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Synthesis of Neoglycoconjugates and Oligosaccharides with Potential anti-*Helicobacter pylori* Activity

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Academic dissertation

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“The avalanche has already started. It is too late for the pebbles to vote.”

Babylon 5

SUMMARY

The significance of carbohydrate-protein interactions in many biological phenomena is now widely acknowledged and carbohydrate based pharmaceuticals are under intensive development. The interactions between monomeric carbohydrate ligands and their receptors are usually of low affinity. To overcome this limitation natural carbohydrate ligands are often organized as multivalent structures. Therefore, artificial carbohydrate pharmaceuticals should be constructed on the same concept, as multivalent carbohydrates or glycoclusters. Infections of specific host tissues by bacteria, viruses, and fungi are among the unfavorable disease processes for which suitably designed carbohydrate inhibitors represent worthy targets.

The bacterium *Helicobacter pylori* colonizes more than half of all people worldwide, causing gastritis, gastric ulcer, and conferring a greater risk of stomach cancer. The present *medication therapy* for *H. pylori* includes the use of antibiotics, which is associated with increasing incidence of bacterial resistance to traditional antibiotics. Therefore, the need for an alternative treatment method is urgent.

In this study, four novel synthesis procedures of multivalent glycoconjugates were created. Three different scaffolds representing linear (chondroitin oligomer), cyclic (γ-cyclodextrin), and globular (dendrimer) molecules were used. Multivalent conjugates were produced using the human milk type oligosaccharides LNDFH I (Lewis-b hexasaccharide), LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), and GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc all representing analogues of the tissue binding epitopes for *H. pylori*. The first synthetic method included the reductive amination of scaffold molecules modified to express primary amine groups, and in the case of dendrimer direct amination to scaffold molecule presenting 64 primary amine groups. The second method described a direct procedure for amidation of glycosylamine modified oligosaccharides to scaffold molecules presenting carboxyl groups. The final two methods that were created both included an oxime-linkage on linkers of different length. All the new synthetic procedures synthesized had the advantage of using unmodified reducing sugars as starting material making it easy to synthesize glycoconjugates of different specificity.

In addition, the binding activity of an array of neoglycolipids to *H. pylori* was studied. Consequently, two new neolacto-based structures, Glcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer and GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer, with binding activity toward *H. pylori* were discovered. Interestingly, *N*-methyl and *N*-ethyl amide modification of the GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer glucuronic acid residue resulted in more effective *H. pylori* binding epitopes than the parent molecule.

TABLE OF CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their roman numerals:

I Weikkolainen, K., Aitio, O., Blomqvist, M., Natunen, J., and Helin, J. Conjugation of oligosaccharides by reductive amination to amine modified chondroitin oligomer and γ-cyclodextrin. *Glycoconjugate Journal*, in press, 2007.[a]

II Weikkolainen, K., Aitio, O., Natunen, J., and Helin, J. Conjugation of oligosaccharides to chondroitin oligomer and γ-cyclodextrin. *Carbohydrate Polymers*, in press, 2007.[b]

III Miller-Podraza, H., Weikkolainen, K., Larsson, T., Johansson, P., Helin, J., Natunen, J., and Karlsson, K.-A. New carbohydrate structures with binding activity for *Helicobacter* pylori. Manuscript in preparation.

In addition, previously unpublished data are also presented.

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[b]Reprinted from *Carbohydrate Polymers* with permission from Elsevier.

ABBREVIATIONS

αGal epitope	Galα1-3Galβ1-4GlcNAcβ
Aoa	Aminooxyacetic acid
CD	Cyclodextrin
Cer	Ceramide
Ch14	Chondroitin 14-mer, $(GlcA\beta1\text{-}3GalNAc\beta1\text{-}4)_6GlcA\beta1\text{-}3GalNAc$
CS	Chondroitin sulphate
DS	Dermatan sulphate
E. coli	*Escherichia coli*
Fuc	L-Fucose
GAG	Glycosaminoglycan
Gal	D-Galactose
GalNAc	*N*-Acetyl-D-galactosamine
Glc	D-Glucose
GlcNAc, Gn	*N*-Acetyl-D-glucosamine
GlcA	D-Glucuronic acid
GnLacNAcLac	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
H-1 antigen	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
HA	Hyaluronic acid
HIV	Human immunodeficiency virus
HP	Heparin
H. pylori	*Helicobacter pylori*
HS	Heparan sulphate
IdoA	L-Iduronic acid
K_a	Association constant
K_d	Dissociation constant
KS	Keratan sulphate
Lac	Lactose, Galβ1-4Glc
LacNAc	Galβ1-4GlcNAc
Le^a	Lewis a, Galβ1-3(Fucα1-4)GlcNAc
Le^b	Lewis b, Fucα1-2Galβ1-3(Fucα1-4)GlcNAc
LNDFH I	Lewis b hexasaccharide, Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc
LNnT	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MALT	Mucosal-associated lymphoid tissue
NMR	Nuclear magnetic resonance
Neu5Gc	*N*-glycolyl-D-neuraminic acid
NeuNAc/Neu5Ac	*N*-acetyl-D-neuraminic acid
PG	Proteoglycan
pNP-β-GlcA	Para-nitrophenyl-β-glucuronide
Ser	Serine
sLe^x	Sialyl Lewis x, NeuNAcα2-3Galβ1-4(Fucα1-3)GlcNAc
SPG	Sialylparagloboside, Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer
Thr	Threonine

1 INTRODUCTION

Carbohydrates or saccharides (Greek: *sakcharon*, sugar) are ubiquitous in cells. They dangle from many of the lipid molecules and nearly all the proteins in the body. The surfaces of all cells are adorned with these glycolipids and glycoproteins, which play a fundamental role in the interaction of cells with other cells and with their surroundings having both structural and regulatory functions. The body uses these molecules to signal something as general as "I am a human tissue, I belong here," or as detailed as "I am injured, send help from the immune system." For example, blood clotting as well as harmful inflammatory reactions are often triggered by carbohydrates. Cancer cells use carbohydrate structures on their surfaces to slip past cells belonging to the immune system, and to infiltrate tissues. In addition, the cell surface serves as a docking site for other cells, pathogens, and the extracellular matrix. For example, certain pathogenic viruses, bacteria and yeasts rely on the glycolipids and glycoproteins on cell surfaces to home in on their tissue of preference, in their favorite host species, and to spread themselves from cell to cell.

Glycobiology is the study of the structure, chemistry, biosynthesis, and biological functions of carbohydrates and their derivates. Glycobiology-based therapies have the potential to revolutionize the treatment of some diseases and carbohydrate-based drugs are expected to be the next major breakthrough in drug discovery. Such drugs may be used to treat, for example, cancer, inflammatory diseases, infections, and transplant rejection. In addition, the development of carbohydrate-based anti-adhesives presents a promising approach for the prevention of susceptible microbial infections. The alarming increase of bacterial strains resistant to antibiotics makes it vitally important to develop such new means of combating bacteria.

Because of the multivalent nature of pathogen binding to its target cells and tissues, an effective carbohydrate-based anti-adhesive should be designed based on the same concept, i.e. structures carrying several copies of the active carbohydrate sequence in the carrier molecule. Understanding the role of carbohydrate structures in adhesion of the infecting organisms to their host cell surfaces through multivalent carbohydrate-protein interaction is currently incomplete. However, the knowledge is constantly growing and there is an enormous need to improve the tools available for characterizing these interactions. There is also a need to develop conjugates which can interfere with these interactions in a beneficial manner, so that they can be applied to the study and treatment of disease. In this thesis, several different kinds of multivalent neoglycoconjugates were constructed based on three structurally different scaffolds representing cyclic, linear and globular scaffold types. All oligosaccharides used for the conjugation were analogs of established *Helicobacter pylori* binding epitopes. In addition, the binding activity of several new carbohydrate structures towards *H. pylori* and their structural requirements were studied.

2 REVIEW OF THE LITERATURE

2.1 Biologically relevant carbohydrate interactions

Carbohydrates are hydrophilic in nature and are consequently generally located on the outside of cell membranes. It is thus hypothesized that the first contact many cells make with each other occurs via interactions of the carbohydrate structures. Consequently, carbohydrates are widely involved in cell-cell recognition, cell-external agent interactions, and cell differentiation events (Varki, 1993). These interactions can initiate both beneficial biological processes, such as immune responses, fertilization, and cell growth and differentiation (e.g. during embryogenesis), as well as detrimental disease events, such as inflammation, cancer metastasis, and bacterial and viral infections (Davis, 2000; Dwek, 1996; Gabius, 1997; Lis and Sharon, 1998; Varki, 1993). Here, only a few examples of relevant interactions will be given and the interested reader in referred to literature cited for a more comprehensive look on the subject (Varki et al., 1999). The focus of this thesis is on carbohydrate interactions between pathogens and host cell surfaces and a more in-depth overview on this subject will be given in section 2.2.

The onset of modern glycobiology began in the late 1980s. Several research groups separately cloned the genes for the three human carbohydrate-binding proteins, which play a pivotal role in attracting white blood cells, or leukocytes to injured sites in the body. These proteins, called selectins, are expressed on leukocytes (L-selectin), on vascular endothelial cells (E- and P-selectin), and on platelets (P-selectin) (Kansas, 1996). The selectins interact with glycoprotein ligands modified to express sialylated, fucosylated glycans. The selectin family of adhesion molecules is responsible for the initial tethering of leukocytes to the walls of blood vessels and maintain leukocyte rolling, facilitating the subsequent firm adhesion and transendothelial migration (Kansas, 1996; Tedder et al., 1995). Although leukocytes start the healing process, they can also lead to inflammation, which itself can damage tissues. Blocking the ability of selectins to bind to their carbohydrate targets using molecules that mimic natural selectin ligand may thus prevent inflammation. For example, using sialyl Lewis^x (sLe^x) as a lead a low molecular weight molecule selectin inhibitor was discovered, which was shown to inhibit E-, P-, and L-selectin dependent adhesion *in vitro* (Davenpeck et al., 2000; Kogan et al., 1998).

Examples of viral and bacterial infections (see also section 2.2) include influenza virus surface proteins complexing with specific membrane-bound oligosaccharides on human cells (Wiley and Skehel, 1987) resulting in viral infections, and *Helicobacter pylori* infection of the human stomach starting by adherence of the bacteria to cell surface receptors (see also section 2.9). Binding of cell surface lectins to its oligosaccharide ligand can also transduce a signaling event, e.g. the strength of the immunoglobulin signaling is regulated by B cell lectin CD22 binding to sialic acid (Jin et al., 2002; Kelm et al., 2002). In addition, a variety of tumor-associated carbohydrate antigens are known, including the carcinoma-associated Thomsen-Freidenreich T or T_F antigen (Galβ1-3GalNAcα-*O*-Ser/Thr), the *O*-linked glycan T_N (GalNAcα-*O*-Ser/Thr), and

sialyl T_N antigen (Siaα2-6GalNAcα-*O*-Ser/Thr) (Brockhausen, 1999; Dennis et al., 1999; Kim and Varki, 1997). Consequently, carbohydrate antigens can serve as diagnostic markers for specific tumor cells and the presence of these antigens has even been correlated with more aggressive disease states (Shigeoka et al., 1999).

Carbohydrates also have a crucial role in xenograft rejection. Exposure of human blood to non-primate cells, tissues, and organs elicits a strong immune response, which is followed by rejection of the foreign tissue. The foremost antigenic epitope responsible for this is the terminal carbohydrate Galα1-3Galβ1-4GlcNAc, also called the "αGal" epitope (Galili et al., 1985; Rother and Squinto, 1996; Sandrin et al., 1993). If this αGal-mediated immunorejection could be countermanded, e.g. by neutralization of anti-αGal antibodies by αGal related oligosaccharides (Cairns et al., 1995; Neethling et al., 1994), organs from other animals could potentially develop into a greatly needed source of tissue grafts for humans. Furthermore, the carbohydrate moieties of glycoproteins influence the overall physical structure of the proteins, and serve as biological tags, marking proteins with different oligosaccharides for different fates. Certain oligosaccharides label a glycoprotein for secretion or insertion into the plasma membrane while others signal transfer to lysosomes. Sialoglycoproteins in the blood that lose their outermost sialic acid residues are targeted for removal from the circulation by the liver cell receptors and are degraded by the liver.

2.2 Glycans and microbial pathogenesis

In pathological processes glycoconjugates serve as receptors for bacteria and viruses attaching to host cells consequently participating in the initial stages of infection. Adhesins are proteins on the surface of viruses or bacteria that bind to ligands present on the surface of higher eukaryotic cells. Microorganisms have evolved adhesins that interact with glycoproteins, glycolipids, and proteoglycans. For infection to occur bacteria and viruses must first pass through the glycocalyx that surrounds the cells, bind to exposed extracellular matrix or cell surfaces, and colonize the target tissue. Some examples of pathogen binding on host cell surface glycan structures are presented below.

2.2.1 Bacterial adhesins and toxins

Most bacteria have multiple adhesins with different carbohydrate specificities helping to define the range of susceptible tissues (i.e. the bacteria's ecological niche). Binding of an adhesin to a receptor is generally of low affinity, but because they both often cluster in the plane of the membrane, the resulting strength of the interaction (avidity) can be quite strong. Several binding epitopes for *Helicobacter pylori* (see section 2.9.2. for more detail), the bacterium that causes gastric ulcers and cancer, have been characterized, including Lewis b (Le^b) antigen present on human gastric epithelium (Ilver et al., 1998). *Streptococcus suis,* which causes septicemia,

meningitis, and pneumonia in pigs, and which is also known to cause bacterial meningitis in humans, binds to galabiose (Galα1-4Gal) moiety found as a constituent on cell surface glycolipids (Haataja et al., 1994). In addition, *Streptococcus suis* and *Mycoplasma pneumoniae* bind to terminal Neu5Acα2-3Gal structures present on erythrocytes (Liukkonen et al., 1992; Loomes et al., 1984), and the receptor saccharide for cholera toxin produced by *Vibrio cholerae* is the pentasaccharide Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc (Merritt et al., 1994). Specific strains of *Pseudomonas aeruginosa*, a serious opportunistic pathogen with several targets, including skin of burn injuries, damaged cornea, and lung epithelium in patients with cystic fibrosis, bind specifically to carbohydrate sequence GalNAcβ1-4Gal (Lee et al., 1994; Sheth et al., 1994). Bacteria can also recognize internal sugars in addition to terminal ones. For example, *Streptococcus pneumoniae* interacts specifically not only with the pentasaccharide NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc, but also with the corresponding trisaccharide GlcNAcβ1-3Galβ1-4Glc and the tetrasaccharide Galβ1-4GlcNAcβ1-3Galβ1-4Glc (Barthelson et al., 1998; Idanpään-Heikkilä et al., 1997).

Escherichia coli is a normal member of the intestinal flora. However, opportunistic infections and rogue strains pose a significant risk to human health. Among susceptible individuals *E. coli* is a cause of traveler's diarrhea, sepsis, urinary tract infections, and newborn meningitis. *E. coli* can possess numerous types of fimbriae, which are classified according to their carbohydrate binding specificities (Sokurenko et al., 1997). These include type 1 fimbriated strains specific for mannose, neural S fimbriated strains specific for NeuAcα2-3Galβ1-3GalNAc, and strains carrying P fimbriae specific for galabiose (Galα1-4Gal). In addition, heat-labile enterotoxin from *E. coli* bind to terminal LacNAc units (Karlsson et al., 1996) and Shiga toxins produced by *E. coli* recognizes Galα1-4Galβ1-4Glc (Lingwood et al., 1987).

2.2.2 Viruses

Influenza is one of the most common viral infectious diseases that is inadequately controlled by modern medicine. The influenza virus glycoprotein hemagglutinin mediates viral adhesion by binding to the cell surface sialic acid residues. The carbohydrate binding specificity of the virus is dependent of the species of origin: Human viruses bind to glycans with terminal Neu5Acα2-6Gal structures (Connor et al., 1994; Rogers and Paulson, 1983), whereas, viruses of avian origin preferentially bind to glycans with terminal Neu5Acα2-3Gal structures (Ha et al., 2001). The next residue of the carbohydrate chain, namely GlcNAc, also takes a considerable part in binding, so that the receptor for all true human A and B influenza strains is actually the trisaccharide Neu5Acα2-6Galβ1-4GlcNAc (Gambaryan et al., 1997). Many other viruses including animal rotavirus, Sendai, and polyomavirus also bind to sialic acid containing residues (Karlsson, 1995). However, both animal and human rotaviruses have also been shown to bind to non-acid glycolipids (Mendez et al., 1993). Dengue flavivirus, the causative agent of dengue hemorrhagic fever, and herpes simplex virus bind to heparan sulphate and heparin, respectively (Chen et al., 1997; Rostand and Esko, 1997).

