IMMOBILIZATION OF OLIGOSACCHARIDES IN AFFINITY CHROMATOGRAPHY AND APPLICATIONS TO SCREENING HUMAN MILK OLIGOSACCHARIDE RECEPTORS

by

Daniel Hernandez Armada

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry

ABSTRACT

A variety of applications in glycobiology exploit affinity chromatography through the immobilization of glycans to a solid support. Although several strategies are available, in many cases there is a lack of satisfactory chemical characterization. In this thesis we describe work towards improved characterization of chemistries with improved efficiencies which may be essential for work with glycans available only in limited quantity. Several chemical approaches were compared to divinyl sulfone based on immobilization efficiency, attachment to the solid support, and binding capacity to a protein receptor. We observed that immobilizations using *p*-Toluene sulfonyl hydrazide offered higher immobilization efficiencies and comparable binding capacities to the DVS method.

We also generated a series of affinity matrices containing individual human milk oligosaccharides (HMO) species. To test the utility of the affinity matrix for identifying glycan receptors, we used the lysate from human cells. Proteins which specifically bound to the affinity matrix were captured, and then identified using LC-MS/MS-based proteomics analysis. This method has successfully identified a number of HMO-receptors, including members if the galectin family.

A mis padres, quienes me han enseñado a nunca darme por vencido

ACKNOWLEDGMENTS

First, I would like to thank my supervisor, Prof. Chris Cairo, for opening the doors of the Cairo lab for me and for his support over the last two years. I am fortunate to have had a mentor that allowed to develop my scientific skills and helped me to grow personally and professionally. Thank you for having a huge passion for science, that always helped me to keep myself motivated.

My sincere appreciation to Prof. Robert Campbell for always having a word of wisdom whenever I ran into a road block.

Mr. Mark Miskolzie and Dr. Angie Morales Izquierdo, thank you for sharing your knowledge about NMR and MS, and for helping me with my experiments.

I am grateful for the guidance and advice received from Dr. Yoram Apelblat and for giving the opportunity and the trust to run a class in front of 400 students. My sincere gratitude to 溫師傅 (Dr. Hayley Wan) and to Ms. Нада Ђокић (Nada Djokic) for making my lab teaching experience so memorable and fun, for always having a candy or chocolate to share, and also for providing a space to forget about research for a minute. Your enthusiasm is really contagious. Mr. Marcel Munroe and Mr. Matthew Kingston, it was really enjoyable to work with you, thanks for always having a joke and a smile to share.

I was very fortunate to work with a great group of people in the Cairo Lab. My special thanks to 邹纯霞 [Chunxia (Cecilia) Zou] and Jobette Santos, without whom the Chapter 3 of this thesis would not exist. I appreciate the help from অর্পণ দন্ডপাট (Arpan Dandapat), नेहा खन्ना (Neha Khanna), and 张屹 (Dr. Yi Zhang) for getting me started in the art of organic synthesis. I also appreciate the help received from 贾峰 (Feng Jia), 李才顺 [Dr. Caishun (Carson) Li], 郑瑞祥

(Blake Zheng) to learn the biology techniques that I required for doing my experiments, and from امجد لبوهي (Dr. Amgad Albohy) for his help with my statistics and his helpful advices.

I was lucky to find a great group of friends that helped to make this experience so enjoyable, especially: Eva Rodríguez López, Dr. Maribel Funes Huacca, Ralph Enterina, 黎欣 (Tiffany Lay), ساناز نیک جاه (Sanaz Nikjah), Claude Larrivée, Taras Rybak, Артур Яровой (Artur Iarovoi), Drew Hawranik, Burçin Akgün. نازنین عاصم‌پور (Nazanin Assempour), نگار مختاری حج (Negar Moktarihaj), رضا جعفری (Reza Jafari), 吴绍鸣 (Simon Ng), Вадим Маточко (Wadim Matochko), and Bren Atienza.

I'm really grateful with the Lazaruik family for opening the doors of their home for me, and helping me when I first arrived to Canada.

Thanks to all the people that has been part of my life for so many years and helped me despite the distance. Rodrigo, Arturo, Carla, Blanca, Ale López, Ale Castañón, Sandra, Linka, Blanca, Goretti, Ernesto, Rafa, Gaby G, Gaby S, Dr. Neha Gandhi, and Dr. Rosario Mejía.

Finalmente mi eterno agradecimiento a mis papás, Arturo y Mónica, por su incondicional amor y apoyo en cada momento de mi vida. Ustedes son un verdadero ejemplo de tenacidad y eso siempre me ha motivado a seguir adelante. Gracias por siempre impulsarme a ser mejor y por ser un ejemplo de ello; sin sus consejos y apoyo creo que no podría estar donde estoy hoy. Gracias por siempre creer en mi y enseñarme a creer en mi mismo; por ustedes esta meta hoy es realidad.

Arturo, gracias por tu constante apoyo y por siempre ayudarme a dar ese paso que tanto me da miedo. Es verdaderamente una fortuna tenerte como hermano y como amigo. Gracias por siempre estar ahí para escuchar mis aventuras, satisfacer un antojo, o ayudar a los titos cuando las cosas se ponen rudas; pero sobre todo gracias por tu apoyo incondicional.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

2'FL	2'-fucosyllactose
ANOVA	Analysis of variance
CAM	Ceric ammonium molibdate
CaR-ESI-MS	Catch-and-release electrospray ionization mass spectrometry
CC	Cyanuric chloride
CNBr	Cyanogen Bromide
CRD	Carbohydrate recognition domain
CTB	Cholera toxin sub-unit B
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DCM	Dichloromethane
DIPEA	di-isopropyl ethyl amine
DMF	Dimethylformamide
DS'LNT	α2,6-linked disialyllacto-*N*-tetraose
DSLNT	Disialyllacto-*N*-tetraose
DSLNnT	Disialyllacto-*N*-neotetraose
DVS	Divinyl sulfone
ECM	Extracellular matrix
EDTA	Ethylenediamineteraacetic acid
ESI	Electrospray Ionization
EI	Electron Impact
FAC-MS	Frontal affinity chromatography coupled to mass spectrometry

FITC	Fluorescein isothiocyanate
Fuc	Fucose
Gal	Galactose
GalNAc	*N*-Acetylgalactosamine
GBPs	Glycan binding proteins
GlcNAc	*N*–Acetylglucosamine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HMO	Human Milk Oligosaccaride
HR-SSNMR	High resolution solid state nuclear magnetic resonance
ITC	Isothermal titration calorimetry
Lac	Lactose
LAD-II	Leukocyte adhesion deficiency type II
LnNT	Lactose-*N*-neotetraose
LNT	Lactose-*N*-tetraose
Man	Mannose
MAS NMR	Magic angle spinning nuclear magnetic resonance
MeOH	Methanol
NEC	Necrotising enterocolitis
Neu5Ac	*N*-acetylneuraminic acid
NMR	Nuclear magnetic resonance
NBS	*N*-Bromo succinimide
NHS	*N*-hydroxysuccinimide

PNC	Platelet-neutrophil complex
PSA	Phenol sulfuric acid
p-TsH	*para*-toluenesulfonyl Hydrazide
rt	room temperature
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SPR	Surface plasmon resonance
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TOF	Time of flight
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
WGA	Wheat germ agglutin

Chapter 1

The Importance of Carbohydrate – Protein Interactions in Biology: the specific case of Human Milk Oligosaccharides

1.1 Biological roles of glycans

There are three main types of bioploymers in nature: nucleic acids, proteins, and carbohydrates. Carbohydrates are the most abundant compounds produced by living organisms. (*1*) It has been known for a long time that carbohydrates are an important source of energy and act as structural components. Carbohydrates are also known to contribute to the development, growth, and functioning of the organism that synthesizes them. (*2-5*) Carbohydrates have also been recognized as serving critical roles in areas such as cell-cell communication, (*6, 7*) cell signalling, (*8, 9*) and host-pathogen interactions. (*10-12*) Most of these processes are mediated by interactions between proteins and glycans.

Proteins that bind to specific glycans are known as glycan binding proteins (GBPs). There are two types of GBPs: endogenous, which recognize glycans from the same organism and exogenous, which recognize glycans from a different organism. (*13*) Endogenous GBPs permit interactions between cells of the same type, and they can also recognize glycans within the same cell. Exogenous GBPs are often toxins, adhesins, and agglutinins present in pathogenic organisms. These proteins play a crucial role in pathogen – host interactions. (*14*) A graphic representation of this type of interaction is shown in **Figure 1.1**.

Human milk oligosaccharides (HMO) are a specific group of carbohydrates highly abundant and unique to human milk. (*15*) These molecules have received a lot of attention in recent years due to the potential health benefits they offer to the breast-fed infant and the lactating mother. The following sections of this chapter will discuss in more detail the different roles that carbohydrates are proposed to play in order to understand the importance of studying the interactions between HMO and GBPs.

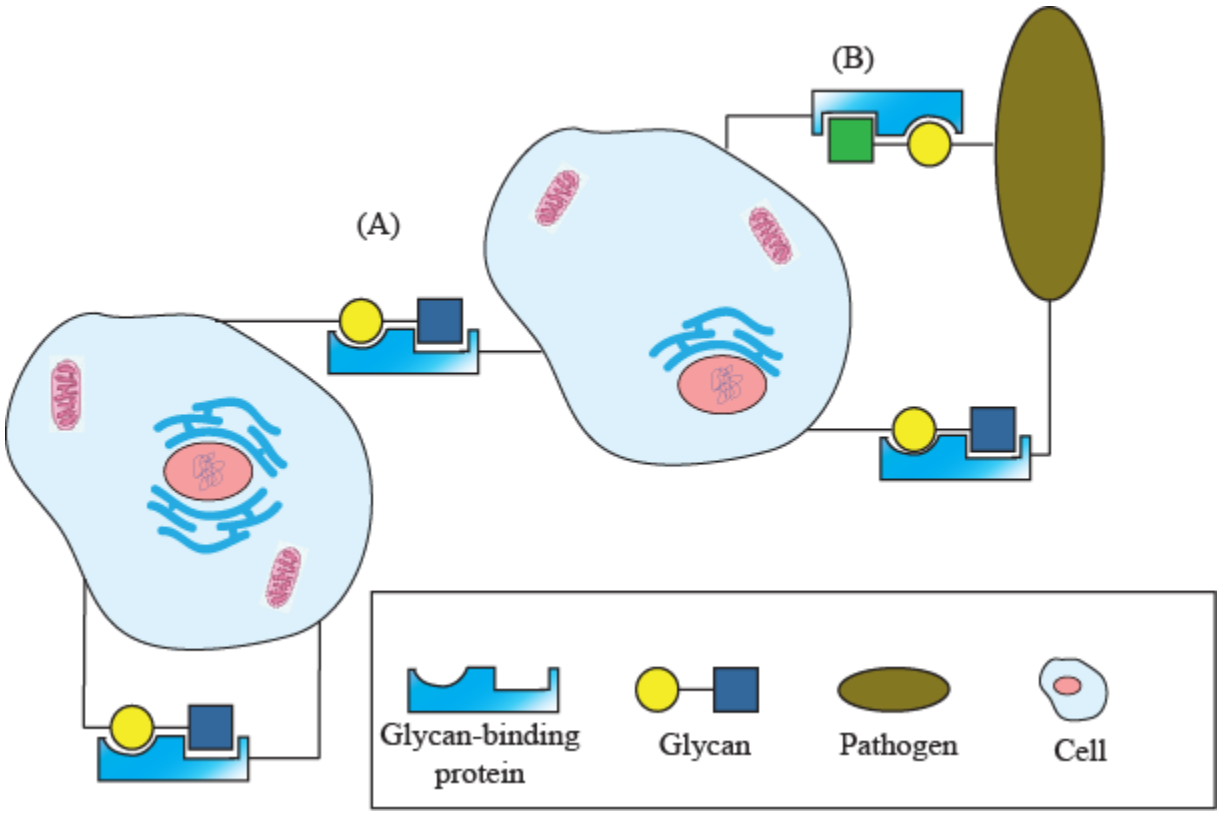


Figure 1.1 Biological roles of glycans.

Intrinsic and extrinsic interactions of lectins with glycan structures; either between (A) the same types of organism or cells or (B) between different types, in this case a cell recognizing a pathogen or a toxin and the pathogen or toxin recognizing the cell. Modified from (*13*).

1.1.1 Structural and modulatory roles of glycans

In cells glycans protect, stabilize, provide organization, and serve as barriers. The best known example of glycans which fits these diverse roles is the glycocalyx. Luft first visualized and described the glycocalyx in 1966 by electron microscopy. (*16*) The glycocalyx is a carbohydrate rich layer that covers all eukaryotic cells and the cell envelope of prokaryotic cells. It serves as a physical barrier between the cell and the external media to limit the flow of

certain molecules to the inside of the cell based on size exclusion. (*17*) The glycocalyx is a network of membrane-bound proteoglycans and glycoproteins. (13) Proteoglycans are comprised of a core protein to which one or more glycosaminoglycan chains are linked. Glycoproteins are proteins that contain oligosaccharide chains. The composition of the glycocalyx around the cell is not uniform. Its components vary at different areas of the cell surface as well as among cell types.

In bacteria, the cell envelope is a complex structure with several layers that serve to protect the organism from the environment. (*18*) Briefly, the first layer is the outer membrane, which is only present in Gram-negative bacteria. The outer membrane is composed mainly of lipopolysaccharide, (*19*) which serves as a barrier, and has also been found to trigger human immune response upon its entrance in the human body as it is recognized by the immune system as an endotoxin. (*20*). The next layer is the peptidoglycan cell wall which serves as a cytoskeleton, hence, determining cell shape. The peptidoglycan is formed from repeating units of *N*-acetyl glucosamine-β1,4-*N*-muramic acid, crosslinked by pentapeptide side-chains. (*21*) The last layer is the inner membrane, which is a phospholipid bilayer where all the membrane-associated functions are performed including lipid biosynthesis, protein secretion, and energy production.

The extracellular matrix (ECM) is comprised of a complex network of polysaccharides, glycoconjugates and proteins secreted by cells. It is a structural element of the cell. The glycoconjugates of the ECM have different binding sites for various sugar chains that play an organizational role. (*9*) Glycosaminoglycans (GAGs) are polysaccharide side chains of proteoglycans formed by linear disaccharide repeating units. (*1*) GAGs are found in abundance in the ECM to maintain surrounding cell hydration. It has been observed that sulfated GAGs,

such as heparin sulfate, interact with fibronectin, an adhesive glycoprotein and collagen fibrous glycoproteins; in this way heparin sulfate can bind to protein ligands. The presence of hyaluronic acid, an anionic non-sulfated glycosaminoglycan, will promote a highly fibrillar and highly hydrated environment that result in cell rounding and locomotion. (*22*) GAGs promote matrix stabilization and participate indirectly in maintaining the morphology and growth rate of differentiated cells.

Another example of the ability of carbohydrates to modulate certain biological processes can be found in plants. Several studies have provided evidence on the role of pectic fragments as regulators of defensive responses, which are generated upon enzymatic digestion of cell wall by plant pathogens. (*23*) The introduction of purified pectic fragments in healthy plant seedlings promoted the activation of a defense mechanisms, the accumulation of oligouronide fragments and the modification of the cell wall structure in sites adjacent to where pectic fragments were introduced. (*24*) These observations support the idea that in plants, the presence of pectic fragments triggers a series of defense reactions

Galectins are a family of proteins with the ability to recognize β-galactosides. (*25*) In mammals, galectins are an example of carbohydrate binding proteins whose interactions are known to regulate many processes. These GBPs bind to extracellular glycoconjugates at the cell surface, which typically contain galactose terminated oligosaccharides. As galectins can bind either bivalently or multivalently, they are able to crosslink glycoconjugates, which can trigger a cascade of transmembrane signaling events. In this fashion, galectins are able to modulate processes such as apoptosis, mitosis, and cell-cycle progression. (*26*)

1.1.2 Glycans as specific ligands for cell-cell interactions

Glycan-containing molecules participate in a wide range of processes at the cellular level and facilitate, trigger, or inhibit certain interactions among cells. Some examples of the roles played by carbohydrates in cell-cell interactions include the formation of colonies in bacteria, the activation of certain immune responses by selectins, and the promotion of metastasis in cancer mediated by galectins. A brief explanation of these interactions is provided in the following paragraphs.

