixture

of thioglycoside **1** (100 mg, 0.14 mmol) and 3-[(*N*-benzyloxycarbonyl)amino]propanol (33 mg, 0.17 mmol) was dried azeotropically with dry toluene and then subjected to high vacuum for 2 h. The mixture was dissolved in dry dichloromethane (10 mL) and stirred at room temperature under argon in the presence of activated molecular sieves (4Å) for 30 min. Then the mixture was cooled to 0°C and reacted with NIS (35 mg, 0.16 mmol) and TESOTf (3  $\mu$ L, 0.01 mmol). After stirring for 10 minutes at 0°C, TLC showed full conversion of the donor. The solution was diluted by dichloromethane (60 mL) and the molecular sieves were removed by filtration through a plug of Celite. The filtrate was washed with aqueous sodium thiosulfate (15%, 4 mL), brine and dried over MgSO<sub>4</sub> and concentrated. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc 2:1) to give the product **2** as a white powder (100 mg, 83%):  $[\alpha]_D -32.2^\circ$  (*c* 1.0, CH<sub>2</sub>Cl<sub>2</sub>); *R*<sub>f</sub> = 0.48 (Hexane/EtOAc 2:1). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.80 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.72-7.20 (m, 16H, Ar-H), 5.74 (d, *J* = 8.3 Hz, 1H, *N*-H), 5.20 (d, *J* = 4.5 Hz, 1H, CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 5.07 (s, 2H, CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 4.86 (t, *J* = 9.4 Hz, 1H, H-3), 4.65-4.51 (m, 4H, ArCH<sub>2</sub>, Troc), 4.48 (d, 1H, *J* = 8.8 Hz, H-1), 4.37 (d, 2H, *J* = 6.3 Hz, Fmoc-CH<sub>2</sub>), 4.29 (t, *J* = 6.3 Hz, 1H, Fmoc-CH), 3.90 (dd, *J* = 2.8, 11.1 Hz, 1H, H-6a), 3.82-3.71 (m, 4H, H-2, H-4, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 3.52-3.41 (m, 2H, H-6b, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub><sup>a</sup>NHCOOCH<sub>2</sub>Ph), 3.20 (m, 1H, H-5), 3.26-3.18 (d, *J* = 1.2 Hz, 1H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub><sup>b</sup>NHCOOCH<sub>2</sub>Ph), 1.72 (m, 2H,

CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 156.9 (NHCOOCH<sub>2</sub>Ph), 155.9 (CHCH<sub>2</sub>OCO, Fmoc), 154.8 (NHCO), 142.5-120.3 (24C, Ar-C), 101.4 (C-1), 95.7 (CCl<sub>3</sub>), 79.4 (C-3), 74.6, 74.1 (2C, OCH<sub>2</sub>Ph, OCH<sub>2</sub>CCl<sub>3</sub>), 73.9 (C-5), 70.9 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 70.7 (CHCH<sub>2</sub>OCO, Fmoc), 70.2 (C-4), 67.5 (C-6), 66.9 (COOCH<sub>2</sub>Ph), 56.2 (C-2), 46.8 (CHCH<sub>2</sub>OCO, Fmoc), 38.0 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 29.9 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>). HR-MALDI-TOF: *m/z*: calcd for C<sub>42</sub>H<sub>43</sub>Cl<sub>3</sub>N<sub>2</sub>O<sub>11</sub>: 856.1932; found: [M + Na<sup>+</sup>] 879.1941.

**3-[(N-Benzyloxycarbonyl)amino]propyl**

**6-O-benzyl-2-deoxy-2[[[(2,2,2-**

**trichloroethoxy)carbonyl]amino]-3-O-(9-fluorenylmethoxycarbonyl)-4-O-(3,4,6-tri-O-**

**benzyl-2-O-levulinoyl- β -D-galactopyranosyl)- β -D-glucopyranoside (4):** A mixture of acceptor **2** (95 mg, 0.11 mmol) and thiogalactoside **3** (80 mg, 0.13 mmol) was dried azeotropically with toluene and then subjected to high vacuum for 2 h. The mixture was dissolved in dry dichloromethane (10 mL) and stirred at room temperature under argon in the presence of activated molecular sieves for 30 min. The mixture was cooled to 0°C and reacted with NIS (33 mg, 0.15 mmol) and TESOTf (3 μL, 0.01 mmol). After 30 min, TLC indicated complete reaction and the mixture was diluted with dichloromethane (60 mL) and filtered through Celite. The filtrate was washed with aqueous sodium thiosulfate (15%, 4 mL) and brine (20 mL), dried over MgSO<sub>4</sub> and concentrated. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc 2:1) to give the product **4** as a white powder (110 mg, 76%). [α]<sub>D</sub> -48.9° (*c* 1.0, CH<sub>2</sub>Cl<sub>2</sub>); R<sub>f</sub> = 0.41 (Hexane/EtOAc 2:1). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 7.76 (d, *J* = 3.0 Hz, 2H, Ar-H), 7.74-7.08 (m, 31H, Ar-H), 5.62 (d, *J* = 8.3 Hz, 1H, *NH*), 5.25 (t, *J* = 9.5 Hz, 1H H-2'), 5.20 (t, *J* = 4.5 Hz, 1H, CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 5.09 (s, 2H, CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 4.93 (t, *J* = 9.5 Hz, 1H, H-3), 4.85 (d, *J* = 11.9 Hz, 1H, ArCH<sub>2</sub>), 4.80-4.61 (m, 3H, ArCH<sub>2</sub>, Troc), 4.58-4.17 (m, 6H, 3×ArCH<sub>2</sub>), 4.48 (d, *J* = 8.2 Hz, 1H, H-1), 4.45 (d, *J* =

7.8 Hz, 1H, H-1'), 4.16 (d,  $J = 6.3$  Hz, 2H, Fmoc-CH<sub>2</sub>), 4.10 (t,  $J = 6.3$  Hz, 1H, Fmoc-CH), 3.90-3.83 (m, 5H, H-4, H-4', H-6, H-6'a), 3.80-3.62 (m, 4H, H-2, H-5, H-6'b, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 3.60-3.41 (m, 2H, H-5, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub><sup>a</sup>NHCOOCH<sub>2</sub>Ph), 3.39 (dd,  $J = 9.0, 1.5$  Hz, 1H, H-3'), 3.26-3.18 (m, 1H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub><sup>b</sup>NHCOOCH<sub>2</sub>Ph), 2.81-2.15 (m, 4H, OCOCH<sub>2</sub>CH<sub>2</sub>, Lev), 2.12 (s, 3H, CH<sub>3</sub>COCH<sub>3</sub>, Lev), 1.72 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta = 206.8$  (CH<sub>3</sub>COCH<sub>2</sub>, Lev), 171.5 (OCOCH<sub>2</sub>CH<sub>2</sub>, Lev), 156.8 (NHCOOCH<sub>2</sub>Ph), 154.9 (CHCH<sub>2</sub>COO), 154.7 (NHCO), 143.7-120.1 (4C, Ar-C), 101.4 (C-1'), 101.2 (C-1), 95.7 (CCl<sub>3</sub>), 80.5 (C-3'), 77.3 (C-3), 75.5 (C-4'), 74.9 (2C, C-5, C-5'), 74.6, 73.8, 73.6, 72.0 (5C, 4×OCH<sub>2</sub>Ph, OCH<sub>2</sub>CCl<sub>3</sub>), 73.4 (C-4), 72.5 (C-2'), 72.1 (C-2), 70.1 (CHCH<sub>2</sub>CO, Fmoc), 68.1, 67.9, 67.4, 66.8 (4C, C-6, C-6', OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>, COOCH<sub>2</sub>Ph), 56.4 (C-2), 46.8 (CHCH<sub>2</sub>CO, Fmoc), 38.0 (2C, OCOCH<sub>2</sub>CH<sub>2</sub>, (Lev), OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 30.1 (CH<sub>2</sub>COCH<sub>3</sub>, Lev), 29.9 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 28.1 (OCOCH<sub>2</sub>CH<sub>2</sub>, Lev). HR-MALDI-TOF:  $m/z$ : calcd for C<sub>74</sub>H<sub>77</sub>Cl<sub>3</sub>N<sub>2</sub>O<sub>18</sub>: 1386.4237; found: [M + Na<sup>+</sup>] 1409.4187.

**3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2[[[(2,2,2-trichloroethoxy) carbonyl]amino]-4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-levulinoyl)-  $\beta$  -D-galactopyranosyl)-  $\beta$  -D-glucopyranoside (5):** Compound **4** (100 mg, 0.072 mmol) was dissolved in a solution of triethylamine in dichloromethane (5 mL, 1/1, v/v). The reaction mixture was stirred at ambient temperature under argon for 18 h, and concentrated to dryness under reduced pressure. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc 2:1) to give the product **5** as a white powder (60 mg, 84%).  $[\alpha]_D -71.2^\circ$  ( $c$  1.0, CH<sub>2</sub>Cl<sub>2</sub>);  $R_f = 0.21$  (Hexane/EtOAc 2:1). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.39-7.26 (m, 25H, Ar-H), 5.40 (d,  $J = 8.3$  Hz, 1H, NH), 5.28 (t,  $J = 6.5$  Hz, 1H H-2'), 5.12 (s, 1H, CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 5.08 (s, 2H, CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 4.92-4.30 (m, 10H, 4×ArCH<sub>2</sub>, Troc), 4.41 (d,  $J = 8.0$  Hz, 1H, H-1), 4.36 (d,  $J = 7.8$  Hz, 1H, H-1'), 3.90 (d,



$J = 2.8$  Hz, 1H, H-4), 3.83 (m, 1H, H-6'a), 3.76-3.70 (m, 3H, H-4, H-6), 3.69-3.51 (m, 4H, H-3, H-5',  $\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOOCH}_2\text{Ph}$ ), 3.49-3.35 (m, 4H, H-2, H-5, H-6'a,  $\text{CH}_2\text{CH}_2\text{CH}_2^{\text{a}}\text{NHCOOCH}_2\text{Ph}$ ), 3.34 (dd,  $J = 8.4, 4.9$  Hz, 1H, H-3'), 3.26-3.18 (m, 1H,  $\text{CH}_2\text{CH}_2\text{CH}_2^{\text{b}}\text{NHCOOCH}_2\text{Ph}$ ), 2.78-2.19 (m, 4H,  $\text{OCOCH}_2\text{CH}_2$ , Lev), 2.12 (s, 3H,  $\text{CH}_2\text{COCH}_3$ , Lev), 1.75 (m 2H,  $\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOOCH}_2\text{Ph}$ ).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta = 171.6$  ( $\text{OCOCH}_2\text{CH}_2$ , Lev), 156.8 ( $\text{NHCOOCH}_2\text{Ph}$ ), 154.8 ( $\text{NHCO}$ ), 138.6-127.1 (30C, Ar-C), 101.6 (C-1'), 101.3 (C-1), 95.9 ( $\text{CCl}_3$ ), 81.2 (C-4), 80.5 (C-3'), 74.8, 74.7, 73.9, 73.7, 72.4 (5C,  $4 \times \text{OCH}_2\text{Ph}$ ,  $\text{OCH}_2\text{CCl}_3$ ), 73.4 (C-5), 74.0 (C-5'), 72.6 (C-3), 72.4 (C-4'), 71.6 (C-2'), 68.4, 67.3, 67.4, 66.8 (4C, C-6, C-6',  $\text{OCH}_2\text{CH}_2\text{CH}_2$ ,  $\text{COOCH}_2\text{Ph}$ ), 57.7 (C-2), 38.1 ( $\text{OCOCH}_2\text{CH}_2$ , Lev), 37.9 ( $\text{OCH}_2\text{CH}_2\text{CH}_2$ ), 30.1 ( $\text{CH}_2\text{COCH}_3$ , Lev), 29.8 ( $\text{OCH}_2\text{CH}_2\text{CH}_2$ ), 29.0 ( $\text{OCOCH}_2\text{CH}_2$ , Lev). HR-MALDI-TOF:  $m/z$ : calcd for;  $\text{C}_{59}\text{H}_{67}\text{Cl}_3\text{N}_2\text{O}_{16}$  1164.3556 ; found:  $[\text{M} + \text{Na}^+]$  1187.3429.