2.3 Multivalency in biological processes

Non-covalent interactions are essential for all processes that take place in living organisms. In comparison to covalent bonds, these transient interactions are weak. However, they are elegantly selective and offer a dynamic framework for adaptation to the environment and self-regulations. The strength and specificity required for recognition in biological processes is high, but the association constants for monovalent carbohydrate-protein interactions are often weak ($K_a=10^{3-4}M^{-1}$) (Bundle and Young, 1992; Kiessling and Pohl, 1996; Lee and Lee, 1995; Mammen et al., 1998; Roy et al., 1996). Consequently, many carbohydrate-protein interactions rely on the amplification of low-affinity interactions by presenting binding epitopes in a multivalent fashion. The multivalent nature of lectin-oligosaccharide interactions allows numerous low-affinity binding events to take place at the same time, resulting in high overall avidity (Dam et al., 2000; Gupta et al., 1996; Hughes, 2001). The interaction of epitopes displayed in a multivalent array with proteins possessing multiple carbohydrate binding sites can result in the formation of several simultaneous binding events with an observed binding affinity greater than the sum of its constituent binding events, a phenomenon known as the cluster glycoside effect. There are several physiological advantages conferred by multivalent binding. First, the kinetics exhibited by multivalent binding events are probably critical for biological systems, for example exhibiting greater reversibility in the presence of competing ligands (Rao et al., 1998). Secondly, multiple weak interactions are less prone to entrap cells in unproductive binding events. Thirdly, low affinity multivalent interactions are expected to be more resistant to shear stress, encountered for instance when cells interact in the bloodstream (Alon et al., 1995). Finally, multivalent interactions have been shown to be highly versatile and specific (Liang et al., 1997; Mortell et al., 1996; Weatherman et al., 1996), and the binding can be modulated by altering saccharide residue spacing or by changing the residues themselves.

Biological events using the multivalency effect include events such as cell-cell communication, tissue differentiation, protein targeting and clearance, and host interaction with pathogens and their secreted toxins (Lundquist and Toone, 2002; Mammen et al., 1998). For example, monomeric affinity of shiga-like toxin is in millimolar range, whereas, the multivalent attachment to the cell has a K_d of ~1 nM (Kitov et al., 2000). Dimerization of L-selectin increases leukocyte adhesion to clustered oligosaccharide residues (Dwir et al., 2002; Ramachandran et al., 2001), and rolling of leukocyte cells in sites of chronic and acute inflammation entails recurring multivalent binding events with rapid binding and release (Dwir et al., 2003; Schwarz and Alon, 2004). The serum mannose-binding protein preferentially recognizes clustered mannose ligands (Lee and Lee, 2000). In addition, multivalent binding of many beneficial and pathogenic strains of bacteria (Hooper and Gordon, 2001; Karlsson, 1995; Schengrund, 2003) is believed to permit colonization by providing resistance to the flow of extracellular fluids (e.g. gastric juices, mucus, etc.).

2.4 Synthetic multivalent neoglycoconjugates as modulators of biological processes

Synthetic multivalent ligands can be divided into two distinct groups of molecules: (1) to inhibitors, in which the multivalent molecule prevents receptor-ligand binding; and (2) to effectors, in which the multivalent molecule induces a cellular response. Multivalent inhibitors can interfere with a broad range of interactions including those concerning cell-extracellular matrix, cell-cell, and cell-pathogen binding. Multivalent effectors, on the other hand, can be used to understand, manipulate, and dissect signal transduction pathways. The range of responses include cellular activation, differentiation, and migration, in particular those initiated by multiple receptor-ligand contacts at the cell surface.

Multivalent inhibitors. The high functional affinities of multivalent ligands have encouraged attempts to develop potent inhibitors of cell-surface binding events. As an example, L-selectin, present on leukocytes, mediates the initial stages on recruitment to the endothelium during inflammation through multivalent binding to its ligand. In addition, pathogenic organisms, including certain pathogenic viruses, bacteria and yeasts, utilize these mechanisms to adhere to mammalian cells and tissues. A deeper understanding of these interactions at the molecular level will enable the development of novel effective and highly selective carbohydrate based pharmaceuticals. Preventing the binding of influenza virus hemagglutinin to host cells was among the first applications of multivalent inhibitors created (Gamian et al., 1991; Glick and Knowles, 1991; Matrosovich et al., 1990; Sabesan et al., 1991; Spaltenstein and Whitesides, 1991). More recently, multivalent sialidase inhibitors build on polyglutamic acid having more potent efficacy in comparison to a monomeric inhibitor *in vivo* and enhanced antiviral activity against influenza virus have been described (Honda et al., 2002). Efforts to create potent multivalent inhibitors, that could function as anti-inflammatory agents, based on the multivalent nature of the naturally-occurring L-selectin ligands, are under study. Several monovalent and multivalent L-selectin ligands have been described (Gordon et al., 2000; Nishida et al., 2000; Sanders et al., 1999; Simanek et al., 1998; Toppila et al., 1999; Turunen et al., 1995) including the study describing the multivalent sLe^x conjugates based on polylactosamine type scaffold, which have been shown to bind selectins with high affinity (Renkonen et al., 1997). In addition, multivalent polymers based on polylysine backbone with sLe^x-mimetics as ligands have been shown to act as superior inhibitors of E-selectin-dependent leukocyte rolling *in vivo* when compared to monovalent antagonists (Ali et al., 2004).

Multivalent effectors. Multivalent binding events are also valuable for eliciting rather than inhibiting biological responses. This role is especially appreciated in the context of generating synthetic carbohydrate-based vaccines. Multiple copies of an antigen on a carrier protein or other suitable scaffold can induce potent immune responses. Examples include glycoconjugate vaccines based on the cell surface carbohydrates of a microorganism, which are proving to be most effective in generating protective immune responses to prevent a wide range of diseases. Such glycoconjugates already licensed include vaccines against *Neisseria meningitidis*, *Haemophilus influenzae* type b, and *Streptococcus pneumoniae* [see (Jones, 2005) for review on

the subject]. Another effort is directed at generation of synthetic anti-cancer vaccines and already complex glycoconjugates with promising anti-cancer activities have been synthesized (Danishefsky and Allen, 2000). In their investigations multivalent conjugates with synthetic oligosaccharides coupled to carrier protein were successful at eliciting both humoral and cellular responses (IgM and IgG antibodies).

2.4.1 Different classes of multivalent model systems

Several classes of model systems have been developed to study multivalent protein-carbohydrate interactions. To illustrate the range of multivalent scaffolds available when designing a glycoconjugate a few examples will be described here. These model systems include cyclodextrins and calixarenes, glycoclusters, dendrimers and dendrons, neoglycoproteins, and glycopolymers (Figure 1).

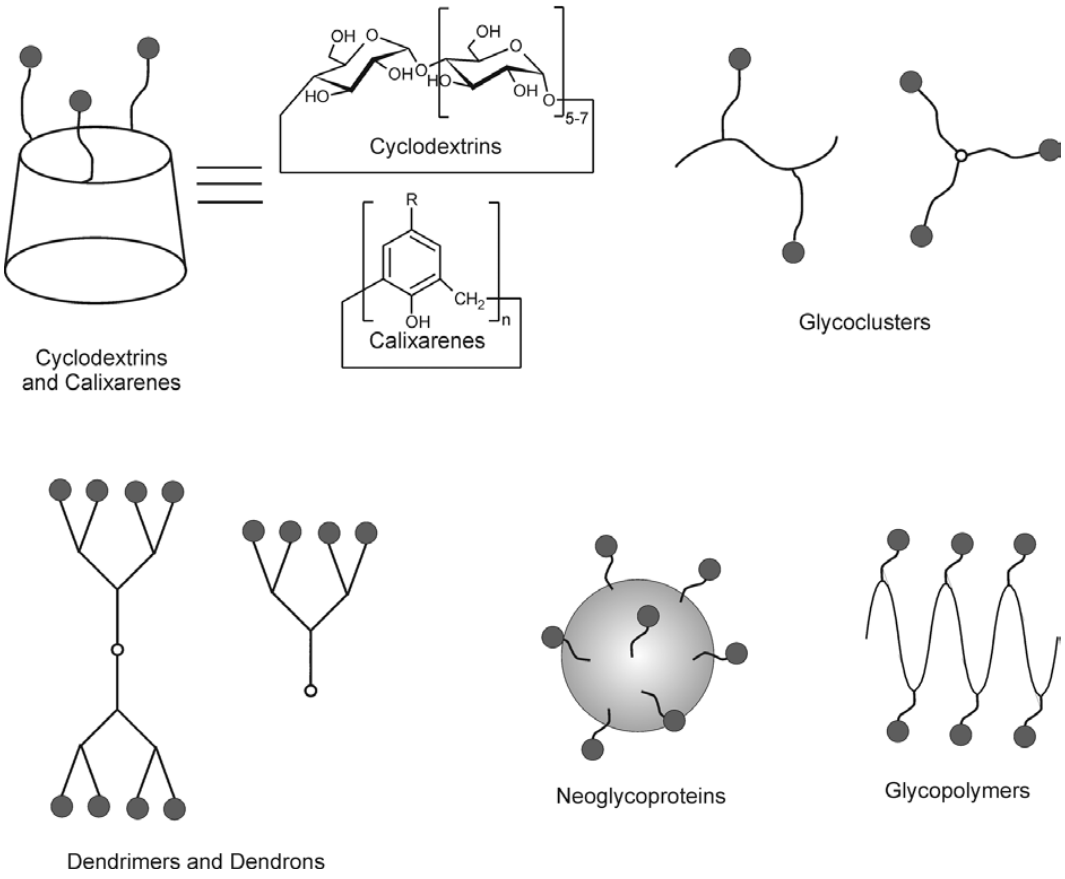


Figure 1. Schematic presentation of multivalent glycoconjugates based on various scaffold structures. The oligosaccharide ligands are presented as grey spheres.

Cyclodextrins and calixarenes. Cyclodextrins are a family of cyclic oligosaccharides containing 6, 7, or 8 α1-4-linked glucopyranosyl units resulting in α-, β-, or γ-cyclodextrin, respectively (see section 2.7. for more details). Synthesis of several neoglycoconjugates based on cyclodextrins with varying chemical linker specificity and length with one or more carbohydrates attached have been described previously [e.g. (Andre et al., 2004; Fulton and Stoddart, 2001; Furuike et al., 2000; Houseman and Mrksich, 2002; Matsuda et al., 1997; Ortiz Mellet et al., 2002)]. Calixarenes in return are cyclic oligomers of substituted aromatic rings commonly derived from the base-induced reaction of phenol with formaldehyde. They are commonly composed of four, six, or eight arene units. Variable size of the cavity, tunable conformation, selective and multiple derivatization, and the ease of preparation in large quantities makes this class of molecules an intriguing choice as multivalent scaffold. Several calixarenes modified with carbohydrate ligands have been described [e.g. (Dondoni et al., 2002; Dondoni et al., 1997; Fujimoto et al., 2000; Fulton and Stoddart, 2001; Roy and Kim, 1999)]. They have also been modified as site-directed drug delivery systems using galactosides to deliver a fluorescent dye to the surface of rat hepatoma cells (Fujimoto et al., 2000).

Glycoclusters. Glycoclusters represent a large group of molecules presenting two to five carbohydrate ligands on a small scaffold (Zanini and Roy, 1998). Scaffolds in this group include butane and pentane (Uchiyama et al., 1995), quaternary carbon (Kretzschmar et al., 1995), ethylene glycol tether (Wittmann et al., 1998), aromatic ligands (Miyauchi et al., 1997), and carbohydrates and peptides (Baisch and Öhrlein, 1996; Kitov et al., 2000; Uchiyama et al., 1995). Studies done with this group of molecules illustrate the importance of ligand to ligand spacing and conformational flexibility in multivalent interactions. For example, a tailored multivalent ligand build on carbohydrate scaffold has been prepared using the crystal structure of the Shiga-like toxin I B-subunit pentamer in complex with an analogue of its carbohydrate receptor as a model, resulting in ligand with subnanomolar inhibitory activity (Kitov et al., 2000). Consequently, the valency and nature of the scaffold molecules in this family are less important than the length of the linker between the carbohydrate ligands and the scaffold.

Dendrimers and dendrons. Dendrimers are a class of polymer structures composed of a core structure that is modified with several regularly hyperbranched units. Dendrons represent a subclass of dendrimers, in which the core structure is modified with single hyperbranched unit (Kim and Zimmerman, 1998). This family of scaffold molecules is continuously growing, with each new generation larger than the previous one. Despite their size, comparable to glycopolymers, glycodendrimers (see section 2.8 for more information on glycodendrimers) are of defined chemical character, and their synthesis, purification, and analysis usually leads to very pure compounds readily suitable for biological tests. The poor valence-dependent binding affinity enhancement toward carbohydrate-binding proteins sometimes observed for these molecules (Zanini and Roy, 1997) is though to reflect an improper geometry and spacing of the carbohydrates presented on these scaffolds (Andre et al., 1999; Lee and Lee, 1997). Other possibility is that large dendrimers and dendrons sterically prevent the cross-linking of carbohydrate-binding proteins.

Neoglycoproteins. Neoglycoproteins present one of the most widely used class of multivalent glycoconjugates. This group consists of naturally occurring proteins, which have been synthetically modified to express carbohydrate ligands. For example, neoglycoproteins constructed by reductive amination of glycans to proteins were first introduced in the early 1980s by Lee and co-workers and are still widely used today (Fadden et al., 2003; Kitov et al., 2000; Lee et al., 1984; Lee et al., 1983; Stambach and Taylor, 2003). Recent variations include attachment of biotinylated glycans to a streptavidin-alkaline phosphate complex (Blixt et al., 2003), and attachment of streptavidin-conjugated glycan to a biotinylated bovine serum album (Yamaji et al., 2003). Neoglycoproteins are suitable as carriers for various therapeutic agents, e.g. as liver targeting devices of an anti-inflammatory agent coupled to lactosaminated or mannosylated human serum albumin to hepatocytes or Kupffer cells, respectively (Franssen et al., 1993). A variety of synthetic methods for glycoprotein preparations exists providing reasonable control over valency (Davis, 1999; Roy, 1994; Roy, 1997; Yi et al., 1998).

Glycopolymers. Polymers present an attractive group of scaffold molecules because their synthetic preparation methods permit control over the density of one or more ligands on the polymer backbone and molecular weight of the molecule (Kadokawa et al., 1999; Roy, 1994; Roy, 1996a; Roy, 1997). The limitations of these neoglycopolymers are that ligands are not presented in defined environments and that the number of interactions is frequently unknown. Polymers in this group include polyacrylamide (Bovin, 1998; Fan et al., 1995; Roy et al., 1992), polylysine (Thoma et al., 1999), and polyglutamine (Zeng et al., 1998), dextran (Yoshitani and Takasaki, 2000), polysaccharides [e.g. GAGs (Sakagami et al., 2000; Soltés et al., 1999) and chitosan (Kato et al., 2001)], and polystyrene (Matsuura et al., 2000; Tsuchida et al., 1998). For example, in an *in vivo* study done in mice polyacrylamide-based glycopolymer inhibitor of influenza virus alleviated the disease symptoms, increased the survival, and decreased lesions in the mouse lungs (Gambaryan et al., 2002), suggesting that synthetic multivalent inhibitors of virus attachment can be employed for the treatment and prevention of influenza.

2.4.2 Effect of scaffold structure on function

When designing a multivalent ligand with a specific activity it is important to consider the wide variety of structural scaffolds available. In order to begin to elucidate the effect of scaffold structure on the mechanism of action, Gestwicki and co-workers evaluated the ability of a structurally diverse collection of 28 multivalent ligands to interact with the lectin concanavalin A (Gestwicki et al., 2002). The scaffolds examined in the study were from 5 different structural classes: (1) low molecular weight compounds, (2) globular proteins, (3) dendrimers, (4) defined linear polymers, and (5) polydisperse polymers. Consequently, structural parameters varied in the study included scaffold size, shape, valency, and density of binding elements. By varying ligand architecture, they were able to identify compounds that preferentially engage in selected binding modes as well as the mechanism of action preferred by the multivalent ligand. For example, the study suggested that ligands with high molecular masses are excellent inhibitors,

whereas, low molecular weight ligands are generally poor inhibitors of ConA binding. Therefore, testing diverse multivalent ligands can be used to help to illuminate the binding modes underlying the activities of specific ligand architectures.

Another example illustrating the effects of the ligand architecture on its specificity and inhibitory activity explored a series of synthetic inhibitors of hemagglutination (Reuter et al., 1999). In their study several dendritic polymeric inhibitors, including linear, linear-dendron copolymers, spheroidal, comb-branched, and dendrigraft polymers bearing Neu5Ac residues were tested for their ability to inhibit virus hemagglutination and to block infection of mammalian cells *in vitro*. The study revealed that the inhibitory potency of the multivalent ligand depended strongly on its three-dimensional structure. Similar results were also obtained using different cyclic glycopeptides to determine the biological activity using hemagglutination assay: Size and three dimensional spatial arrangement were of significant importance in the production of effective multivalent ligands (Ohta et al., 2003). In addition, effects of polymer structure on the inhibition of cholera toxin by a series of polyglutamatic acid-based glycopolymers once again illustrated the effects of variation in the linker length and density of the pendant carbohydrate ligands (Polizzotti and Kiick, 2006).