The glycocalyx plays a very important role in bacteria as it promotes cell-cell interactions to form colonies. The formation of colonies allows bacteria to continue growing while offering protection from antibacterial agents, surfactants, bacteriocins, bacteriophages, antibodies, antibiotics, and phagocytic cells. (*27*)

In humans, some growth factors acquire their binding abilities upon glycosylation while others lose theirs. The β-human chorionic gonadotropin is able to bind its receptor when it is not glycosylated, but it fails to interact and stimulate adenylate cyclase when the carbohydrate moiety is missing. (*28*)

Selectins are another example of carbohydrate-mediated cell-cell interactions. These are a family of cell-adhesion molecules responsible for initiating the binding of leukocytes to endothelium and control of lymphocyte homing and leukocyte entry into inflamed tissue. The receptors for these proteins is a Neu5Ac-α2,3-Gal-β1,4(Fuc-α1,3)-GlcNAc oligosaccharide, known as the sialyl-Lewis(x) antigen. (*29*) Individuals that have leukocyte adhesion deficiency type II (LAD-II) cannot incorporate fucose effectively within selectin ligands. As a consequence, leukocytes of LAD-II patients cannot bind selectins which ultimately leads to

recurrent bacterial infections in mucosal membranes and skin, and defects in mental and psychomotor development. (*29, 30*)

GBPs such as galectins have been found to play a role in tumour metastasis. Metastasis is the migration and invasion of tumour cells into surrounding tissues. This event depends on cell-cell and cell-extracellular matrix interactions. Galectin-3, which binds galactose-containing glycoproteins disrupts the cell-cell adhesion interactions between tumour cells or tumour cells to the ECM by binding to the molecules involved in the adhesion. As such, this allows the detachment of the tumour cell from the primary tumour. The detached tumour cell can then use its secreted galectin-3 to form new attachments to other cells to form the secondary tumour. (*26*) Evidence supports that galectin-3 is clustered at the sites of cell-cell contacts, providing more evidence that the protein is indeed involved in homotypic tumour cell adhesion. (*31*)

As it has been described above, carbohydrates facilitate the interaction between different molecules. For bacteria the formation of colonies is essential for their survival; while in humans the ability of β-human chorionic gonadotropin to bind its receptor is influenced by its glycosidation state. If this process is inhibited, the growth of the fetus may be compromised. These two examples illustrate the relevance of studying and understanding the roles that carbohydrates play in cell-cell interactions.

1.1.3 Glycans as specific ligands for cell-microbe interactions

A diverse number of microorganisms recognize glycans as specific binding sites on host cells. These glycan sites are targets for plant and bacterial toxins. The recognition specificity that these pathogens have for the sequence of the glycan involved is remarkable.

(*14*) Some examples of the carbohydrate-pathogen binding specificity are presented in **Table 1.1**.

Binding to carbohydrates is often the first step of pathogenesis for bacterial pathogens, viruses, and parasites. This is especially important in areas of the human body with constant movement, such as the gastrointestinal tract, where surfaces are constantly washed by fluids. (*32*) In order to colonize, the pathogen must be able to adhere to the surface. The best understood mechanism for surface adherence is attachment through lectins. This process is specific, which is critical as the availability of suitable receptors is often dependent on the host's age and the site of the infection. (*5, 32*) Some well-known pathogens which bind to carbohydrates on the surface of human cells are *Kleibsiella pneumoniae*, which has high affinity to mannosyl glycans present in respiratory tissue and uropathogenic *Escherichia coli*, with affinity towards mannose-containing glycans in the urinary tract. (*33*) For viruses, the Dengue fever virus is known to bind to heparin sulfate and chondroitin sulfate at early stages of the disease. (*34*) Moreover, Norwalk, Snow Mountain and Hawaii viruses, are also known to bind to carbohydrate moieties found in cells. These viruses bind ABH histo-blood group antigens as part of their invasion process. (*35*)

Pathogen	Carbohydrate containing receptor	Reference
Campylobacter jejuni	Fuc-α1,2-Gal-β1,4-GlcNAc	(*36*)
Clostridium botulinum toxin	Gangliosides	(*37*)
Escherichia coli Type 1	Man-α1,3-Man-α1,6-Man	(*33*)
E. coli P	Gal-α1,4-Gal	(*33*)
E. coli S	Neu5Ac-α2,3-Gal-β1,3-GalNAc	(*33*)
E. coli CFA/1	Neu5Ac-α2,8	(*33*)
E. coli F1C	GalNAc-β1,4-Gal	(*38*)
E. coli F17	GlcNAc	(*39*)
E. coli O157:H7	Man	(*40*)
Haemophilus influenzae	Neu5Ac-α2,3-Gal-β1,4-GlcNAc-β1,3-Gal-β1,4-GlcNAc	(*41*)
Helicobacter pylori	Neu5Ac, Lewis antigens, sialyllactose,	(*42*)
Kleibsiella pneumoniae	Gangliosides	(*33*)

Table 1.1 Examples of host-pathogen interactions including their binding specificity. The pathogens listed are responsible for common diseases.

Pathogen	Carbohydrate containing receptor	Reference
Neisseria gonorrhoeae	Man	(*43*)
Neisseria meningitidis	Gal-β1,4-GlcNAc	(*43*)
Pseudomonas aeruginosa	Gal-β1,4-GlcNAc-β1,3-Gal-β1,4GlcNAc	(*44*)
	L-Fuc	
Salmonella typhimurium	Man	(*45*)
Shigella (toxin)	Globotriaosylceramide (Gb3)	(*46*)
	Gal-α1,4-Gal-β1,4-Glc	
Vibrio cholerae	Man, L-Fuc, GlcNAc	(*47*)

Table 1.1 cont. Examples of host-pathogen interactions including their binding specificity. Many of the pathogens listed are responsible for common human diseases.

Table 1.1 presents an abridged list of pathogens that have known carbohydrate binding specificities, showing the variability of these interactions. These binding specificities have served as targets for the development of antiadhesives to prevent the infection. One particular group of molecules that has received a large amount attention for their potential as anti-adhesive antimicrobials is human milk oligosaccharides (HMO). The evidence for HMO antiadhesive and other biological activities will be discussed in the following section.

1.2 Human Milk Oligosaccharides

Human milk oligosaccharides (HMO) are a family of diverse, unconjugated glycans that are highly abundant in and unique to human milk. The concentration of oligosaccharides in human milk is considerably higher in comparison to bovine milk (20 g/L versus 0.05 g/L, respectively). (*48*) This abundance has led investigators to propose biological, physiological and protective functions for these molecules. (*49, 50*) HMO are composed of five monomers: D-Glucose, D-Galactose, *N*-acetyl-D-glucosamine, L-fucose, and sialic acid [*N*-acetyl-D-neuraminic acid (Neu5Ac)]. The structure of HMO tends to include a lactose core at the reducing end which is elongated by *N*-acetyllactosamine units. Structural diversity is achieved by extensive fucosylation and sialylation at the terminal positions. (*50, 51*) More than one hundred different HMO have been identified to date; however, there is variability in the HMO produced between individuals. (*15*)

An interesting fact about HMO is that they are not metabolized by the breast-fed infant. Once ingested, HMO resist the acidity in the gastrointestinal tract and brush border enzymes. (*52*) Several groups have reported that HMO reach the distal small intestine and colon without being modified, and most of them are excreted in the infant's feces. (*53, 54*) However, other groups have noted that the amount and type of HMO present in feces changes as the child develops. HMO entirely disappear from the infant feces once they begin to be weaned. (*55*) It has also been shown that a small percentage (approximately 1%) of HMO are absorbed in the infant's intestine and reach the systemic circulation. This finding was provided by Rudlof et al., who showed that intact HMO appear in the urine of breast-fed infants. (*56, 57*) However, data published this year suggest that neutral HMO might be processed differentially once it is

absorbed in the gut, and their effects may be exerted beyond the intestine and the infant's microbial composition alone. (*58*)

1.2.1 HMO as prebiotics

A prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confers benefits upon host well-being and health." (*59*) The ability of HMO for reaching the distal colon without being modified has allowed researchers to hypothesize that they may play a significant role in shaping the microbiome of breast-fed infants. It has been observed that babies that are breast-fed have an unusual abundance of bacteria, generally characterized as *Bifidobacterium*. (*60*) Moreover, other groups have observed that *Bifidobacterium longum* subspecies *infantis* can easily grow when HMO are offered as the sole carbohydrate source. (*61*) Other studies have reported a predominance of *B. infantis* over other *Bifidobacterium* species when lactose-*N*-neotetraose (LNnT) is present. A different study identified tha *B. infantis* could inhibit bacteria from the genera *Salmonella*, *Listeria*, *Campylobacter*, and *Shigella*. (*62*) These results may imply that *B. infantis* inhibit potentially harmful bacteria by competing for limited nutrient supply. It has also been observed that *B. infantis* produces short-chain fatty acids and other metabolites that create a favourable environment for the growth of commensals while inhibiting potential pathogens. (*63*)

The observations described above have led to the introduction of the term "bifidus factor" to refer to the property of milk that facilitates the growth of this type of bacteria. It is of considerable interest to understand what components of breast milk lead to the unusual accumulation and persistence of the protective microbiome of breast-fed infants. (*51*)

1.2.2 HMO as antiadhesive antimicrobials

Besides the competitive advantage that HMO provide to non-pathogenic bacteria, there is evidence that the oligosaccharides and other glycoconjugates present in human milk have an inhibitory effect on certain virulence-related abilities of pathogenic microorganisms. (*50, 64*) As discussed above, many pathogens rely on their ability to adhere to mucosal surfaces to colonize the host and cause disease. Some HMO prevent intestinal attachment of microorganisms working as soluble decoy receptors competing with epithelial ligands for pathogen binding. (*36, 65, 66*)

One of the best understood pathogens is *Campylobacter jejuni*, whose infections are one of the most common causes of bacterial diarrhea and infant mortality. Blocking of the binding capacity of this pathogen to cultured cells and human mucosa was achieved by adding soluble α1,2-fucolsylated HMO. (*36*) This blocking capacity has been confirmed *in vivo* with mother-infant pairs from Mexico City. *Campylobacter jejuni*-associated diarrhea occurred significantly less often in infants whose mother's milk contained high concentrations of 2'-fucosyllactose (2'FL). (*67*)

In a similar way, neutral oligosaccharides present in human milk were able to protect the intestinal tract of newborns from infection by *Vibrio cholerae*. (*68*) In a different research study, Andersson et al. showed that the adhesion of pneumococci or *Haemophilus influenza*e, which usually adhere to specific carbohydrate structures (from the neolactoseries) of pharyngeal or buccal epithelial cells, was inhibited with human milk. (*69*) These two studies highlight the importance of HMO in providing protection all along the gastrointestinal tract.

The microorganisms discussed above express lectins that bind to glycans on the host cell. Other microorganisms reverse this arrangement by expressing glycans that bind to lectins on host cells. A relevant example of this group of microorganisms is the human immunodeficiency virus (HIV), which binds to the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) on human dendritic cells. (*70*) The envelope glycoprotein gp120 must bind to DC-SIGN in order for HIV to enter through mucosal barriers during mother-to-child transmission of HIV. (*71*) DC-SIGN has a high affinity to high-mannose glycans on gp120 but it has higher affinity to receptors with either a Gal-β1,3(Fuc-α1,4)-GlcNAc oligosaccharide or a Fuc-α1,2-Gal-β1,3(Fuc-α1,4)-GlcNAc oligosaccharide, known as Le blood group antigens (*72*) that can be found in human milk and will compete with gp120 for binding to DC-SIGN. (*71*) This affinity may explain the low occurrence (around 10%) of mother-to-child HIV transmission through breastfeeding, despite the continuous exposure to the virus for several months.

HMO not only provide protection against bacteria and viruses, but there are also reports of their antiadhesive antimicrobial action against parasites. One of the parasites that has been extensively studied is *Entamoeba histolytica*. This parasite has infected nearly 50 million people, and causes 100,000 deaths annually. Jantscher-Krenn et al. observed that some HMO significantly reduce *E. histolyica* attachment and cytotoxicity in co-cultures with human intestinal epithelial cell lines. (*73*) This observation is particularly interesting since *E. histolytica* colonization requires the parasite to attach to the host's colonic mucosa. If the parasite is not able to attach, it is carried downstream and excreted with the feces without causing disease. (*15*)

There are several other examples where HMO exhibit antiadhesive antimicrobial effects, but only some examples have been included here. (*15, 40*) It is important to highlight that HMO contribute to lower incidence of intestinal, upper respiratory, and urinary tract infections in breast-fed infants, and not only in the intestine or colon as could be expected. HMO also exhibit a wide spectrum of protection since they possess antiadhesive antimicrobial activity against bacteria, viruses, and parasites. (*15, 36, 68, 71, 73*)

Finally, it has also been observed that some of the HMO present in milk have antipathogenic effects when they are conjugated to biomolecules. One example is the sialic acid-containing gangliosides that inhibited the enterotoxin from *E. coli* and *V. cholera*. (*74, 75*) The milk ganglioside, GM1, was identified as the component responsible for exerting this effect. (*76*) Moreover, milk gangliosides can also bind Shiga toxin (*77, 78*) cholera toxin, (*79*) and botulinum neurotoxin type A. (*80*)

1.2.3 HMO as immunomodulators

Although HMO have not been detected in the blood of breast-fed infants, (*81*) there is evidence that a small amount of them is absorbed from the gastrointestinal tract and taken into the circulatory system since small amounts of HMO have been detected in urine. (*56*) It follows that HMO may alter protein-carbohydrate interactions at a systemic level. Recent studies suggest that HMO interfere with the adhesion of neutrophils to vascular endothelial cells (*82*) and platelets. (*83*) The hypothesis behind this observation is that HMO resemble the glycoprotein ligands of selectins. (See **Section 1.1.2**)

Selectins bind to glycans with Le blood group epitopes, which are sialylated and fucosylated lacto-*N*-bioses (Gal-β1,3-GlcNAc), or *N*-acetyllactosamines (Gal-β1,4-GlcNAc),

very similar to HMO. It is known that HMO contain Le blood group antigens, (*84*) and they are able to inhibit selectin mediated cell-cell interactions. (*83*) Sialylated HMO reduce the formation of platelet-neutrophil complexes (PNC) and inhibit neutrophil activation in an *ex-vivo* model with whole human blood. (*83*) PNC are a subpopulation of highly reactive neutrophils primed for adhesion, phagocytosis, and enhanced production of reactive-oxygen species. PNC has an anti-inflammatory effect that is inhibited when the binding of selectins to the surface of endothelial cells and platelets is inhibited. (*83*)

At the moment, it is unknown which receptors and signaling pathways involved in lymphocyte cytokine production or macrophage stimulation which are affected by HMO. A large number of lectins are involved in the immune system, and the glycan binding specificity of many human lectins may suggest that HMO could interfere with these processes. For example, siglecs are a family of sialic-acid-binding immunoglobulin-like lectins that are thought to promote cell–cell interactions and regulate the functions of cells in the innate and adaptive immune systems through glycan recognition Siglecs have been shown to bind sialylated HMO. (*85*) This observation is important since Siglecs are regulators of the immune system. (*86*)

Some studies have shown that the incidence of necrotizing enterocolitis (NEC), a condition that is considered to be an exaggerated immune response caused by increased mucosal neutrophil infiltration and activation, is 85% lower in breast-fed than formula fed infants. (*87*) This finding is consistent with an anti-inflammatory effect of absorbed HMO. (*88*) Studies carried out in a rat model, show that HMO provide protection from NEC. In these studies, the survival rate and pathology scores significantly improve when HMO were added to the orally gavaged formula. (*89*) The HMO responsible for this effect was identified as

disialyllacto-*N*-tetraose (DSLNT). A follow up article was recently published where the authors found that the disialyl component is important for the beneficial effect of DSLNT, after testing monosialylated and disialilylated analogs of DSLNT. They found disialyllacto-*N*-neotetraose (DSLNnT), and α2,6-linked disialyllacto-*N*-tetraose (DS'LNT) are also good candidates for preclinical experiments in treating NEC. It still remains unknown how this carbohydrate provides protection from NEC and whether the results observed in the animal model can translate to human neonates. (*90*)

1.2.4 Other proposed benefits of HMO

It has been observed that breast-fed infants have superior developmental scores at 18 months of age and higher intelligence quotients at the age of 7. (*91*) There is evidence that Neu5Ac-containing gangliosides and poly-Neu5Ac containing glycoproteins contribute to brain development and cognition. (*92*) Moreover, post-mortem analysis of human neonates showed that ganglioside and protein-bound Neu5Ac concentration were significantly higher in the brains of breast-fed infants compared with infants fed with formula that contained lower amounts of Neu5Ac than human milk. (*93*)

A lot of effort has been devoted to establishing the potential beneficial effects of HMO to the neonate. However; there is evidence that the lactating mother may also benefit from producing the breast milk. Human milk is not sterile and contains bacterial populations that are specific to each woman. (*94*) The bacteria present in milk could potentially serve as natural probiotics to inoculate the infant's intestinal microbiota, but they may also contribute to the regulation of the mother's milk composition or pathogens that cause diseases like mastitis. Some strains of *Staphylococcus*, the bacteria responsible for mastitis, have shown affinity

towards 2'FL in a biosensor based assay. (*95*) Yet there is no evidence to support that HMO reduce the likelihood of acquiring mastitis. (*95*) Additionally, HMO have been found in pregnant women's urine shortly before giving birth which indicates retrograde "leakage" to the circulation, and suggests potential systemic effects for the breast-feeding woman. (*96*)

There is strong evidence of the potential beneficial effects of HMO for both the neonate and the lactating mother. However, if we consider the evidence discussed, there is a common denominator: most of the studies offer no molecular explanation of the mechanisms or the type of interactions underlying the beneficial effects claimed. Although HMO may mediate biological effects through protein-glycan interactions, the target of these interactions are not known. Below we discuss strategies for identifying protein carbohydrate interactions, which must underpin the mechanisms of HMO-mediated effects.