**3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2[[[(2,2,2-trichloroethoxy)carbonyl]amino]-4-*O*-(3,4,6-tri-*O*-benzyl-  $\beta$ -D-galatopyranosyl)-  $\beta$ -D-glucopyranoside (6):**

A solution of hydrazine acetate (3 mL, 0.5M in methanol) was added drop-wise to a stirred mixture of compound **5** (50 mg, 0.043 mmol) in dichloromethane (10 mL). The reaction was kept at room temperature for 2 hrs, quenched by addition of acetonylacetone (0.2 mL) and diluted by dichloromethane (40 mL). The organic phase was washed with brine, dried over  $\text{MgSO}_4$  and concentrated. The residue was purified by column chromatography ( $\text{SiO}_2$  Hexane/EtOAc 2:1) to give the product **6** as a white powder (40 mg, 87%).  $[\alpha]_{\text{D}} -40.7^\circ$  ( $c$  1.0,  $\text{CH}_2\text{Cl}_2$ );  $R_f = 0.31$  (Hexane/EtOAc 2:1).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.39-7.28 (m, 25H, Ar-H), 5.45 (d,  $J = 8.3$  Hz, 1H, NH), 5.22 (s, 1H,  $\text{CH}_2\text{NHCOOCH}_2\text{Ph}$ ), 5.08 (s, 2H,  $\text{CH}_2\text{NHCOOCH}_2\text{Ph}$ ), 4.90-4.31 (m, 10H,  $4 \times \text{ArCH}_2$ , Troc), 4.61 (d,  $J = 8.9$  Hz, 1H, H-1), 4.26 (d,  $J = 7.6$  Hz 1H, H-1'), 3.99 (dd,  $J = 11.2, 8.4$  Hz, 1H, H-2'), 3.89 (m, 1H, H-6'a), 3.85 (t,  $J =$

1.9, 1H, H-4'), 3.86-3.65 (m, 6H, H-3, H-6, H-5', CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 3.59 (d, *J* = 10.2 Hz, H-5), 3.50-3.32 (m, 2H, H-4, H-6'b), 3.36 (dd, *J* = 10.2, 2.3 Hz, 1H, H-3'), 3.22 (ddd, 1H, H-2), 3.20 (m, 1H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub><sup>a</sup>NHCOOCH<sub>2</sub>Ph), 3.26-3.18 (m, 1H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub><sup>b</sup>NHCOOCH<sub>2</sub>Ph), 1.75 (m 2H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ = 156.7 (NHCOOCH<sub>2</sub>Ph), 154.8 (NHCO), 138.4-127.9 (30C, Ar-C), 104.5 (C-1'), 101.3 (C-1), 95.9 (CCl<sub>3</sub>), 83.3 (C-4), 82.1 (C-3'), 74.8, 74.7, 73.7, 73.6, 72.8 (5C, 4×OCH<sub>2</sub>Ph, OCH<sub>2</sub>CCl<sub>3</sub>), 74.2 (C-5), 73.9 (C-5'), 72.8 (C-3), 72.7 (C-4'), 71.3 (C-2'), 69.6, 68.6, 67.4, 66.8 (4C, C-6, C-6', OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>, COOCH<sub>2</sub>Ph), 57.6 (C-2), 38.1 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 29.8 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>). HR-MALDI-TOF: *m/z*: calcd for; C<sub>54</sub>H<sub>61</sub>Cl<sub>3</sub>N<sub>2</sub>O<sub>14</sub> 1066.3188; found: [M + Na<sup>+</sup>] 1089.3102.

**3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2[[[(2,2,2-trichloroethoxy)carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl-α-L-fucopyranosyl)-4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl-α-L-fucopyranosyl)-β-D-galactopyranosyl)-β-D-glucopyranoside (8):** A solution of compound **6** (50 mg, 0.05 mmol) and compound **7** (72 mg, 0.19 mmol) was dried azeotropically with toluene and then subjected to high vacuum for 2 h. The mixture was dissolved in dry dichloromethane (10 mL) and stirred at room temperature under argon in the presence of activated molecular sieves for 30 minutes. The mixture was cooled to 0°C and NIS (46 mg, 0.20 mmol) and TESOTf (4 μL, 0.01 mmol) were added. The reaction mixture was stirred at 0°C for 30 min., diluted by dichloromethane (60 mL) and filtered through Celite. The filtrate was washed with aqueous sodium thiosulfate (15%, 4 mL) and brine and dried over MgSO<sub>4</sub> and concentrated. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc 2:1) to give the compound **8** as a white powder (45 mg, 61%). [α]<sub>D</sub> -87.0° (*c* 1.0, CH<sub>2</sub>Cl<sub>2</sub>); *R*<sub>f</sub> = 0.31 (Hexane/EtOAc 2:1). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 7.39-6.82 (m, 35H, Ar-

H), 5.54 (d,  $J=3.5$  Hz, 1H, H-1'''), 5.22 (s, 1H, NH), 5.39-4.13 (m, 5H, H-1'', H-3'', H-3''', H-4'', H-4'''), 5.11-4.86 (m, 2H, H-1, H-5'''), 4.82-4.65 (m, 3H, H-3, H-4, H-5''), 4.49 (d, 1H, H-1'), 4.28-3.86 (m, 8H, H-2', H-2'', H-2''', H-4', H-6, H-6'), 3.39-3.05 (m, 5H, H-5', H-5, H-3', CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 3.02-2.86 (m, 1H, H-2), 2.07, 2.06, 1.97, 1.96 (s, 4H, 4×CH<sub>3</sub>CO), 1.69 (s, 1H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.15 (d,  $J=6.7$  Hz, 3H, H-6'''), 0.93 (d,  $J = 6.5$  Hz, 3H, H-6''). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): δ 170.8, 170.6, 170.5, 170.3 (4C, 4×CH<sub>3</sub>CO), 156.7 (NHCOOCH<sub>2</sub>Ph), 153.8 (NHCO), 143.9-120.1 (42C, Ar-C), 99.7 (C-1'), 99.4 (C-1), 98.4 (C-1''), 97.6 (C-1'''), 95.7 (CCl<sub>3</sub>), 83.7 (C-3'), 75.9 (C-5), 75.3 (C-3), 74.7, 74.0, 73.3, 73.0, 72.8, 71.9, 71.6, 71.2, (8C, 6×OCH<sub>2</sub>Ph, OCH<sub>2</sub>CCl<sub>3</sub>, COOCH<sub>2</sub>Ph), 74.6, 74.1, 73.7, 72.6, 72.4, 72.1 (7C, C-2', C-2'', C-2''', C-4, C-4', C-4'', C-4'''), 73.7 (C-5'), 70.9 (C-3'''), 70.0 (C-3''), 68.0, 65.0, 64.8 (C-6, C-6', OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 67.3 (C-5'''), 66.7 (C-5''), 60.0 (C-2), 35.2 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 25.0 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 21.2, 21.1, 20.9, 20.8 (4C, 4×CH<sub>3</sub>CO), 15.7 (C-6''), 15.5 (C-6'''). HR-MALDI-TOF:  $m/z$ : calcd for C<sub>80</sub>H<sub>95</sub>Cl<sub>3</sub>N<sub>2</sub>O<sub>24</sub> 1572.5340; found: [M + Na<sup>+</sup>] = 1595.5398.

**Aminopropyl 2-deoxy-2-acetamido-3-*O*-α-L-fucopyranosyl-4-*O*-(2-*O*-α-L-fucopyranosyl)- β-D-galactopyranosyl- β-D-glucopyranoside (9):** Zinc (10 mg, 0.15 mmol, nanosize powder) was added to a stirred solution of tetrasaccharide **8** (40 mg, 0.02 mmol) in acetic acid (2 mL). After 20 minutes, the zinc was removed by filtering through Celite and the filtrate was concentrated to dryness. The residue was dissolved in pyridine (2 mL) and acetic anhydride (1 mL) and the mixture was stirred at room temperature over night. Methanol (2 mL) was added to quench the reaction. The solution was diluted by dichloromethane (60 mL) and was washed successively with 1M HCl solution, aqueous sodium hydrogen carbonate (15%), and brine. The organic layer was dried over MgSO<sub>4</sub> and concentrated. The obtained residue was dissolved by methanol (5 mL) and sodium methoxide (1M in methanol) was added until pH = 10. The

solution was stirred at room temperature for 24 h, neutralized with Dowex 50 H<sup>+</sup> resin, diluted by methanol (50 mL), filtered and concentrated. The residue was purified by column chromatography (SiO<sub>2</sub> EtOAc/Methanol 10:1). The obtained compound was dissolved in acetic acid (5 mL) and ethanol (1 mL). The mixture was hydrogenolysed over Pd/C (10%, 20 mg) at ambient temperature. After 24 h, the mixture was filtered through Celite to remove the catalyst and concentrated to dryness under reduced pressure. The residue was purified by size exclusion column chromatography (Biogel P2 column, eluted with H<sub>2</sub>O containing 1% n-BuOH) to give the product **9** as a white powder (9 mg, 52%).  $[\alpha]_D -62.9^\circ$  (*c* 1.0, MeOH). <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O, 30°C):  $\delta$  5.20 (d, *J* = 3.0 Hz, 1H), 5.02 (d, *J* = 3.9 Hz, 1H), 4.79 (q, *J* = 6.4 Hz, 1H), 4.42 (d, *J* = 8.0 Hz, 2H), 4.19 (q, *J* = 6.4 Hz, 1H), 3.95-3.58 (brs, 31H), 3.36 (brs, 1H), 3.24 (m, 1H), 3.08-3.02 (m, 2H), 1.98-1.79 (m, 5H), 1.16 (d, *J* = 6.4 Hz, 3H), 1.12 (d, *J* = 6.4 Hz, 3H). <sup>13</sup>C NMR data of anomeric carbons (125 MHz, D<sub>2</sub>O, 30°C):  $\delta$  = 101.3, 100.4, 99.6, 98.8. HR-MALDI-TOF; *m/z*: calcd for C<sub>29</sub>H<sub>52</sub>N<sub>2</sub>O<sub>19</sub> 732.3164; found: [M + Na<sup>+</sup>] 755.2945.

**3-(S-Acetylthioglycolylamino)-propyl 2-deoxy-2-acetamido-3-O- $\alpha$ -L-fucopyranosyl-4-O-(2-O- $\alpha$ -L-fucopyranosyl)-  $\beta$ -D-galactopyranosyl-  $\beta$ -D-glucopyranoside (10):** Compound **8** (6 mg, 0.01 mmol) was dried under vacuum overnight. The sugar was slurried in dry DMF. SAMA-OPfp (5.3 mg, 0.02 mmol) was added. TEA (2.3  $\mu$ L, 0.02 mmol) was added drop-wise into the mixture. After stirring at room temperature for 2 h, the mixture was concentrated and the residue was purified by size exclusion chromatography (Biogel P2 column eluted with H<sub>2</sub>O containing 1% n-BuOH) to give thioacetate **10** as a white powder (4 mg, 58%). <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O, 30°C):  $\delta$  5.21 (d, *J*=3.0 Hz, 1H), 4.95 (d, *J* = 3.9 Hz, 1H), 4.78 (q, *J* = 6.4 Hz, 1H), 4.40 (d, *J* = 8.0 Hz, 2H), 4.11 (q, *J* = 6.4 Hz, 1H), 3.85-3.51 (brs, 33H), 3.36 (brs, 1H), 3.24 (m, 1H), 3.08-

3.02 (m, 2H), 1.98-1.79 (m, 8H), 1.17 (d,  $J = 6.4$  Hz, 3H), 1.12(d,  $J = 6.4$  Hz, 3H). HR-MALDI-TOF:  $m/z$ : calcd for  $C_{33}H_{56}N_2O_{21}S$  848.3096; found:  $[M + Na^+]$  871.2983.