2.4.3 Designing a multivalent ligand

Empirical approach. To design effective multivalent ligands, detailed information about the native structure of the proteins that adhere to them is advantageous. However, if this information is not available an empirical approach to the development of ligands is necessary. Without knowing the optimal valency or spacing of the lectins in question, one can develop an array of different potent oligovalent carbohydrate derivates. Parameters that influence the mechanisms by which a multivalent ligand acts include the structure of the scaffold, spacers, conjugation chemistry, the identity of the binding epitopes, number of binding groups, and density of binding elements. Altering a single structural feature of a multivalent ligand, such as the arrangement of its binding sites, density of the binding epitopes, or its valency can change its activity [e.g. (Allen et al., 2001; Cairo et al., 2002; Cochran and Stern, 2000; Dintzis et al., 1976; Gestwicki et al., 2000; Kudryashov et al., 2001; Reuter et al., 1999; Roy et al., 1998; Woller and Cloninger, 2002)]. Valuable insight into the construction of compounds with defined biological activities can be provided by the collected information of ligands of various architectures. The biological and chemical relevance of many of these studies have been reviewed (Bertozzi and Kiessling, 2001; Davis, 2000; Fan et al., 2000a; Kiessling et al., 2000; Kiessling and Pohl, 1996; Koeller and Wong, 2000; Lee and Lee, 1995; Lindhorst and Welsch, 2001; Lundquist and Toone, 2002; Mammen et al., 1998; Roy et al., 1996; Wright and Usher, 2001; Yarema and Bertozzi, 1998). Screening of the biological activities of the resulting glycoconjugates with different structures can then be used to gather information about the productive display of carbohydrate residues for the system under study. By systematic ligand variation, it is possible to identify the mechanism

of action preferred by a multivalent ligand (Gestwicki et al., 2002), which can lead to potent ligands (Hansen et al., 1997; Kötter et al., 1998; Lindhorst et al., 1998).

Rational design and empirical optimization. The number of precisely known lectin structures is continuously increasing leading to the syntheses of new multivalent inhibitors based on rational design and to empirical optimization strategies of multivalent molecules previously described. Several reports show that it is possible to design ligands capable of occupying simultaneously all the carbohydrate binding sites of a lectin, provided that the precise three-dimensional structure of the oligomeric lectin is known [e.g. (Fan et al., 2000a; Fan et al., 2000b; Kitov et al., 2000; Lee et al., 1984)]. Alternatively, surprisingly small differences in the scaffold structure may be unfavorable to the conjugate's biological activity (Ohta et al., 2003). Analysis of previous reports should be useful in the design of new rational synthetic multivalent inhibitors of protein-carbohydrate interactions and/or of empirical optimization strategies. To illustrate the use of rational design and/or of empirical optimization strategies to develop potent multivalent inhibitors of protein-carbohydrate interactions a few examples will be discussed here.

The family of AB_5 bacterial toxins is characterized by a single A subunit in the center surrounded by a pentagonal arrangement of five B subunits (Merritt and Hol, 1995). This protein family includes cholera toxin, shiga toxin, shiga-like toxins, pertussis toxin, and heat-labile enterotoxin. These toxins, which are responsible for millions of deaths each year (Holmgren and Svennerholm, 1992), invade the cells by multivalent binding of the B subunit to the carbohydrate residues of gangliosides. Consequently, a rational strategy is to design a multivalent ligand that can occupy all five binding sites simultaneously. Kitov and co-workers used the crystal structure of the B_5 subunit of *Escherichia coli* Shiga-like toxin I in complex with an analogue of its carbohydrate receptor (Galα1-4Galβ1-4Glcβ-*O*-Cer) (Karmali et al., 1985; Ling et al., 1998) to design a decavalent carbohydrate ligand (named STARFISH) (Kitov et al., 2000). This STARFISH ligand exhibited a subnanomolar inhibitory activity. The *in vitro* inhibitory activity was 1-10-million-fold higher than that of the trisaccharide alone, which lies within the range desired for an anti-adhesive therapeutic agent. Variants of this design for multivalent inhibitors of Shiga-like toxin and cholera toxin imply that the spacing of glycan ligands attached to the scaffold is more important than the actual core structure (Mulvey et al., 2003; Nishikawa et al., 2002; Zhang et al., 2002). Fan and co-workers in return designed pentavalent ligands for the *E. coli* heat-labile enterotoxin (Fan et al., 2000b). The distance between nonadjacent binding sites is known from the available atomic structural data (Sixma et al., 1992). They used this data as a basis for their structure-based design of several pentavalent molecules in which the capping carbohydrates were attached to the fivefold symmetry center using linkers of different length. Again the structure-based design of ligands produced a potent antagonist of heat-labile enterotoxin, a pentavalent ligand 10^5 times more active than the corresponding monovalent derivative.

Influenza virus infection is initiated by the binding of hemagglutinin to sialic acid-containing oligosaccharides on the host cell surfaces. Hemagglutinin is a trimeric protein with each subunit containing one ligand binding site. In order to fashion a tailored blocker of influenza virus hemagglutinin Ohta and co-workers created cyclic peptide scaffolds containing tridentate oligosaccharide units (Ohta et al., 2003), a design based on the three-dimensional structure of hemagglutinin (Weis et al., 1988; Wilson et al., 1981). They found that the amino acid sequence of the peptides had a marked influence on the flexibility and direction of ligand presentation.

An optimization of chemotactic response of *E. coli* has been achieved by tuning the valency of galactose-functionalized oligomers (Gestwicki et al., 2000). Consequently, a second generation of multivalent chemoattractants designed using molecular modeling resulted in ligands with a responsive threshold concentrations 10-fold lower than those required with the original displays (Gestwicki et al., 2001).

2.5 Carbohydrates as targeting vehicles

Another exciting area for carbohydrate conjugates is their use as specific targeting vehicles. The concept of targetable glycosylated drug delivery systems employs the extensive involvement of oligosaccharide in various recognition processes (Monsigny et al., 1988; Ouchi and Ohya, 1994; Wadhwa and Rice, 1995). Targeted drug delivery system can be used to promote specific accumulation of bioactive molecules with low therapeutic index into disease sites, which results in enhanced therapeutic efficacy and reduced systemic side effects. Targeting system is achieved by conjugating therapeutic agents to a macromolecular carrier functionalized to express carbohydrate epitopes specifically recognized by cell receptors (Matthews et al., 1996; Takakura and Hashida, 1996). For example, liver cells can be targeted with structures expressing terminal galactose units (Neufeld and Ashwell, 1979; Wall et al., 1980), which are specifically recognized by asialoglycoprotein receptors on liver cells (Ashwell and Harford, 1982; Ashwell and Morell, 1974). In addition, receptors present on Kupffer cells specific for mannose residues can induce receptor mediated endocytosis (Dragsten et al., 1987; Fallon and Schwartz, 1989; Franssen et al., 1993; Jansen et al., 1991; Seymour, 1994; Seymour et al., 1991), L1210 leukemia cells have fucose specific receptors at their cell surface (Monsigny et al., 1984), and mannosyl/fucosyl receptors present at the surface on macrophages are all attractive choices for targeting purposes (Duffels et al., 2000; Rice, 1997).

Due to their toxicity and high pharmacological activity, antiviral and anticancer agents are excellent candidates for utilization of drug targeting systems. Models supporting this idea include effective accumulation of cisplatin-dextran complex containing tetravalent galactose conjugates to human hepatoma cells *in vitro* (Ohya et al., 2001), targeting of β-CD-polymannoside dendrimer carrying docetaxel as a guest molecule to macrophages *in vitro*, and targeting of glycosylated HPMA copolymer-doxorubicin into colon cancer cells *in vitro* (David et al., 2004). In addition, carrier-mediated targeting also holds great promise in delivering

synthetic oligonucleotide drugs [e.g. (Duff et al., 2000; Maier et al., 2003; Zanta et al., 1997) Unmodified oligonucleotides suffer from poor cellular uptake and lack stability against enzymatic degradation. Attachment to a multivalent carbohydrate cluster significantly improves the cellular uptake of oligonucleotides and thus the therapeutic efficacy of antisense oligonucleotides *in vivo*.

However, the *in vivo* performance of the therapeutic system, i.e. the drug release and the pharmacokinetic profile may depend severely on the bioconjugated moieties such as targeting agents and drug. In a recent study (Cavallaro et al., 2004) two macromolecular conjugates build on polyaspartamide scaffold with either galactose or mannose as targeting sugars and as drug components either acyclovir or ganciclovir (Colla et al., 1983; Fan-Havard et al., 1989; Faulds and Heel, 1990; O'Brien and Campoli-Richards, 1989; Schaeffer et al., 1978) were shown to display significant difference in their *in vivo* behavior. In particular, the mannosyl derivatization promoted the rapid disposition of the conjugate in the liver, while the galactosyl derivatization showed no difference to disposition in the liver of that of a naked polyaspartamide polymer. It was concluded that slight physico-chemical differences of the conjugates (architecture, structure, solubility, etc.) may reflect in this impressive biological difference with particular reference the different quantitative composition of the targeting sugar moieties. Consequently, to obtain successful therapeutic targeting systems things to be considered include the appropriate choice of the components and the proper chemical synthesis process, which allows derivatives with the required characteristics to be obtained.

2.6 Glycosaminoglycans

Glycosaminoglycans (GAGs) are linear, polydisperse, acidic polysaccharides, which are often covalently linked to a protein core to form proteoglycans (PGs). GAG polysaccharides extend from a protein core in a brush-like composition. To core proteins, from 10 kDa to >500 kDa in size, the number of GAG chains attached ranges from 1 up to >100 (Kjellen and Lindahl, 1991). The most common GAGs are chondroitin sulphate (CS), dermatan sulphate (DS), heparin (HP), heparan sulphate (HS), keratan sulphate (KS), and hyaluronic acid (HA). Except for HA, all GAGs are biosynthesized as PGs. PGs occur in the membranes of all animal tissues, extracellularly in the matrix, or intracellularly in certain cells (usually in secretory granules). Chondroitin sulphate is the most common GAG in the body, and occurs in both soft and skeletal tissues. It is also found in neural tissues (Margolis and Margolis, 1997) and on cell surfaces (Fransson, 1987). Vertebrates utilize glycosaminoglycans in structural, adhesion, recognition, and signaling roles. GAGs are also increasingly thought to have a role in regulating a wide variety of biological processes, including the inflammatory response and tumor cell metastasis.

Glycosaminoglycans are synthesized with alternating hexosamine (either GalNAc or GlcNAc) and uronic acid (GlcA or IdoA) residues, in which the set of monosaccharide units gives rise to a number of complex sequences by variable substitution with *N*-sulphate, *O*-sulphate, and *N*-acetyl groups (Table 1). Considerable variations occur in the positions of both IdoA and sulphation. HA lacks any sulphate groups, but the rest of the glycosaminoglycans contain sulphates.

Table 1. Glycosaminoglycans.

Glycosaminoglycan	Synonym	Structure of main repeating disaccharide
Chondroitin-4-sulphate	Chondroitin sulfate A	-3GalNAc(4-OSO_3^-)β1-4GlcAβ1-
Chondroitin-6-sulphate	Chondroitin sulfate C	-3GalNAc(6-OSO_3^-)β1-4GlcAβ1-
Dermatan sulphate	Chondroitin sulfate B	-3GalNAc(4-OSO_3^-)β1-4IdoAα1-
Heparin		-4GlcNSO_3^-(6-OSO_3^-)β1-4IdoA(2-OSO_3^-)α1-
Heparan sulphate		-4GlcNAcα1-4GlcAβ1-
Hyaluronan		-3GlcNAcβ1-4GlcAβ1-
Keratan sulphate		-4GlcNAc(6-OSO_3^-)β1-3Galβ1-

Hyaluronan is the simplest GAG consisting of $[\text{-GlcNAc}\beta1\text{-}4\text{GlcA}\beta1\text{-}3]_n$ disaccharide units, where *n* can be up to 25000. Keratan sulphate oligo- or polysaccharides contain repeating sulphated $[\text{-GlcNAc}\beta1\text{-}3\text{Gal}\beta1\text{-}4]_n$ disaccharides. It is generally of relatively low molecular weight, with nearly equal amounts of sulpho groups on the 6-positions of GlcNAc and Gal. Chondroitin sulphate consists of repeating $[\text{-GalNAcS}\beta1\text{-}4\text{GlcA}\beta1\text{-}3]_n$ units polymerized into long chains. The size of the CS chain varies greatly, with an average size of 20 kDa (~40 disaccharides) found for cartilage proteoglycan (Iozzo, 1985). Two common chondroitin sulphates are chondroitin-4-sulphate or chondroitin sulphate type A (CSA) and chondroitin-6-sulphate or chondroitin sulphate type C (CSC). CSA has 90% of the GalNAc residues sulphated at the 4-position with 10% at the 6-position, and CSC has 90% of the GalNAc residues sulphated at the 6-position with 10% sulphated at the 4-position. Dermatan sulphate (chondroitin sulphate B) is a polydisperse microheterogenous copolymer of GalNAc and primarily IdoA acid $[\text{-GalNAc}\beta1\text{-}4\text{IdoA}\alpha1\text{-}3]_n$. *O*-sulpho groups are generally found on 4-position of GalNAc and infrequently on the 6-position of GalNAc and the 2-position of IdoA. Heparan sulfate (HS)/heparin are polydisperse, highly sulphated, linear polysaccharides made up of GlcNAcα1-4GlcA(β1-4)/IdoA(α1-4) repeating units. While structurally similar, HS and heparin have different ratios of *O*-sulpho and *N*-acetyl groups.

2.6.1 Pharmaceutical use of GAGs

Many pharmaceutical products based on GAGs themselves have been previously prepared of which the most well known is heparin, which is utilized therapeutically as an antithrombotic drug and anticoagulant and which is readily available in good quantities. Over 33 metric tons of heparin is manufactured worldwide each year (e.g. of bovine and porcine origin), amount representing over 500 million doses (Linhardt, 1991). In addition to its best known application, heparin also has a wide variety of other activities (Table 2).

Table 2. Current and potential therapeutic applications of heparin and heparin analogues.

Application	Reference
Anticoagulant / antithrombotic	(Frangos et al., 2000)
Complement inhibitors	(Edens et al., 1993)
Antiatherosclerotics	(Jaeger, 2001)
Anti-inflammatory	(Fath et al., 1998)
Anticancer agents	(Hettiarachchi et al., 1999)
Antiviral agents	(Chen et al., 1997)
Antiangiogenic agents	(Smorenburg and Van Noorden, 2001)
Anti-Alzheimer agents	(Dudas et al., 2002)

Other GAGs have also found their use in therapeutic applications. Like HS, dermatan sulfate is also anticoagulant, although of lower potency than heparin (Tollefsen et al., 1982; Tollefsen et al., 1983). DS has been useful in the development of artificial tissues (Miwa and Matsuda, 1994) and CS has been used as a component of artificial skin (Osborne et al., 1999). CS (e.g. of bovine origin) has also been used as a therapeutic medicine for the chronic inflammatory diseases such as rheumatoid arthritis, cirrhosis, and chronic photo damage (Conte et al., 1995; Fawthrop et al., 1997; Gressner et al., 1980). In addition, CS from human milk has been found to inhibit HIV glycoprotein gp120 binding to its host cell CD4 receptor, *in vitro* (Konlee, 1998). Hyaluronic acid has been used in a wide array of medical applications, for example, to treat patients with osteoarthritis (Manek and Lane, 2000; Rosier and O'Keefe, 2000), as a cloth-like material to prevent adhesions after surgery (Panay and Lower, 1999), and as an aerosol to prevent elastase-mediated injury in pulmonary emphysema (Cantor et al., 1998).

2.7 Cyclodextrins

Cyclodextrins (CDs) are formed (Bender, 1986) during the degradation of the linear amylose fraction of starch by cyclodextrin glucosyltransferases, isolated from the bacterium *Bacillus macerans*, yielding a family of cyclic α-(1-4)-linked oligosaccharides consisting mainly of six, seven or eight D-glucose units resulting in α-, β- or γ-cyclodextrin, respectively (Figure 2). This process also forms the basis for the production of CDs in industrial scale (Schmidt, 1985). The cyclic arrangement of CDs creates a hydrophilic exterior and a hydrophobic "open cavity" region (Stella and Rajewski, 1997). Cyclodextrins resemble truncated, hollow cones in which all glucose units adopt the commonly observed $^4C_1$-chair conformation. Primary hydroxyl groups (O6) of cyclodextrins are on the narrow side of the cone, and the secondary hydroxyl groups (O2, O3) are on the wider side of the cone (Figure 2) forming intramolecular, interglucose O2···O3' hydrogen bonds stabilizing the conformation of the molecule (Czugler et al., 1981; Harata, 1986; Mentzafos et al., 1991; Saenger, 1984).

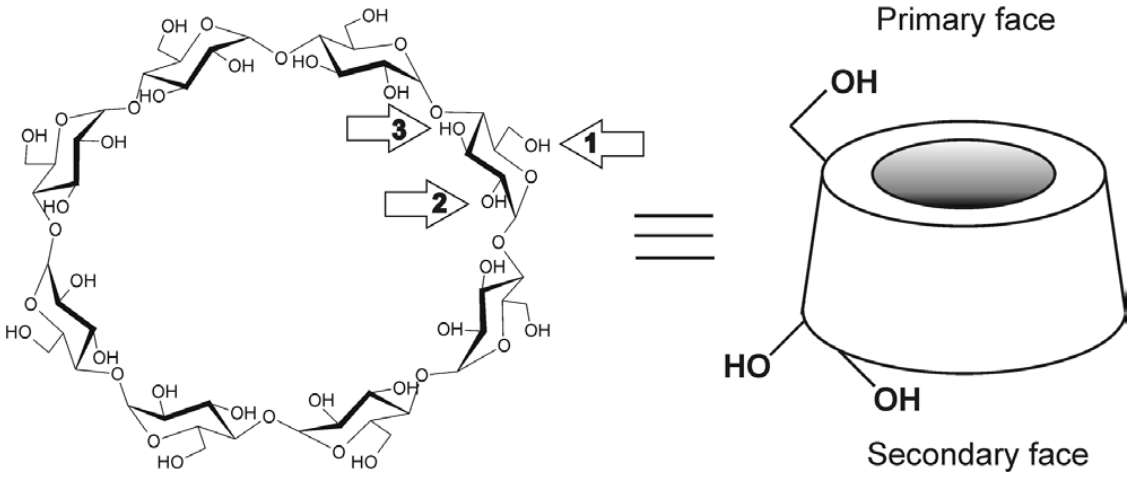


Figure 2. Structural and a truncated cone presentation of γ-cyclodextrin. The wider side formed by the secondary 2- and 3-hydroxyl groups and the narrower side formed by the primary 6-hydroxyl groups are indicated.