1.3 Techniques for studying carbohydrate – protein interactions

The study of carbohydrate-protein interactions is challenging due to the complexity of the carbohydrates, the amphiphilic character of carbohydrates results in different types of interactions with surrounding water molecules. The hydrophobic ring forces densely packed water molecules, whereas the hydroxyl groups force the binding water molecules to arrante in a configuration that is less favourable. (*97*) Moreover, the weak affinities of the binding between these two types of molecules makes the problem more complex. The interactions between carbohydrates and proteins are usually weaker in comparison to protein – protein interactions in terms of equilibrium dissociation constants (K_D). The K_D of a lectin binding with a monosaccharide is usually in the mM range (*98, 99*) for example: Concanavalin A with mannopyranosides (K_D ~ 0.1 mM) (*100*), MBP to several monosaccharides (K_D from 0.1 to 1

mM). (*100, 101*) These affinities are usually improved when the lectins bind to oligosaccharides. For example, the K_a of Concanavalin A binding to a trimannoside oligosaccharide ($K_a = 4.9 \times 10^5\ M^{-1}$) is 60 times larger than the mannose monomer ($K_a = 8.2 \times 10^3\ M^{-1}$). (*102*) The same is true for MBP, with a K_a ($6.25 \times 10^6\ M^{-1}$) for maltose (Glu-α1,4-Glu) and it shows no binding to glucose as a monomer. (*103*) In order to improve these affinities, nature uses multivalent interactions – simultaneous interactions between the clusters of carbohydrates and proteins that have multiple carbohydrate recognition domains. (*104-106*) Multivalency has been reported to increase the strength of the interactions up to a 10,000-fold. (*107*) With the incorporation of high densities of carbohydrates on the cell surface, the multivalent contact points (hydrogen bonds, van der Waals interactions, and hydrophobic interactions) between the carbohydrates and proteins can result in the so-called cluster glycoside effect, which can result in almost logarithmic enhancements of binding potencies. (*101*)

The detection of carbohydrate – protein interactions, the determination of their structure, and the measurement of their thermodynamic and kinetic parameters is fundamental for the design of carbohydrate-based therapeutics for the treatment of diseases. Several analytical techniques have been developed for identifying and quantifying carbohydrate – protein interactions in vitro, each with strengths and weaknesses.

Isothermal titration calorimetry (ITC) is the gold standard for quantifying the thermodynamic parameters of complex formation, and it is the only method that directly provides the enthalpy of association. One of the major drawbacks from this technique is that the conventional instruments require large amounts of pure protein and ligand. Newer instruments, such as nano ITC, have improved sensitivity and lowered sample requirements.

(*108, 109*) Another drawback of this technique is that it only allows the study of one protein interacting with one carbohydrate at a time, which could be time and labour intensive considering a system where both the interacting ligand-receptor pair is unknown.

Surface Plasmon Resonance (SPR) can be used to measure the affinity of an immobilized binding partner to one in solution. (*110*) A potential limitation for this approach is that one of the molecules involved in the binding needs to be immobilized on the sensor chip, which may affect the binding interaction. There are some examples where ITC and SPR have yielded divergent binding data for the same protein – ligand complex. (*111*) SPR has certain limitations, the surface immobilization method needs to be designed to control the surface density so it mimics multivalent presentations found in nature. In addition, the immobilization method should not disrupt the carbohydrate-protein interaction of interest. (*112, 113*)

Carbohydrate microarrays have evolved as a popular tool for high-throughput studies of binding interactions. The arrays consist of oligosaccharides that are attached to a surface via a covalent linker. (*114*) Potential binders are incubated with the array, followed by a washing step, and the binding complexes are identified using a secondary reporter, usually a fluorophore. (*115*) The need of a secondary reporter increases the cost of this type of assay, and they can prevent quantitative measurements. A dense presentation of the oligosaccharides serves to mimic the situation encountered on cell surfaces that allows for multivalent interactions with low-affinity carbohydrate ligands. (*116*) This approach offers the simultaneous screening of multiple glycans for a single receptor. Arrays also require having multiple pure oligosaccharides with the corresponding receptor in order to obtain useful data. Moreover, it is known that glycan arrays may produce false negatives and are sensitive to the nature of the linker used to immobilize the glycans. (*117, 118*) Furthermore, glycan

microarrays represent a limited number of glycans compared to the naturally occurring structures. The ability to detect a GBP will depend on the platform used. (*119*) Hsu et al. used a different approach to study glycan-protein interactions with microarrays. They immobilized the lectins and exposed the resulting lectin array to different sugars and detected the binding indirectly. This approach, however, is labour intensive since the lectins had to be recombinantly produced due to the fact that some lectins contain carbohydrate moieties that may disrupt the binding or enhance it leading to false positives or false negatives. (*120*)

The direct ESI-MS assay is gaining popularity for studying carbohydrate-protein interactions due to its versatility and convenience for quantifying association constants. (*121-123*) The catch-and-release (CaR-ESI-MS) format permits the screening of hundreds of carbohydrates against target proteins. The method involves incubating the protein with the ligands, followed by direct ESI-MS analysis to detect the protein binding with the highest affinity ligands. When the complex mass cannot be determined, the ligand is released as ions, and its mass determined alone or in combination with fragmentation or ion mobility separation. (*124*) The main drawback of this method is the necessity of knowing the protein and ligand concentrations for calculating the K_a.(*125*)

Affinity chromatography combined with mass spectrometry provides a very powerful method for the analysis of mixtures of compounds for specific protein interactions. The method involves the immobilization of one of the binding partners (e.g. the carbohydrate) to a solid support while the binding partner is in a mobile phase as part of a complex mixture (e.g. cell lysate). The capture step is followed by washing, to remove non-specific or weak binders; and elution, to disrupt the binding and obtain the binding partners in solution again. (*126*) The proteins that were bound can be identified by mass spectrometry by obtaining proteolytic

peptide fragments followed by its analysis using database search algorithms for identification. (*127*) One of the advantages of this method is that it provides a high-throughput screening of ligands. Moreover, its application in a broad range of biological systems makes it very convenient for the screening and identification of new potential drug leads. This technique has the potential to provide the corresponding K_a values when frontal affinity chromatography – mass spectrometry (FAC-MS) method is used. (*128*) However, the method traditionally involves feeding ligands continuously into the column where the receptor has been immobilized on a solid support. Those ligands with affinity will be retained and they will not come out of the column until their breakthrough volume is achieved, at this point they are identified by means of mass spectrometry. (*129*)

The immobilization of carbohydrates onto solid supports has been studied in the past, and several methods are currently available. The immobilization of carbohydrates using divinyl sulfone, a bi-functional reagent, has been used since the 70's due to the simplicity of the method, and the possibility of doing the coupling in aqueous solution. (*130*) However, the method has some drawbacks: the protocol requires a capping step, which increases the possibility of non-specific interactions; and also the immobilization is not regioselective, it can occur in any of the hydroxyl groups available which may interfere with the binding. (*90*) A more detailed discussion on affinity chromatography and the different methods for the immobilization of carbohydrates and proteins can be found in **Sections 2.1.1** and **2.1.2**.

1.4 Hypothesis and project objectives

As discussed above, HMO are thought to be important contributors to the health and development of the breast-fed infant. The precise molecular mechanisms underlying these

proposed effects are not well understood, and more data on the molecular interactions of these ligands with their target receptors is needed.

For example, in 2012 the Bode group showed evidence that disialyllacto-*N*-tetraose (DSLNT) can reduce the incidence of necrotizing enterocolitis (NEC) in a neonatal rat model. The study involved the fractionation of milk based on charge, characterisation, and testing in the animal model. The fraction with the greatest beneficial effect was further fractionated until they were able to identify DSLNT as the specific HMO responsible for reducing the incidence of necrotizing enterocolitis. However, the mechanism of how DSLNT reduces NEC remains to be elucidated. (*89*)

In a different study, the binding antigen of *Campylobacter jejuni* was identified. The authors hypothesized that the receptor binding to this bacterium had to be one that recognized fucose since the presence of the saccharide at high concentrations inhibits the binding of *C. jejuni* to epithelial cells. After a series of experiments that involved the production of neoglycoproteins, the transfection of cells, and several *in vivo* and *ex vivo* studies the authors were able to identify Fuc-α1,2-Gal-β1,4-GlcNAc (known as H(O) blood group) as a critical human intestinal ligand for *Campylobacter jejuni*. (*36*)

In the examples described above, we can notice that the identification of a single HMO – receptor pair is a lengthy, labour-intensive process that also requires having access to significant amounts of HMO, both as a mixture and in pure form. Bearing this in mind and taking into consideration the methods currently available for studying interactions between carbohydrates and proteins, we hypothesized we could develop a high-throughput method based on affinity chromatography coupled to mass spectrometry. The main difference of our method would be that the ligand is immobilized in the solid support and the receptors, coming

from human and bacterial sources, will be flowed through the column. The receptors binding to the HMO attached in the solid support can be identified after elution from the column, and analyzed using electrospray-ionization mass spectrometry (ESI-MS). Knowing the identity of both carbohydrate and protein opens up the possibility of doing more studies, among them: determining the K_a, carrying out inhibition studies or other biological tests to observe the effect of the interaction in cells, tissues or animal models with the objective of better understanding the nature of the interactions and their implications in health research.

In **Chapter 2** we envisioned that we could develop a method for selectively immobilizing the HMO to a solid support through the reducing end using *p*-Toluenesulfonyl hydrazide activation without requiring any protective groups. (*131, 132*). Considering that HMO diversity arises from extensive fucosylation and sialylation starting from a common lactose core, (*50, 51*) we believe that the immobilization from the reducing end would be the least disruptive to binding between HMO and the corresponding receptor. The method developed can be used to immobilize HMO in a way that preserves the binding epitope, and can be used to capture human and bacterial receptors, as it is described in **Chapter 3**.

To test these hypotheses we decided to compare our approach for carbohydrate immobilization against two benchmark methods: immobilization using divinyl sulfone (DVS) and immobilization using cyanuric chloride (CC). We decided to compare the three methods on the basis of efficiency by measuring the amount of carbohydrate immobilized into the solid support; binding capacity by determining the amount of protein that 1 mg of resin could capture; and also compared the stereoselectivity, if any, that each method provided by running a series of model reactions in solution and comparing their NMR spectra. These studies revealed that the approach taken for the carbohydrate immobilization was successful since we

can get comparable binding capacity with our method using almost half the amount of carbohydrate, which in the case of HMO is very valuable due to their limited availability.

In **Chapter 3** we tested our approach for the identification of HMO binding receptors building a series of affinity chromatography columns using five commercially available HMO, (**Figure 1.2**) we then optimized a procedure for liquid chromatography to identify binding receptors. We tested two approaches for the identification of the proteins binding to the immobilized HMO. The receptor-ligand pairs that we identified support the idea that our approach can identify multiple ligands using a simple and straightforward method, opening the possibility of screening large number of ligands in relatively short periods of time. As we envisioned, the method allowed us to identify several receptor-ligand pairs.

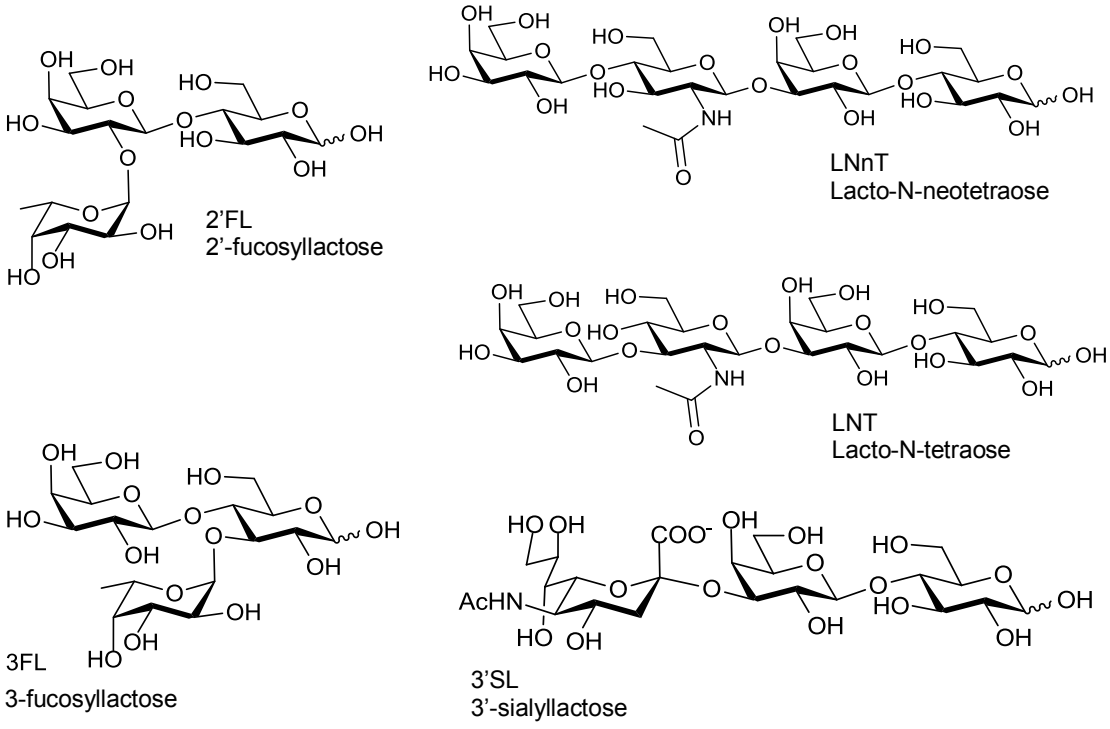


Figure 1.2 HMO immobilized in solid support for binding studies in Chapter 3.

1.5 References

1. Esko, J. D., Kimata, K., and Lindahl, U. (2009) Chapter 16 Proteoglycans and Sulfated Glycosaminoglycans, In *Essentials of Glycobiology* (Varki, A., Cummings, R., Esko, J. D., Freeze, H. H., Stanley, C. M., Bertozzi, C. R., Hart, G. W., and Etzler, M. E., Eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
2. Esko, J. D. (1991) Genetic analysis of proteoglycan structure, function and metabolism, *Current Opinion in Cell Biology 3*, 805-816.
3. Hart, G. W. (1992) Glycosylation, *Current Opinion in Cell Biology 4*, 1017-1023.
4. Paulson, J. C. (1989) Glycoproteins: what are the sugar chains for?, *Trends in Biochemical Sciences 14*, 272-276.
5. Rademacher, T. W., Parekh, R. B., and Dwek, R. A. (1988) Glycobiology, *Annual Review of Biochemistry 57*, 785-838.
6. McMillan, S. J., and Crocker, P. R. (2008) CD33-related sialic-acid-binding immunoglobulin-like lectins in health and disease, *Carbohydrate Research 343*, 2050-2056.
7. Brandley, B. K., and Schnaar, R. L. (1986) Cell-surface carbohydrates in cell recognition and response, *Journal of Leukocyte Biology 40*, 97-111.
8. Sacchettini, J. C., Baum, L. G., and Brewer, C. F. (2001) Multivalent Protein−Carbohydrate Interactions. A New Paradigm for Supermolecular Assembly and Signal Transduction, *Biochemistry 40*, 3009-3015.
9. Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct, *Glycobiology 3*, 97-130.

10. Kumari, K., Gulati, S., Smith, D., Gulati, U., Cummings, R., and Air, G. (2007) Receptor binding specificity of recent human H3N2 influenza viruses, *Virology Journal 4*, 42-53.

11. Ko, J., and Splitter, G. A. (2003) Molecular Host-Pathogen Interaction in Brucellosis: Current Understanding and Future Approaches to Vaccine Development for Mice and Humans, *Clinical Microbiology Reviews 16*, 65-78.

12. Sequeira, L. (1978) Lectins and their role in host-pathogen specificity, *Annual Review of Phytopathology 16*, 453-481.

13. Gagneux, P., and Varki, A. (1999) Evolutionary considerations in relating oligosaccharide diversity to biological function, *Glycobiology 9*, 747-755.

14. Varki, A., and Lowe, J. B. (2009) Biological Roles of Glycans, In *Essentials of Glycobiology* (Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hart, G. W., and Etzler, M. E., Eds.) 2nd ed., pp 75-88, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).

15. Bode, L. (2012) Human milk oligosaccharides: Every baby needs a sugar mama, *Glycobiology 22*, 1147-1162.

16. Luft, J. H. (1966) Fine structures of capillary and endocapillary layer as revealed by ruthenium red, *Federation proceedings 25*.

17. van Haaren, P. M. A., VanBavel, E., Vink, H., and Spaan, J. A. E. (2003) Localization of the permeability barrier to solutes in isolated arteries by confocal microscopy, *American Journal of Physiology - Heart and Circulatory Physiology 285*, H2848-H2856.

18. Silhavy, T. J., Kahne, D., and Walker, S. (2010) The Bacterial Cell Envelope, *Cold Spring Harbor Perspectives in Biology 2*.

19. Kamio, Y., and Nikaido, H. (1976) Outer membrane of Salmonella typhimurium: accessibility of phospholipid head groups to phospholipase C and cyanogen bromide activated dextran in the external medium, *Biochemistry 15*, 2561-2570.