**3-(mercaptoacetamido)-propyl 2-deoxy-2-acetamido-3- $O$ - $\alpha$ -L-fucopyranosyl-4- $O$ -(2- $O$ - $\alpha$ -L-fucopyranosyl)-  $\beta$  -D-galactopyranosyl-  $\beta$  -D-glucopyranoside (11):** 7%  $NH_3$  (g) in DMF solution (50  $\mu$ L) was added to a solution of thioacetate 10 (1 mg) in ddH<sub>2</sub>O (15  $\mu$ L) and the mixture was stirred under argon atmosphere. The reaction was monitored by MALDI-TOF showing the product peak of  $[M+Na]$ . After 45 min the mixture was concentrated under reduced pressure and co-evaporated twice with toluene. The thiol dried under high vacuum for 30 minutes and then used immediately in conjugation without further purification.

**3-[4-( $N$ -maleimidomethyl)cyclohexane-1-carboxylamino]-propyl 2-deoxy-2-acetamido-3- $O$ - $\alpha$ -L-fucopyranosyl-4- $O$ -(2- $O$ - $\alpha$ -L-fucopyranosyl)- $\beta$ -D- galactopyranosyl-  $\beta$  -D - glucopyranoside (12):** A mixture of tetrasaccharide **9** (1.2 mg, 1.64  $\mu$ mol) and sulfo-SMCC (1.07 mg, 2.46  $\mu$ mol) in 0.1 M sodium phosphate buffer, 0.15 M NaCl, pH 7.2 (600  $\mu$ L) was stirred at ambient temperature for 2 h. The compound was purified by size-exclusion chromatography on a Sephadex G10 column equilibrated in 0.1 M sodium phosphate buffer, 5mM EDTA, pH 6.2. Fractions containing compound 12, as determined by TLC and MALDI-TOF  $[M+Na^+ 976.5]$ , were pooled and used immediately without further characterization in conjugation to thiolated BSA or KLH.

Conjugation of Le<sup>y</sup> derivative **11** to mcKLH-MI and BSA-MI: The conjugations were performed as suggested by Pierce Endogen. In short, thiol **11** (2.5 equiv. excess to available MI-groups on the protein) deprotected just prior to conjugation as described above, was dissolved in ddH<sub>2</sub>O (100  $\mu$ L) and added to a solution of maleimide activated protein (2 mg) that had been restored with ddH<sub>2</sub>O (200  $\mu$ L) to give the protein in the conjugation buffer, sodium phosphate

pH 7.2 containing EDTA and sodium azide. The mixture was incubated for 2 h at room temperature and then purified by Millipore centrifugal filter device with a 10.000 molecular cut-off. All centrifugations were made at 15°C for 20 min., spinning at 13xg. The reaction mixture was centrifuged off and the filter washed with 10 mM Hepes buffer pH 6.5 (3x200  $\mu$ L). The filtrates were checked for presence of carbohydrate by TLC. The conjugate was retrieved and taken up in 10 mM Hepes buffer pH 6.5 (1 mL). The average number of copies of Le<sup>y</sup> attached to mK<sub>1</sub>LH and BSA was determined to be 620 and 9, respectively, according to Dubois' phenol-sulfuric acid total carbohydrate assay and Lowry protein concentration test.

***p*-(*p*-benzoyloxy)-benzyl 6-*O*-benzyl-2-deoxy-2-[(2,2,2-trichloroethoxy)carbonyl]amino]-3-*O*-(9-fluorenylmethoxycarbonyl)- $\beta$ -D-glucopyranoside (**12**):**

A solution of thioglycoside **1** (4.77 g, 6.73 mmol) and spacer compound **2** (3.07 g, 13.47 mmol) was dried azeotropically with toluene and then subjected to high vacuum for 2 hours. The mixture was stirred at room temperature under Argon in the presence of activated molecular sieves for 30 minutes. The mixture was cooled to 0°C and then reacted with NIS (1.67 g, 7.41 mmol) and TESOTf (0.15 mL, 0.67 mmol). The reaction mixture was stirred at 0°C for 30 minutes until TLC showed full conversion of the donor. The solution was diluted by dichloromethane (60 mL) and the molecular sieves were removed by filtration. The filtrate was washed with aqueous sodium thiosulfate (15%, 4 mL). The organic phase was washed with brine and dried over MgSO<sub>4</sub> and concentrated. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc=2/1) to give the product **12** as a white powder (5.05 g, 86%): [ $\alpha$ ]<sub>D</sub>-20.6° (*c* 1.0, CH<sub>2</sub>Cl<sub>2</sub>); R<sub>f</sub> 0.28 (1:2 EtOAc-hexane). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  8.20 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.76-7.09 (m, 20H, Ar-H), 5.49 (d, *J* = 8.3 Hz, 1H, *NH*), 4.92 (d, *J* = 8.8 Hz, 1H, H-1), 4.86 (t, *J* = 9.4 Hz, 1H, H-3), 4.66-4.54 (m, 6H, ArCH<sub>2</sub>, Troc), 4.38 (d, *J* = 6.3 Hz, 2H, Fmoc-

$\text{CH}_2$ ), 4.22 (t,  $J = 6.3$  Hz, 1H, Fmoc-CH), 3.85-3.80 (m, 4H, H-6, H-4, H-2), 3.65-3.52 (m, 1H, H-5).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta = 165.6$  (ArCO), 156.0 ( $\text{CHCH}_2\text{OCO}$ , Fmoc), 154.7 (Ar-C), 150.6 (NHCO), 150.7-120.3 (29C, Ar-C), 99.2 (C-1), 95.9 ( $\text{CCl}_3$ ), 79.6(C-3), 74.7(C-5), 74.5 ( $\text{OCH}_2\text{Cl}_3$ , Troc), 73.9 ( $\text{OCH}_2\text{PhOBz}$ ), 70.7(C-4), 70.1( $\text{OCH}_2\text{Ph}$ ), 69.9( $\text{CHCH}_2\text{OCO}$ , Fmoc) 64.8(C-6), 56.1(C-2), 46.4( $\text{CHCH}_2\text{OCO}$ , Fmoc). HR-MALDI-TOF MS  $m/z$ : calcd for  $\text{C}_{45}\text{H}_{40}\text{Cl}_3\text{NO}_{11}$  (875.1667); MS MALDI-TOF:  $(\text{M} + \text{Na}^+) = 898.1659$ .

***p*-(*p*-benzoyloxy)-benzyl 6-*O*-benzyl-2-deoxy-2[[[(2,2,2-trichloroethoxy)carbonyl]amino]-3-*O*-(9-fluorenylmethoxycarbonyl)-4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-levulinoyl- $\beta$ -D-**

**galatopyranosyl)- $\beta$ -D-glucopyranoside (13):** A solution of the compound **12** (4.0 g, 4.57 mmol) and donor compound **3** (4.06 g, 6.86 mmol) was dried azeotropically with toluene and then subjected to high vacuum for 2 hours. The mixture was stirred at room temperature under Argon in the presence of activated molecular sieves for 30 minutes. The mixture was cooled to  $0^\circ\text{C}$  and reacted with NIS (1.69 g, 7.54 mmol) and TESOTf (0.16 mL, 0.69 mmol). The reaction mixture was stirred at  $0^\circ\text{C}$  for 0.5 hour after which TLC showed full conversion of the donor. The solution was diluted by dichloromethane (60 mL) and the molecular sieves were removed by filtration. The filtrate was washed with aqueous sodium thiosulfate (15%, 4 mL). The organic phase was washed with brine and dried over  $\text{MgSO}_4$  and concentrated. The residue was purified by column chromatography ( $\text{SiO}_2$  Hexane/EtOAc=2/1) to give the product **13** as a white powder (5.19 g, 81%).  $[\alpha]_{\text{D}} -64.2^\circ$  ( $c$  1.0,  $\text{CH}_2\text{Cl}_2$ );  $R_f$  0.38 (1:2 EtOAc-hexane).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta = 8.20$  (d, 2H,  $J = 8.4$  Hz, Ar-H), 7.75-7.12 (m, 35H, Ar-H), 5.34 (t, 1H,  $J = 9.2$  Hz, H-2'), 5.18 (d, 1H,  $J = 9.8$  Hz, N-H), 4.96 (t, 1H,  $J = 9.4$  Hz, H-3), 4.88 (d, 1H,  $J = 12.0$  Hz, ArCH<sub>2</sub>), 4.85 (d, 1H,  $J = 12.0$  Hz, ArCH<sub>2</sub>), 4.61 (d,  $J = 8.2$  Hz, 1H, H-1), 4.60-4.30 (m, 10H, 3 $\times$ ArCH<sub>2</sub>, Troc,  $\text{CH}_2\text{PhOBz}$ ), 4.44 (d, 1H,  $J = 7.7$  Hz, H-1'), 4.28 (d,  $J = 6.8$  Hz, 2H, Fmoc-

CH<sub>2</sub>), 4.12 (t,  $J$  = 6.0 Hz, 1H, Fmoc-CH), 4.02-3.71 (m, 4H, H-4, H-4', H-6), 3.60-3.51 (m, 3H, H-2, H-5, H-6a'), 3.69-3.51 (m, 2H, H-5', H-6b'), 3.44 (dd, 1H,  $J$  = 9.2, 1.6 Hz, H-3'), 3.36 (d,  $J$  = 9.1 Hz, 1H, H-5'), 2.69-2.36 (m, 4H, CH<sub>2</sub>CH<sub>2</sub>, Lev), 2.13 (s, 3H, COCH<sub>3</sub>, Lev); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 206.6 (CH<sub>3</sub>COCH<sub>2</sub>, Lev), 171.5 (OCOCH<sub>2</sub>CH<sub>2</sub>, Lev), 165.3 (ArCO), 155.0 (CHCH<sub>2</sub>CO, Fmoc), 154.3 (Ac-C), 150.7 (NHCO), 146.0-120.1 (47C, Ar-C), 100.5 (C-1'), 100.0 (C-1), 95.6 (CCl<sub>3</sub>), 80.5 (C-5'), 77.3 (C-3), 75.4 (C-4), 75.0 (C-5), 74.7, 73.9, 72.0, (6C, 4×OCH<sub>2</sub>Ph, OCH<sub>2</sub>CCl<sub>3</sub>, OCH<sub>2</sub>PhOBz), 73.5 (C-3'), 72.6 (C-4'), 72.1 (C-2'), 70.2 (CHCH<sub>2</sub>CO, Fmoc), 68.2, 67.9 (2C, C-6, C-6'), 56.5 (C-2), 46.8 (CHCH<sub>2</sub>CO, Fmoc), 38.0 (OCOCH<sub>2</sub>CH<sub>2</sub>, Lev), 30.1 (CH<sub>2</sub>COCH<sub>3</sub>, Lev), 28.1 (OCOCH<sub>2</sub>CH<sub>2</sub>, Lev). HR-MALDI-TOF:  $m/z$ : calcd for C<sub>74</sub>H<sub>77</sub>Cl<sub>3</sub>N<sub>2</sub>O<sub>18</sub>: 1405.3972; found: [M + Na<sup>+</sup>] 1428.4265.