Cyclodextrin's hydrophobic cavity has the remarkable property of forming inclusion complexes both in solution and in the solid state with a large variety of guest molecules and ions that have a suitable size and shape to be fully or partially accommodated (Bender and Komiyama, 1978; Saenger, 1980; Saenger, 1984; Szejtli, 1988; Uekama and Irie, 1987). This feature is utilized in food and pharmaceutical industries (Frömming and Szejtli, 1994) to encapsulate compounds that have a low solubility in water, are sensitive to the environment, or are volatile and have more favorable properties as CD inclusion complexes. The number of glucose molecules determines the size of the central cavity capable of forming inclusion complexes with hydrophobic

molecules. β-CD is currently the most widely available and the CD of lowest cost. Relative to β-CD, α-CD has a higher water solubility (14.5 versus 1.9 g/ml at 25°C) and a smaller cavity (5.3 versus 6.6 Å). Similarly, γ-CD has a higher water solubility (23.2 g/ml at 25°C) and a larger (8.4 Å) and a more flexible cavity (Szente and Szejtli, 1999).

Selective chemical modification of the hydroxyl groups in order to prepare new compounds with specific properties is a challenge due to large number of hydroxyl groups. Numerous mono- to persubstituted CDs have been synthesized (Khan et al., 1998) to introduce catalytic activity or to improve the inclusion selectivity of the host. Modification of the hydroxyl groups by acyl, hydroxyalkyl, or longer alkyl groups decreases the solubility of the β-CDs, but their solubility may increase again in the presence of a guest molecules (Lindberg et al., 1991). In addition, their host/guest forming capability has been used to create specific drug delivery systems. Cyclodextrins are able in alleviating the undesirable properties of drug molecules through the formation of inclusion complexes. Attachment of bio-recognizable saccharides onto CDs specific site delivery of pharmacologically active compounds has been faced (Benito et al., 2004; de Robertis et al., 1994; Kassab et al., 1997; Lainé et al., 1995; Leray et al., 1995; Uekama et al., 1998). Finally, their primary and secondary hydroxyl groups make them ideal candidates as scaffolds to create multivalent glycoconjugates [e.g. (Andre et al., 2004; Fulton and Stoddart, 2001; Furuike et al., 2000; Matsuda et al., 1997; Ortiz Mellet et al., 2002)] and the multivalency effect of CD-carbohydrate glycoconjugates has been demonstrated in several studies (Andre et al., 2004; Furuike et al., 2000; Ichikawa et al., 2000).

2.8 Glycodendrimers

Dendrimers are fully synthetic macromolecules comprised of perfectly branched repeat units in layers (with each layer synthesized depicted as a new generation) emanating radially from a central core (see also Figure 6). There are two defined methods of dendrimer synthesis: The molecule can be assembled from the core to the periphery using a divergent synthesis method or from the outside to termination at the core using a convergent synthesis method. Dendrimers are defined by their three components: A central core, the branches (an interior dedritic structure), and a surface that can contain a variety of substituents. Dendrimers are globular, extremely well-defined synthetic polymers with a number of characteristics, which make them useful in biological systems.

Glycodendrimers (Roy et al., 1993) are a relatively novel class of glycoconjugates, which have been proposed to afford a better understanding of multiple protein-carbohydrate interactions at a molecular level (Andre et al., 1999; Zanini and Roy, 1996; Zanini and Roy, 1998). Glycodendrimers are generally classified in three different categories: (1) carbohydrate-coated; (2) carbohydrate-based; and (3) carbohydrate-centered structures. Here, only glycodendrimers belonging to the first category will be discussed and the interested reader is referred to a recent review for more detail (Turnbull and Stoddart, 2002). Glycodendrimers have several surface

group carbohydrate epitopes available for multiple binding interactions. In addition, the chemical nature of the synthetic linker used to construct dendrimers and the type of glycosidic linkages used to attach oligosaccharides to scaffold can be varied. This class of glycoconjugates are structurally similar to naturally occurring multiantennary glycoproteins built on dendritic scaffolds (Archut and Vögtle, 1998; Bosman et al., 1999; Chow et al., 1998; Hawker and Frechet, 1990; Seebach et al., 1998; Smith and Diederich, 1998; Tomalia and Durst, 1993; Tomalia et al., 1990; Zeng and Zimmerman, 1997). The defined molecular architecture and nonimmunogenic nature of these molecules makes them interesting tools as multivalent glycoconjugates for the inhibition of host cell infections by pathogens, even though they are not as effective as neoglycoproteins and glycopolymers in inhibition studies.

Glycodendrimers appear to be potent inhibitors for a number of carbohydrate-protein assays *in vitro*. For example, effective ligands for cholera toxin (Thompson and Schengrund, 1997), various lectins (Dam et al., 2002; Page and Roy, 1997), human immunodeficiency virus type 1 (HIV-1) (Kensinger et al., 2004), and influenza virus (Reuter et al., 1999) have been successfully synthesized by conjugating cell surface carbohydrate ligands to spherical hyperbranched dendrimers. Systematic study exploring the behavior of Starburst dendrimers, modified to express terminal β-D-lactosyl epitopes, towards lectins with identical monosaccharide specificity, but with differential binding site orientation, concluded that the intimate details of topology in both the ligand display on different generations of core assembly and presentation of receptor binding sites determine the potential of glycodendrimers as lectin targeting device (Andre et al., 1999). Several reviews concerning the design, synthesis, and biomedical use of glycodendrimers with a thorough evaluation of a number of neoglycoconjugate types have been published (Boas and Heegaard, 2004; Lindhorst, 2001; Roy, 1996b; Röckendorf and Lindhorst, 2001; Turnbull and Stoddart, 2002). These exciting properties make this family of molecules an interesting choice in the human trials involving protection against malignancies and microbial infections.

2.9 *Helicobacter pylori*

In 1982 Barry J. Marshall and J. Robin Warren discovered the gram-negative bacterium *Helicobacter pylori* (Figure 3) and elucidated its role in peptic ulcer disease and gastritis. Before that lifestyle and stress were considered to be the major causes of peptic ulcer disease. It is now firmly established that *Helicobacter pylori* causes up to 80% of gastric ulcers and more than 90% of duodenal ulcers. In 2005 Marshall and Warren were awarded the Nobel Prize in Physiology or Medicine for their discovery: “The bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease”.

More than half of the world's population is infected with *H. pylori*. Frequency of *H. pylori* infection varies with country of origin and socioeconomic status. Prevalence of infection is thought to be 30-50% in industrialized countries and 80-90% in developing countries (Goodman and Cockburn, 2001). *H. pylori* is almost always acquired during childhood (usually before the age of 10) (De Giacomo et al., 2002) and if untreated infection is lifelong. It is not known how the bacteria are transmitted or why some patients become symptomatic while others do not. Bacterium most likely spreads from person to person through oral-oral, fecal-oral, or gastric-oral routes, while contaminated water sources are possible environmental reservoirs.

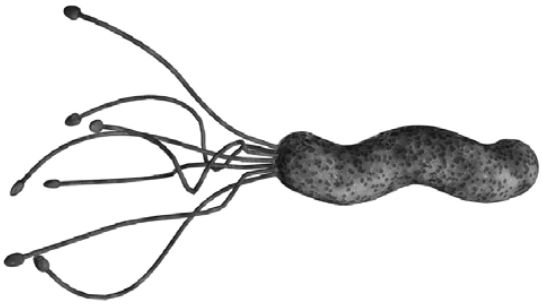


Figure 3. *Helicobacter pylori.*
H.pylori image courtesy of www.hpylori.com.au

2.9.1 Diseases associated with *Helicobacter pylori* infection

H. pylori colonizes the gastric mucosa and elicits both immune and inflammatory lifelong responses, including the release of various host-dependent and bacterial cytotoxic substances (Peterson and Graham, 1998). Most of the infected persons never suffer any symptoms related to this infection. However, *H. pylori* causes duodenal and gastric ulcers, chronic gastritis (Dunn et al., 1997; Figueiredo et al., 2005; McGee and Mobley, 1999), mucosal-associated lymphoid tissue (MALT) lymphoma ((EHPSG), 1997; NIH, 1994), and increases the risk of neoplastic diseases of the gastrointestinal tract (Kikuchi and Dore, 2005; Mueller et al., 2005; Peek and Blaser, 2002). In addition, the epidemiological data suggests that *H. pylori* gastritis is associated with gastric carcinogenesis (Plummer et al., 2004; Sipponen and Marshall, 2000) and *H. pylori* infection has been recognized as a risk factor for both intestinal and diffuse types of gastric cancer (Xue et al., 2001). Disease severity in *H. pylori*-infected hosts has been shown to depend both upon the bacterial (Blaser et al., 1995; Censini et al., 1996; Peek et al., 1995; Wu et al., 2003) and host (El-Omar et al., 2000) factors. In 1994 the bacterium itself was classified as a class I carcinogen by the World Health Organization (WHO) and International Agency for Research on Cancer Consensus Group (IARC, 1994). Interestingly, *H. pylori* may protect against the development of gastroesophageal reflux disease and diseases to which this might lead, i.e. Barrett's esophagus and esophaegeal adenocarcinoma (Blaser, 1999)

2.9.1.1 Current diagnostic and treatment methods

Diagnosis. There are two types of diagnostic tests used to detect *H. pylori* infection: Invasive and noninvasive. Invasive test include endoscopy: During endoscopy biopsy specimens of the stomach and duodenum are obtained. This enables to determine either the presence or absence of infection and the severity and extent of mucosal injury. Noninvasive tests include blood test, stool test, and urea breath test. The blood test is a serological test that measures specific IgG antibodies against *H. pylori* present in the blood. Test shows whether the individual has been infected or whether *H. pylori* was present in the past and is now cleared. The stool test uses monoclonal or polyclonal anti-*H. pylori* antibodies to detect *H. pylori* antigens. The urea breath test is the most recent diagnostic method: The patient is given either ^{13}C- or ^{14}C-labeled urea to drink, an effective enzyme in the bacteria metabolizes the urea into carbon dioxide after which labeled carbon can then be measured as CO_2 in the patient's expired breath to determine whether *H. pylori* is present. The use of upper gastrointestinal endoscopy with gastric biopsy is considered the golden standard of diagnostic tests for *H. pylori.*

Treatment. Therapy for *H. pylori* infection consists of 7 to 14 days of one or two effective antibiotics, such as amoxicillin, metronidazole, clarithromycin, or tetracycline, plus either bismuth subsalicylate, ranitidine bismuth citrate, or a proton pump inhibitor. Acid suppression by proton pump inhibitor or H2-receptor blocker in conjunction with the antibiotics may enhance the efficacy of the antibiotics against *H. pylori* at the gastric mucosal surface, helps to alleviate ulcer-related symptoms (i.e., abdominal pain, nausea), and helps the healing gastric mucosal inflammation. However, eradication is not always successful (in adults the greatest rates of bacterial eradication is >80%) and harmful side effects of these drugs may be encountered. Moreover, the current antibiotics based treatment is directly connected to risks of emergence of antibiotic resistant strains and eradication of *H. pylori* using this method is not sensible in large populations (Van Der Wouden et al., 2000; Wu et al., 2005). Therefore, nonantibiotic agents that are both safe and effective are required. An alternative way of treatment based on the oral use of anti-adhesion molecules is under discussion (Karlsson, 2000).

2.9.2 Attachment of *Helicobacter pylori* on host cell surface

Helicobacter pylori is a spiral-shaped bacterium (Figure 3) that is found adherent at the epithelial lining of the stomach or in the gastric mucous layer. *H. pylori* colonization of the stomach starts by adherence of the bacteria to the gastric epithelium. Adhesin proteins expressed on *H. pylori* outer membrane bind to specific host-cell receptors. *H. pylori* recognizes carbohydrates, probably mediating essential attachment to host cells (Karlsson, 1998; Karlsson, 2000). The most prominent bacterial adhesins described so far are the Le^b epitope binding adhesin BabA (Ilver et al., 1998) and the sialic acid-binding adhesin SabA (Mahdavi et al., 2002). Several different carbohydrate receptor candidates for *H. pylori* binding have been documented (Table 3). In addition, in a recent study the binding of *babA/sabA* double mutant *H. pylori* strain to

glycoproteins fibronectin and lactoferrin was shown to be only abolished by denaturation rather than deglycosylation indicating that the protein moiety also plays a role in receptor recognition (Walz et al., 2005). One recent hypothesis states (Roche et al., 2004) that the initial binding of *H. pylori* is achieved through binding to carbohydrate epitopes present in the normal gastric epithelium, e.g. the Le^b antigen and lactotetraosylceramide. The following inflammation leads to upregulation of the expression of sialic acid-containing glycoconjugates, which are normally present only at very low concentrations (Madrid et al., 1990; Miller-Podraza et al., 1997). This enhanced expression in turn provides novel binding sites for *H. pylori* SabA adhesin, thus contributing to the chronicity of the infection.

As illustrated by the wide range of different structures in Table 3, *H. pylori* is highly variable in its binding activities to respective carbohydrate epitopes. Expression of different specificities depends both on growth conditions and bacterial strains. Dependence of binding to elements related to lactotetraosylceramide, neolacto structures, fucosylated, and sialic acid-containing structures will be described here in more detail.

Table 3. Carbohydrate receptor candidates recognized by *H. pylori*

Name	Structure	References
Blood group antigens, e.g. Le^b	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ-R	(Boren et al., 1993)
Sialic acid-containing structures	Neu5Ac-R	(Evans et al., 1988; Hirmo et al., 1996; Miller-Podraza et al., 1997)
Galactosylceramide	Galβ1-Cer	(Abul-Milh et al., 2001)
Lactosylceramide	Galβ1-4Glcβ1-Cer	(Ångström et al., 1998)
Lactotetraosylceramide	Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-Cer	(Teneberg et al., 2002)
Neolacto structures	R_1-Galβ1-4GlcNAcβ1-3Galβ1-4Glc-R_2	(Miller-Podraza et al., 2005)
Sulphatide	SO_3-Galβ1-Cer	(Saitoh et al., 1991)
Ganglio structures	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer GalNAcβ1-4Galβ1-4Glcβ1-Cer	(Lingwood et al., 1992)

Lacto-based structures. A study using glycosphingolipids structurally related to lactotetraosyl-ceramide investigated for their *H. pylori* binding activity illustrated that all the substitutions tested abolished the binding of the only active glycosphingolipid, lactotetraosylceramide (Teneberg et al., 2002). Particularly, the terminal disaccharide Galβ1-3GlcNAcβ1-3 with an intact acetoamino group was evaluated to constitute as the binding epitope. Moreover, binding to lactotetraosylceramide was also achieved with strains devoid of Le^b binding activity indicating that the *H. pylori* binding to these two epitope structures is not due to cross-binding but represents two separate binding specificities.

Neolacto-based structures. In a another study *H. pylori* binding to neolacto glycolipids and various glycolipids with related structures was investigated (Miller-Podraza et al., 2005). The tetra-, penta-, and hexaglycosylceramides were all shown to bind *H. pylori* with pentaglycosylceramide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer being the strongest binder. The terminal trisaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3 was indicated as the sequence required for maximal activity. Interestingly, the terminal GlcNAcβ of pentaglycosylceramide could be exchanged for either Galα3, GalNAcβ3, or GalNAcα3 without losing activity. However, molecular modeling studies indicated that *H. pylori* binding to these four molecules are a result of molecular mimicry rather than being due to separate specificities.

Fucosylated structures. *H. pylori* binding to fucosylated structures Le^b [Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc] and H-1 (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc) is restricted to the terminally fucosylated lacto series type 1 chains (Boren et al., 1993). In addition, the branched fucose residue on the Le^b chain is important for optimal receptor-bacterium interaction as indicated by weaker binding activity of the H-1 antigen (Boren et al., 1993). The terminal structure Fucα1-2Gal has been shown sufficient for binding of *H. pylori* although significantly stronger binding has been observed using the terminal trisaccharide Fucα1-2Galβ1-3GlcNAc (Walz et al., 2005). Moreover, Le^a (Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc) lacking the terminal fucose shows no binding activity against *H. pylori* (Boren et al., 1993).