20. Raetz, C. R. H., and Whitfield, C. (2002) Lipopolysaccharide Endotoxins, *Annual Review of Biochemistry 71*, 635-700.

21. Vollmer, W. (2008) Structural variation in the glycan strands of bacterial peptidoglycan, *FEMS Microbiology Reviews 32*, 287-306.

22. Hook, M., Kjellen, L., Johansson, S., and Robinson, J. (1984) Cell-Surface Glycosaminoglycans, *Annual Review of Biochemistry 53*, 847-869.

23. Ryan, C. A., and Farmer, E. E. (1991) Oligosaccharide Signals in Plants: A Current Assessment, *Annual Review of Plant Physiology and Plant Molecular Biology 42*, 651-674.

24. Benhamou, N., Chamberland, H., and Pauzé, F. J. (1990) Implication of Pectic Components in Cell Surface Interactions between Tomato Root Cells and Fusarium oxysporum f. sp. radicis-lycopersici: A Cytochemical Study by Means of a Lectin with Polygalacturonic Acid-Binding Specificity, *Plant Physiology 92*, 995-1003.

25. Barondes, S. H., Castronovo, V., Cooper, D. N. W., Cummings, R. D., Drickamer, K., Felzi, T., Gitt, M. A., Hirabayashi, J., Hughes, C., Kasai, K.-i., Leffler, H., Liu, F.-T., Lotan, R., Mercurio, A. M., Monsigny, M., Pillai, S., Poirer, F., Raz, A., Rigby, P. W. J., Rini, J. M., and Wang, J. L. (1994) Galectins: A family of animal β-galactoside-binding lectins, *Cell 76*, 597-598.

26. Liu, F.-T., and Rabinovich, G. A. (2005) Galectins as modulators of tumour progression, *Nature Reviews Cancer 5*, 29-41.

27. Costerton, J. W., Irvin, R. T., and Cheng, K. J. (1981) The Bacterial Glycocalyx in Nature and Disease, *Annual Review of Microbiology 35*, 299-324.

28. Matzuk, M. M., Krieger, M., Corless, C. L., and Boime, I. (1987) Effects of preventing O-glycosylation on the secretion of human chorionic gonadotropin in Chinese hamster ovary cells, *Proceedings of the National Academy of Sciences of the United States of America 84*, 6354-6358.

29. Luhn, K., Wild, M. K., Eckhardt, M., Gerardy-Schahn, R., and Vestweber, D. (2001) The gene defective in leukocyte adhesion deficiency II encodes a putative GDP-fucose transporter, *Nature Genetics 28*, 69-72.

30. Ley, K. (2003) The role of selectins in inflammation and disease, *Trends in Molecular Medicine 9*, 263-268.

31. Glinsky, V. V., Glinsky, G. V., Glinskii, O. V., Huxley, V. H., Turk, J. R., Mossine, V. V., Deutscher, S. L., Pienta, K. J., and Quinn, T. P. (2003) Intravascular Metastatic Cancer Cell Homotypic Aggregation at the Sites of Primary Attachment to the Endothelium, *Cancer Research 63*, 3805-3811.

32. Dai, D., Nanthkumar, N. N., Newburg, D. S., and Walker, W. A. (2000) Role of Oligosaccharides and Glycoconjugates in Intestinal Host Defense, *Journal of Pediatric Gastroenterology and Nutrition 30*, S23-S33.

33. Sharon, N. (2006) Carbohydrates as future anti-adhesion drugs for infectious diseases, *Biochimica et Biophysica Acta (BBA) - General Subjects 1760*, 527-537.

34. Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J., and Marks, R. M. (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate, *Nature Medicine 3*, 866-871.

35. Harrington, P. R., Lindesmith, L., Yount, B., Moe, C. L., and Baric, R. S. (2002) Binding of Norwalk Virus-Like Particles to ABH Histo-Blood Group Antigens Is Blocked by Antisera from Infected Human Volunteers or Experimentally Vaccinated Mice, *Journal of Virology 76*, 12335-12343.

36. Ruiz-Palacios, G. M., Cervantes, L. E., Ramos, P., Chavez-Munguia, B., and Newburg, D. S. (2003) Campylobacter jejuni Binds Intestinal H(O) Antigen (Fucα1, 2Galβ1, 4GlcNAc), and Fucosyloligosaccharides of Human Milk Inhibit Its Binding and Infection, *Journal of Biological Chemistry 278*, 14112-14120.

37. Lacy, D. B., and Stevens, R. C. (1999) Sequence homology and structural analysis of the clostridial neurotoxins, *Journal of Molecular Biology 291*, 1091-1104.

38. Khan, A. S., Kniep, B., Oelschlaeger, T. A., Van Die, I., Korhonen, T., and Hacker, J. (2000) Receptor Structure for F1C Fimbriae of Uropathogenic Escherichia coli, *Infection and Immunity 68*, 3541-3547.

39. Buts, L., Bouckaert, J., De Genst, E., Loris, R., Oscarson, S., Lahmann, M., Messens, J., Brosens, E., Wyns, L., and De Greve, H. (2003) The fimbrial adhesin F17-G of enterotoxigenic Escherichia coli has an immunoglobulin-like lectin domain that binds N-acetylglucosamine, *Molecular Microbiology 49*, 705-715.

40. Ashkenazi, S. (1994) Review of the Effect of Human-Milk Fractions on the Adherence of Diarrheogenic Escherichia coli to the Gut in an Animal-Model., *Israel Journal of Medical Sciences 30*, 335-338.

41. Ukkonen, P., Varis, K., Jernfors, M., Herva, E., Jokinen, J., Ruokokoski, E., Zopf, D., and Kilpi, T. (2000) Treatment of acute otitis media with an antiadhesive oligosaccharide: a randomised, double-blind, placebo-controlled trial, *The Lancet 356*, 1398-1402.

42. Mysore, J. V., Wigginton, T., Simon, P. M., Zopf, D., Heman-Ackah, L. M., and Dubois, A. Treatment of Helicobacter pylori infection in rhesus monkeys using a novel antiadhesion compound, *Gastroenterology 117*, 1316-1325.

43. Mandrell, R. E., Griffiss, J. M., and Macher, B. A. (1988) Lipooligosaccharides (LOS) of Neisseria gonorrhoeae and Neisseria meningitidis have components that are immunochemically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes, *The Journal of Experimental Medicine 168*, 107-126.

44. Imberty, A., Wimmerová, M., Mitchell, E. P., and Gilboa-Garber, N. (2004) Structures of the lectins from Pseudomonas aeruginosa: insights into the molecular basis for host glycan recognition, *Microbes and Infection 6*, 221-228.

45. Weinstein, D. L., O'Neill, B. L., Hone, D. M., and Metcalf, E. S. (1998) Differential Early Interactions between Salmonella enterica Serovar Typhi and Two Other Pathogenic Salmonella Serovars with Intestinal Epithelial Cells, *Infection and Immunity 66*, 2310-2318.

46. Newburg, D. S., Ashkenazi, S., and Cleary, T. G. (1992) Human Milk Contains the Shiga Toxin and Shiga-like Toxin Receptor Glycolipid Gb3, *The Journal of Infectious Diseases 166*, 832-836.

47. Bénitez, J. A., Spelbrink, R. G., Silva, A., Phillips, T. E., Stanley, C. M., Boesman-Finkelstein, M., and Finkelstein, R. A. (1997) Adherence of Vibrio cholerae to cultured differentiated human intestinal cells: an in vitro colonization model, *Infection and Immunity 65*, 3474-3477.

48. Kunz, C., Rudloff, S., Schad, W., and Braun, D. (1999) Lactose-derived oligosaccharides in the milk of elephants: comparison with human milk, *British Journal of Nutrition 82*, 391-399.

49. Coppa, G. V., Gabrielli, O., Pierani, P., Catassi, C., Carlucci, A., and Giorgi, P. L. (1993) Changes in Carbohydrate Composition in Human Milk Over 4 Months of Lactation, *Pediatrics 91*, 637-641.

50. Kunz, C., Rudloff, S., Baier, W., Klein, N., and Strobel, S. (2000) OLIGOSACCHARIDES IN HUMAN MILK: Structural, Functional, and Metabolic Aspects, *Annual Review of Nutrition 20*, 699-722.

51. German, J. B., Freeman, S. L., Lebrilla, C. B., and Mills, D. A. (2007) Human Milk Oligosaccharides: evolution, structures and bioselectivity as substrates for intestinal bacteria, In *62nd Nestle Nutrition Workshop* (Bier, D. M., German, J. B., and B., L., Eds.), pp 205-222, Nestec Ltd, Helsinki.

52. Engfer, M. B., Stahl, B., Finke, B., Sawatzki, G., and Daniel, H. (2000) Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract, *The American Journal of Clinical Nutrition 71*, 1589-1596.

53. Coppa, G. V., Pierani, P., Zampini, L., Bruni, S., Carloni, I., and Gabrielli, O. (2001) Characterization of Oligosaccharides in Milk and Feces of Breast-Fed Infants by High-

Performance Anion-Exchange Chromatography, In *Bioactive Components of Human Milk* (Newburg, D., Ed.), pp 307-314, Springer US.

54. Chaturvedi, P., Warren, C., Buescher, C., Pickering, L., and Newburg, D. (2001) Survival of Human Milk Oligosaccharides in the Intestine of Infants, In *Bioactive Components of Human Milk* (Newburg, D., Ed.), pp 315-323, Springer US.

55. Albrecht, S., Schols, H. A., van den Heuvel, E. G. H. M., Voragen, A. G. J., and Gruppen, H. (2011) Occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk, *Carbohydrate Research 346*, 2540-2550.

56. Rudloff, S., Pohlentz, G., Diekmann, L., Egge, H., and Kunz, C. (1996) Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula, *Acta Pædiatrica 85*, 598-603.

57. Rudloff, S., Pohlentz, G., Borsch, C., Lentze, M. J., and Kunz, C. (2012) Urinary excretion of in vivo 13C-labelled milk oligosaccharides in breastfed infants, *British Journal of Nutrition 107*, 957-963.

58. Dotz, V., Rudloff, S., Blank, D., Lochnit, G., Geyer, R., and Kunz, C. (2014) 13C-labeled oligosaccharides in breastfed infants' urine: Individual-, structure- and time-dependent differences in the excretion, *Glycobiology 24*, 185-194.

59. Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., and Roberfroid, M. B. (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics, *Nutrition Research Reviews 17*, 259-275.

60. Harmsen, H. J. M., Wildeboer–Veloo, A. C. M., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G., and Welling, G. W. (2000) Analysis of Intestinal Flora

Development in Breast-Fed and Formula-Fed Infants by Using Molecular Identification and Detection Methods, *Journal of Pediatric Gastroenterology and Nutrition 30*, 61-67.

61. LoCascio, R. G., Ninonuevo, M. R., Freeman, S. L., Sela, D. A., Grimm, R., Lebrilla, C. B., Mills, D. A., and German, J. B. (2007) Glycoprofiling of Bifidobacterial Consumption of Human Milk Oligosaccharides Demonstrates Strain Specific, Preferential Consumption of Small Chain Glycans Secreted in Early Human Lactation, *Journal of Agricultural and Food Chemistry 55*, 8914-8919.

62. Gibson, G., and Wang, X. (1994) Regulatory effects of bifidobacteria on the growth of other colonic bacteria, *Journal of Applied Bacteriology (United Kingdom)*.

63. Gibson, G. R., and Wang, X. (1994) Regulatory effects of bifidobacteria on the growth of other colonic bacteria, *Journal of Applied Bacteriology 77*, 412-420.

64. Schroten, H., Hanisch, F. G., Plogmann, R., Hacker, J., Uhlenbruck, G., Nobis-Bosch, R., and Wahn, V. (1992) Inhibition of adhesion of S-fimbriated Escherichia coli to buccal epithelial cells by human milk fat globule membrane components: a novel aspect of the protective function of mucins in the nonimmunoglobulin fraction, *Infection and Immunity 60*, 2893-2899.

65. Simon, P. M., Goode, P. L., Mobasseri, A., and Zopf, D. (1997) Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides, *Infection and Immunity 65*, 750-757.

66. Gustafsson, A., Hultberg, A., Sjöström, R., Kacskovics, I., Breimer, M. E., Borén, T., Hammarström, L., and Holgersson, J. (2006) Carbohydrate-dependent inhibition of Helicobacter pylori colonization using porcine milk, *Glycobiology 16*, 1-10.

67. Morrow, A. L., Ruiz-Palacios, G. M., Altaye, M., Jiang, X., Lourdes Guerrero, M., Meinzen-Derr, J. K., Farkas, T., Chaturvedi, P., Pickering, L. K., and Newburg, D. S. (2004) Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants, *The Journal of Pediatrics 145*, 297-303.

68. Holmgren, J., Svennerholm, A. M., and Lindblad, M. (1983) Receptor-like glycocompounds in human milk that inhibit classical and El Tor Vibrio cholerae cell adherence (hemagglutination), *Infection and Immunity 39*, 147-154.

69. Andersson, B., Porras, O., Hanson, L. Å., Lagergård, T., and Svanborg-Edén, C. (1986) Inhibition of Attachment of Streptococcus pneumoniae and Haemophilus influenzae by Human Milk and Receptor Oligosaccharides, *Journal of Infectious Diseases 153*, 232-237.

70. Su, S. V., Gurney, K. B., and Benhur, L. (2003) Sugar and Spice: Viral Envelope-DC-SIGN Interactions in HIV Pathogenesis, *Current HIV Research 1*, 87-99.

71. Hong, P., Ninonuevo, M. R., Lee, B., Lebrilla, C., and Bode, L. (2009) Human milk oligosaccharides reduce HIV-1-gp120 binding to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), *British Journal of Nutrition 101*, 482-486.

72. Naarding, M. A., Ludwig, I. S., Groot, F., Berkhout, B., Geijtenbeek, T. B. H., Pollakis, G., and Paxton, W. A. (2005) Lewis X component in human milk binds DC-SIGN and inhibits HIV-1 transfer to CD4+ T lymphocytes, *The Journal of Clinical Investigation 115*, 3256-3264.

73. Jantscher-Krenn, E., Lauwaet, T., Bliss, L. A., Reed, S. L., Gillin, F. D., and Bode, L. (2012) Human milk oligosaccharides reduce Entamoeba histolytica attachment and cytotoxicity in vitro, *British Journal of Nutrition 108*, 1839-1846.

74. Otnaess, A. B., Laegreid, A., and Ertresvåg, K. (1983) Inhibition of enterotoxin from Escherichia coli and Vibrio cholerae by gangliosides from human milk, *Infection and Immunity 40*, 563-569.

75. Sanchez-Juanes, F., Alonso, J. M., Zancada, L., and Hueso, P. (2009) Glycosphingolipids from bovine milk and milk fat globule membranes: a comparative study. Adhesion to enterotoxigenic Escherichia coli strain, *Biological Chemistry 390*, 31-40.

76. Lægreid, A., and Kolstø Otnæss, A.-B. (1987) Trace amounts of ganglioside GM1 in human milk inhibit enterotoxins from Vibrio cholerae and Escherichia coli, *Life Sciences 40*, 55-62.

77. Herrera-Insua, I., Gomez, H., Diaz-Gonzalez, V., Chaturvedi, P., Newburg, D., and Cleary, T. (2001) Human Milk Lipids Bind Shiga Toxin, In *Bioactive Components of Human Milk* (Newburg, D., Ed.), pp 333-339, Springer US.

78. Kitova, E. N., Kitov, P. I., Paszkiewicz, E., Kim, J., Mulvey, G. L., Armstrong, G. D., Bundle, D. R., and Klassen, J. S. (2007) Affinities of Shiga toxins 1 and 2 for univalent and oligovalent Pk-trisaccharide analogs measured by electrospray ionization mass spectrometry, *Glycobiology 17*, 1127-1137.

79. Lin, H., Kitova, E., and Klassen, J. (2014) Measuring Positive Cooperativity Using the Direct ESI-MS Assay. Cholera Toxin B Subunit Homopentamer Binding to GM1 Pentasaccharide, *J. Am. Soc. Mass Spectrom. 25*, 104-110.

80. Iwamori, M., Takamizawa, K., Momoeda, M., Iwamori, Y., and Taketani, Y. (2008) Gangliosides in human, cow and goat milk, and their abilities as to neutralization of cholera toxin and botulinum type A neurotoxin, *Glycoconj J 25*, 675-683.

81. Bode, L. (2006) Recent Advances on Structure, Metabolism, and Function of Human Milk Oligosaccharides, *The Journal of Nutrition 136*, 2127-2130.

82. Klein, N., Schwertmann, A., Peters, M., Kunz, C., and Strobe, S. (2002) Immunomodulatory Effects of Breast Milk Oligosaccharides, In *Short and Long Term Effects of Breast Feeding on Child Health* (Koletzko, B., Michaelsen, K., and Hernell, O., Eds.), pp 251-259, Springer US.

83. Bode, L., Rudloff, S., Kunz, C., Strobel, S., and Klein, N. (2004) Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a decrease in neutrophil β 2 integrin expression, *Journal of Leukocyte Biology 76*, 820-826.