***p*-(*p*-benzoyloxy)-benzyl 6-*O*-benzyl-2-deoxy-2[[[(2,2,2-trichloroethoxy) carbonyl]amino]-4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-levulinoyl)-  $\beta$ -D-galactopyranosyl)-  $\beta$ -D-glucopyranoside (14):**

The compound **13** (500 mg, 0.36 mmol) was dissolved in 20% triethylamine solution (5 mL) in dichloromethane. The solution was stirred at room temperature under argon for 18 hr. and concentrated to dryness under reduced pressure. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc=2/1) to give the product **14** as a white powder (400 mg, 95%). [ $\alpha$ ]<sub>D</sub>-27.7° ( $c$  1.0, CH<sub>2</sub>Cl<sub>2</sub>);  $R_f$  0.21 (1:2 EtOAc-hexane). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 8.21 (d,  $J$  = 7.4 Hz, 2H, Ar-H), 7.54-7.16 (m, 27H, Ar-H), 5.38 (dd,  $J$  = 10.1, 8.3 Hz, 1H, H-2'), 5.08 (d,  $J$  = 8.5 Hz, 1H, N-H), 4.89 (dd,  $J$  = 8.8, 2.1 Hz, 2H, CH<sub>2</sub>Ar), 4.59 (d,  $J$  = 8.2 Hz, 1H, H-1), 4.80-4.23 (m, 10H, 3×CH<sub>2</sub>Ar, Troc, CH<sub>2</sub>PhOBz), 4.40 (d, 1H,  $J$  = 7.7 Hz, H-1'), 3.84 (d,  $J$  = 2.4 Hz, 1H, H-4'), 3.89-3.73 (m, 3H, H-3, H-6'), 3.70-3.62 (m, 2H, H-6a, H-4), 3.60 (dd,  $J$  = 9.0, 5.1 Hz, H-5), 3.58-3.49 (m, 3H, H-2, H-5', H-6b), 3.46 (dd,  $J$  = 8.9, 5.2 Hz, 1H, H-3'), 2.87-2.21 (m, 4H, OCOCH<sub>2</sub>CH<sub>2</sub>, Lev), 2.10 (s, 3H, CH<sub>2</sub>COCH<sub>3</sub>, Lev). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):



$\delta$  = 206.4 (CH<sub>2</sub> COCH<sub>3</sub>, Lev), 171.6 (OCOCH<sub>2</sub>CH<sub>2</sub>, Lev), 165.3 (ArCO), 154.4 (Ar-C), 150.7 (NHCO), 138.7-121.9 (35C, Ar-C), 101.7 (C-1'), 100.0 (C-1), 95.8 (CCl<sub>3</sub>), 81.2 (C-4), 80.4 (C-3'), 74.8, 74.7, 73.9, 73.8, 72.5, 72.4 (6C, 4×OCH<sub>2</sub>Ph, OCH<sub>2</sub>CCl<sub>3</sub>, OCH<sub>2</sub>PhOBz), 74.5 (C-5), 74.0 (C-5'), 72.6 (C-3), 72.3 (C-4'), 71.7 (C-2'), 70.3, 68.5 (2C, C-6, C-6'), 57.9 (C-2), 37.9 (OCOCH<sub>2</sub>CH<sub>2</sub>, Lev), 30.1 (CH<sub>2</sub>COCH<sub>3</sub>, Lev), 28.1 (OCOCH<sub>2</sub>CH<sub>2</sub>, Lev). HR-MALDI-TOF: *m/z*: calcd for; C<sub>59</sub>H<sub>67</sub>Cl<sub>3</sub>N<sub>2</sub>O<sub>16</sub> 1183.3291; found: [M + Na<sup>+</sup>] 1206.3286.

***p*-(*p*-benzoyloxy)-benzyl 6-*O*-benzyl-2-deoxy-2[[*(2,2,2*-trichloroethoxy) carbonyl]amino]-4-*O*-(3,4,6-tri-*O*-benzyl- $\beta$ -D-galatopyranosyl)- $\beta$ -D-glucopyranoside (15):** Compound **14** (200 mg, 0.17 mmol) was dissolved in dichloromethane (10 mL). A solution of hydrazine acetate (12 mL, 0.5 M) in methanol was added. The reaction mixture was stirred at room temperature for 2 hours. The reaction was quenched by adding acetonylacetone (0.8 ml), then was diluted by dichloromethane (40 mL). The organic phase was washed with brine (20 mL), dried over MgSO<sub>4</sub> and concentrated under reduced pressure. The residue was purified by column chromatography (SiO<sub>2</sub>, Hexane/EtOAc=2/1) to give **15** as a white powder (160 mg, 87%).  $[\alpha]_D$  -26.5° (*c* 1.0, CH<sub>2</sub>Cl<sub>2</sub>); *R<sub>f</sub>* = 0.30 (1:2 EtOAc-hexane). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  8.22 (d, *J* = 7.8 Hz, 2H, Ar-H), 7.67 (d, *J* = 1.5 Hz, Ar-H), 7.64 (d, *J* = 1.5 Hz, Ar-H), 7.54-7.17 (m, 25H, Ar-H), 5.28 (d, *J* = 8.5 Hz, 1H, N-H), 4.89 (dd, *J* = 9.8, 3.1 Hz, 2H, ArCH<sub>2</sub>), 4.85 (d, *J* = 8.2 Hz, 1H, H-1), 4.82-4.43 (m, 10H, 3×ArCH<sub>2</sub>, Troc, CH<sub>2</sub>PhOBz), 4.42 (d, 1H, *J* = 7.7 Hz, H-1'), 3.94 (t, *J* = 2.4 Hz, 1H, H-4'), 3.86-3.80 (m, 3H, H-3, H-6'), 3.82 (dd, *J* = 10.2, 8.6 Hz, 1H, H-2'), 3.70-3.62 (m, 4H, H-6a, H-2, H-4), 3.60 (dd, *J* = 8.6, 5.1 Hz, 1H, H-5'), 3.58-3.49 (m, 2H, H-5, H-6b), 3.39 (dd, *J* = 9.9, 2.2 Hz, 1H, H-3'). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 167.3 (ArCO), 156.7 (Ar-C), 153.2 (NHCO), 138.2-121.8 (35C, Ar-C), 104.7 (C-1'), 100.0 (C-1), 96.8 (CCl<sub>3</sub>), 82.2 (C-4), 82.0 (C-3'), 75.2, 74.9, 73.8, 73.6, 72.4, 72.0 (6C, 4×OCH<sub>2</sub>Ar, OCH<sub>2</sub>CCl<sub>3</sub>, OCH<sub>2</sub>PhOBz), 74.3

(C-5), 73.9 (C-5'), 72.8 (C-3), 71.9 (C-4'), 71.4 (C-2'), 70.3, 68.7 (2C, C-6, C-6'), 57.9 (C-2).

HR-MALDI-TOF:  $m/z$ : calcd for;  $C_{59}H_{67}Cl_3N_2O_{16}$  1085.2923; found:  $[M + Na^+]$  1108.2845.

***p*-(*p*-benzoyloxy)-benzyl 6-*O*-benzyl-2-deoxy-2[[*(2,2,2*-trichloroethoxy) carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)--4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)- $\beta$ -D-galatopyranosyl)- $\beta$ -D-glucopyranoside (**16**):**

A solution of compound **15** (120 mg, 0.11 mmol) and **7** (126 mg, 0.33mmol) was dried azeotropically with toluene and then subjected to high vacuum for 2 hours. The mixture was stirred at room temperature under Argon in the presence of activated molecular sieves for 30 minutes. The mixture was cooled to 0°C, then NIS (81.7 mg, 0.36 mmol) and TESOTf (7.5  $\mu$ l, 0.03mmol) were added. The reaction mixture was stirred at 0°C for 0.5 hour and TLC showed full conversion of the donor. The solution was diluted by dichloromethane (60 ml) and the molecular sieves were removed by filtration. The filtrate was washed with aqueous sodium thiosulfate (15%, 4 ml). The organic phase was washed with brine and dried over  $MgSO_4$  and concentrated. The residue was purified by column chromatography ( $SiO_2$  Hexane/EtOAc=2/1) to give the product **16** as a white powder (164 mg, 86%).  $[\alpha]_D -82.7^\circ$  ( $c$  1.0,  $CH_2Cl_2$ );  $R_f$  0.48 (1:2 EtOAc-hexane).  $^1H$  NMR (500 MHz,  $CDCl_3$ ):  $\delta$  = 8.20 (d,  $J$  = 7.7 Hz, 1H, Ar-H), 7.53-6.93 (m, Ar-H, 37H,  $6 \times OCH_2Ph$ ,  $OCH_2PhOBz$ ), 5.65 (d,  $J$  = 3.0 Hz, 1H, H-1'''), 5.49 (dd,  $J$  = 10.6, 2.7 Hz, 1H, H-3''), 5.43 (dd,  $J$  = 10.8, 2.7 Hz, 1H, H-3'''), 5.42-5.20 (m, 3H, H-4'', H-4''', NH), 5.18 (d,  $J$  = 3.9 Hz, 1H, H-1''), 5.07 (q,  $J$  = 6.0 Hz, 1H, H-5''), 4.76 (d,  $J$  = 9.0 Hz, 1H, H-1), 4.80-4.15 (m, 20H,  $6 \times OCH_2Ph$ ,  $OCH_2PhOH$ ,  $OCH_2CCl_3$ , H-5''', H-1', H-4, H-3), 4.05 (m, 2H, H-6), 3.96 (t,  $J$  = 8.2 Hz, 1H, H-2'), 3.95 (d,  $J$  = 3.1 Hz, 1H, H-4'), 3.88-3.84 (m, 2H, H-6), 3.84 (dd,  $J$  = 10.8, 3.0 Hz, 1H, H-2'''), 3.76 (dd,  $J$  = 10.6, 3.9 Hz, 1H, H-2''), 3.38 (dd,  $J$  = 8.2, 3.1 Hz, 1H, H-3'), 3.25-3.26 (m, 2H, H-5', H-5), 3.09 (ddd, 1H, H-2), 2.20 (s, 3H,  $CH_3CO$ ), 2.07 (s,

3H, CH<sub>3</sub>CO), 1.99 (s, 3H, CH<sub>3</sub>CO), 1.96 (s, 3H, CH<sub>3</sub>CO), 1.17 (d, *J* = 6.3 Hz, 3 H, H-6'''), 0.92 (d, *J* = 6.0 Hz, 3H, H-6''); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ=170.8, 170.5, 170.4, 169.7 (4 C, 4×CH<sub>3</sub>CO), 165.3 (1 C, Ar-C), 153.6 (NHCO), 150.7 (PhCO), 138.8-121.8 (47C; Ar-C), 99.7 (C-1'), 98.8 (C-1''), 98.3 (C-1'''), 97.6 (C-1), 95.6 (CCl<sub>3</sub>), 83.8 (C-3'), 75.4 (C-5), 75.2 (C-3), 74.7, 73.9, 73.7, 73.6, 73.3, 73.1 73.0, 72.9 (8 C, 6×OCH<sub>2</sub>Ph, OCH<sub>2</sub>PhOBz, OCH<sub>2</sub>CCl<sub>3</sub>), 73.4 (C-2'''), 73.4 (C-2'), 72.9 (C-5'), 72.6 (C-4), 72.2 (C-2''), 72.0 (2C, 4'', C-4'''), 71.4 (C-4'), 70.9 (C-3'''), 70.7 (C-3''), 68.1 (2C, C-6, C-6'), 64.9 (C-5''), 64.8 (C-5'''), 59.7 (C-2), 21.2, 21.1, 20.9, 20.8 (4 C, 4×CH<sub>3</sub>CO), 15.7 (C-6'''), 15.6 (C-6''). HR-MALDI-TOF MS *m/z*: calcd for C<sub>91</sub>H<sub>98</sub>Cl<sub>3</sub>NO<sub>26</sub> 1725.5443; found: [M + Na<sup>+</sup>] 1748.6041.