Sialic acid-containing structures. The structural requirements for binding of *H. pylori* to complex gangliosides has been investigated in several studies. *H. pylori* strains bind to *N*-acetyllactosamine-based gangliosides (with preference for Galβ1-4GlcNAc over Galβ1-3GlcNAc in the core structure) with α3-linked Neu5Ac, while terminal NeuAcα6, NeuAcα8NeuAcα3, or NeuGcα3 are not recognized (Evans et al., 1988; Hirmo et al., 1996; Johansson et al., 1999; Johansson and Miller-Podraza, 1998; Miller-Podraza et al., 2004; Miller-Podraza et al., 1997; Roche et al., 2004; Roche et al., 2001). In addition, fucose substitution of the *N*-acetyllactosamine core chain, the branches of the carbohydrate chains, and length of the carbohydrate chain have been indicated as factors affecting binding affinity (Roche et al., 2004). Studies done to investigate the dependence of different parts of the carbohydrate epitope, trisaccharide Neu5Acα2-3Galβ1-4GlcNAc, revealed an important role for Neu5Ac and its carboxyl and glycerol side chains and *N*-acetoamino group (Johansson et al., 2005; Miller-Podraza et al., 2004). Moreover, parts of Gal seems to be necessary, whereas, GlcNAc appears to have a secondary role serving as a guiding carrier for the ending epitope (Johansson et al., 2005).

3 AIMS OF THE STUDY

Adhesion of bacteria to host cells and tissues is a prerequisite for most infections to occur. In various cases, this is initiated by bacterial surface lectins that bind to complementary carbohydrates structures on the surface of the host cells and tissues. Consequently, carbohydrate based anti-adhesion therapy presents a highly promising approach for combating bacteria: Suitable carbohydrate structures prevent the adhesion of the bacteria to host cells, or detach them at the early stages of infection. It is however generally accepted that to augment the activity of the weak individual protein-carbohydrate interactions infecting organisms engage in multipoint attachment. Based on same concept effective anti-adhesives should be constructed by coupling several bioactive carbohydrate drug units to a single backbone carrier (multivalent presentation of carbohydrates).

The aims of the present study were:

1. To synthesize multivalent neoglycoconjugates with varying linker chemistry on a γ-cyclodextrin backbone (**I**, **II**, and unpublished results).

2. To synthesize multivalent neoglycoconjugates with varying linker chemistry on a chondroitin oligomer backbone (**I**, **II**).

3. To synthesize multivalent neoglycoconjugates on a dendrimer scaffold (unpublished results).

4. To synthesize and analyze the binding activity of an array of novel neolacto based carbohydrate structures towards *Helicobacter pylori* (**III**).

4 MATERIALS AND METHODS

4.1 Commercial oligosaccharides (I-III)

LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) (**I-III**) and GnLacNAcLac (GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc) (**I**) were from Kyowa Hakko (Japan). Lewis b hexasaccharide LNDFH I (Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc) (**I**) was purchased from IsoSep (Lund, Sweden). γ-CD (**I, II**), Chondroitin sulphate A (CSA) (from bovine trachea) (**I, II**), and para-nitrophenyl-β-glucuronide (pNP-β-GlcA) (**I**, **III**) were from Calbiochem. GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer (**III**) was from Wako Pure Chemicals Industries. GlcNAcβ1-3Man and GlcNAcβ1-6Gal (**III**) were from Sigma.

4.2 Chemical and enzymatic methods (I-III)

Table 4. Conjugation reactions used for neoglycoconjugate syntheses in the present study.

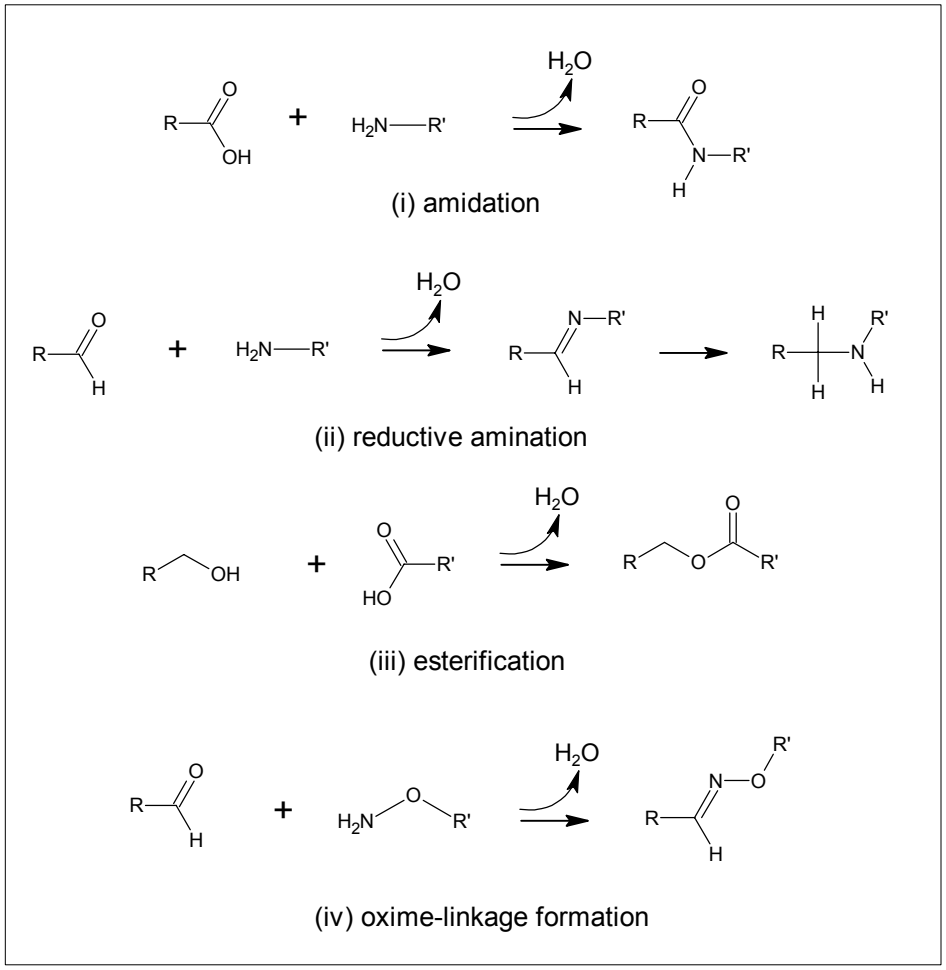


Table 5. A list of methods used in the present study for syntheses of multivalent conjugates. Detailed description of the methods, and the reagents used in them, are found in the original publications (**I-III**) as indicated and in references therein.

Method	Described in publication
Reductive amination	I, III
Desulphation and hydrolysis	I, II
Amidation	I-III
Oxidation	I, II
α2,6-sialylation	I
Esterification	II
Oxime formation	II
Stability analysis	II
Glycosylamidation	II
Preparation of glycolipid derivatives	III
Modifications of glycolipids	III
Preparation of sialylated derivatives	III
Synthesis of neoglycolipids	III
Enzymatic synthesis of oligosaccharides	III

4.3 Preparation of multivalent PAMAM dendrimers (unpublished results)

Glycodendrimers based on generation 4.0 polyamido amine dendrimer (PAMAM'64 dendrimer) (Starburst^{TM} dendrimers, Aldrich) (Tomalia et al., 1985) were prepared as follows: 250 nmol PAMAM, 50 µmol oligosaccharide (LNnT or GnLacNAcLac), and 250 µmol $NaCNBH_4$ were dissolved in 250 µl of 0.1 M Na-borate pH 8.5. Both reactions were performed at room temperature for 24 hours under constant magnetic stirring. Products were isolated by gel filtration chromatography and analyzed by MALDI-TOF MS and ^{1}H-NMR.

4.4 Modification of LNnT using aminooxyacetic acid and amidation to DAP-ox-γ-CD (unpublished results)

LNnT was modified using aminooxyacetic acid (Aoa) as follows: 100 µmol LNnT and 200 µmol aminooxyacetic acid (Sigma) were dissolved in 2.4 ml 0.2 M Na-acetate buffer, pH 4.0 and incubated at room temperature for 48 hours. The oligosaccharide fraction was isolated by gel filtration chromatography in a column of Superdex 30 (5 × 95 cm) run in 200 mM NH_4HCO_3. The DAP-ox-γ-CD (described in part **I**) was amidated with LNnT-Aoa as follows: 5 µmol of DAP-ox-γ-CD, 500 µmol DIPEA, 500 µmol HBTU, and 100 µmol crude LNnT-Aoa were dissolved in pyridine containing 10% H_2O. Reaction was performed at room temperature, in the dark, and under constant magnetic stirring for three days. Reaction mixture was evaporated to

dryness with rotary evaporator and purified using Superdex 30 (5 × 95 cm) run in 200 mM NH_4HCO_3.

4.5 *Helicobacter pylori* strains (III)

CCUG 17874 and CCUG 17875 (Culture Collection, Göteborg University, Sweden), the babA1A2-knock out mutant of CCUG 17875 (from Dr. Thomas Borén, Umeå University, Sweden), and S-032 (acquired from a patient with duodenal ulcer at the Örebro Medical Center, Örebro, Sweden). Growth conditions were as described previously (Miller-Podraza et al., 1996).

4.6 Chromatographic methods (I-III)

Table 6. List of purification methods employed in the present study. Full description of the chromatographic methods, and the reagents used in them, are found in the original publications (**I-III**) as indicated and in references therein.

Method	Described in publication
Size-exclusion chromatography on a Superdex 30 (5 × 95 cm) or Superdex Peptide HR 10/30 (10 × 300 mm) column	I-III
Anion-exchange chromatography on a Resource^{TM}Q column	III
Thin-layer chromatography	III
Hydrophobic silica-based purification on a BondElut C-18 column	III
Reversed-phase chromatography on a Hypercarb column	III
Desalting of glycolipids using a small column filled with Sephadex G-25 or by Sephadex LH-20 chromatography	III
Desalting and deproteinization of reaction mixtures in a mixed bed of Dowex AG 1-X8 (Ac^-) and AG 50W-X8 H^+	III

4.7 Mass spectrometry (I-III)

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra (MS) (**I-III**) were recorded on a Voyager-DE^{TM} STR BioSpectrometry^{TM} (PerSeptive Biosystems) time-of-flight instrument. Samples were analyzed in either negative ion delayed extraction linear mode using 2,4,6-trihydroxyacetophenone (THAP) (Fluka) (3 mg/ml in acetonitrile / 20 mM aqueous diammonium citrate, 1:1, by volume) or positive ion delayed extraction reflector mode using 2,5-dihydroxybenzoic acid (DHB) (Aldrich) matrix (10 mg/ml in H_2O).

Fast atom bombardment mass spectrometry (FAB MS) (**III**) was performed on a JEOL SX-102 mass spectrometer in the negative ion mode. The spectra were produced by Xe atoms using triethanolamine as a matrix.

4.8 Nuclear magnetic resonance spectroscopy (I-III and unpublished results)

Prior to the 1D ^{1}H NMR experiments, the samples were lyophilized twice from D_2O (99.9%) (Aldrich) and then dissolved in 38 µl D_2O. The 1D ^{1}H NMR spectra were recorded in D_2O (Aldrich, 99.9%) with a Varian Unity 500 spectrometer (Varian Inc., CA, USA) at 23°C using a gHX nano-NMR probe (Varian Inc., CA, USA). The ^{1}H chemical shifts are presented by reference to internal acetone (δ=2.225 ppm).

5 RESULTS

5.1 Synthesis of multivalent neoglycoconjugates (I, II, and unpublished results)

Three different scaffold molecules were chosen for multivalent glycoconjugates: (1) γ-cyclodextrin, (2) a chondroitin 14-mer fraction, and (3) a commercial PAMAM'64 dendrimer, representing cyclic, linear, and globular scaffolds, respectively. Multivalent glycoconjugates were produced using the human milk type oligosaccharides LNDFH I (Lewis-b hexasaccharide), LNnT, or GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc (Table 7), all representing analogs of the tissue binding epitopes for the human gastric pathogen *Helicobacter pylori*. Four different methods were created to attach these oligosaccharides to their scaffold molecules.

Table 7. A list of ligand oligosaccharides attached to chondroitin oligomer, γ-CD, or PAMAM scaffolds

Ligand	Ch14	γ-CD	PAMAM
Galβ1-4GlcNAcβ1-3Galβ1-4Glc (**LNnT**)	**I, II**	**I**, **II**, unpublished results	Unpublished results
GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc (**GnLacNAcLac**)	**I**		Unpublished results
Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc (**LNDFH I**)	**I**		

5.1.1 Reductive amination of oligosaccharides to amine modified scaffolds (I)

Chondroitin oligomer as scaffold. In order to create linear multivalent neoglycoconjugates chondroitin sulphate A (CSA) (from bovine trachea) was chosen as starting material. A fraction containing the chondroitin 14-mer (Ch-14) oligosaccharide (GlcAβ1-3GalNAcβ1-4)$_6$GlcAβ1-3GalNAc (compound **1**, Figure 4) as the major compound was prepared by CSA desulphation and hydrolysis. Product was purified and isolated with gel permeation chromatography, and identified by MALDI-TOF MS and 1D ^{1}H NMR spectroscopy. Amine function was introduced to scaffold molecule by amidation of 1,3-diaminopropane to chondroitin 14-mer glucuronic acid residues (**1**→**2**). DAP-amidated Ch14 (DAP-Ch14, compound **2**) was isolated and analyzed as above. A substitution level of 4.5 DAP-units per Ch14 molecule was determined from NMR spectrum. Three different oligosaccharides (Table 7), LNnT, GnLacNAcLac or LNDFH I were then linked to DAP-Ch14 by reductive amination (**2**→**3a** for LNnT). Products LNnT-DAP-Ch14, GnLacNAcLac-DAP-Ch14 and LNDFH I-DAP-Ch14 (compounds **3a-c**, respectively) were isolated and analyzed as above. MALDI-TOF mass spectrum of each product indicated 2-6 oligosaccharides attached to the DAP-Ch14 backbone. The NMR studies showed that α/βH1 signals of reducing end Glc from LNnT, GnLacNAcLac, or LNDFH I, were all missing indicating that no free reducing oligosaccharides remain in the sample. In all spectra αH1/βH1

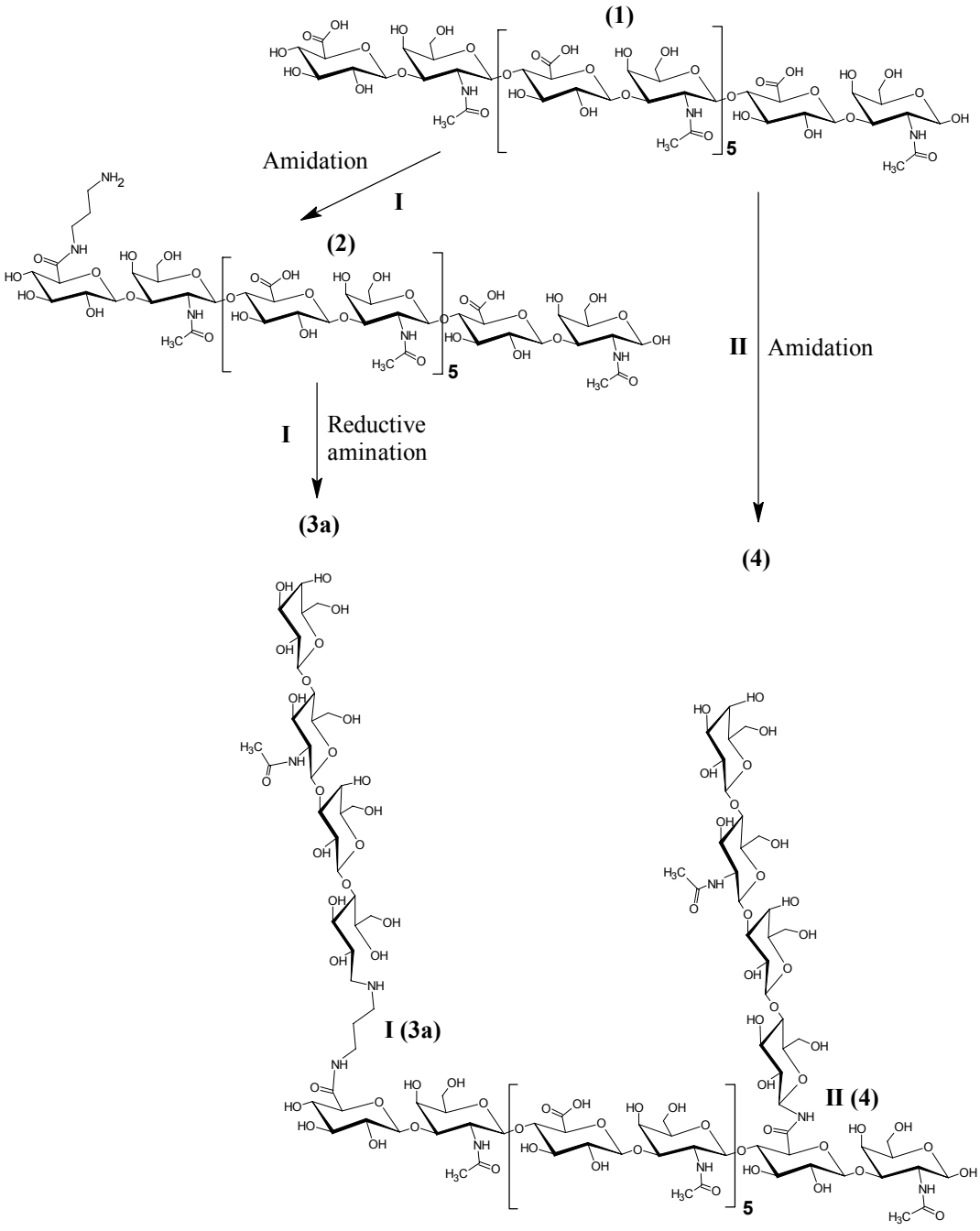


Figure 4. Synthetic methods employed to construct chondroitin oligomer based multivalent products (**I** and **II**). To simplify illustration only single modification event for each reaction is shown and both products are combined on a single Ch14 scaffold (in actual conjugates oligosaccharides are presumably attached randomly to 6'-position carboxyl-groups along the scaffold). Both oligosaccharides attached to Ch14 backbone using different methods is LNnT.

signals for Gal adjacent to this Glc had shifted downfield due to reductive amination. The more distant part βH1 signals were consistent with those reported for the free LNnT, GnLacNAcLac, or LNDFH I, molecules. In addition, from LNDFH I-DAP-Ch14 spectrum an average substitution level of 4.6 LNDFH I oligosaccharides per DAP-Ch14 molecule was obtained indicating that the reductive amination reaction was essentially complete.