84. Rudloff, S., Stefan, C., Pohlentz, G., and Kunz, C. (2002) Detection of ligands for selectins in the oligosaccharide fraction of human milk, *Eur J Nutr 41*, 85-92.

85. Koliwer-Brandl, H., Siegert, N., Umnus, K., Kelm, A., Tolkach, A., Kulozik, U., Kuballa, J., Cartellieri, S., and Kelm, S. (2011) Lectin inhibition assays for the analysis of bioactive milk sialoglycoconjugates, *International Dairy Journal 21*, 413-420.

86. Crocker, P. R., Paulson, J. C., and Varki, A. (2007) Siglecs and their roles in the immune system, *Nature Reviews Immunology 7*, 255-266.

87. Stefanutti, G., Lister, P., Smith, V. V., Peters, M. J., Klein, N. J., Pierro, A., and Eaton, S. (2005) P-selectin expression, neutrophil infiltration, and histologic injury in neonates with necrotizing enterocolitis, *Journal of Pediatric Surgery 40*, 942-948.

88. Lucas, A., and Cole, T. J. (1990) Breast milk and neonatal necrotising enterocolitis, *The Lancet 336*, 1519-1523.

89. Jantscher-Krenn, E., Zherebtsov, M., Nissan, C., Goth, K., Guner, Y. S., Naidu, N., Choudhury, B., Grishin, A. V., Ford, H. R., and Bode, L. (2012) The human milk

oligosaccharide disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats, *Gut 61*, 1417-1425.

90. Yu, H., Lau, K., Thon, V., Autran, C. A., Jantscher-Krenn, E., Xue, M., Li, Y., Sugiarto, G., Qu, J., Mu, S., Ding, L., Bode, L., and Chen, X. (2014) Synthetic Disialyl Hexasaccharides Protect Neonatal Rats from Necrotizing Enterocolitis, *Angewandte Chemie 126*, 6805-6809.

91. Lucas, A., Morley, R., Cole, T. J., Lucas, P. J., Gore, S. M., Crowle, P., Pearse, R., Boon, A. J., and Powell, R. (1990) Early diet in preterm babies and developmental status at 18 months, *The Lancet 335*, 1477-1481.

92. Wang, B. (2009) Sialic Acid Is an Essential Nutrient for Brain Development and Cognition, *Annual Review of Nutrition 29*, 177-222.

93. Wang, B., McVeagh, P., Petocz, P., and Brand-Miller, J. (2003) Brain ganglioside and glycoprotein sialic acid in breastfed compared with formula-fed infants, *The American Journal of Clinical Nutrition 78*, 1024-1029.

94. Martín, R., Heilig, H. G. H. J., Zoetendal, E. G., Jiménez, E., Fernández, L., Smidt, H., and Rodríguez, J. M. (2007) Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women, *Research in Microbiology 158*, 31-37.

95. Lane, J. A., Mehra, R. K., Carrington, S. D., and Hickey, R. M. (2011) Development of biosensor-based assays to identify anti-infective oligosaccharides, *Analytical Biochemistry 410*, 200-205.

96. Hallgren, P., Lindberg, B. S., and Lundblad, A. (1977) Quantitation of some urinary oligosaccharides during pregnancy and lactation, *Journal of Biological Chemistry 252*, 1034-1040.

97. Holgersson, J., Gustafsson, A., and Breimer, M. E. (2005) Characteristics of protein-carbohydrate interactions as a basis for developing novel carbohydrate-based antirejection therapies, *Immunol Cell Biol 83*, 694-708.

98. Goldstein, I. J., Reichert, C. M., and Misaki, A. (1974) Interaction of Concavalin A with model substrates, *Annals of the New York Academy of Sciences 234*, 283-296.

99. Ambrosi, M., Cameron, N. R., and Davis, B. G. (2005) Lectins: tools for the molecular understanding of the glycocode, *Organic & Biomolecular Chemistry 3*, 1593-1608.

100. Charville, H., Jin, J., Evans, C. W., Brimble, M. A., and Williams, D. E. (2013) The synthesis and lectin-binding properties of novel mannose-functionalised polymers, *RSC Advances 3*, 15435-15441.

101. Lee, Y. C., and Lee, R. T. (1995) Carbohydrate-Protein Interactions: Basis of Glycobiology, *Accounts of Chemical Research 28*, 321-327.

102. Dam, T. K., and Brewer, C. F. (2002) Thermodynamic Studies of Lectin−Carbohydrate Interactions by Isothermal Titration Calorimetry, *Chemical Reviews 102*, 387-430.

103. Spurlino, J. C., Lu, G. Y., and Quiocho, F. A. (1991) The 2.3-A resolution structure of the maltose- or maltodextrin-binding protein, a primary receptor of bacterial active transport and chemotaxis, *Journal of Biological Chemistry 266*, 5202-5219.

104. Boggs, J. M., Wang, H., Gao, W., Arvanitis, D. N., Gong, Y., and Min, W. (2004) A glycosynapse in myelin?, *Glycoconj J 21*, 97-110.

105. Pohl, N. L., and Kiessling, L. L. (1999) Scope of multivalent ligand function: lactose-bearing neoglycopolymers by ring-opening metathesis polymerization, *Synthesis*, 1515-1519.

106. Mammen, M., Choi, S.-K., and Whitesides, G. M. (1998) Polyvalent Interactions in Biological Systems: Implications for Design and Use of Multivalent Ligands and Inhibitors, *Angewandte Chemie International Edition 37*, 2754-2794.

107. Shinohara, Y., Hasegawa, Y., Kaku, H., and Shibuya, N. (1997) Elucidation of the mechanism enhancing the avidity of lectin with oligosaccharides on the solid phase surface, *Glycobiology 7*, 1201-1208.

108. Leavitt, S., and Freire, E. (2001) Direct measurement of protein binding energetics by isothermal titration calorimetry, *Current Opinion in Structural Biology 11*, 560-566.

109. Utsuno, K., and Uludağ, H. (2010) Thermodynamics of Polyethylenimine-DNA Binding and DNA Condensation, *Biophysical Journal 99*, 201-207.

110. O'Shannessy, D. J., Brighamburke, M., Soneson, K. K., Hensley, P., and Brooks, I. (1993) Determination of Rate and Equilibrium Binding Constants for Macromolecular Interactions Using Surface Plasmon Resonance: Use of Nonlinear Least Squares Analysis Methods, *Analytical Biochemistry 212*, 457-468.

111. Moll, D., Schweinsberg, S., Hammann, C., and Herberg, F. W. (2007) Comparative thermodynamic analysis of cyclic nucleotide binding to protein kinase A, *Biological Chemistry 388*, 163-172.

112. Seo, J. H., Adachi, K., Lee, B. K., Kang, D. G., Kim, Y. K., Kim, K. R., Lee, H. Y., Kawai, T., and Cha, H. J. (2007) Facile and Rapid Direct Gold Surface Immobilization with Controlled Orientation for Carbohydrates, *Bioconjugate Chemistry 18*, 2197-2201.

113. Bamdad, C. (1998) The Use of Variable Density Self-Assembled Monolayers to Probe the Structure of a Target Molecule, *Biophysical Journal 75*, 1989-1996.

114. Horlacher, T., and Seeberger, P. H. (2008) Carbohydrate arrays as tools for research and diagnostics, *Chemical Society Reviews 37*, 1414-1422.

115. Liu, Y., Palma, A. S., and Feizi, T. (2009) Carbohydrate microarrays: key developments in glycobiology, *Biological Chemistry 390*, 647-656.

116. Disney, M. D., and Seeberger, P. H. (2004) Carbohydrate arrays as tools for the glycomics revolution, *Drug Discovery Today: TARGETS 3*, 151-158.

117. He, X. G., Gerona-Navarro, G., and Jaffrey, S. R. (2005) Ligand Discovery Using Small Molecule Microarrays, *Journal of Pharmacology and Experimental Therapeutics 313*, 1-7.

118. Grant, O. C., Smith, H. M., Firsova, D., Fadda, E., and Woods, R. J. (2014) Presentation, presentation, presentation! Molecular-level insight into linker effects on glycan array screening data, *Glycobiology 24*, 17-25.

119. Wang, L., Cummings, R. D., Smith, D. F., Huflejt, M., Campbell, C. T., Gildersleeve, J. C., Gerlach, J. Q., Kilcoyne, M., Joshi, L., Serna, S., Reichardt, N.-C., Parera Pera, N., Pieters, R. J., Eng, W., and Mahal, L. K. (2014) Cross-platform comparison of glycan microarray formats, *Glycobiology 24*, 507-517.

120. Hsu, K.-L., Gildersleeve, J. C., and Mahal, L. K. (2008) A simple strategy for the creation of a recombinant lectin microarray, *Molecular BioSystems 4*, 654-662.

121. Soya, N., Shoemaker, G. K., Palcic, M. M., and Klassen, J. S. (2009) Comparative study of substrate and product binding to the human ABO(H) blood group glycosyltransferases, *Glycobiology 19*, 1224-1234.

122. Shoemaker, G. K., Soya, N., Palcic, M. M., and Klassen, J. S. (2008) Temperature-dependent cooperativity in donor–acceptor substrate binding to the human blood group glycosyltransferases, *Glycobiology 18*, 587-592.

123. Hofstadler, S. A., and Sannes-Lowery, K. A. (2006) Applications of ESI-MS in drug discovery: interrogation of noncovalent complexes, *Nature Reviewas Drug Discovery 5*, 585-595.

124. El-Hawiet, A., Shoemaker, G. K., Daneshfar, R., Kitova, E. N., and Klassen, J. S. (2011) Applications of a Catch and Release Electrospray Ionization Mass Spectrometry Assay for Carbohydrate Library Screening, *Analytical Chemistry 84*, 50-58.

125. Kitova, E., El-Hawiet, A., and Klassen, J. (2014) Screening Carbohydrate Libraries for Protein Interactions Using the Direct ESI-MS Assay. Applications to Libraries of Unknown Concentration, *J. Am. Soc. Mass Spectrom.*, 1-9.

126. Urh, M., Simpson, D., and Zhao, K. (2009) Chapter 26 Affinity Chromatography: General Methods, In *Methods in Enzymology* (Richard, R. B., and Murray, P. D., Eds.), pp 417-438, Academic Press.

127. Trauger, S. A., Webb, W., and Siuzdak, G. (2002) Peptide and protein analysis with mass spectrometry, *Spectroscopy: An International Journal 16*, 15-28.

128. Ng, E. S. M., Chan, N. W. C., Lewis, D. F., Hindsgaul, O., and Schriemer, D. C. (2007) Frontal affinity chromatography[mdash]mass spectrometry, *Nature Protocols 2*, 1907-1917.

129. Slon-Usakiewicz, J. J., Ng, W., Dai, J.-R., Pasternak, A., and Redden, P. R. (2005) Frontal affinity chromatography with MS detection (FAC-MS) in drug discovery, *Drug Discovery Today 10*, 409-416.

130. Fornstedt, N., and Porath, J. (1975) Characterization studies on a new lectin found in seeds of Vicia ervilia, *FEBS Letters 57*, 187-191.

131. Edgar, L. J. G., Dasgupta, S., and Nitz, M. (2012) Protecting-Group-Free Synthesis of Glycosyl 1–Phosphates, *Organic Letters 14*, 4226-4229.

132. Gudmundsdottir, A. V., and Nitz, M. (2008) Protecting Group Free Glycosidations Using p-Toluenesulfonohydrazide Donors, *Organic Letters 10*, 3461-3463.

Chapter 2

Protecting Group-free Immobilization of Carbohydrate Epitopes Using p-Toluenesulfonyl Hydrazide for Affinity Chromatography[1]

[1] The SS-NMR experiments were carried out with the help of Mark Miskolzie, NMR Laboratory. University of Alberta

2.1 Introduction

2.1.1 Affinity Chromatography

Affinity chromatography is a method for the purification of biomolecules based on specific and reversible binding of a receptor and its ligand. We will define the ligand as the binding partner which is immobilized onto an insoluble support and the target as the binding partner dissolved in a mobile phase. The principle behind this type of separation is very simple: when the two components are mixed they will form a reversible bimolecular complex associated with the solid support. This allows for enrichment of the targets as non-binders will flow easily through the column, whereas the target will be retained based on its affinity for the ligand. The target can be eluted by flowing through the column a solution containing competing target in solution (competitive elution), or by using buffer conditions that may disrupt the interactions between ligand and target. (*1, 2*)

This method, developed almost 45 years ago, gained popularity and revolutionized the entire fields of modern biology, chemistry, molecular biology, and biotechnology. (*3*) A search for the topic of affinity chromatography in the Web of Science currently yields 156,455 results; out of those, 50,716 have been published in the last ten years. The broad scope of the method has resulted in the development of specialized techniques. Among the most popular methods, we can find immunoaffinity chromatography, where an antibody is immobilized into the solid support, and the target antigen is purified upon loading and subsequent elution from the column. (*4, 5*) The inclusion of affinity tags is also a popular method for the purification of recombinant proteins, the two most common systems being avidin-biotin (*6*) and the His-tag and nickel. (*7, 8*) For the purification of lectins, immobilized carbohydrates have been used. (*9,*

10) There are several other specialized techniques that have derived from affinity chromatography; however, they are out of the scope of this thesis.

The selection of the affinity matrix is an important step when developing an affinity chromatography method. The insoluble matrix used for affinity chromatography should have minimal non-specific interactions with the target, it should be macroporous to allow entry of large biomolecules, it should be physically and chemically stable, and should be of uniform size.(*1, 2*) Commercially available matrices include agarose, cellulose, silica, polyacrylamide, polystyrene, and dextrose (*2*)

Moreover, supports like polystyrene are known to have high non-specific interactions with proteins due to its hydrophobicity; and silica has the same effect due to the negative charge present in its surface (*2*) Sepharose, an agarose (Gal-β1,4-[3,6]-anhydro-L-Gal) support, is often used for affinity chromatography because it has low non-specific binding, can be used under a wide range of pH, and tolerates most organic solvents. (*1, 11*) Sepharose CL is a crosslinked derivative of Sepharose prepared by reacting agarose with 1,3-dibromo-2-propanol under strongly alkaline conditions providing a gel with a very low content of ionisable groups. (*12*) A partial structure of Sepharose CL is shown in **Figure 2.1**.

For our studies we chose Sepharose CL because the support complies with most of the characteristics described above. It is stable under a wide range of pH, which will allow us to used several binding elution conditions; it allows the attachment of ligands using mild conditions; retains favourable flow properties during use, and it has minimal interactions with proteins, our main target. (*11*)

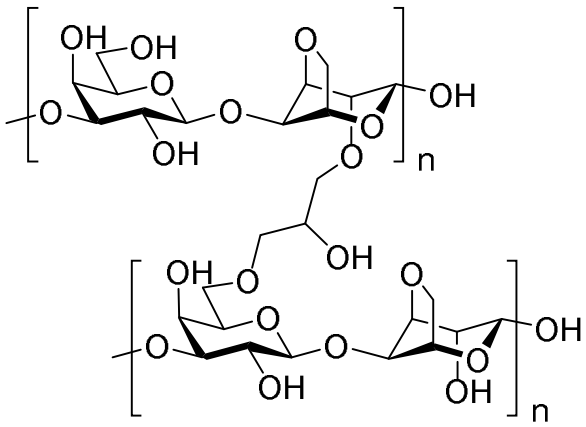


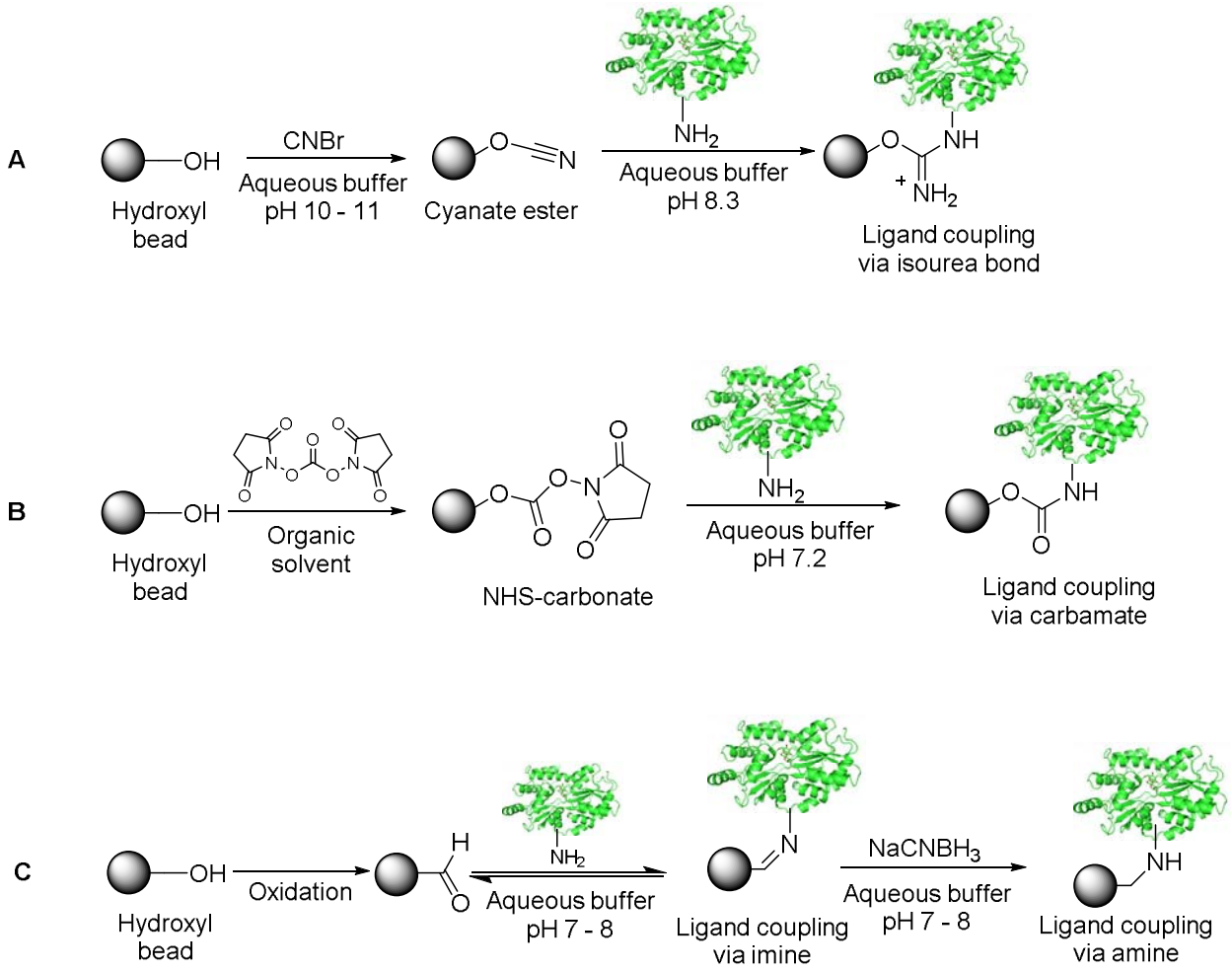
Figure 2.1 Chemical structure of Sepharose CL Sepharose is a cross-linked agarose (D-galactose and 3,6-anhydro-L-galactose) polymer where most of the hydroxyl groups are blocked or are used for cross-linking the repeating units.