***p*-Hydroxybenzyl 6-*O*-benzyl-2-deoxy-2[[[(2,2,2-trichloroethoxy)carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl-α-L-fucopyranosyl)--4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl-α-L-fucopyranosyl)-β-D-galactopyranosyl)-β-D-glucopyranoside (17):**

Compound **16** (160 mg, 0.093 mmol) was dissolved in a mixture of THF (8 mL) and Et<sub>3</sub>N (400 μl) and H<sub>2</sub>O<sub>2</sub> (50% in water, 200 μL). After the reaction mixture was stirred under Argon at room temperature for 18h, the solution was concentrated to dryness in *vacuo*. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc=2/1) to give the **17** as a white powder (123 mg, 82%). [α]<sub>D</sub> -83.9° (*c* 1.0, CH<sub>2</sub>Cl<sub>2</sub>); R<sub>f</sub> 0.31 (1:2 EtOAc-hexane). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.37-6.78 (m, Ar-H, 34 H, 6×OCH<sub>2</sub>Ph, OCH<sub>2</sub>PhOH), 5.68 (d, *J* = 2.9 Hz, 1 H, H-1'''), 5.29 (dd, *J* = 10.8, 2.9 Hz, 1 H, H-3''), 5.23 (dd, *J* = 11.0, 2.9 Hz, 1 H, H-3'''), 5.22-5.20 (m, 3 H, H-4'', H-4''', NH), 5.12 (d, *J* = 3.9 Hz, 1 H, H-1''), 5.03 (q, *J* = 6.3 Hz, 1 H, H-5''), 4.76 (d, *J* = 8.2 Hz, 1 H, H-1), 4.76-4.12 (m, 20 H, 6×OCH<sub>2</sub>Ph, OCH<sub>2</sub>PhOH, OCH<sub>2</sub>CCl<sub>3</sub>, H-5''', H-1', H-4, H-3), 4.05 (dd, *J* = 10.1, 9.3 Hz, 1 H, H-6a), 3.96 (dd, *J* = 10.5, 8.2 Hz, 1 H, H-2'), 3.90 (d, *J* = 2.8 Hz, 1 H, H-4'), 3.88-3.84 (m, 3 H, H-6', H-6b), 3.84 (dd, *J* = 11.0, 2.9 Hz, 1 H,

H-2'''), 3.76 (dd,  $J = 10.8, 3.9$  Hz, 1 H, H-2''), 3.38 (dd,  $J = 10.5, 2.8$  Hz, 1 H, H-3'), 3.25 (dd,  $J = 8.6, 2.8$  Hz, 1 H, H-5'), 3.26 (d,  $J = 9.3$  Hz, 1 H, H-5), 3.09 (ddd, 1 H, H-2), 2.10 (s, 3 H,  $\text{CH}_3\text{CO}$ ), 2.07 (s, 3 H,  $\text{CH}_3\text{CO}$ ), 2.00 (s, 6 H,  $2 \times \text{CH}_3\text{CO}$ ), 1.14 (d,  $J = 6.7$  Hz, 3 H, H-6'''), 0.92 (d,  $J = 6.3$  Hz, 3 H, H-6'');  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$ =171.4, 170.7, 170.6, 169.6 (4 C,  $4 \times \text{CH}_3\text{CO}$ ), 156.2 (1 C, Ar-C), 153.5 (NHCO), 138.6-115.6 (41C, Ar-C), 99.6 (C-1'), 98.1 (C-1''), 97.4 (C-1'''), 97.3 (C-1), 95.5 ( $\text{CCl}_3$ ), 83.7 (C-3'), 75.5 (C-5), 75.3 (C-3), 74.7, 73.9, 73.7, 73.6, 73.3, 73.1 72.9, 73.6, 72.7, 72.4, 71.8, 71.1, 70.9, 70.7 (16 C,  $6 \times \text{OCH}_2\text{Ph}$ ,  $\text{OCH}_2\text{PhOH}$ ,  $\text{OCH}_2\text{CCl}_3$ , C-2''', C-2', C-5', C-4, C-2'', 4'', C-4''', C-4'), 70.8 (C-3'''), 70.1 (C-3''), 68.0 (2 C, C-6, C-6'), 64.7 (C-5''), 64.6 (C-5'''), 59.7 (C-2), 21.6, 21.2, 21.1, 20.9 (4 C,  $4 \times \text{CH}_3\text{CO}$ ), 15.6 (C-6'''), 15.5 (C-6''). HR-MALDI-TOF MS  $m/z$ : calcd for  $\text{C}_{84}\text{H}_{94}\text{Cl}_3\text{NO}_{25}$  1621.5181; found:  $[\text{M} + \text{Na}^+]$  1644.5204.

**6-*O*-benzyl-2-deoxy-2[[[(2,2,2-trichloroethoxy) carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)--4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)- $\beta$ -D-galatopyranosyl)- $\beta$ -D-glucopyranosyl trichloroacetimidate 19**

DDQ (40 mg, 0.136 mmol) was added to a stirred mixture of compound **17** (110 mg, 0.068 mmol) in dichloromethane (3.8 mL) and water (0.2 mL). The mixture was stirred in the dark for 1h. The solution was diluted by dichloromethane (40 mL). The organic phase was washed with brine and dried over  $\text{MgSO}_4$  and concentrated. The residue was purified by column chromatography ( $\text{SiO}_2$  Hexane/EtOAc=2/1) to give compound **18** as a colorless syrup (80 mg, 78.0%). Compound **18** (80 mg, 0.053 mmol) was dissolved in 5 mL dichloromethane and  $\text{CCl}_3\text{CN}$  (0.5 mL). DBU (5  $\mu\text{L}$ ) was added. After 5min, the solution was concentrated to dryness. The residue was purified by column chromatography ( $\text{SiO}_2$  Hexane/EtOAc/triethylamine=1/1/0.01) to give the **19** as colorless syrup (80 mg, 91%).

**(2'R, 3'R) Ethyl 2,3-O-(2',3'-dimethoxybutane-2',3'-diyl)-1-thiol-β-D-galactopyranoside (21):** Galactoside **20** (14.7 g, 65.7 mmol), butane-2,3-dione (6.9 mL, 78.8 mmol), trimethylorthoformate (23 mL, 197 mmol) and camphorsulfonic acid (1.5 g, 6.5 mmol) in MeOH (200 mL) were heated under reflux for 16 h. The reaction mixture was cooled to room temperature and triethylamine (2 mL) was added to quench the reaction. After the solution was concentrated to dryness, the residue was purified by column chromatography (SiO<sub>2</sub>, EtOAc/hexane 3:2) to furnish diol **21** (17.2 g, 78 %) as a white foam: *R*<sub>f</sub> = 0.35 (EtOAc/hexane 3:2); [α]<sub>D</sub> 147.1 (c 1.0 in CHCl<sub>3</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 4.57 (d, *J* = 9.6 Hz, 1H, H-1), 4.10 (t, *J* = 9.6 Hz, 1H, H-2), 4.00 (d, *J* = 2.1 Hz, 1H, H-4), 3.93 (dd, *J* = 5.5, 11.8 Hz, H-6), 3.79 (dd, *J* = 5.5, 11.8 Hz, 1H, H-6), 3.74 (dd, *J* = 2.1, 9.6 Hz, 1H, H-3), 3.61 (t, *J* = 5.5 Hz, 1H, H-5), 3.28 (s, 3H, OCH<sub>3</sub>-BDA), 3.26 (s, 3H, OCH<sub>3</sub>-BDA), 2.75 (q, *J* = 1.32, 2H, 7.42 Hz, SCH<sub>2</sub>CH<sub>3</sub>), 1.32 (s, 3H, CH<sub>3</sub>-BDA), 1.31 (s, 3H, CH<sub>3</sub>-BDA), 1.30 (t, 3H, *J* = 7.42 Hz, SCH<sub>2</sub>CH<sub>3</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): 100.51, 100.48 (2×C-BDA), 83.4 (C-1), 78.9 (C-5), 71.9 (C-3), 68.2 (C-4), 66.3 (C-2), 62.3 (C-6), 48.3 (2×C, OCH<sub>3</sub>-BDA), 24.7 (SCH<sub>2</sub>CH<sub>3</sub>), 17.9 (CH<sub>3</sub>-BDA), 17.7 (CH<sub>3</sub>-BDA), 14.7 (SCH<sub>2</sub>CH<sub>3</sub>). HR-MALDI-TOF MS *m/z*: calcd for C<sub>14</sub>H<sub>26</sub>O<sub>7</sub>S 338.1399; found: [M + Na<sup>+</sup>] 361.1307.

**(2'R, 3'R) Ethyl 4,6-di-O-benzyl-2,3-O-(2',3'-dimethoxybutane-2',3'-diyl)-1-thiol-β-D-galactopyranoside (22):** Compound **21** (5 g, 14.8 mmol) and NaH (0.78 g, 32.5 mmol) were dissolved in dry DMF and the solution was cooled to 0°C. Benzyl bromide (3.87 mL, 32.5 mmol) was added portionwise. The reaction mixture was stirred at RT for 2 h after which, MeOH (3 mL) was added to quench the reaction. The solvent was partly removed under reduced pressure. The remaining mixture (1 mL) was diluted with ethyl acetate with water, dried over MgSO<sub>4</sub> and concentrated. The residue was purified by column chromatography (SiO<sub>2</sub>,

EtOAc/Hexane 3:2) to give compound **22** (6.5 g, 85%) as a white foam  $R_f = 0.88$  (EtOAc/Hexane 3:2);  $[\alpha]_D^{25} 147.1$  (c 1.0 in  $\text{CHCl}_3$ );  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 7.42-7.26 (m, 10H, Ar-H), 4.96 (d,  $J = 11.6$  Hz, 1H,  $\text{ArCH}_2$ ), 4.59 (d,  $J = 11.6$  Hz, 1H,  $\text{ArCH}_2$ ), 4.48 (q,  $J = 11.8, 8.0$  Hz, 2H,  $\text{ArCH}_2$ ), 4.26 (d,  $J = 9.4$  Hz, 1H, H-1), 4.07 (t,  $J = 9.4$  Hz, 1H, H-2), 3.81 (m, 2H, H-6), 3.75 (d,  $J = 2.1$  Hz, 1H, H-3), 3.68-3.63 (m, 2H, H-4, H-5), 3.30 (s, 3H,  $\text{OCH}_3\text{-BDA}$ ), 3.27 (s, 3H,  $\text{OCH}_3\text{-BDA}$ ), 2.78-2.66 (m, 2H,  $\text{SCH}_2\text{CH}_3$ ), 1.29 (t,  $J = 7.4$  Hz, 3H,  $\text{SCH}_2\text{CH}_3$ ), 1.28 (s, 3H,  $\text{CH}_3\text{-BDA}$ ), 1.27 (s, 3H,  $\text{CH}_3\text{-BDA}$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz): 139.2-127.5 (12C, Ar-C), 100.2, 100.0 (2 $\times$ C-BDA), 83.6 (C-1), 78.2 (C-4), 74.2 (C-3), 73.9 ( $\text{ArCH}_2$ ), 73.8 ( $\text{ArCH}_2$ ), 73.5 (C-5), 69.2 (C-6), 67.0 (C-2), 48.2 (2 $\times$ C,  $\text{OCH}_3\text{-BDA}$ ), 24.7 ( $\text{SCH}_2\text{CH}_3$ ), 17.9 ( $\text{CH}_3\text{-BDA}$ ), 17.7 ( $\text{CH}_3\text{-BDA}$ ), 15.3 ( $\text{SCH}_2\text{CH}_3$ ). HR-MALDI-TOF MS  $m/z$ : calcd for  $\text{C}_{28}\text{H}_{38}\text{O}_7\text{S}$ : calcd 518.2338; found  $[\text{M} + \text{Na}^+]$  541.2315