γ-cyclodextrin as scaffold. In order to create cyclic multivalent neoglycoconjugates γ-cyclodextrin (γ-CD) was chosen as starting material. First, primary alcohol groups of γ-CD were partially oxidized by TEMPO (2,2,6,6-tetramethylpiperidine-1-oxy radical) mediated process to introduce carboxyl groups to scaffold (**5→6**) (Figure 5). Product, ox-γ-CD (compound **6**), was isolated using gel filtration and identified by mass spectrometry. MALDI-TOF MS analysis revealed an oxidized product with an average of 6 carboxylate groups. These 6'-position carboxyl-groups were further modified by amidation with 1,3-diaminopropane to form a γ-CD derivative carrying primary amine groups (**6→7**). Product, DAP-ox-γ-CD (compound **7**), was isolated and analyzed as above. A substitution level of 2.5-3 DAP-units attached to the ox-γ-CD scaffold was determined from MALDI-TOF mass spectra. LNnT was then bound to modified carrier by reductive amination (**7→10**) and product, LNnT-DAP-ox-γ-CD (compound **10**), was isolated and analyzed by MALDI-TOF MS and 1D ^{1}H NMR. MALDI-TOF mass spectrum of product indicated 1-5 oligosaccharides attached to the DAP-ox-γ-CD backbone. The NMR showed that α/βH1 signals of reducing end Glc from LNnT were missing (as observed for LNnT-DAP-Ch14, GnLacNAcLac-DAP-Ch14, and LNDFH I-DAP-Ch14) indicating that no reducing end LNnT remains in the sample. In addition, compared to the free LNnT the βH1 signal for Gal adjacent to this Glc had shifted downfield due to reductive amination. The more distant part βH1 signals were consistent with those reported for the free LNnT molecule.

Sialylation of multivalent product. The LNnT-DAP-ox-γ-CD conjugate was also shown to act as an acceptor for α2,6-sialyltransferase. The sialylated product (SA-LNnT-DAP-ox-γ-CD) was isolated by gel filtration and analyzed using MALDI-TOF mass spectrometry. The spectrum showed a major peak containing the fully sialylated SA_3-$LNnT_3$-DAP-ox-γ-CD product, in addition to minor peaks representing different degrees of sialylation on $(LNnT)_{2\text{-}4}$-DAP-ox-γ-CD backbone. The average sialylation level for the reaction was approximately 70 %.

5.1.2 Reductive amination of oligosaccharides to PAMAM dendrimer scaffold (unpublished results)

LNnT and GnLacNAcLac were attached to PAMAM'64 dendrimer (compound **13**) by reductive amination (Figure 6). The glycodendrimers were isolated by gel filtration chromatography and analyzed by MALDI-TOF MS and 1D ^{1}H NMR. The mass spectrum indicates that the LNnT-PAMAM'64 conjugate (compound **14a**) carried about 56 oligosaccharide units (Figure 7A). The MALDI-TOF MS analysis of the GnLacNAcLac-PAMAM'64 conjugate (compound **14b**) revealed an equal substitution level (data not shown). The ^{1}H NMR spectrum of LNnT-

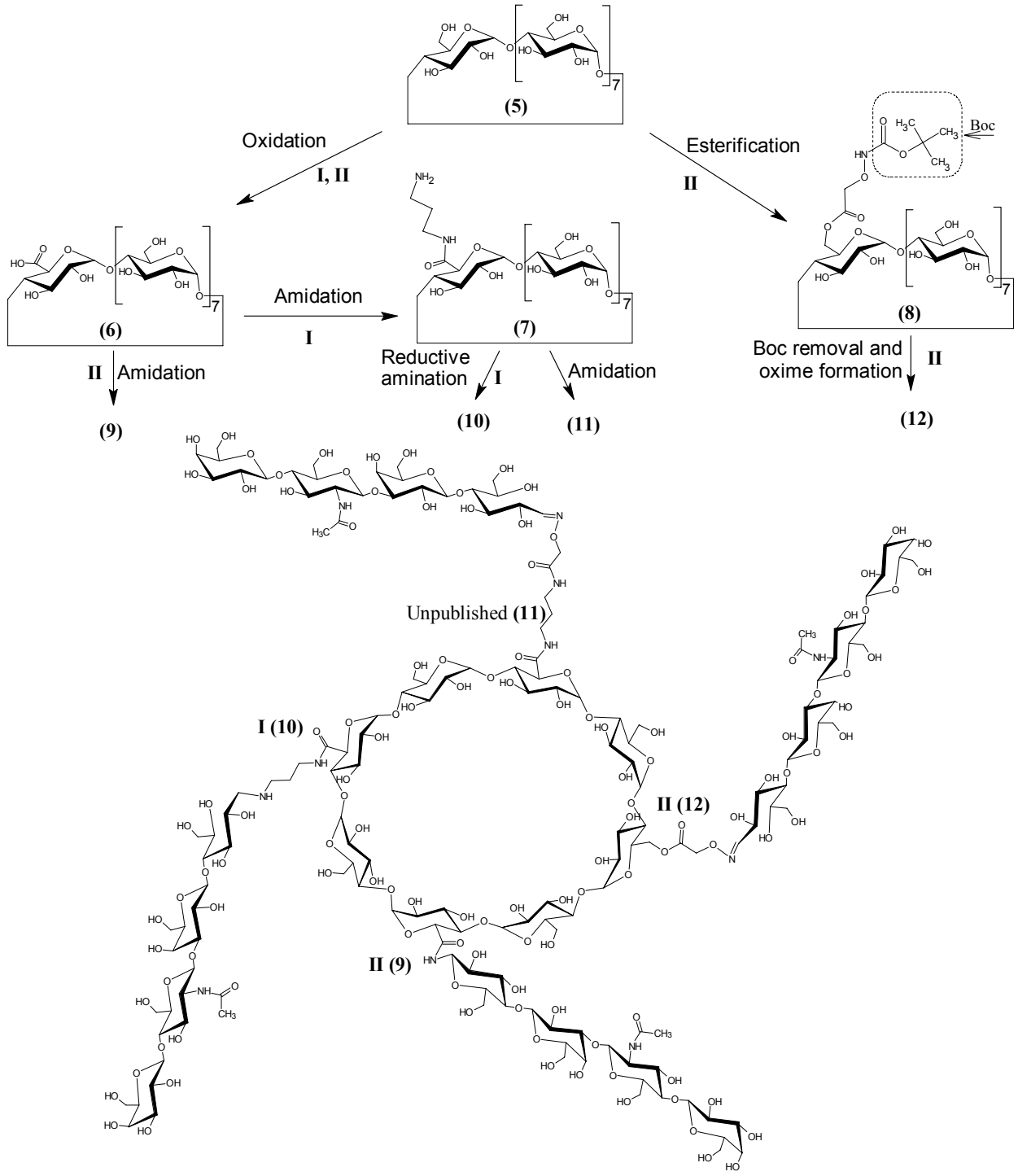


Figure 5. Synthetic methods used to construct γ-cyclodextrin based multivalent products (parts **I**, **II**, and unpublished results). To simplify illustration only a single modification event for each reaction is shown and all four products are combined on a single γ-CD scaffold. The oligosaccharide attached to the γ-CD backbone in each case is LNnT.

PAMAM'64 (Figure 7B) shows the same pattern for LNnT signals as already described for LNnT-DAP-Ch14 and LNnT-DAP-ox-γ-CD conjugates. The PAMAM´64 scaffold protons are seen at 2.4-3.0 ppm. The LNnT-PAMAM'64 conjugate carried an average of 2 LNnT units as calculated by comparing the intensity of ***C***-GlcNAc *N*-acetyl proton signals to those of PAMAM proton signals. The LNnT-PAMAM'64 conjugate (~50 nmol sample) ^{1}H NMR spectrum was recorded with 600 nmol of L-fucose as internal quantification standard. Quantification gave as a result 39 nmol of LNnT-PAMAM'64 conjugate (yield 78%). This was calculated by comparing the integrated intensities of LNnT ***C***-GlcNAc *N*-acetyl signals or PAMAM'64 backbone proton signals to the intensity of internal standard L-Fuc α/βCH3 signals. Similar results were obtained also with the GnLacNAcLac-PAMAM'64 conjugate recorded with L-fucose: Conjugate carried an average of 53 GnLacNAcLac units attached to PAMAM´64 backbone and quantification gave as a result 37 nmol of PAMAM'64 conjugate (yield 74%) (data not shown).

5.1.3 Amidation of oligosaccharide glycosylamines to scaffold carboxylic acid units (II)

Chondroitin oligomer as scaffold. LNnT converted to its glycosylamine form (LNnT-NH_2) was conjugated to Ch14 oligomer (**1→4)** by amidation to 6'-position carboxyl-groups (Figure 4). Product, LNnT-NH-Ch14 (compound **4**) was isolated by gel filtration and analyzed using MALDI-TOF MS and 1D ^{1}H NMR. Mass spectra of LNnT-NH-Ch14 indicated 0-3 oligosaccharides attached to the Ch14 backbone, with the main product carrying one oligosaccharide chain. The NMR study showed that α/βH1 signals of reducing end Glc from LNnT were missing indicating that no free reducing oligosaccharides remained in the sample. In addition, compared to the free LNnT the βH1 signal for Gal adjacent to this Glc had shifted downfield. The average substitution level deduced from NMR spectrum was 1.6 LNnT oligosaccharides per Ch14 molecule.

γ-cyclodextrin as scaffold. LNnT converted to its glycosylamine form was conjugated to ox-γ-CD scaffold (**5→6**, described in section 5.1.1.) by amidation to 6'-position carboxyl-groups (**6→9**, Figure 5). Product, LNnT-NH-ox-γ-CD (compound **9**) was isolated by gel filtration and analyzed using MALDI-TOF MS and 1D ^{1}H NMR. Mass spectrum of LNnT-NH-ox-γ-CD indicated 1-4 oligosaccharides attached to the ox-γ-CD molecule, and the main products were di- and trisubstituted species. The NMR study resulted in similar results as obtained for LNnT-NH-Ch14: The α/βH-1 signals of reducing end Glc unit from LNnT were missing indicating that no free reducing LNnT remains in the sample, and compared to the free tetrasaccharide the βH1 of Gal adjacent this amidated Glc had shifted downfield. The average substitution level could not be established from the spectrum because the heterogeneous nature of the αH1 signals of the modified γ-CD resulted in unreliable integration of this area.

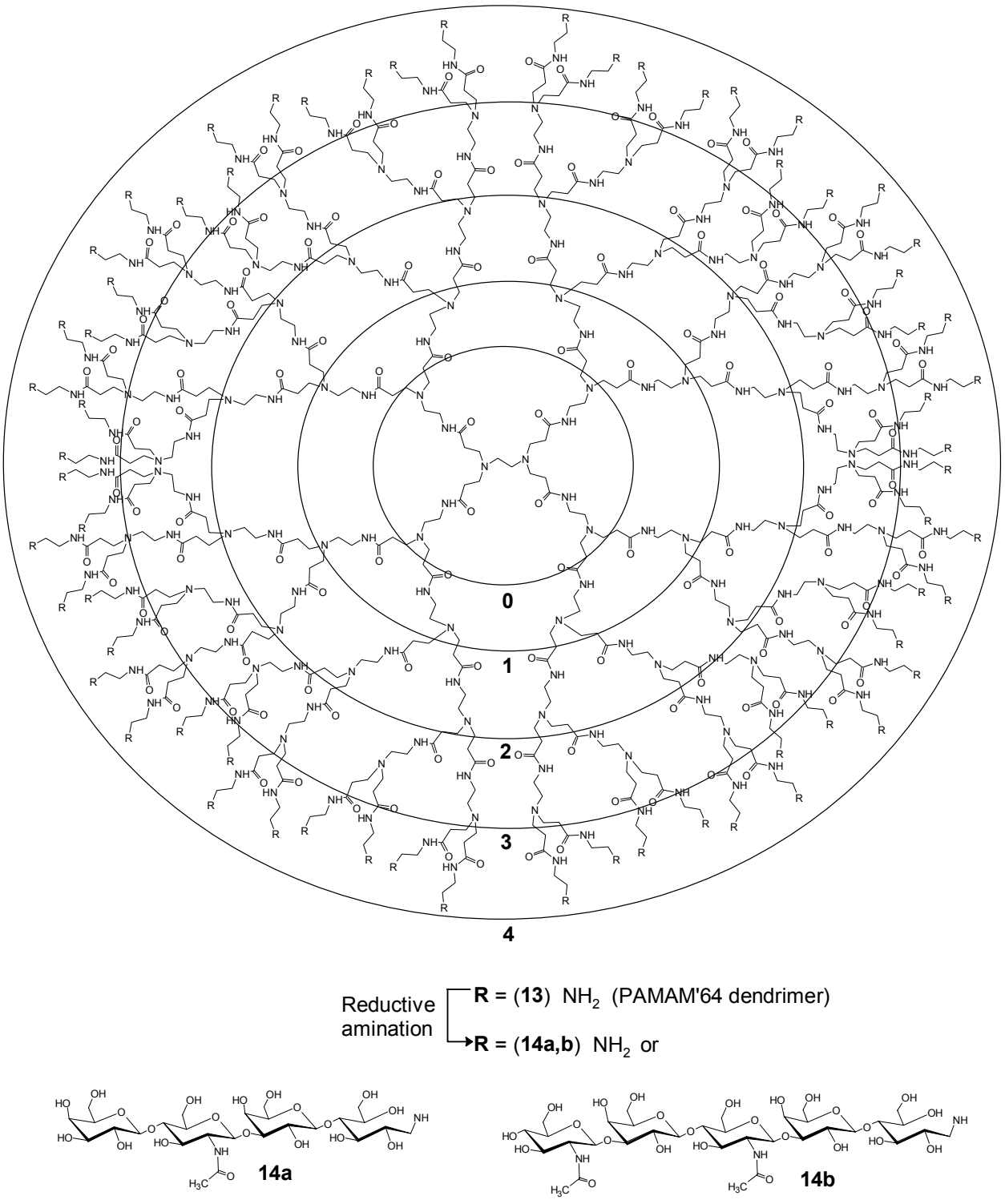


Figure 6. Reductive amination of oligosaccharides to the polyamido amine dendrimer scaffold (PAMAM'64, Generation 4.0). Generations 0-4 are specified and numbered

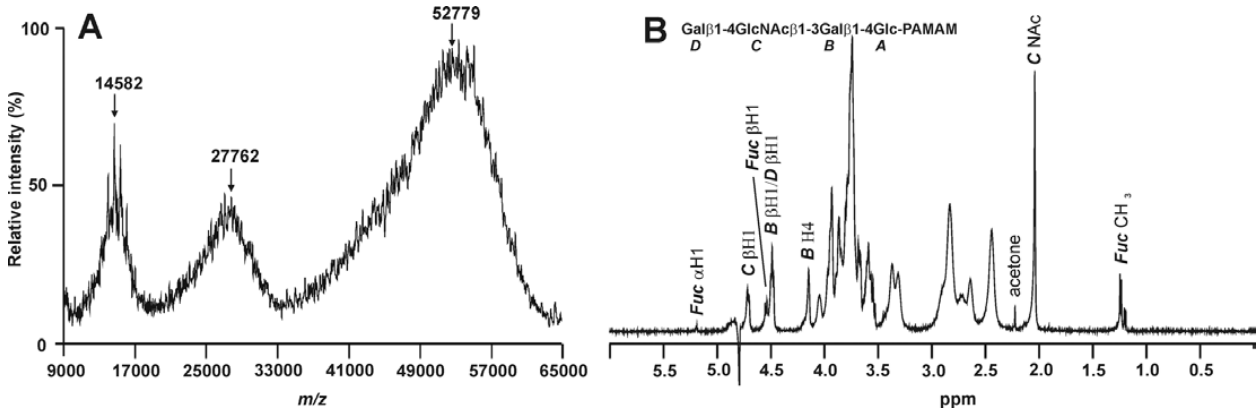


Figure 7. (A) MALDI-TOF mass spectrum of LNnT-PAMAM glycodendrimer (compound **14a**) measured in linear positive-ion mode. The signal at *m/z* 52779 corresponds to singly-charged $[M+Na]^+$ glycodendrimer carrying approximately 56 LNnT oligosaccharides attached to PAMAM´64 backbone. Signals at *m/z* 27762 and 14582 represent doubly- and triply-charged species, respectively. (B) 1D ^{1}H-NMR spectrum of LNnT-PAMAM'64 (compound **14a**) with fucose as internal quantification standard.