2.1.2 Methods for substrate immobilization

The immobilization of peptides, and proteins is usually carried out through the formation of covalent bonds using the amine groups in the ligand reacting with a functionality on the resin surface (**Scheme 2.1**). A commonly employed method is the reaction of a cyanogen bromide (CNBr) activated surface. The resin is then reacted with a protein, which can react through a lysine side chain. (*2, 13*) The main drawback of this method is the toxicity of the CNBr reagent; however, commercially available resins that are already activated have reduced the difficulty of this method. The principal advantage of this approach is that it can be used under aqueous conditions. (*14*) The lysine chains of proteins can also react with supports bearing active esters, such as *N*-hydroxysuccinimide (NHS). A disadvantage of this method is the low immobilization yields due to competing hydrolysis from aqueous buffers. (*14*) An alternate method is coupling to a surface with an aldehyde group to obtain an imine linkage

(*15*) that can be reduced with sodium cyanoborohydride to form a stable secondary amine linkage.

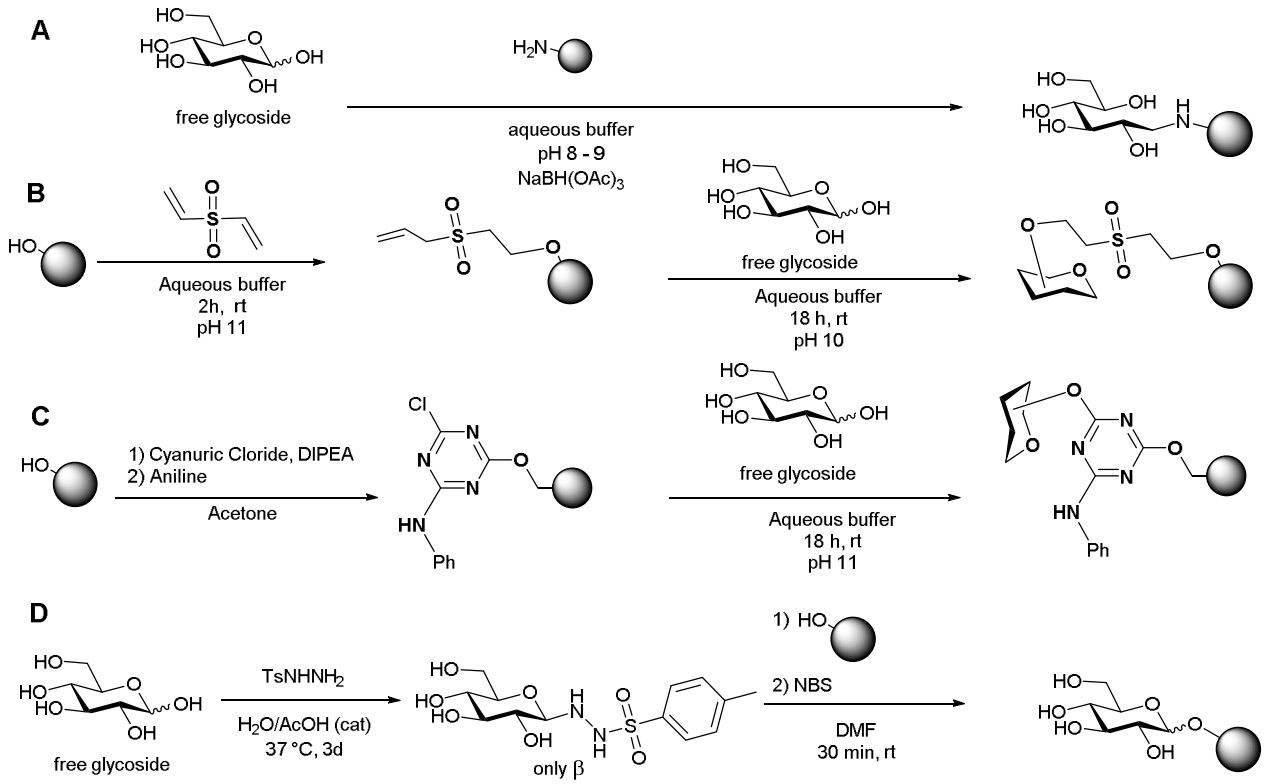
It is possible to take advantage of other amino acid side chains present in proteins for immobilization chemistry. For example, the thiol group of cysteine residues holding can easily react with α,β-unsturated carbonyls (e.g. maleimides) to form thioether bonds. Thiols can also react with epoxides and NHS esters; however, these reactions are slower and the resulting linkage is susceptible to degradation. (*16*) Carboxylic acid-bearing residues, such as aspartic and glutamic acid, can also be used for immobilization. The strategy involves the conversion of the carbonyl to the corresponding activated ester using a carbodiimide coupling agent (e.g. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) while NHS is used to generate the active ester. This procedure can be carried out in water; however, the carbodiimides and the active ester in aqueous media are sometimes low yielding. Moreover the active esters formed in the protein can further react with other protein molecules, through lysine chains, to form polymers. (*16*)



Scheme 2.1 Common ligation methods for the immobilization of proteins to a solid support. These methods use amine nucleophiles from from the protein. Reaction of the resin with **A.** CNBr, **B.** NHS ester, or **C.** Aldehydes are shown.

Although affinity chromatography is commonly used for protein-based ligands, there are some issues specific to the use of carbohydrate-based ligands. Specifically, carbohydrate ligands do not typically contain free amine groups. Instead, chemistries that can form covalent linkages between the hydroxyl groups of the solid support and those of the ligand are required.

Commonly employed strategies to address this problem are described below and summarized in **Scheme 2.2**.



Scheme 2.2 Methods for the immobilization of carbohydrates to a solid support. Some of the methods currently available include **A.** Reductive amination, **B.** Divinyl sulfone, **C.** Cyanuric Chloride, and the method that we implemente **D.** *p*-toluenesulfonyl hydrazide.

2.1.2.1 Reductive amination

Reductive amination (**Scheme 2.2 A**) relies in the ability of sodium cyanoborohydride to selectively reduce the imine formed from the reaction between an aldehyde and an amine under neutral conditions. (*17*) An amine functionalized resin is required along with a reducing carbohydrate, in equilibrium with its open chain form (*18, 19*) The reaction suffers an important drawback, the coupling times can be sometimes very long. The reaction can be sped

up by adding excess carbohydrate, but the requirement of large amounts ligand is a potential limitation. (*19*)

2.1.2.2 Divinyl sulfone

Divinyl sulfone (DVS) (**Scheme 2.2 B**) is an electrophilic homobifuncitional reagent capable of crosslinking hydroxyl nucleophiles. DVS can be used to activate a hydroxyl-containing solid support through a Michael-type addition to one of the vinyl groups. Crosslinking can be minimized if the reaction is carried out under a large excess of DVS relative to the nucleophile. (*20, 21*)

A range of functional groups can react with DVS as nucleophiles, including amine and hydroxyl groups. In the case of hydroxyl nucleophiles, such as carbohydrates, the reaction must be carried out at a pH greater than 10 to form a covalent ether linkage. DVS-coupled affinity ligands should not be exposed to conditions exceeding pH 8.5 to avoid potential retro Michael reaction. (*22, 23*) DVS activation has been used in several applications including carbohydrate immobilization onto surfaces (*24*) and immobilization onto chromatographic supports for affinity chromatography. (*9*) Some of the limitations of this method are the need of a capping step to block unreacted active sites and the need of relatively large amounts of carbohydrate.

2.1.2.3 Cyanuric Chloride

Cyanuric chloride (CC) (**Scheme 2.2 C**) is a trifunctional, heterocyclic compound that can be used for the coupling of dyes to fabric. Because of its high reactivity, it has also been used in the activation of chromatography resins with hydroxyl groups in the immobilization of affinity ligands. The reactivity of CC decreases as the chlorines are substituted by other atoms. (*20*) The main advantage that CC offers as a coupling reagent is the stability of the bond

formed at high pH values, which is a problem for the DVS approach. (*25*) Similar to DVS, CC has been used for the immobilization of affinity ligands, (*20*) and was also recently used for anchoring carbohydrates into surfaces for the preparation of carbohydrate microarrays. (*26*) Some flaws of this method are the need of a capping step to block unreacted sites, and the decrease in reactivity as substitution of the triazine ring increases.

2.1.2.4 Tosyl Hydrazide

Recently, Nitz and coworkers have reported a method for protecting-group free glycosidation reactions based on the use of a glycosyl hydrazide (**Scheme 2.2 D**). (*27, 28*) This method results in reaction at the anomeric position of the donor exclusively. Additionally, in the case of 2-acetimido sugars, such as N-acetyl-glucosamine (GlcNAc), the reaction selectively forms the β-anomer. The conditions for the reaction are mild (37 °C) for the formation of the activated carbohydrate, and room temperature for the glycosidation reaction. The mechanism for the glycosidation is believed to occur via oxidation of the glycosyl donors with NBS to form a glycosyl diazene. The formation of an oxocarbenium ion occurs due to the elimination of sulfinic acid and nitrogen gas. The oxocarbenium ion is then trapped by the incoming alcohol wherein the stereochemistry of the attack will be directed by the neighbouring acetamido group (*27*).

We considered that this chemistry could be ideal for immobilization of carbohydrates onto solid supports. In this configuration, the hydroxyl groups of the solid support would act as the glycosyl acceptor and the glycosyl hydrazide would act as the donor. This method would have several advantages over standard immobilization chemistries for carbohydrates. First, the method would not require an activation of the resin and could be performed under very mild conditions. Moreover, no capping or inactivation step would be required at the end of the

reaction, which may reduce the likelihood of non-specific interactions. (*2*) Most importantly, this strategy would offer the advantage of being regioselective, reacting only at the anomeric position of the glycoside, in contrast to the chemistries discussed above.

Herein we develop a protocol for the immobilization of carbohydrate ligands to a cross-linked agarose support (Sepharose CL-6B) using *para*-toluenesulfonyl hydrazide (p-TsH) activation. We envisioned that this method could provide similar levels of immobilization to existing chemistries, but would be complementary due to its region- and stereo-specificity. We compared our method with two benchmark chemistries: DVS and CC. Each method was compared based on its immobilization efficiency and carbohydrate binding capacity of the resulting matrix.

2.2 Results & Discussion

2.2.1 Model reactions in solution

Our goal was to perform crosslinking reactions in a heterogeneous phase; however, to provide a clear point of comparison for each method we first established a solution phase protocol using model compounds. The reactions carried out in solution would provide essential data for establishment of the reaction conditions required for the immobilization strategies. (*29, 30*)

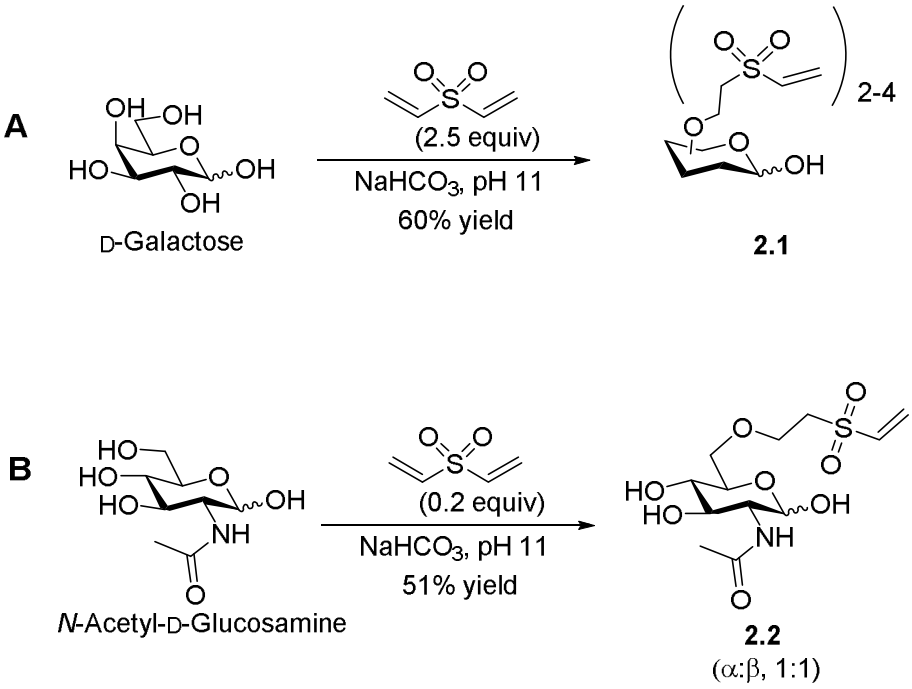
2.2.2 Functionalization of monosaccharides with DVS in solution

We took as a starting point the method reported by Fornstedt and Porath in 1975 (*9*) for the immobilization of D-mannose onto a solid support using DVS as a linker. Initially we carried out the reaction shown in the **Scheme 2.3** using one equivalent of Gal to 2.5 equivalents of DVS. These conditions afforded a mixture of products that included mono, di,

tri, and tetra-DVS-functionalized glycosides of Gal (**2.1**) in 60% yield. By reducing the number of equivalents of DVS we isolated singly-modified glycosides of GlcNAc (**2.2**) in 51% yield. We used GlcNAc in this step in order to have the same substrate that afforded the best site-selectivity with the p-TsH chemistry (described in **Section 2.2.4)**. The optimal ratio used for obtaining the mono-functionalized product, attached through the hydroxyl in the sixth position, was 5 equivalents of monosaccharide per equivalent of DVS. (*21*) Using ^{1}H NMR and ^{1}H GCOSY (shown in **Appendix A,** results summarized in **Table 2.1**) we determined that the substitution occurs mainly at position 6 of the monosaccharide; however, the ^{1}H NMR data shows two sets of vinylic and anomeric peaks whereas the mass only identifies one product. The ^{1}H GCOSY revealed that H-6 shifted downfield from ~3.8 to ~3.9 ppm. This effect is expected due to the electron-withdrawing character of the sulfone. These data suggest that the DVS attachment occurred through more than one of the hydroxyls available, providing evidence of the poor site-selectivity of this method.