**Ethyl 4,6-di-*O*-benzyl-1-thiol- $\beta$ -D-galactopyranoside (**23**):**

The product **22** (5 g, 9.65 mmol) was dissolved in TFA/ $\text{H}_2\text{O}$  (200 mL, 9/1, v/v). After 2 min the solvent was removed under reduced pressure. The remaining solid was purified by column chromatography ( $\text{SiO}_2$ , EtOAc/Hexane 3:2) to provide diol **23** (4.2 g, 10.4 mmol, 67%) as a white foam:  $R_f = 0.40$  (EtOAc/Hexane 3:2);  $[\alpha]_D^{25} 147.1$  (c 1.0 in  $\text{CHCl}_3$ );  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 7.36-7.26 (m, 10H, Ar-H), 4.71 (q,  $J = 11.8, 12.4$  Hz, 2H,  $\text{ArCH}_2$ ), 4.48 (q,  $J = 11.8, 8.0$  Hz, 2H,  $\text{ArCH}_2$ ), 4.29 (d,  $J = 9.4$  Hz, 1H, H-1), 3.90 (d,  $J = 3.0$  Hz, 1H, H-4), 3.74-3.58 (m, 5H, H-2, H-3, H-5, H-6), 2.78-2.66 (m, 2H,  $\text{SCH}_2\text{CH}_3$ ), 1.29 (t,  $J = 7.4$  Hz, 3H,  $\text{SCH}_2\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz): 138.6-18.0 (12C, Ar-C), 86.4 (C-1), 77.8 (C-2), 76.4 (C-4), 75.6 (C-3), 75.4 ( $\text{ArCH}_2$ ), 73.8 ( $\text{ArCH}_2$ ), 71.1 (C-5), 68.7 (C-6), 24.7 ( $\text{SCH}_2\text{CH}_3$ ), 15.5 ( $\text{SCH}_2\text{CH}_3$ ). HR-MALDI-TOF MS  $m/z$ : calcd for  $\text{C}_{22}\text{H}_{28}\text{O}_5\text{S}$ : calcd 404.1657; found  $[\text{M} + \text{Na}^+]$  427.1620

**Ethyl 3-*O*-diethylisopropylsilyli-4, 6-di-*O*-benzyl-1-thiol- $\beta$ -D-galactopyranoside (24):** Diol **23** (4.0 g, 9.90 mmol) and imidazole (0.67 g, 100 mmol) were dissolved in dry THF (5 mL). TBDPSCl (2.63 mL, 9.90 mmol) was added dropwise to the above solution. The reaction mixture was stirred at room temperature for 4h. MeOH (2 mL) was added to quench the reaction. The mixture was diluted with ether (30 mL), washed with water and dried over MgSO<sub>4</sub> and concentrated. The residue was purified by column chromatography (SiO<sub>2</sub>, Hexane/EtOAc 9:1) to furnish silyl ether **23** (4.05 g, 78%) as a white foam:  $R_f$  = 0.58 (Hexane/EtOAc 11:2);  $[\alpha]_D$  (c 1.0 in CHCl<sub>3</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 7.34-7.30 (m, 10H, Ar-H), 5.04 (d,  $J$  = 11.3 Hz, 1H, ArCH<sub>2</sub>), 4.58 (d,  $J$  = 11.3 Hz, 1H, ArCH<sub>2</sub>), 4.25 (q,  $J$  = 6.6, 9.8 Hz, 2H, ArCH<sub>2</sub>), 4.31 (d,  $J$  = 9.4 Hz, 1H, H-1), 3.85 (dd,  $J$  = 5.7, 2.0 Hz, 1H, H-3), 3.78 (d,  $J$  = 2.0 Hz, 1H, H-4), 3.74 (dd,  $J$  = 9.4, 5.7 Hz, 1H, H-2), 3.65 (m, 3H, H-5, H-6), 2.78 (m, 2H, SCH<sub>2</sub>CH<sub>3</sub>), 1.30 (t,  $J$  = 7.4 Hz, 3H, SCH<sub>2</sub>CH<sub>3</sub>), 1.05 (m, 12H, CH<sub>3</sub>-DEIPS), 0.75 (m, 5H, CH<sub>2</sub>, CH-DEIPS); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): 139.3-127.6 (12C, Ar-C), 86.8 (C-1), 77.7 (C-3), 77.4 (C-6), 76.9 (C-2), 75.3 (CH<sub>2</sub>Ph), 73.8 (CH<sub>2</sub>Ph), 70.7 (C-5), 69.2 (C-6), 24.5 (SCH<sub>2</sub>CH<sub>3</sub>), 17.7 (SCH<sub>2</sub>CH<sub>3</sub>), 17.7, 15.6, 13.3, 7.5, 7.4 (5C, CH, CH<sub>3</sub>-DEIPS), 4.24 (2C, CH<sub>2</sub>-DEIPS). HR-MALDI-TOF MS  $m/z$ : calcd for C<sub>29</sub>H<sub>44</sub>O<sub>5</sub>SSi 532.2679; found [M + Na<sup>+</sup>] 555.2696

**Ethyl 2-*O*-benzoyl-3-*O*-diethylisopropylsilyli-4, 6-di-*O*-benzyl-1-thiol- $\beta$ -D-galactopyranoside (26):**

To a solution of the alcohol **25** (309 mg, 0.58 mmol), in CH<sub>2</sub>CCl<sub>2</sub> (2 mL) at room temperature was added TEA (0.38 mL, 2.7 mmol), BzCl (0.32 mL, 2.7 mmol), and DMAP (671 mg, 5.5 mmol). The mixture was stirred at room temperature for 20 h after which it was poured into EtOAc (50 mL). The organic phase was washed with 1 M HCl, water, sat. NaHCO<sub>3</sub> aq and brine. The organic phase was dried (MgSO<sub>4</sub>) and concentrated in *vacuo*. The mixture was purified by

column chromatography (SiO<sub>2</sub>, EtOAc/Haxane 3:2) to furnish **26** (328mg, 51.5 mmol, 89%) as a white foam: R<sub>f</sub> = 0.48 (EtOAc/Haxane 3:2); [α]<sub>D</sub> (c 1.0 in CHCl<sub>3</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 8.05 (d, *J* = 7.2 Hz, 2H, Ar-H), 7.57-7.26 (m, 13H, Ar-H), 5.62 (t, *J* = 8.8 Hz, 1H, H-2), 5.04 (d, *J* = 11.6 Hz, 1H, ArCH<sub>2</sub>), 4.86 (d, *J* = 11.6 Hz, 1H, ArCH<sub>2</sub>), 4.25 (m, 2H, ArCH<sub>2</sub>), 4.20 (d, *J* = 9.6 Hz, 1H, H-1), 4.01 (dd, *J* = 5.8, 1.2 Hz, 1H, H-3), 3.83 (d, *J* = 1.2 Hz, 1H, H-4), 3.65 (q, *J* = 3.2 Hz, 1H, H-5), 3.61 (m, 2H, H-6), 2.68 (m, 2H, SCH<sub>2</sub>CH<sub>3</sub>), 1.19 (t, *J* = 7.4 Hz, 3H, SCH<sub>2</sub>CH<sub>3</sub>), 0.95 (m, 12H, CH<sub>3</sub>-DEIPS), 0.54 (m, 5H, CH<sub>2</sub>, CH-DEIPS) <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): 165.7 (COAr), 139.2-125.6 (18C, Ar-C), 84.0 (C-1), 77.9 (C-5), 77.8 (C-4), 76.1 (C-3), 75.5 (ArCH<sub>2</sub>), 73.8 (ArCH<sub>2</sub>), 71.5 (C-2), 68.9 (C-6), 23.7 (SCH<sub>2</sub>CH<sub>3</sub>), 17.5, 17.4, 15.1, 13.1 (4C, CH<sub>3</sub>-DEIPS), 17.7 (SCH<sub>2</sub>CH<sub>3</sub>), 7.3, 4.3, 4.0 (3C, CH<sub>2</sub>, CH-DEIPS). HR-MALDI-TOF MS *m/z*: calcd for C<sub>36</sub>H<sub>48</sub>O<sub>6</sub>SSi 636.2941; found [M + Na<sup>+</sup>] 661.2965

***p*-(*p*-benzoyloxy)-benzyl 6-*O*-benzyl-2-deoxy-2[[*(2,2,2*-trichloroethoxy)carbonyl]amino]-3-*O*-(9-fluorenylmethoxycarbonyl)-4-*O*-(2-*O*-benzoyl-3-*O*-diethylisopropylsilyl)-4, 6-di-*O*-benzyl -β-*D*-galatopyranosyl)-β-*D*-glucopyranoside (**27**):** A solution of the glycosyl donor **26** (150 mg, 0.24 mmol) and the glycosyl acceptor **12** (175 mg, 0.2 mmol) was dissolved in DCM (2 mL). The mixture was stirred at room temperature under Argon in the presence of activated molecular sieves for 30 minutes. The mixture was cooled to 0 °C and NIS (594 mg, 0.26 mmol) was added followed by TESOTf (5.5 μL, 0.024 mmol). After the donor was fully converted, the reaction mixture was diluted with DCM (50 mL) and molecular sieves were filtered out. Aqueous Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (15%, 2 mL) was added and the organic layer was dried by MgSO<sub>4</sub> and concentrated under reduced pressure. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc 2:1) to give the **27** as a white powder (200 mg, 69%): [α]<sub>D</sub> -28.9 ° (c 1.0, CH<sub>2</sub>Cl<sub>2</sub>); R<sub>f</sub> = 0.57 (Hexane/EtOAc 2:1). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 8.20 (d, 2H, *J* = 7.7 Hz,



Ar-H), 8.05 (d, 2H,  $J = 7.7$  Hz, Ar-H), 7.77-7.13 (m, 33H, Ar-H), 5.57 (t, 1H,  $J = 8.8$  Hz, H-2'), 5.19 (d, 1H,  $J = 9.4$  Hz, N-H), 5.01 (t, 1H,  $J = 9.4$  Hz, H-3), 4.98 (d, 1H,  $J = 11.6$  Hz, Bn), 4.85 (d, 1H,  $J = 11.6$  Hz, Bn), 4.68 (d,  $J = 8.6$ , 1H, H-1), 4.60-4.31 (m, 8H, 2×Bn, Troc, CH<sub>2</sub>PhOBz), 4.52 (d, 1H,  $J = 8.9$ , H-1'), 4.35 (d,  $J = 6.0$  Hz, 2H, Fmoc-CH<sub>2</sub>), 4.18 (t,  $J = 6.0$  Hz, 1H, Fmoc-CH), 4.10 (dd, 1H,  $J = 5.9, 1.6$  Hz, H-3'), 4.08-3.81 (m, 4H, H-2, H-4, H-6), 3.80 (d, 1H,  $J = 1.6$  Hz, H-4'), 3.69-3.51 (m, 3H, H-5', H-6'), 3.61 (d,  $J = 9.1$  Hz, 1H, H-5), 0.95 (m, 12H, CH<sub>3</sub>-DEIPS), 0.54 (m, 5H, CH<sub>2</sub>, CH-DEIPS); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): 165.3 (COPh), 165.1 (ArCO), 154.9 (CHCH<sub>2</sub>OCO, Fmoc), 154.4 (Ar-C), 150.7 (NHCO), 143.7-12.2 (47C, Ar-C), 101.4 (C-1'), 100.0 (C-1), 95.6 (CCl<sub>3</sub>), 77.2 (C-3'), 77.1 (C-4'), 75.7 (C-3), 75.5, 74.7, 73.8, 73.4, 73.3 (4C, 3×CH<sub>2</sub>Ph, Troc, CH<sub>2</sub>PhOBz) 73.6 (2C, C-2', C-4), 73.5 (C-5'), 70.2 (CH<sub>2</sub>-Fmoc), 68.2, 68.0 (2C, C-6, C-6'), 60.6 (C-2), 46.8 (CH-Fmoc), 17.5, 17.3, 14.5, 13.0 (4C, CH<sub>3</sub>-DEIPS), 7.3 (CH-DEIPS), 4.3, 4.0 (2C, CH<sub>2</sub>-DEIPS). HR-MALDI-TOF MS  $m/z$ : calcd for C<sub>79</sub>H<sub>86</sub>Cl<sub>3</sub>NO<sub>17</sub>Si 1449.4418; found [M + Na<sup>+</sup>] 1472.4530.