5.1.4 Synthesis of oxime-linked multivalent oligosaccharides (II and unpublished results)

Two different methods were created to conjugate oligosaccharides to γ-CD backbone using an oxime linkage. In the first method (**II**) Boc-Aoa was ester-linked to primary alcohol group of γ-CD (**5→8**) (Figure 5). Reaction mixture was purified using dialysis. The average Boc-Aoa substitution level was 3.5 as determined by MALDI-TOF MS analysis. In addition, signals representing molecular species where protecting group Boc had undergone hydrolysis, thus revealing amine groups, were also observed. Next, the protecting Boc groups were removed and LNnT was chemoselectively ligated to unprotected Aoa-γ-CD (**8→12**). Product (LNnT-Aoa-γ-CD, compound **12**) was isolated using gel filtration chromatography and analyzed by MALDI-TOF MS and 1D ^{1}H NMR. The mass spectrum of LNnT-Aoa-γ-CD conjugate indicated 2-5 oligosaccharide units attached to the Aoa-γ-CD scaffold. In addition, molecular species, where the amine groups have probably been lost from the aminooxy units revealing hydroxyl groups (O-NH_2 converted to OH = *m/z* -15), were observed. The NMR study showed that α/βH1 signals of reducing end Glc from LNnT were missing indicating that no free reducing oligosaccharides remained in the sample. In addition, the βH1 signal for Gal adjacent to this Glc had shifted downfield when compared to the free LNnT oligosaccharide. Signal representing oxime proton Glc H1 was also observed. The average substitution level was 3.1 LNnT oligosaccharides per modified γ-CD molecule as calculated by comparing the integrated intensities of αH1 signals of the modified γ-CD and LNnT βH1 of GlcNAc.

The second method (unpublished results) included the oxidized and amidated γ-CD scaffold described in section 5.1.1. (**5→6→7**) (Figure 5). This DAP-ox-γ-CD (compound **7**) was amidated with aminooxyacetic acid modified LNnT (LNnT-Aoa) to create the multivalent product (**7→11**). Product (LNnT-Aoa-DAP-ox-γ-CD, compound **11**) was isolated with gel

filtration chromatography and analyzed using MALDI-TOF MS and ^{1}H NMR (Figure 8). From mass spectrum (Figure 8A) the indicated signals were tentatively identified as $LNnT_1$-Aoa_2-DAP_4-ox_7-γ-CD (*m/z* 2493 $[M-3H+2Na]^-$), $LNnT_2$-Aoa_3-DAP_4-ox_7-γ-CD (*m/z* 3214 $[M-H]^-$), and $LNnT_3$-Aoa_3-DAP_5-ox_6-γ-CD (*m/z* 3950 $[M-H]^-$). The heterogeneity in the spectrum is due to variable levels of oxidation and amidation.

The ^{1}H NMR spectrum of LNnT-Aoa-DAP-ox-γ-CD (Figure 8B), show in the anomeric region H1 resonances βH1 of ***D***-Gal (4.480 ppm) and βH1 of ***C***-GlcNAc (4.713 ppm), and H4 of 3-substituted ***B***-Gal (4.157 ppm) consistent with those reported for the free LNnT molecule. αH1 resonances of the modified γ-CD are seen around 5.1 ppm. When compared to the spectrum of unmodified γ-CD where αH1 signals (Glcα1-4) resonate at the same frequency (5.09 ppm), the αH1 signal area of LNnT-Aoa-DAP-ox-γ-CD is very heterogeneous due to the complex nature of the molecule. Compared to the free LNnT tetrasaccharide the βH1 of ***B***-Gal had shifted downfield from 4.436 ppm to 4.518 ppm due to modification of the ***A***-Glc unit. The α/βH-1 signals of ***A***-Glc are missing indicating that no free reducing LNnT remains in the sample. The average substitution level could not be established from the spectrum because the heterogeneous nature of the αH1 signals of the modified γ-CD resulted in unreliable integration of this area.

Stability of the oxime linkage. The stability of sugar-oxime linkage was investigated under highly acidic conditions that orally administered molecules would probably experience in the stomach (+37°C pH ~1). Samples containing approximately 40% LNnT and 60% LNnT-Aoa were incubated in highly acidic conditions (at pH 0 or pH 1) at room temperature or at +37°C. At selected time points aliquots were removed and analyzed using MALDI-TOF MS. At +37°C pH ~1 the half-life of approximately 3 hours and even at +37°C pH 0 the half-life of about 1 hour were observed for LNnT-Aoa.

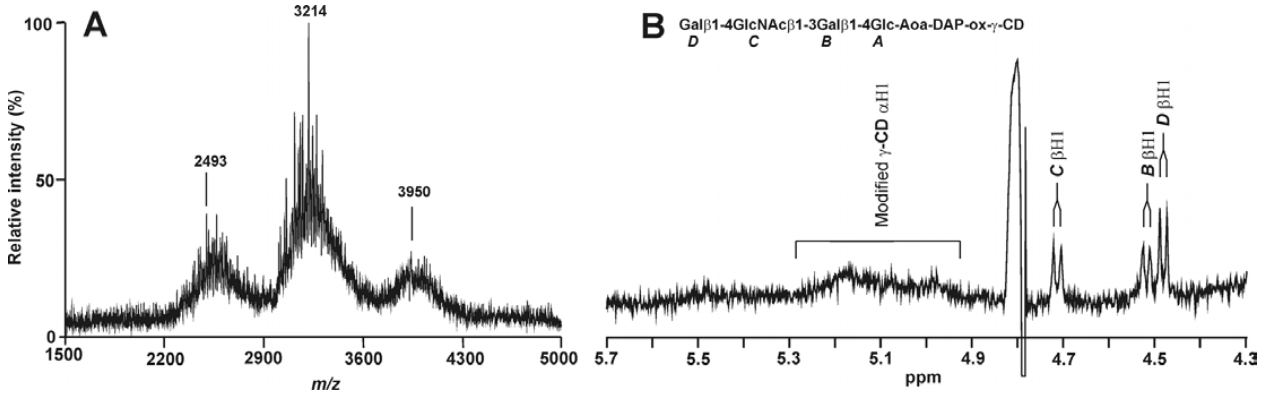


Figure 8. (A) MALDI-TOF MS of LNnT-Aoa-DAP-ox-γ-CD (compound **11)** measured in the linear negative ion mode. (B) 1D ^{1}H NMR spectrum of LNnT-Aoa-DAP-ox-γ-CD.

5.2 Characterization of novel *Helicobacter pylori* binding carbohydrate structures (III)

An array of glycolipid derivatives and neoglycolipids were prepared, followed by analysis (on TLC plates) of their binding activity against *H. pylori* and the structural requirements involved (Table 8):

Discovery of new carbohydrate structures with binding activity towards H. pylori. GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer (compound **15**) and Glcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer (compound **19**) represent two novel neolacto based carbohydrate structures with binding activity against *H. pylori*. In addition, *N*-methyl (compound **16**) and *N*-ethyl (compound **17**) amidation of GlcA residue of compound **13** promoted this binding.

The role of N-acetoamido groups in H. pylori binding to neutral neolacto epitope. De-*N*-acetylation of either (compound **22**) or both (compound **23**) of the *N*-acetoamido groups of Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer (compound **20**) resulted in inactive structures. The same result was also obtained by ring-opening of the terminal Gal residue (compound **21**).

Binding of H. pylori to hydrophobic derivatives of sialylparagloboside (SPG). Coupling of C18 aliphatic group to the terminal Neu5Ac of the Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer (compound **24**) glycolipid all resulted in binding-active (of different strengths) derivatives (compounds **25**-**27**).

Binding of H. pylori to a number of neoglycolipids. Neoglycolipids were prepared by chemical coupling of reducing oligosaccharides to two different aminated lipids: Hexadecylaniline (HDA, $CH_3(CH_2)_{15}C_6H_4NH_2$), or a branched palmitate-lysine-based conjugate (C-42, Pal-Lys(Pal)CONH$(CH_2)_4$-NH_2). Analysis on TLC plates of these neoglycolipids confirmed that neolacto structures with terminal GlcA(methyl amide) (compound **38**/**39**) or GlcNAc (compounds **28**/**29** and **32**/**33**) are among the most active epitopes against *H. pylori*. Moreover, the importance of more distal sugar residues was seen. The most active derivatives were obtained by linking the epitope by β6 linkage to GlcNAc or Gal (compounds **28**/**29**, **32**/**33**, **38**/**39**), whereas replacing β6 linkage by α6 (**30**/**31**) or β3 (**42**/**43**) linkage negatively influenced the binding. In addition, lipid parts containing different hydrophobic moieties (HDA or C42) also appear to influence binding.

Table 8. Binding of *H. pylori* to glycolipid derivatives and neoglycolipids.

Structure	Compound	Binding
GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	**15**	+
GlcA(methyl amide)β1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	**16**	+++
GlcA(ethyl amide)β1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	**17**	+++
GlcA(benzyl amide)β1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	**18**	+
Glcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	**19**	+
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	**20**	++
Gal(oxid/red)β1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	**21**	-
Galβ1-4GlcNβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer and Galβ1-4GlcNAcβ1-3Galβ1-4GlcNβ1-3Galβ1-4Glcβ1-Cer	**22**	-
Galβ1-4GlcNβ1-3Galβ1-4GlcNβ1-3Galβ1-4Glcβ1-Cer	**23**	-
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	**24**	+++
Neu5Ac(octadecyl)α2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer (R-COOH → R-CO-NH-$(CH_2)_{17}CH_3$)	**25**	++(+)
Neu5Ac(octadecyl)α2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer (R-CHOH-CHOH-CH_2OH → R-CHOH-CH_2NH-$(CH_2)_{17}CH_3$ + R-CH_2-NH-$(CH_2)_{17}CH_3$)	**26**	+++
Neu5Ac_{red}(octadecyl)α2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer (R-COOH → R-CO-NH-$(CH_2)_{17}CH_3$ R-CHOH-CHOH-CH_2OH → R-CHOH-CH_2OH)	**27**	(+)
GlcNAcβ1-3Galβ1-4GlcNAcβ1-6GlcNAc-HDA / -C42	**28 / 29**	+++ / ++
GlcNAcβ1-3Galβ1-4GlcNAcα1-6GlcNAc-HDA / -C42	**30 / 31**	(+) / (+)
GlcNAcβ1-3Galβ1-4GlcNAcβ1-6Gal-HDA / -C42	**32 / 33**	+++ / ++
GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Man-HDA / -C42	**34 / 35**	+ / +
GlcAβ1-3Galβ1-4GlcNAcβ1-6GlcNAc-HDA / -C42	**36 / 37**	+ / +
GlcA(methyl amide)β1-3Galβ1-4GlcNAcβ1-6GlcNAc-HDA / -C42	**38 / 39**	+++ / ++
GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-HDA / -C42	**40 / 41**	(+) / +
GlcA(methyl amide)β1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-HDA / -C42	**42 / 43**	(+) / (+)

"+++" stands for high frequency of binding, "++" and "+" for less strong binding, "(+)" for occasional binding, and "–" for no binding

6 DISCUSSION

Helicobacter pylori persistently infects the gastric mucosa of a majority of the global human population and is implicated in several diseases of the gastrointestinal tract including chronic gastritis, duodenal and gastric ulcers, and gastric adenocarcinoma (Israel and Peek, 2001; Peek and Blaser, 2002). The current treatment of *H. pylori*, based on the use of antibiotics, although effective is connected with risks of emergence of antibiotic resistant strains. There is therefore a need for alternative therapeutic treatment for eradication therapy of which method based on the oral use of anti-adhesion molecules (Figure 9) is under discussion (Karlsson, 2000). Indeed, it has been reported that high doses of sialyllactose (Neu5Acα2-3Galβ1-4Glc) could cure *H. pylori* infection in Rhesus monkeys (Mysore et al., 1999) and bovine milk glycoconjugates inhibited the *in vivo* infection in mouse model (Wang et al., 2001).

The general principle of anti-adhesion therapy is the inhibition of micro-organism adhesion to the host cell with the help of soluble receptor analog (Figure 9). This is achieved by administration of natural or synthetic carbohydrate derivatives having a high affinity for the microorganism lectin [e.g. (Zopf and Roth, 1996)]. Consequently, the microorganism is no longer able to interact with the host cell surface carbohydrate structures, and as a result it will pass through the body stopping the ongoing infection or without ever initiating infection. Such anti-adhesives agents occurring naturally include human breast milk, which contains various soluble oligosaccharides providing newborn babies with a mechanism for aborting the course of infection (Mouricout et al., 1990). Thus, carbohydrates are ideal candidates as therapeutic agents since they are unlikely to be immunogenic or toxic, and in particular since various carbohydrate structures that inhibit bacterial adhesion are found as normal constituents of body fluids (such as human milk) or on cell surfaces.

All oligosaccharide ligands used to construct multivalent glycoconjugates in the present study (**I**, **II**, and unpublished results) were established analogs of *H. pylori* binding epitopes (Boren et al., 1993; Miller-Podraza et al., 2005). In addition, structural details of neolacto-based structures were studied further (**III**), which can prove to be useful for possible future development of new high affinity anti-adhesion molecules.

6.1 Synthesis of multivalent glycoconjugates (I, II and unpublished results)

Monovalent carbohydrate molecules bind weakly, which makes even the most optimized carbohydrate analog of the human *Helicobacter* receptor of limited use. Consequently, adhesion of *Helicobacter pylori* binding to gastrointestinal epithelial cells was inhibited by monomeric 3´-sialyllactose at millimolar concentrations whereas multivalent neoglycoproteins bearing 3´-sialyllactose were 1000-fold more potent (Simon et al., 1997). In addition, the expression of the sialyl Le^x and Le^b epitopes on pig milk glycoproteins has been shown to correlate to the ability

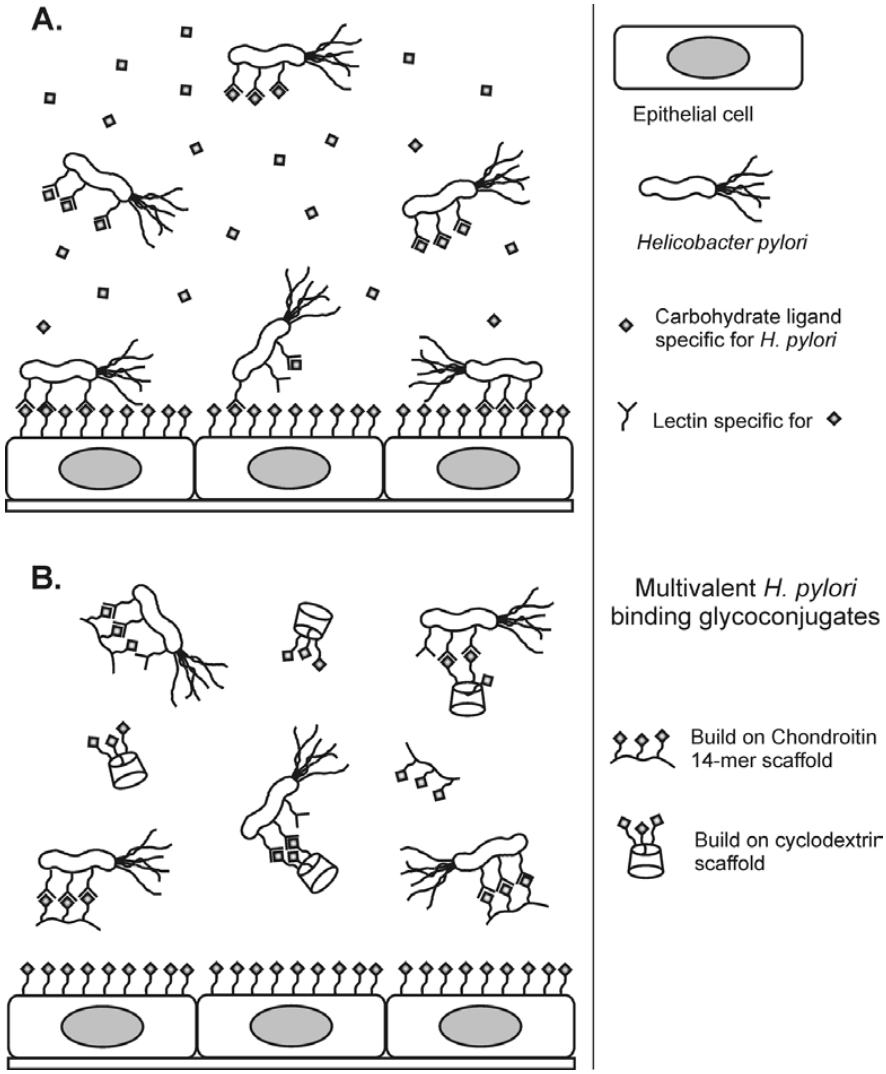


Figure 9. Basis of anti-adhesion therapy against *Helicobacter pylori*. (A) Monovalent oligosaccharide epitopes are ineffective. (B) Multivalent receptor analogs effectively compete with oligosaccharides on the cell surface resulting in *H. pylori* clearance from the body.

of porcine milk to inhibit *H. pylori* adhesion *in vitro* and lower degree of colonization *in vivo* (Gustafsson et al., 2006).