Proton	**GlcNAc** **Chemical shift (ppm)**	**2.2** **Chemical shift (ppm)**
H-1 (α/β)	5.1 / 4.6	5.1 / 4.4
H-2	3.85 – 3.76	3.73 – 3.60
H-3	3.72 - 3.66	3.90 – 3.80
H-4	3.72 - 3.66	3.74 – 3.60
H-5	3.38	3.48 – 3.31
H-6	3.85 – 3.76	3.90 – 3.80

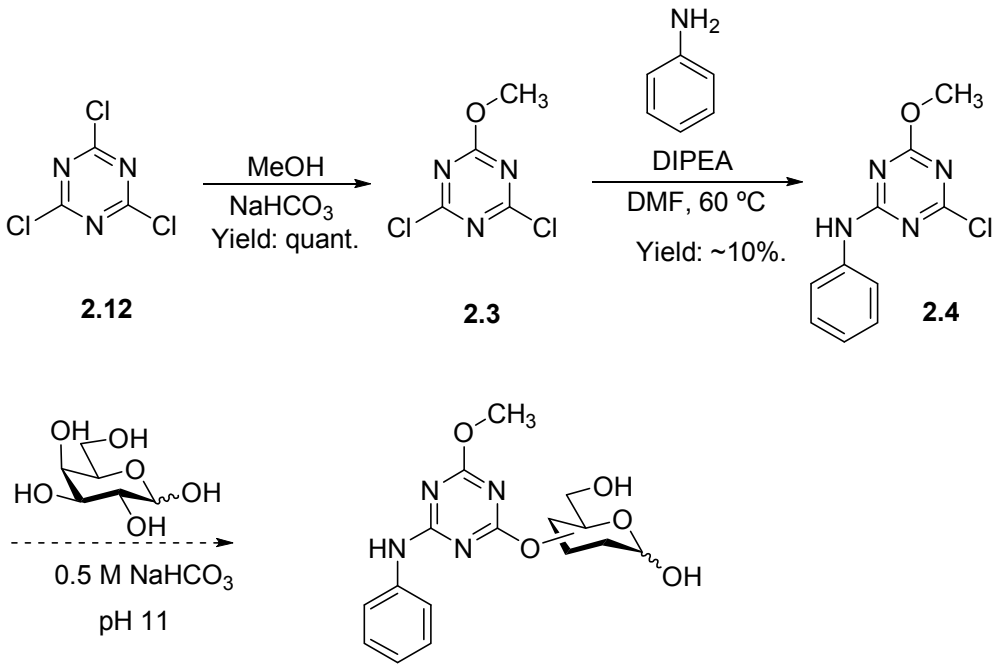
Table 2.1 Comparison of chemical shifts for GlcNAc and molecule 2.2



Scheme 2.3. DVS-functionalization of monosaccharides in solution. Monosaccharides **A** Gal and **B** GlcNAc were reacted with DVS in alkaline conditions to provide a mixture (**2.1**) and single-modified derivatives (**2.2**).

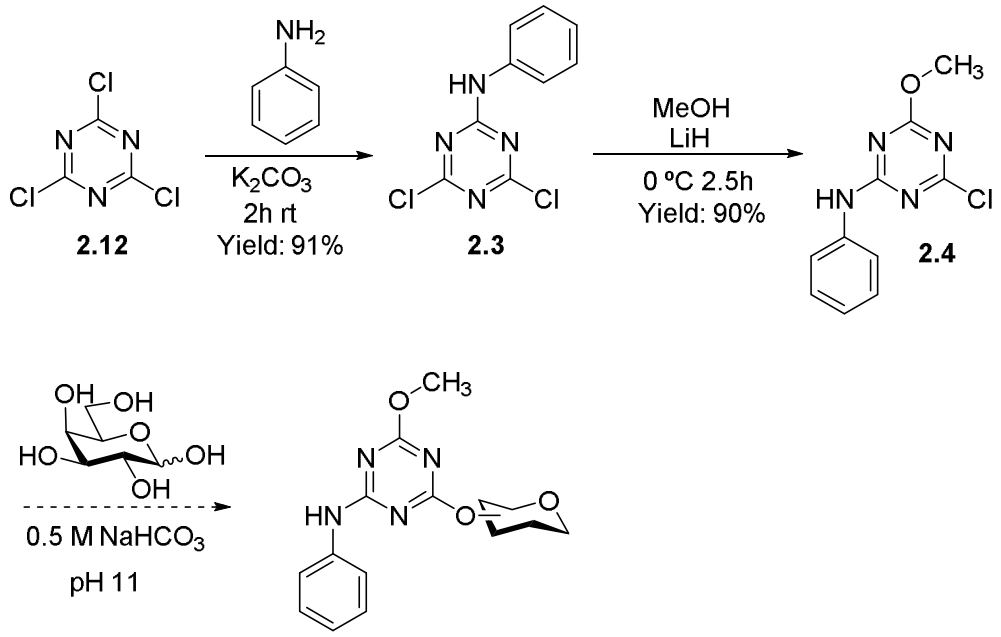
2.2.3 Functionalization of monosaccharides with CC in solution

Cyanuric chloride (CC, **2.12**) has been used for immobilization of carbohydrates to solid support. (*26*) We followed conditions developed by Finlay et al. (*20, 31*), shown in **Scheme 2.4**. In our hands, these conditions were low yielding and often resulted in hydrolysis of the cyanuric chloride to afford the corresponding acid.



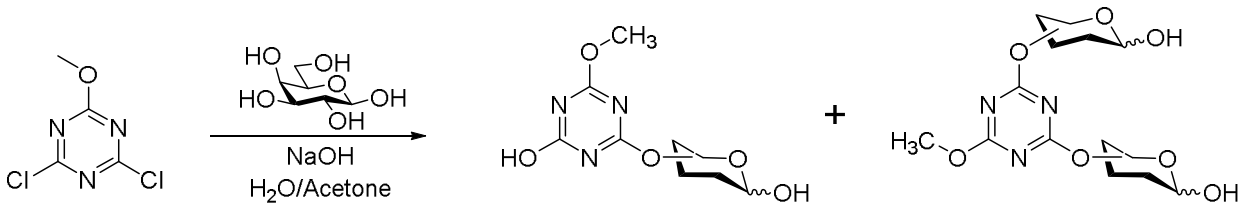
Scheme 2.4 CC-functionalization of glycosides. Conditions for CC coupling in solution. (*31*) The last step was not tested due to the low yields in the first two steps.

We considered an alternative method, shown in **Scheme 2.5**, which relied on initial activation of the triazine to the aniline conjugate, **2.3**, followed by reaction with the desired alcohol. In our case, we chose to use MeOH as a stand-in for the resin acceptor, forming compound **2.4**. (*32, 33*) This method gave improved yields (81% yield overall, after two steps) as compared to **Scheme 2.4**; however, the carbohydrate coupling step was not achieved. The reaction was tested using $NaHCO_3$ and NaOH as a base and with and without heating for periods up to 1 week. The product obtained in all cases was the hydrolysis product of compound **2.4** and no observable traces of the desired product based on NMR and mass spectrometry analysis.



Scheme 2.5. CC-functionalization of glycosides – route 2. Method based on the procedures reported by Kumar et al. and Belyakov et al.(*32, 33*). The first two steps gave improved yields (80% overall). However, the glycosidation step was not successful, affording the hydrolysis product of compound **2.4**.

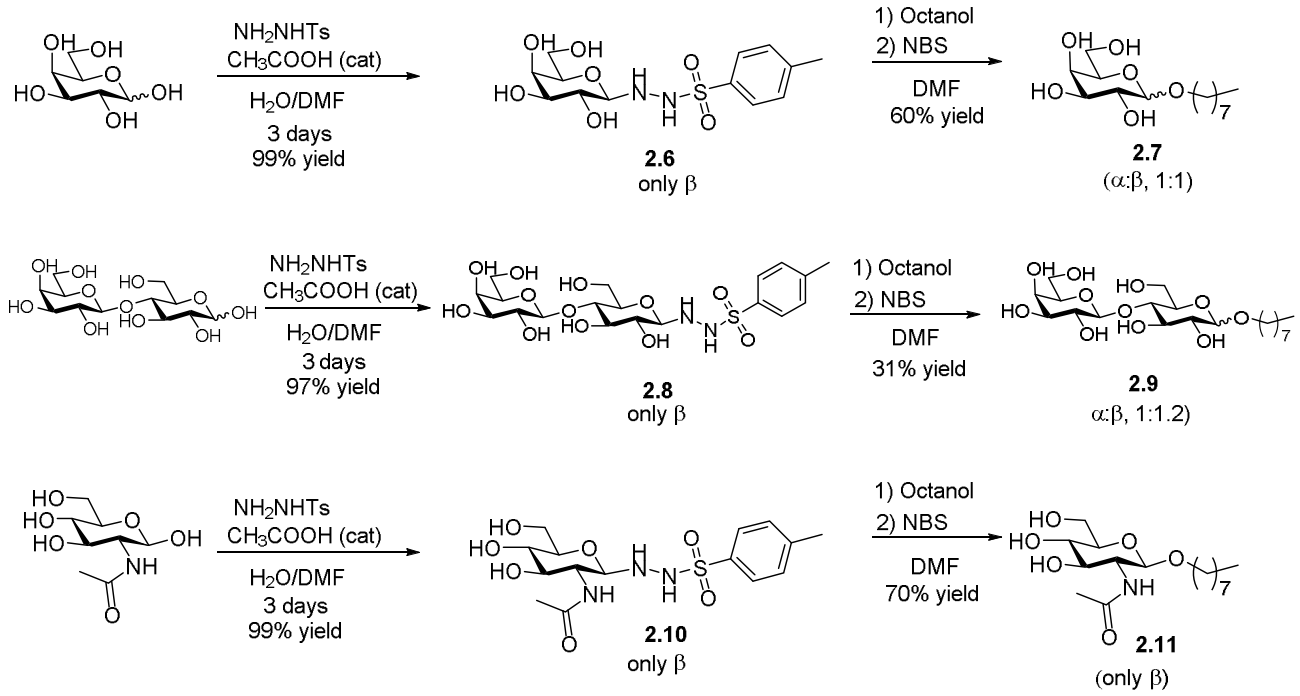
To investigate the site of attachment and the efficiency of the immobilization of carbohydrates using CC, we tested the conditions proposed by Liang and Chen (*26*) and described in the **Scheme 2.6**. The reaction described in **Scheme 2.6** afforded a mixture of two products: the monosubstituted triazine (**2.5a**) with the chlorine being hydrolyzed and the di-substituted triazine (**2.5b**). The products were isolated using preparative TLC as described in the method section. The ^{1}H NMR spectrum of the compounds isolated shows a very complex set of peaks between 5.5 and 4.4 ppm indicating substitution at more than one of the hydroxyl groups on the monosaccharide. These results are in accordance with the reported by Liang and Chen and indicate a poor immobilization site-selectivity of the method. The presence of hydrolysis products opens up the possibility of having non-specific interactions if the method is used in binding studies with proteins.



Scheme 2.6. CC-functionalization of glycosides – conditions for site attachment analysis. Using conditions previously reported (*26*) we were able to determine the site-selectivity of the CC chemistry. The reaction afforded a mixture of mono- (**2.5a**) and a di-substituted product (**2.5b**). The ^{1}H NMR spectrum suggests substitution at more than one hydroxyl group.

2.2.4 *p*-Toluenesulfonyl Hydrazide (p-TsH) functionalization of carbohydrates in solution

Previous reports have used p-TsH chemistry as a method for protecting-group free glycosidations in solution. (*27, 28*) Using reported conditions, we generated glycosides using octanol as an acceptor. A typical reaction sequence is presented in **Scheme 2.7**. We generated octyl glycosides of Gal, lactose (Lac), and GlcNAc (compounds **2.7**, **2.9**, and **2.11** respectively) using reported protocols. (*27, 28*) The glycosidation reactions were carried in anhydrous conditions and under inert atmosphere to avoid the hydrolysis of the activated saccharide. The corresponding octyl saccharides were obtained in good purity with yields ranging from 31 to 70 %, with a slight preference for the formation of the β anomer in the case of galactose and lactose, and exclusively the β anomer for the GlcNAc. This set of experiments provides further evidence that the glycosidation only occurs at the anomeric position. Moreover, we were also able to observe that amino sugars favour the formation of the β anomer whereas non-amino sugars have a tendency to form a mixture of anomers with a very slight preference for the β-anomer.



Scheme 2.7. Glycosidation reactions with pTsH-glycosides in solution. Glycosidation of Gal, Lac, and GlcNAc using p-TsH activation.

The yields and anomeric selectivity obtained in the reactions carried out in solution provided an indication of their suitability for carrying out the reaction in heterogeneous phase (solid-liquid). We observed that the main disadvantage of the DVS method was the formation of polymers or polyfunctionalization of one monosaccharide. In the case of CC, the method was very low yielding in solution, and was prone to the formation of the corresponding acid via hydrolysis. The p-TsH method was straightforward and provided moderate yields; its main disadvantage was the need for anhydrous conditions in order to preserve the glycosyl donor. Based on these observations we expected the DVS and the p-TsH method to perform best in reactions carried out in heterogeneous phase.

2.2.5 Immobilization of carbohydrate epitopes to solid support

As discussed above, our aim in the current study was to compare methods of carbohydrate immobilization. We chose to use Gal, Lac, and GlcNAc saccharides as the ligands for our study, and Sepharose CL-6B as the solid support. The immobilization of carbohydrates using DVS or CC as linkers is well established, and we followed established protocols for their use. (*9, 31*) For p-TsH immobilization, we based our protocol around the solution studies described above. The resin was transferred from a slurry in aqueous solution to dry DMF by washing with increasing concentrations of DMF in water (25, 50, 75 and 100%). A final wash with 100% DMF was carried out twice more. While washing the resin, we never allowed the resin to dry out completely. The resin was re-suspended in DMF and transferred to a dry round bottom flask under inert atmosphere. The mixture was stirred slowly in an orbital shaker, and the glycosyl acceptor added as a solution in dry DMF and allowed to mix for five minutes. Finally NBS dissolved in DMF was added drop-wise to the reaction mixture and allowed to react for 30 minutes. The resin was transferred to a sintered glass funnel and washed with DMF followed by decreasing concentrations of DMF in water (75, 50 and 25%), followed by two washes with distilled water. In all cases we compared similar amounts of the carbohydrate ligand when testing alternative immobilization strategies. We generated resin samples for the three saccharides and the three immobilization chemistries as described in Materials & Methods. In the case of DVS and CC chemistry, we had to use a quenching step to destroy any unreacted sites on the resin. In order to maintain the stability and structure of the cross linked agarose we paid special attention to transfer of the support from the aqueous to the organic phase. We also avoided mechanical agitation of the gel to maintain the structural integrity of the resin.

2.2.6 High-Resolution Magic Angle Spinning Nuclear Magnetic Resonance (HR MAS-NMR)

Magic Angle Spinning Nuclear Magnetic Resonance (MAS-NMR) is a technique for obtaining high resolution NMR from solids. (*34*) Unlike liquid NMR, the molecules forming a solid are not in constant movement and their orientations do not change rapidly. Thus, anisotropic interactions are not averaged making the spectra more complex. Interactions such as the chemical shift and dipolar coupling dominate, producing broad spectral lines. By rapidly spinning a polycrystalline powder around an axis at the magic angle, $\theta = 54.74^{\circ}$, one can suppress this chemical shift broadening. (*34, 35*) Recent progress in solid-state NMR (SS NMR) has allowed for the study of structure and dynamics in many different systems. Catalysts, glasses, polymers, and proteins are some of the most common applications of SS NMR. (*35*) We envisioned that HR MAS-NMR could be used to provide information about the regio- or stereochemistry of immobilized carbohydrate ligands on the solid support.

We collected ^{1}H NMR spectra of resin samples using HR MAS-NMR and compared them to unmodified or control resin to look for resolved resonances that could be attributed to the immobilized ligands. One inherent challenge from this approach is that the Sepharose support itself is a carbohydrate polymer; thus, a considerable portion of the carbohydrate region in ^{1}H NMR (4-2 ppm) is obscured. We first examined DVS-immobilized carbohydrates on Sepharose CL-6B (**Figure 2.2**). The DVS-activated resin samples show the appearance of vinylic protons of the sulfone with good resolution at 6.8 and 6.3 ppm. We next examined samples of DVS-activated resin reacted with Gal, and were pleased to observe the appearance of new peaks ($\delta = 5.2$ and 4.5 ppm) close to the anomeric signals observed for Gal in solution ($\delta = 5.3$ and 4.6 ppm). Notably, chemical shift and coupling constants (indicated in **Figure 2.2**)

of the anomeric (both alpha and beta anomers) and vinylic protons correspond to the data obtained in solution. Hence, the HR MAS-^{1}H NMR data provided chemical information about the point of attachment of the immobilized ligand.

A summary of the HR-MAS ^{1}H NMR results obtained is presented in **Table 2.2**. We present a comparison of the chemical shifts and coupling constants for H1 of the monosaccharides in solution and the corresponding values observed for the immobilized ligand attached to Sepharose CL-6B. The corresponding NMR spectra are shown in **Figure 2.2** and **Figure 2.3**.