***p*-(*p*-benzoyloxy)-benzyl 6-*O*-benzyl-2-deoxy-2[[[(2,2,2-trichloroethoxy)carbonyl]amino]-4-*O*-(2-*O*-benzoyl-3-*O*-diethylisopropylsilyl)-4, 6-di-*O*-benzyl -β-D-galactopyranosyl)-β-D-glucopyranoside (28):** Compound **27** (100 mg, 0.07 mmol) was dissolved in dichloromethane (4 mL) and triethylamine (1 mL) was added. The reaction mixture was stirred at room temperature overnight and then concentrated under reduced pressure. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc 2:1) to give the **28** as a white powder (80 mg, 95%): [α]<sub>D</sub> -26.4° (*c* 1.0, CH<sub>2</sub>Cl<sub>2</sub>); R<sub>f</sub>=0.32 (Hexane/EtOAc 2:1). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 8.21 (d, 2H,  $J = 7.7$  Hz, Ar-H), 8.07 (d, 2H,  $J = 7.7$  Hz, Ar-H), 7.67-7.03 (m, 25H, Ar-H), 5.60 (t, 1H,  $J = 8.5$  Hz, H-2'), 5.20 (d,  $J = 9.0$  Hz, 1H, NH), 5.08 (d, 2H,  $J = 11.6$  Hz, ArCH<sub>2</sub>), 4.82-4.06 (m, 10H, 3×ArCH<sub>2</sub>, CH<sub>2</sub>PhOBz, Troc), 4.38 (d,  $J = 8.9$ , 1H, H-1), 4.28 (d, 1H,  $J = 8.7$ , H-1'), 4.08

(dd, 1H,  $J = 1.6, 8.0$  Hz, H-3'), 3.80 (d, 1H,  $J = 2.0$  Hz, H-4), 3.79-3.74 (m, 3H, H-2, H-6), 3.62 (m, 1H, H-3), 3.60 (d, 1H,  $J = 1.6$  Hz, H-4'), 3.59-3.51 (m, 2H, H-5', H-6'), 3.41-3.36 (m, 2H, H-5, H-6'), 0.98 (m, 12H, CH<sub>3</sub>-DEIPS), 0.64 (m, 5H, CH<sub>2</sub>, CH-DEIPS). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): 165.7 (COPh), 165.1 (ArCO), 154.4 (Ar-C), 150.7 (NHCO), 138.7-121.2 (35C, Ar-C), 101.7 (C-1'), 99.9 (C-1), 95.8 (CCl<sub>3</sub>), 81.0 (C-3'), 75.5 (C-4'), 74.7 (C-3), 74.8, 74.6, 72.6, 72.2 (4C, 3×CH<sub>2</sub>Ph, Troc, CH<sub>2</sub>PhOBz), 74.2 (C-2'), 73.3 (C-5'), 70.3 (C-4), 68.8, 68.6 (2C, C-6, C-6'), 57.9 (C-2), 17.5, 17.4, 17.3, 14.4 (4C, CH<sub>3</sub>-DEIPS), 7.4 (CH-DEIPS), 4.4, 4.0 (2C, CH<sub>2</sub>-DEIPS). HR-MALDI-TOF MS  $m/z$ : calcd for C<sub>64</sub>H<sub>72</sub>Cl<sub>3</sub>NO<sub>15</sub>Si 1227.3737; found [M + Na<sup>+</sup>] 1250.3685.

***p*-(*p*-benzoyloxy)-benzyl 6-*O*-benzyl-2-deoxy-2[[[(2,2,2-trichloroethoxy) carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)-4-*O*-(2-*O*-benzoyl-3-*O*-diethylisopropylsilyl)-4, 6-di-*O*-benzyl - $\beta$ -D-galatopyranosyl) - $\beta$ -D-glucopyranoside (29):**

A solution of the glycosyl donor **9** (53.5 mg, 0.14 mmol) and the glycosyl acceptor **28** (80 mg, 0.07 mmol) was dissolved in DCM (3 mL). The mixture was stirred at room temperature under Argon in the presence of activated molecular sieves for 30 minutes. The mixture was cooled to 0 °C and NIS (34.7 mg, 0.15 mmol) was added followed by TESOTf (3.3  $\mu$ L, 0.015 mmol). After the donor was fully converted, the reaction mixture was diluted with DCM (50 mL) and molecular sieves were filtered off. Aqueous Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (15%, 2 mL) was added and the organic layer was dried by MgSO<sub>4</sub> and concentrated under reduced pressure. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc 2:1) to give the product **29** as a white powder (80 mg, 73.9%): [ $\alpha$ ]<sub>D</sub> -42.6° ( $c$  1.0, CH<sub>2</sub>Cl<sub>2</sub>); R<sub>f</sub> = 0.54 (Hexane/EtOAc 2:1). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 8.20 (d, 2H,  $J = 7.4$  Hz, Ar-H), 8.07 (d, 2H,  $J = 7.4$  Hz, Ar-H), 7.66-7.05 (m, 30H, Ar-H), 5.45 (t, 1H,  $J = 8.5$  Hz, H-2'), 5.33 (d, 1H,  $J = 8.9$  Hz, N-H), 5.25 (dd, 1H,  $J = 10.6, 3.3$  Hz,

H-3''), 5.17 (d,  $J = 3.6$  Hz, 1-H, H-1''), 5.14 (d,  $J = 3.3$  Hz, 1H, H-4''), 4.88 (q,  $J = 6.0$  Hz, 1-H, H-5''), 4.84 (d,  $J = 8.6$ , 1H, H-1), 4.76-4.37 (m, 12H, 4×Bn, Troc, CH<sub>2</sub>PhOBz), 4.62 (d, 1H,  $J = 9.0$ , H-1'), 4.10 (d,  $J = 3.6$  Hz, 1H, H-4'), 4.01 (t,  $J = 9.0$  Hz, 1H, H-3), 3.85-3.67 (m, 6H, H-4, H-6a, H-3', H-6', H-2''), 3.58 (d,  $J = 8.5$  Hz, 1H, H-6b), 3.44 (q, 1H,  $J = 3.6$  Hz, H-5'), 3.19-3.05 (m, 2H, H-5, H-2), 2.08 (s, 3H, COCH<sub>3</sub>), 2.00 (s, 3H, COCH<sub>3</sub>), 0.97 (d,  $J = 6.0$  Hz, 3H, H-6''), 0.95 (m, 12H, CH<sub>3</sub>-DEIPS), 0.54 (m, 5H, CH<sub>2</sub>, CH-DEIPS); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): 170.6, 169.7 (2C, 2×CH<sub>3</sub>CO), 165.3 (COPhOBz), 165.0 (ArCO), 153.7 (Ar-C), 150.6 (NHCO), 138.9-121.7 (41C, Ar-C), 100.1 (C-1), 99.0 (C-1), 97.5 (C-1''), 95.6 (CCl<sub>3</sub>), 77.5 (C-3'), 75.6 (C-5), 75.3 (C-4'), 75.1, 74.3, 73.3, 72.6, 70.7 (6C, 4×CH<sub>2</sub>Ph, Troc, CH<sub>2</sub>PhOBz), 74.6 (C-5'), 74.3 (C-3), 73.7 (C-2''), 73.6 (C-2'), 73.5 (C-4), 73.4 (C-4''), 70.7, 70.6 (2C, C-6, C-6'), 70.5 (C-3''), 64.9 (C-5''), 59.1 (C-2), 21.2, 21.0 (2C, 2×CH<sub>3</sub>CO), 17.6 (C-6''), 17.5, 17.4, 15.3, 13.0 (4C, CH<sub>3</sub>-DEIPS), 7.3 (CH-DEIPS), 4.3, 4.0 (2C, CH<sub>2</sub>-DEIPS). HR-MALDI-TOF MS  $m/z$ : calcd for C<sub>81</sub>H<sub>92</sub>Cl<sub>3</sub>NO<sub>21</sub>Si 1547.4997; found [M + Na<sup>+</sup>] 1570.4900.

**3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2[[*(2,2,2*-trichloroethoxy) carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)-4-*O*-(2-*O*-benzoyl-3-*O*-diethylisopropylsilyl)-4, 6-di-*O*-benzyl - $\beta$ -D-galatopyranosyl) - $\beta$ -D-glucopyranoside (33):**

Compound **29** (80 mg, 0.05 mmol) was dissolved in THF (5 mL) and Et<sub>3</sub>N (2.5 mL) and H<sub>2</sub>O<sub>2</sub> (30% in water, 0.13 mL) were added. After stirring the reaction mixture at room temperature for 30 minutes, it was concentrated under reduced pressure. The obtained residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc 2:1) to give compound **30** (60 mg, 80%), which was used directly in the next step.

The compound **30** (60 mg, 0.04 mmol) was dissolved in dichloromethane (5% water 4 mL) and DDQ (9 mg, 0.04 mmol) was added. The reaction mixture was stirred in dark at room

temperature for 1h. The reaction mixture was diluted with DCM (50 mL) and was washed with aqueous sodium hydrogen carbonate (15%, 25 mL) and brine (25mL). The organic phase was dried and concentrated to dryness. The residue was purified by silicon column chromatography (ethyl acetate/hexane 2/3) to give compound **31** (45 mg, 81%).

Compound **31** was dissolved in dichloromethane (5 mL).  $\text{CCl}_3\text{CN}$  (0.5 mL) and DBU (5  $\mu\text{L}$ ) were added to the solution. After 5min, the solution was concentrated to dryness and the residue was purified by column chromatography ( $\text{SiO}_2$  Hexane/EtOAc/triethylamine=1/1/0.01) to give the **32** as colorless syrup (45 mg, 90%).

Compound **32** and 3-[(*N*-benzyloxycarbonyl) amino]propanol (11 mg, 0.06 mmol) was dissolved in dry dichloromethane (2 ml) with molecular sieves. After stirring for 30 min,  $\text{BF}_3 \cdot \text{Et}_2\text{O}$  (1  $\mu\text{L}$ ) was added. The reaction was kept stirring at room temperature for 10 min. After the reaction was quenched by adding triethylamine (20  $\mu\text{L}$ ), it was diluted with dichloromethane (60 mL). The molecular sieves were removed by filtration. The filtrate was washed with brine, dried over  $\text{MgSO}_4$  and concentrated. The residue was purified by column chromatography ( $\text{SiO}_2$  Hexane/EtOAc = 1/1) to give the **33** as a colorless syrup (40 mg, 86%).  $[\alpha]_D -51.2^\circ$  ( $c$  1.0,  $\text{CH}_2\text{Cl}_2$ );  $R_f$  = 0.49 (Hexane/EtOAc 2:1).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ): 7.88 (d, 2H,  $J$  = 7.8 Hz, Ar-H), 7.50-7.12 (m, 28H, Ar-H), 5.50 (d, 1H,  $J$  = 8.7 Hz, *NH*), 5.35 (t, 1H,  $J$  = 9.3 Hz, H-2'), 5.25 (dd, 1H,  $J$  = 8.8, 2.9 Hz, H-3''), 5.10 (d,  $J$  = 3.5 Hz, 1-H, H-1''), 5.04 (d,  $J$  = 2.9 Hz, 1H, H-4''), 4.97 (s, 2H,  $\text{CH}_2\text{NHCOOCH}_2\text{Ph}$ ), 4.80 (q,  $J$  = 6.5 Hz, 1-H, H-5''), 4.64 (d,  $J$  = 9.0 Hz, 1H, H-1), 4.76-4.37 (m, 10H, 4 $\times$ Bn, Troc), 4.58 (d, 1H,  $J$  = 8.6 Hz, H-1'), 3.85 (d,  $J$  = 3.6 Hz, 1H, H-4'), 3.83 (m, 1H, H-3), 3.80-3.65 (m, 6H, H-4, H-6a, H-6', H-3', H-2''), 3.58 (d,  $J$  = 8.5 Hz, 2H,  $\text{OCH}_2\text{CH}_2\text{CH}_2\text{N}$ ), 3.44 (d,  $J$  = 8.8 Hz, 1H, H-6b), 3.38 (q, 1H,  $J$  = 3.8 Hz, H-5'), 3.30-3.20 (m, 1H,  $\text{OCH}_2\text{CH}_2\text{CH}_2\text{N}$ ), 3.19-3.10 (m, 1H,  $\text{OCH}_2\text{CH}_2\text{CH}_2\text{N}$ ), 3.09-3.01 (m, 2H, H-5, H-2), 2.00