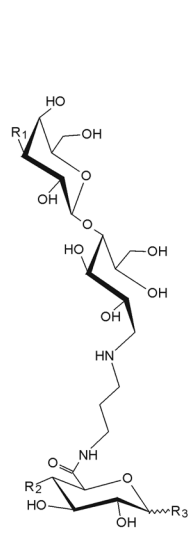
In the present study (**I**, **II**, and unpublished results) four different methods were created to synthesize new types of multivalent glycoconjugates. When choosing possible scaffold molecules the focus was on assembling our multivalent ligands on carbohydrate based scaffolds which may offer better biocompability. Furthermore, the configurational, conformational, and constitutional diversity of carbohydrates enables control over the presentation of ligands.

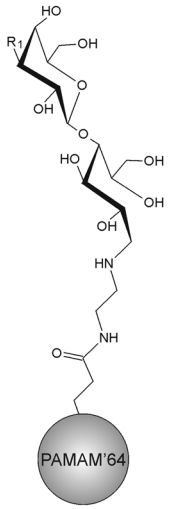
Previously described carbohydrate based multivalent molecules include conjugates constructed e.g. on cyclodextrins (Fulton and Stoddart, 2001; Houseman and Mrksich, 2002; Ortiz Mellet et al., 2002), heparin (Sakagami et al., 2000), hyaluronic acid (Soltés et al., 1999), and chitosan (Murata et al., 1996; Sakagami et al., 2000).

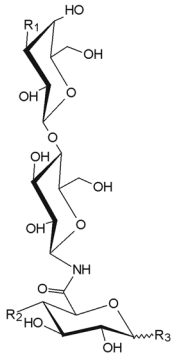
The three different scaffolds used in this study, (1) the cyclic γ-CD, (2) the linear Ch14, and (3) globular dendrimer, were chosen because they present their ligands in a diverse manner. (1) cyclodextrin based conjugates are expected to present their ligands in a relatively rigid fashion, and may find preferential use in binding to influenza virus hemagglutinin type proteins and bacterial toxins (Kitov et al., 2000; Ohta et al., 2003). (2) GAGs are valuable scaffold molecules for constructing multivalent glycoconjugates because their carboxyl-groups are easily functionalized for subsequent derivatization with carbohydrate units. The chondroitin oligomer based conjugates described in this thesis present their oligosaccharide ligands on a linear scaffold, which may mimic e.g. polylactosaminoglycans and natural mucins. Chondroitin based neoglycoconjugates may find favored use in e.g. selectin inhibitor area: Polyvalent sLex conjugates based on polylactosamine scaffolds with high affinity towards selectins have been previously described (Renkonen et al., 1997). (3) Carbohydrate coated dendrimers (glycodendrimers) involve modification of pre-existing dendrimers in a convenient way to make multivalent glycoconjugate in minimal number of steps. Glycodendrimers are established tools in glycobiology, and therefore it is important to compare the binding properties of multivalent glycoconjugates based on novel carrier structures with glycodendrimers. In addition, glycodendrimers may mimic the complex multi-antennary carbohydrate moieties of glycoconjugates.

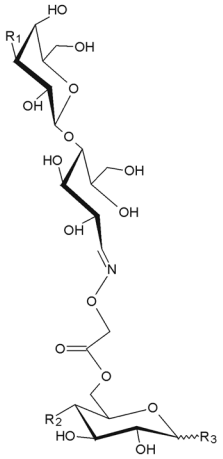
In part **I** and in unpublished results reductive amination was used to conjugate unmodified reducing oligosaccharides to the three selected scaffold molecules (Table 9). Reductive amination is an established method in neoglycoconjugate synthesis, which allows the reactions to be performed in the absence of protective groups and under aqueous conditions. For example, it has previously been used to attach 64 galactose units to PAMAM dendrimer (Bhadra et al., 2005) and up to 64 galactose or lactose units has been attached to polypropylene imine dendrimers by means of amide bond (Ashton et al., 1997). The scaffold molecules used here included, chondroitin 14-mer and γ-cyclodextrin both modified to express primary amines, and a commercial PAMAM´64 dendrimer carrying 64 primary amino groups (unpublished results). MALDI-TOF MS analysis of multivalent product LNDFH I-DAP-Ch14 indicated that 2-6 oligosaccharides were attached to the modified Ch14 backbone, whereas from NMR analysis an average substitution level of 4.6 LNDFH I oligosaccharides per Ch14 scaffold was obtained. Comparable results were also acquired for LNnT-DAP-Ch14 and GnLacNAcLac-DAP-Ch14. Similarly MALDI-TOF MS analysis of LNnT-DAP-ox-γ-CD indicated that 1-5 LNnT units were attached to the carrier molecule, resulting in an average substitution level of ~2.5. Finally, MALDI-TOF MS and ^{1}H-NMR spectroscopy analyses of LNnT-PAMAM'64 and GnLacNAcLac-PAMAM'64 indicated an average substitution of >50 oligosaccharide units attached to the scaffold molecule.

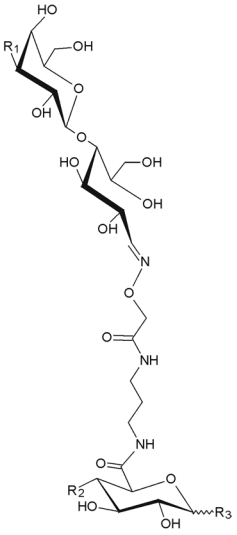
Table 9. Properties of multivalent neoglycoconjugates created in this thesis.











Described in	Part I	Unpublished Results	Part II	Part II	Unpublished results
Compounds synthesized	LNnT-DAP-Ch14 (**3a**) GnLacNAcLac-DAP-Ch14 (**3b**) LNDFH I-DAP-Ch14 (**3c**) LNnT-DAP-ox-γ-CD (**10**)	LNnT-PAMAM'64 (**14a**) GnLacNAcLac-PAMAM'64 (**14b**)	LNnT-NH-Ch14 (**4**) LNnT-NH-ox-γ-CD (**9**)	LNnT-Aoa-γ-CD (**12**)	LNnT-Aoa-DAP-ox-γ-CD (**11**)
Reducing sugar unit of ligand in ring conformation	No	No	Yes	Both open / ring conformations observed (ratio not known)	Both open / ring conformations observed (ratio not known)
Substitution level as determined from:					
MALDI-TOF MS or	2-6 (**3a-c**); 1-5 (**10**)	~56 (**14a,b**)	0-3 (**4**); 1-4 (**9**)	2-5 (**12**)	1-3 (**11**)
1D ^{1}H NMR data	n.d. (**3a,b**); ~4.6 (**3c**); n.d. (**10**)	~52 (**14a**) ~53 (**14b**)	~1.6 (**4**); n.d. (**9**)	~3.1 (**12**)	n.d. (**11**)

R_1: Galβ1-4GlcNAcβ1- (for LNnT), GlcNAcβ1-3Galβ1-4GlcNAcβ1- (for GnLacNAcLac), or Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1- (for LNDFH I); R_2 and R_3 depend on scaffold molecule used (see Figures 4 and 5 for more details); n.d. not determined.

The multivalent conjugate LNnT-DAP-ox-γ-CD was also shown to act as an acceptor in α2,6-sialyltransferase reaction, yielding a multivalent sialylglycoconjugate. All LNnT sites in the conjugate could be sialylated indicating that they are available for biological recognition. Based on the same concept a chemo-enzymatic approach for oligosaccharide-branched cyclodextrins have been previously described (Furuike et al., 2005; Matsuda et al., 1997). Moreover, the structural data from influenza virus hemagglutinin has been used to design cyclic peptide scaffolds presenting three sialotrisaccharide epitopes. Interestingly, these conjugates were shown to exhibit different binding affinities against hemagglutinin depending on the scaffold structure (Ohta et al., 2003). On similar approach, the new chemoenzymatic method described in this study can be used to create cyclic carbohydrate based (α-, β-, or γ-CD) conjugates presenting hemagglutinin binding oligosaccharides (e.g. sialotrisaccharide).

In part **II** multiple copies of glycosylamine modified LNnT were bound to 6'-position carboxyl-groups of either chondroitin oligomer or oxidized γ-cyclodextrin by amidation (Table 9). Oligosaccharide derivatization through a β-glycosylamide linkage is an recognized method in glycobiology (Chiu et al., 1995; Wong et al., 1993). MALDI-TOF MS analysis of multivalent product LNnT-NH-Ch14 indicated that 0-3 oligosaccharides were attached to the modified Ch14 backbone, whereas from NMR analysis an average substitution level of 1.6 LNnT oligosaccharides per Ch14 scaffold was obtained. Amidation of oxidized γ-CD was more efficient: MALDI-TOF MS analysis of product LNnT-NH-ox-γ-CD indicated that 1-4 oligosaccharides were attached to the modified γ-CD backbone, resulting in an average substitution level of ~2.5. These glycoconjugates have the benefit that their degradation products are devoid of any added linker structures. Interestingly, using an analogous method, synthesis of glycosylated calixarene through the formation of amide bonds using calix[4]arene diacid and galactosamine has been previously attempted (Schädel et al., 2005). It is notable that in their study no glycosylated calixarenes were obtained using this approach due to steric effects and longer spacers were required for successful reactions.

In addition, in part **II** and in unpublished results two novel synthesis methods for oxime-linked sugar-sugar conjugates were described (Table 9). Synthesis of several glycopeptide analogues containing this sugar-peptide oxime-linkage has been reported previously (Brask and Jensen, 2000; Marcaurelle et al., 1998; Marcaurelle et al., 2001; Peri et al., 1999; Peri et al., 1998; Renaudet and Dumy, 2001; Rodriguez et al., 1998; Singh et al., 2005). The first multivalent glycoconjugate in this group was synthesized as follows: γ-CD was effectively esterified with Boc-Aoa and after Boc removal unprotected reducing LNnT was attached by oxime linkage in good yield to the modified γ-CD. MALDI-TOF MS analysis of product LNnT-Aoa-γ-CD indicated that 2-5 oligosaccharides were attached to the modified γ-CD backbone, whereas from NMR analysis an average substitution level of 3.1 LNnT oligosaccharides per γ-CD scaffold was obtained. The second oxime-linked multivalent molecule was synthesized as follows: Aminooxyacetic acid modified LNnT was amidated to modified γ-CD scaffold. MALDI-TOF MS analysis of product LNnT-Aoa-DAP-ox-γ-CD indicated that 1-3 oligosaccharides were attached to the modified γ-CD backbone, resulting in an average substitution level of ~2.0.

Because the site of biological action is at the epithelial cell surfaces of the stomach, multivalent ligand against *Helicobacter pylori* should be orally delivered. Peptide-oximes, while stable under mildly acidic and neutral conditions, are unstable at high pH (Rose, 1994; Shao and Tam, 1995). Stability analysis of sugar oxime conjugates under highly acidic conditions (+37°C pH ~1) analyzed in this study resulted in a half-life of approximately 3 hours for sugar oxime-linkage. As the residence time of compounds in the stomach has been reported to be as low as 0.5 h (Sakkinen et al., 2006) the stability of oxime-linkage in these compounds is expected to be sufficient for therapeutic gastric applications.

By comparing the different methods created in this study (see also Table 9), it can be concluded that reductive amination is clearly the most successful technique (**I**). The disadvantage of this process is that it results in open ring conformation of the reducing sugar unit attached to the scaffold, which may influence the ability of the oligosaccharide to function as a ligand. In contrast, direct amidation of glycosylamine modified oligosaccharides to GlcA carboxyl groups preserves the reducing sugar in its ring conformation (**II**). However, this method resulted in the lowest substitution level observed here, which may be due to steric hindrance. Both methods employing the oxime-linkage worked reasonably well (**II** and unpublished results). Of these, the oxidation and amidation of γ-CD, followed by amidation of oxime modified oligosaccharide to the scaffold may prove too laborious for practical applications.

6.2 Binding activity of an array of neolacto-based structures against *H. pylori* (III)

Helicobacter pylori binding to a wide range of natural and chemically modified neutral and sialylated neolacto-based carbohydrate structures has been investigated in previous studies [e.g. (Johansson et al., 2005; Miller-Podraza et al., 2004; Miller-Podraza et al., 2005). GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer has proven as an effective binder, where terminal GlcNAcβ3 can be replaced by GalNAcβ3, GalNAcα3 or Galα3 without losing activity (Miller-Podraza et al., 2005). In this study, an array of neolacto-based carbohydrate chains were synthesized and studied for their *in vitro* binding activity towards *H. pylori* using TLC overlay assay. As a result two new neolacto-based pentaglycosylceramides with binding activity for *H. pylori* were discovered, GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer and Glcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer. In addition, amidation of the terminal GlcA using methylamide or ethylamide resulted in enhanced binding activity.

A previous study indicates that terminal extension of the active neolacto-based GlcNAcβ1-3Galβ1-4GlcNAc oligosaccharide by Galβ4 and NeuAcα2-3Galβ4 are tolerated by *H. pylori* (Miller-Podraza et al., 2005). In this thesis, it was shown that oxidation/reduction of terminal galactose in Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer abolishes binding activity confirming that vicinal sugars may influence binding. In addition, TLC binding analysis using glycolipids with different core structures revealed an important role for both types of monosaccharides of distal parts of the chains and glycosidic linkages.

Octadecylamide derivative of sialylparagloboside (SPG, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer) is *H. pylori* binding-active on TLC plates (Miller-Podraza et al., 2004). To investigate the possibility that this binding is based on the interaction with the C18 lipid tail, other hydrophobic derivatives of SPG with octadecyl chains were studied. It is known that ceramide is of importance for the interaction of *H. pylori* in some cases (Abul-Milh et al., 2001; Tang et al., 2001; Ångström et al., 1998) and it has been proven that *H. pylori* binds to some phospholipids (Lingwood et al., 1992). Results here verify the importance of the glycerol tail of Neu5Ac and also indicate that the interaction of *H. pylori* with hydrophobic derivatives of SPG is dependent on the presence of sialic acid suggesting a less probable role for hydrophobic aliphatic chains in *H. pylori* binding.

H. pylori has the ability to interact with various terminal neolacto-based oligosaccharides. However, of neolacto-based structures studied here only Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc and Neu5Acα2-3Galβ1-4GlcNAc are ubiquitous components of human cells. The rest have not been described as normal part of carbohydrates structures present in human stomach and other possible *H. pylori* target tissues. Nonetheless, it can not be excluded that carbohydrate chains present on body fluids and other human cells as well as some inner carbohydrate sequences might turn out to be of consequence for the interaction with *H. pylori*. For example, gastric mucosa layer contains glycosaminoglycans such as hyaluronic acid, dermatan sulphate, and heparin sulphate (Theocharis et al., 2003) all of which have both uronic acids and hexosamines, monosaccharide building blocks present in oligosaccharides analyzed in this study. *H. pylori* infection decreases gastric mucin synthesis by inhibition of galactosyltransferase (Tanaka et al., 2003), which may consequently result in increase of *H. pylori* binding to mucin chains carrying GlcNAcβ1-3Galβ1-4GlcNAc epitopes (Miller-Podraza et al., 2005). Accordingly, mucins carrying GlcNAcβ1-3Galβ1-4GlcNAc terminating structures have been described from deep gastric and duodenal glands as well as from neoplasia and metaplasia with gastric differentiation (Hanisch et al., 1993). Additionally, mucins MG1 and MG2, present in the saliva, carry diverse sialylated and neutral carbohydrates based on lacto, neolacto, and GalNAc-containing structures, thus generating a wide range of potential binding sites for microorganisms (Prakobphol et al., 1998; Thompson et al., 2002).

7 CONCLUDING REMARKS

Anti-adhesive therapy based on multivalent sugar receptor analogs can be used both to prevent infection and detach adherent bacteria. The successful treatment of infections in the gastrointestinal tract presents a realistic objective for therapy with receptor analogs. In order to interfere effectively with the multivalent microbe-host cell interaction the inhibitor should be multivalent in nature as well. Consequently, optimized carbohydrate analogs may become too large to be resorbed into the blood, whereas retaining their activity against pathogens in the gastrointestinal lumen.

More than half of world population is infected with *Helicobacter pylori*. Although the current treatment based on antibiotics is quite effective, the eradication of this organism worldwide is complicated by the emergence of resistant strains. Moreover, practical and economic problems preclude intensive and widespread use of antibiotics in most developing countries. Still, *H. pylori* infection poses a considerable health risk and alternative treatment method based on glycobiology is currently under investigation in several laboratories and biocompanies. It is generally accepted that this method would be based on multivalent presentation of *H. pylori* receptor analogs on a suitable scaffold, a concept adopted from natural processes.

In this thesis synthesis of multivalent molecules based on linear (chondroitin 14-mer), cyclic (γ-CD), and globular (dendrimer) scaffolds all presenting several copies of established carbohydrate analogs of the human *Helicobacter* receptor were described. It will be of great interest to assess whether the binding epitope arrangements in the present conjugates results in a clear multivalency effect *in vitro*. The different synthetic methods described here can also be used to attach reducing sugars to create multivalent inhibitory ligands of different specificities. Also, other scaffolds can be easily used, including other GAG structures, other dendrimers, as well as other CDs (α-, β-, and γ-CD). In addition, the structural requirements for binding of *H. pylori* to neolacto-based oligosaccharide chains, *in vitro*, was investigated further. Given account the continuously growing list of saccharide structures known to be active as binding molecules toward *H. pylori* one could speculate, that the best anti-adhesive result will probably be achieved using a mixture of molecules with different epitope activities on a suitable multivalent scaffold.

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