(s, 3H, COCH<sub>3</sub>), 1.88 (s, 3H, COCH<sub>3</sub>), 1.19 (s, 2H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 0.87 (d, *J* = 6.5 Hz, 3H, H-6''), 0.85 (m, 12H, CH<sub>3</sub>-DEIPS), 0.54 (m, 5H, CH<sub>2</sub>, CH-DEIPS); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz): 170.6, 169.6 (2C, 2×CH<sub>3</sub>CO), 165.0 (ArCO), 156.7 (NHCOOCH<sub>2</sub>Ph), 154.6 (NHCO), 138.9-127.7 (36C, Ar-C), 100.0 (C-1), 97.7 (C-1'), 96.5 (C-1''), 95.4 (CCl<sub>3</sub>), 77.5 (C-3'), 75.8 (C-5), 75.5 (C-4'), 75.0, 74.3, 73.3, 72.6, 70.7 (6C, 4×CH<sub>2</sub>Ph, Troc, NHCOOCH<sub>2</sub>Ph), 74.7 (C-5'), 74.6 (C-3), 73.7 (C-2''), 73.6 (C-2'), 73.5 (C-4), 73.4 (C-4''), 73.3 (C-3''), 68.3, 68.2 (2C, C-6, C-6'), 66.9 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 65.0 (C-5''), 59.0 (C-2), 37.8 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 30.0 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 21.2, 21.0 (2C, 2×CH<sub>3</sub>CO), 17.5 (C-6''), 17.5, 17.3, 15.3, 13.0 (4C, CH<sub>3</sub>-DEIPS), 7.3 (CH-DEIPS), 4.3, 4.0 (2C, CH<sub>2</sub>-DEIPS). HR-MALDI-TOF MS *m/z*: calcd for C<sub>78</sub>H<sub>95</sub>Cl<sub>3</sub>N<sub>2</sub>O<sub>21</sub>Si 1528.5262; found [M + Na<sup>+</sup>] 1551.5194.

**3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2[[*(2,2,2*-trichloroethoxy)carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)-4-*O*-(2-*O*-benzoyl-4,6-di-*O*-benzyl- $\beta$ -D-galatopyranosyl)- $\beta$ -D-glucopyranoside (**34**):**

Compound **33** (40 mg, 0.03mmol) was dissolved in dry THF (2 mL), and then AcOH (0.2 mL) and a solution of TBAF in THF (1M, 0.2 mL) were added. The reaction mixture was stirred at room temperature for two days. The reaction mixture was then diluted with EtOAc (50 mL) and washed with water (10 mL), aqueous NaHCO<sub>3</sub> (15%, 10 mL) and brine (10 mL). The organic phase was dried by MgSO<sub>4</sub> and then concentrated under vacuum. The obtained residue was further purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc = 1/1) to give the **34** as a white powder (30 mg, 82%). [ $\alpha$ ]<sub>D</sub> -49.3° (*c* 1.0, CH<sub>2</sub>Cl<sub>2</sub>); R<sub>f</sub> = 0.38 (Hexane/EtOAc 2:1). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): 7.92 (d, 2H, *J* = 7.8 Hz, Ar-H), 7.65-7.10 (m, 28H, Ar-H), 5.61 (d, 1H, *J* = 9.0 Hz, N-H), 5.38 (t, 1H, *J* = 8.0 Hz, H-2'), 5.28 (dd, 1H, *J* = 9.6, 3.0 Hz, H-3''), 5.18 (d, *J* = 3.5 Hz, 1-H, H-1''), 5.09 (d, *J* = 3.1 Hz, 1H, H-4''), 5.01 (s, 2H, CH<sub>2</sub>NHCOOCH<sub>2</sub>Ph), 4.82 (q, *J* =

6.5 Hz, 1-H, H-5''), 4.59 (d,  $J = 8.9$  Hz, 1H, H-1), 4.76-4.37 (m, 10H, 4×Bn, Troc), 4.40 (d, 1H,  $J = 9.2$  Hz, H-1'), 4.10 (d,  $J = 3.8$  Hz, 1H, H-4'), 4.03 (m, 1H, H-3), 4.02-3.95 (m, 5H, H-4, H-6', H-3', H-2''), 3.85 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 3.64 (d, 2H,  $J = 3.8$  Hz, H-6), 3.58 (q, 1H,  $J = 4.0$  Hz, H-5'), 3.30 (m, 1H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2a</sub>N), 3.19-3.10 (m, 3H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2b</sub>N, H-5, H-2), 2.20 (s, 3H, COCH<sub>3</sub>), 2.00 (s, 3H, COCH<sub>3</sub>), 1.29 (s, 2H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 1.15 (d,  $J = 6.5$  Hz, 3H, H-6''); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz): 170.8, 169.8 (2C, 2×CH<sub>3</sub>CO), 166.1 (ArCO), 156.7 (NHCOOCH<sub>2</sub>Ph), 154.0 (NHCO), 138.6-127.9 (36C, Ar-C), 100.3 (C-1), 98.0 (C-1'), 95.7 (C-1''), 95.4 (CCl<sub>3</sub>), 77.4 (C-3'), 76.4 (C-5), 75.9 (C-4'), 75.7, 74.8, 74.7, 72.5, 70.8 (6C, 4×CH<sub>2</sub>Ph, Troc, NHCOOCH<sub>2</sub>Ph), 74.3 (C-5'), 74.3 (C-3), 73.3 (C-2''), 72.6 (C-2'), 73.3 (C-4), 72.6 (C-4''), 71.7 (C-3''), 67.8, 66.9 (2C, C-6, C-6'), 66.8 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 65.0 (C-5''), 59.4 (C-2), 37.8 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 30.0 (OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 21.2, 21.0 (2C, 2×CH<sub>3</sub>CO), 15.8 (C-6''). HR-MALDI-TOF MS  $m/z$ : calcd for C<sub>71</sub>H<sub>79</sub>Cl<sub>3</sub>N<sub>2</sub>O<sub>21</sub> 1400.4241; found [M + Na<sup>+</sup>] 1423.4268.

**3-[(*N*-Benzyloxycarbonyl)amino]propyl 6-*O*-benzyl-2-deoxy-2-[(2,2,2-trichloroethoxy) carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)-4-*O*-(2-*O*-benzoyl-4,6-di-*O*-benzyl)-3-*O*-(6-*O*-benzyl-2-deoxy-2-[(2,2,2-trichloroethoxy) carbonyl]amino]-3-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)-4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-acetyl-2-*O*-benzyl- $\alpha$ -L-fucopyranosyl)- $\beta$ -D-galatopyranosyl)- $\beta$ -D-glucopyranoside)- $\beta$ -D-galatopyranosyl)- $\beta$ -D-glucopyranoside (35):**

Glycosyl donor **19** (45 mg, 0.027 mmol) and glycosyl acceptor **34** (35 mg, 0.025 mmol) were dissolved in DCM (1 mL). The reaction mixture was stirred at room temperature under Argon in the presence of activated molecular sieves for 30 minutes. The mixture was cooled to -30 °C and TBSOTf (1.0  $\mu$ L) was added. After the donor was fully converted, the reaction mixture was diluted with DCM (50 mL) and molecular sieves were filtered off. The organic layer was dried

by MgSO<sub>4</sub> and concentrated under vacuum. The residue was purified by column chromatography (SiO<sub>2</sub> Hexane/EtOAc 1:1) to give the **35** as a white powder (36 mg, 62%) [ $\alpha$ ]<sub>D</sub> -104.3° (*c* 1.0, CH<sub>2</sub>Cl<sub>2</sub>); R<sub>f</sub> = 0.28 (Hexane/EtOAc 1:1).

The <sup>1</sup>H NMR (800MHz, CDCl<sub>3</sub>) spectral data for compound **35** was listed as following:

	Gal'	Fuc''	GlcN'	Fuc'	Gal	GlcN	Fuc
H-1	4.67	5.64	5.02	5.10	4.50	4.31	5.18
H-2	5.36	3.76	3.20	3.66	5.38	2.81	3.80
H-3	3.89	5.23	4.04	4.95	3.76	4.06	5.14
H-4	4.01	5.20	3.89	4.90	4.05	3.89	5.18
H-5	3.67	4.69	3.10	4.90	3.76	3.09	4.75
H-6	4.68	1.02	3.50	0.81	4.62	3.52	0.98

HR-MALDI-TOF MS *m/z*: calcd for C<sub>148</sub>H<sub>165</sub>Cl<sub>6</sub>N<sub>3</sub>O<sub>44</sub> 2897.8897; found [M + Na<sup>+</sup>] 2920.9002.

**Amino propyl 2-deoxy-2-acetamido-3-O- $\alpha$ -L-fucopyranosyl-(4-O-(3-O-2-deoxy-2-acetamido-3-O-( $\alpha$ -L-fucopyranosyl)-4-O-(2-O- $\alpha$ -L-fucopyranosyl)- $\beta$ -D-galatopyranosyl)- $\beta$ -D-glucopyranoside)- $\beta$ -D-galatopyranosyl)- $\beta$ -D-glucopyranoside (37):**

Zinc (10 mg, 0.15 mmol, nanosize powder) was added to a stirred solution of tetrasaccharide **36** (20 mg, 0.02 mmol) in acetic acid (2 mL). After 20 minutes, the zinc dust was filtered out through Celite and the filtrate was concentrated to dryness. The residue was dissolved in pyridine (2 mL) and acetic anhydride (1 mL) and the mixture was stirred at room temperature over night. Then the reaction was quenched by addition of methanol (2 mL). The solution was diluted by dichloromethane (60 mL) and was washed successively with 1 M HCl solution, aqueous sodium

hydrogencarbonate (15%), and brine. The organic layer was dried over  $\text{MgSO}_4$  and concentrated. The residue was dissolved in ethanol and acetic acid (5/1), and the mixture was hydrogenolysed over  $\text{Pd}(\text{OAc})_2$  (20 mg) at room temperature. After 24 h the mixture was filtered through Celite to remove the catalyst and concentrated to dryness under reduced pressure. The obtained residue was dissolved by methanol (5 mL) and sodium methoxide (1 M in methanol) was added until pH = 10. The solution was stirred at room temperature for 24 h, neutralized with Dowex 50  $\text{H}^+$  resin, diluted by methanol (50 mL), filtered and concentrated. The residue was purified by size exclusion column chromatography (Biogel P2 column, eluted with  $\text{H}_2\text{O}$  containing 1% n-BuOH) to give the product **37** as a white powder (4 mg, 52%).  $[\alpha]_D -99.4$  (*c* 1.0, MeOH).  $^1\text{H}$  NMR (800 MHz,  $\text{D}_2\text{O}$ ): 5.42 (d, 1H,  $J = 3.5$  Hz), 5.05 (d, 1H,  $J = 4.0$  Hz), 5.04 (d, 1H,  $J = 3.7$  Hz), 4.83 (q, 1H,  $J = 6.5$  Hz), 4.67 (q, 1H,  $J = 6.5$  Hz), 4.64 (d, 1H,  $J = 7.6$  Hz), 4.45 (d, 2H,  $J = 8.0$  Hz), 4.41 (d,  $J = 7.8$  Hz), 4.22 (q, 1H,  $J = 6.5$ , 1H), 2.01 (s, 6H,  $2 \times \text{NHCOCH}_3$ ), 1.21, 1.22, 1.24 (3d, 9H(Fuc),  $J = 6.5$  Hz).  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 200 MHz): 105.1, 104.2, 103.4, 103.0, 102.5, 101.0 (7C, anomeric centers). HR-MALDI-TOF MS  $m/z$ : calcd for  $\text{C}_{49}\text{H}_{85}\text{N}_3\text{O}_{33}$  1243.5065; found  $[\text{M} + \text{Na}^+]$  1266.5523.



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