Sample	**H1-alpha**		**H1-beta**	
	δ (ppm)	***J* (Hz)**	**δ (ppm)**	***J* (Hz)**
Gal	5.40	3.2	4.59	8.0
Sep-DVS-Gal	5.18	3.7	4.51	7.9
Octyl-GlcNAc	4.94*	3.5*	4.39	7.8
Sep-GlcNAc	Not observed	Not observed	4.40	7.9

Table 2.2 Comparison of chemical shifts and coupling constants for H-1 in solution and attached to a solid support glycosides. * Expected values taken from literature. (*36*)

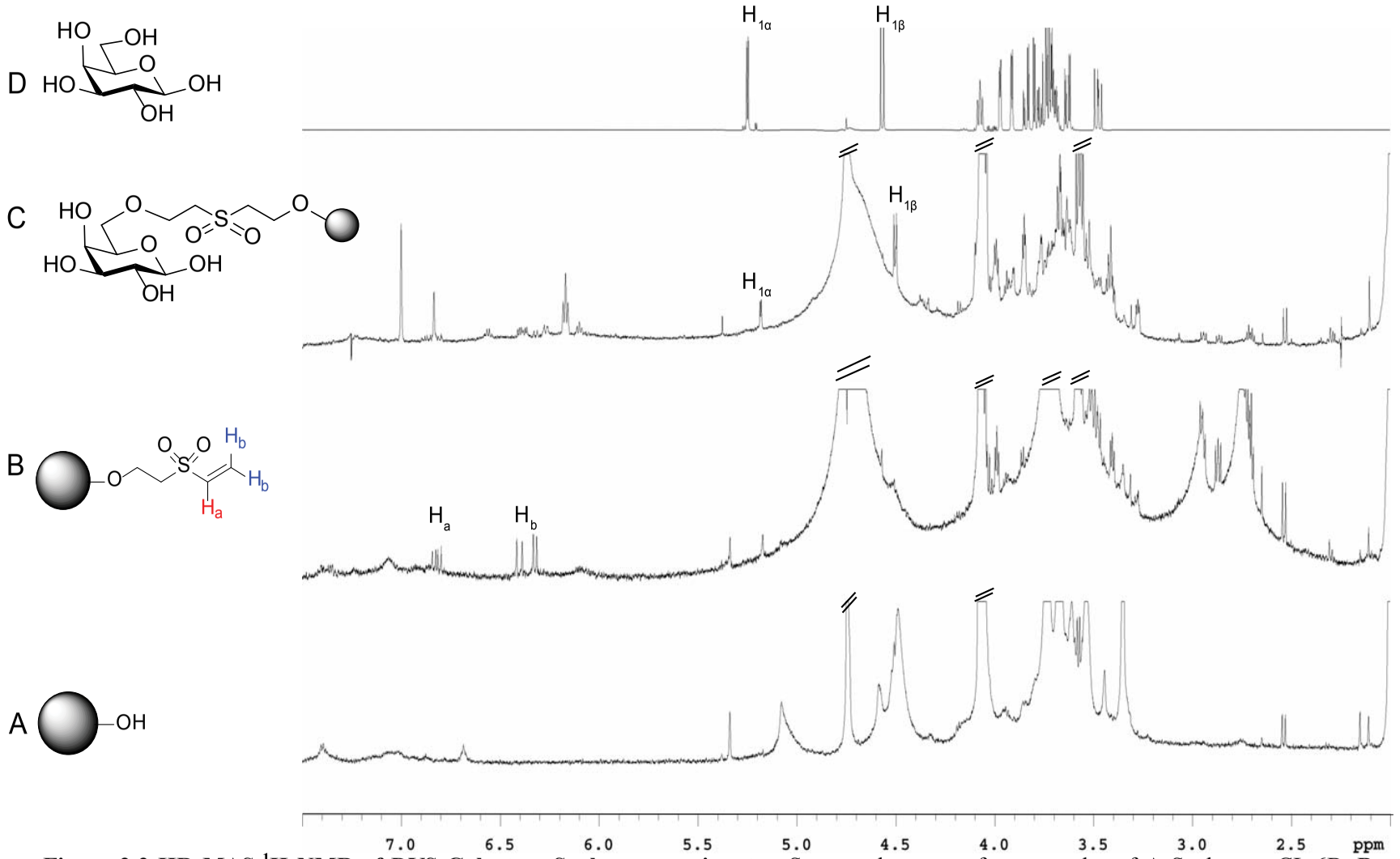


Figure 2.2 HR MAS-^{1}H NMR of DVS-Galactose-Sepharose conjugates. Spectra shown are from samples of **A** Sepharose CL-6B, **B** Sepharose CL-6B activated with DVS, **C** Sepharose CL-6B activated with DVS and coupled to Gal, **D** Gal in solution (D_2O). New peaks observed in the modified resin correspond to: peaks in **B** δ = 6.80 (*CH*=CH2, dd, *J* = 16.5, 9.4 Hz*),* 6.35 (CH=*CH2,* dd, *J* = 16.5, 9.4 Hz*),* peaks in **C** 5.24 ($H_{1\alpha}$, 5.24, *J* = 3.7 *Hz),* 4.5 ($H_{1\beta}$, *J* = 8 *Hz)*. Spectra were acquired at 2 kHz spinning frequency at room temperature in D_2O.

We also obtained HR MAS-NMR data for GlcNAc-Sepharose conjugates formed using p-TsH chemistry (**Figure 2.3**). Peaks observed in the anomeric region were consistent with exclusive formation of the β anomer on the solid support, as a peak at 4.4 ppm could be observed with the appropriate scalar coupling for β-D-GlcNAc.

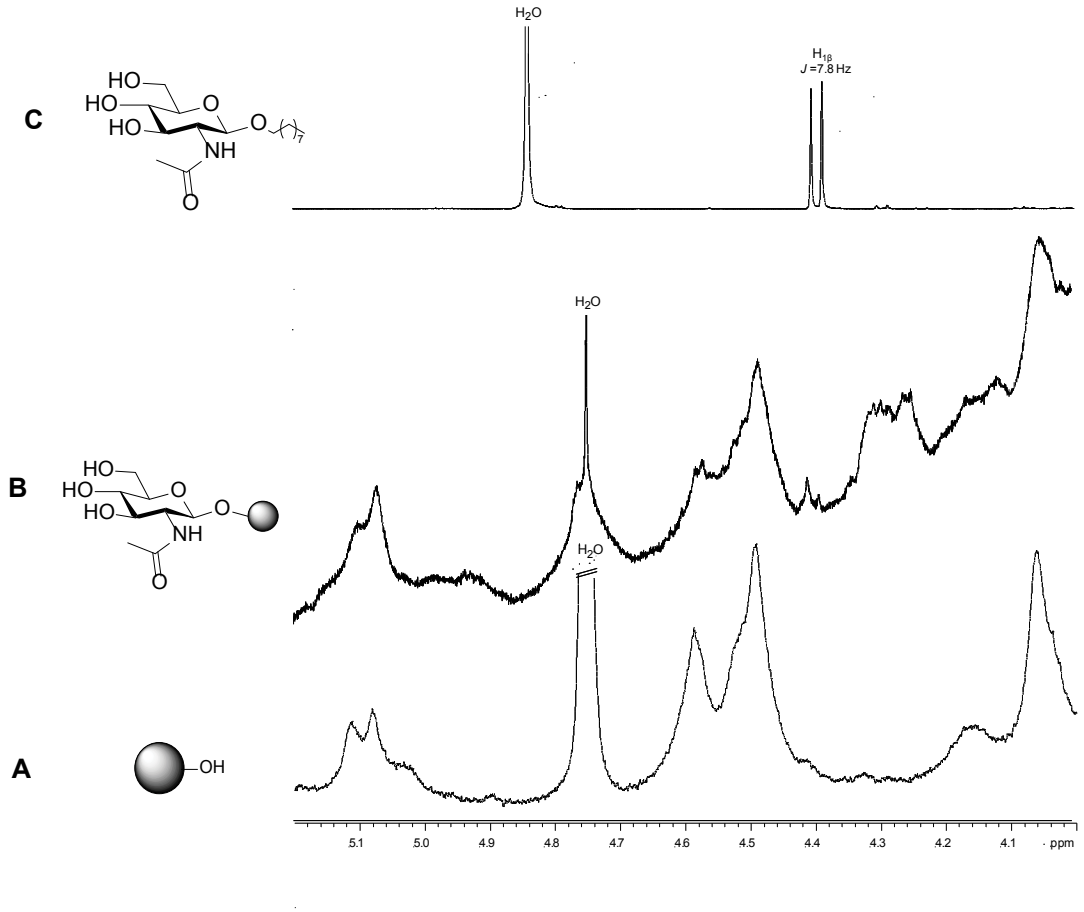


Figure 2.3 HR MAS-^{1}H NMR of Sepharose GlcNAc conjugates generated by Tosyl hydrazide coupling. The anomeric region is shown for samples of **A.** Sepharose CL-6B, **B.** Sepharose-GlcNAc, and **C**. 1-octyl-β-D-GlcNAc in solution (D_2O). Peaks were observed at δ = 4.4 ppm ($H_{1\beta}$ *J* = 7.9 *Hz*) for the conjugate, and δ = 4.4 ppm ($H_{1\beta}$ *J* = 7.8 *Hz)* in solution. Peaks for H1α were not observed in any of the samples coupled by p-TsH. Spectra **A** and **B** were acquired at 2 kHz spinning at room temperature in D_2O.

Immobilization efficiency

2.2.7 Quantification of carbohydrate bound using the phenol sulfuric acid

There are several methods for the determination of carbohydrate content in aqueous solutions. Among these, colorimetric methods are most commonly used because of their versatility, low cost, and simplicity. (*37*) The reaction between the hydrolyzed carbohydrate and a colour developing reagent is the basis of these methods. Common colouring reagents include phenol, (*38*) alkaline ferricyanide ($K_4Fe(CN)_6$), (*39*) and anthrone. (*29*)

Initially, we tested the anthrone method for quantification of immobilized carbohydrate on the solid support. We were able to build a calibration curve, determine a linear range, and test the method against laboratory prepared solutions. However, analysis of resin samples consistently gave higher amounts of carbohydrate in blanks than in sample despite immobilization of carbohydrate. We next examined the phenol-sulfuric acid assay (PSA). The PSA method relies on the acid hydrolysis of carbohydrates, followed by dehydration to afford furfural derivatives. (*30, 38*) The reaction between the furfural and phenol produces a yellow-orange colour with maximum absorbance at wavelength of 490 nm (*38, 40*) that can easily be measured with a spectrophotometer.

Coupling technique	Sample	PSA [nmol Gal mg^{-1} resin] Mean ± SEM	Difference [nmol Gal mg^{-1} resin]
Divinyl Sulfone (DVS)	Support-DVS	1740 ± 30	-
	Support-DVS-Gal	1820 ± 40	270
	Support-DVS-Lac	1860 ± 70	500
	Support-DVS-GlcNAc	1600 ± 70	-
Cyanuric Chloride (CC)	Support-CC-Aniline	1700 ± 20	-
	Support-CC-Aniline-Gal	1960 ± 110	330
	Support-CC-Aniline-Lac	2200 ± 70	480
	Support-CC-Aniline-GlcNAc	1610 ± 20	-
p-TsH	Support	1990 ± 40	-
	Support-Gal	2580 ± 100	260
	Support-Lac	3030 ± 100	500
	Support-GlcNAc	2200 ± 130	200

Table 2.3 Carbohydrate coupling efficiency determined using the PSA assay. The results are presented as Mean ± SEM. All resin samples were 5 mg, and were prepared with 1.1 mmol of carbohydrate mL^{-1} of solid support. Three batches of each conjugate were produced and the carbohydrate quantification was done in triplicate (N = 9).

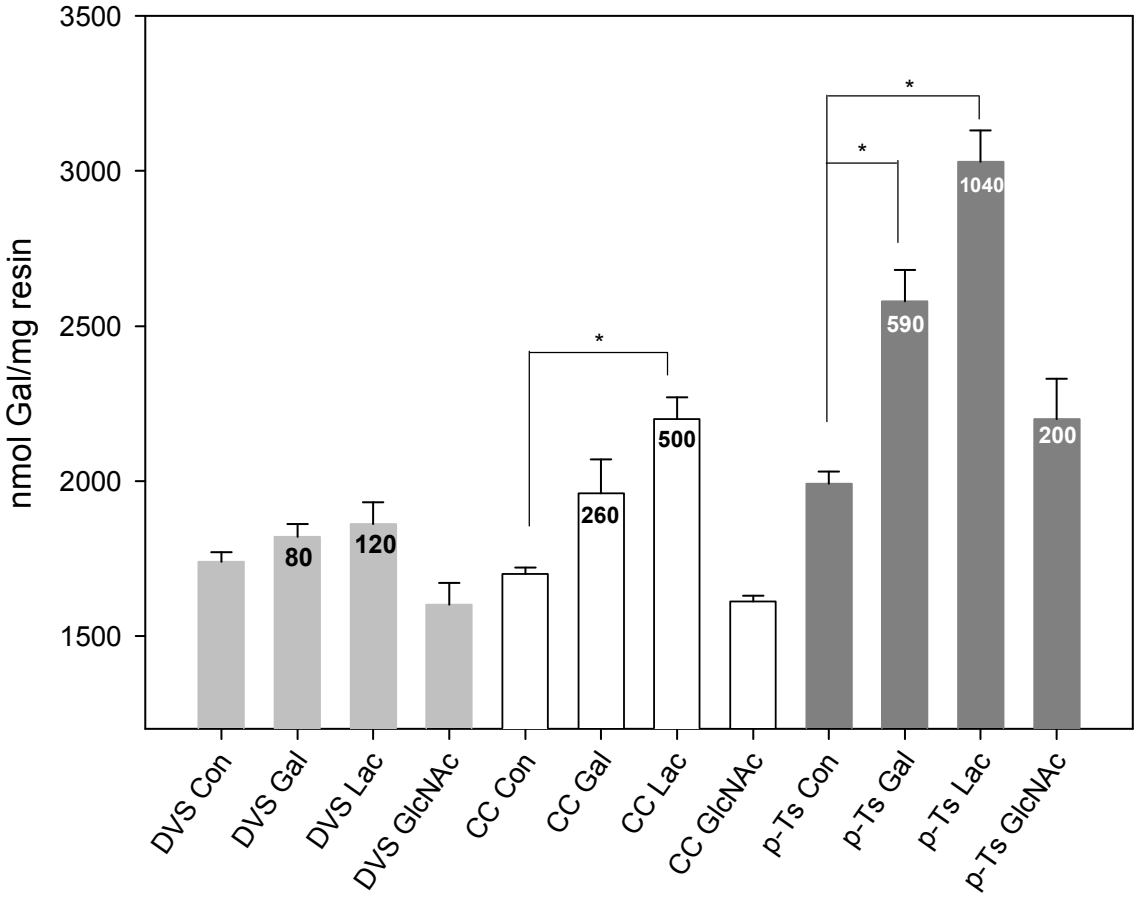


Figure 2.4 Immobilization efficiency of Gal and Lac and GlcNAc determined by PSA. To determine differences in immobilization for each sample, they were compared against the corresponding control (DVS Con, CC Con or p-TsH Con, accordingly) using a two-tailed student's t-test (* = $p < 0.001$ with $\alpha = 0.05$). The control samples for DVS or CC were pre-activated and quenched using 2-mercaptoethanol. The number on each bar represents the difference between the sample and the corresponding control. The results are presented as Mean ± SEM. All resin samples were 1 mg, and were prepared with 1.1 mmol of carbohydrate mL^{-1} of solid support.

The results presented in **Table 2.3** and **Figure 2.4** show that the p-TsH method was the one with the highest immobilization efficiency for all three sugars tested. In comparison with the DVS method, p-TsH immobilized five to nine times more carbohydrate per mg of resin, and approximately double the amount in comparison to the CC method. A possible explanation for the reduced efficiency of the DVS and CC method is consumption of the reactive sites on resin due to the aqueous condition. The p-TsH method circumvents this problem by carrying the reaction under anhydrous conditions, reducing the likelihood of the activated carbohydrate reacting with water. This increased efficiency could be particularly relevant for samples where the availability of carbohydrate is limited. Samples where GlcNAc was immobilized gave significantly lower concentration of total carbohydrate content. This discrepancy is likely due to the reduced reactivity of amino sugars in colorimetric methods such as the PSA as compared to neutral sugars. (*41, 42*)

2.2.8 Determination of FITC-Jacalin binding capacity

After quantifying the amount of carbohydrate immobilized by each method, we considered that different methods could give different amounts of active carbohydrate epitopes. For example, if a binding interaction is dependent upon the 6-OH of Gal, there may be fewer active sites in a DVS-immobilized sample than would be expected based on the mass of the sugar. Additionally, it may be possible in some methods for multiple sites of attachment to occur for a single ligand, which could lead to inactivation of the ligand. Thus, we sought to test the binding of proteins containing carbohydrate recognition domains (CRDs) to test the availability of the immobilized epitopes. We employed an immuno-precipitation-type assay, where a small amount of solid support was incubated with a known mass of protein in solution

followed by elution and measurement of the bound protein. By using a commercially available fluorescent labeled protein, the amount of bound protein was determined using fluorescence spectroscopy.

We selected two readily available lectins, Jacalin and the wheat-germ agglutinin (WGA), for our binding measurements. Jacalin is a lectin obtained from the seeds of *Artocaspus integrifolia,* commonly known as the jackfruit. This lectin has a tetrameric structure with a molecular weight of approximately 66 kDa. Jacalin was selected due to its specificity towards Gal and GalNAc. (*43*) It has been reported that Jacalin binds preferentially to the α-anomer of these carbohydrates. (*44*) WGA is a member or the cereal lectin family, it is a mixture of three isolectins, composed of subunits with almost identical amino acid sequences. (*43, 45, 46*) The WGA lectin binds specifically to GlcNAc, di-*N*-acetyl-chitibiose, and β-linked oligosaccharides of *N*-acetyl glucosamine, with a preference for longer oligosaccharides. (*45, 46*) Moreover this lectin has no binding preference for α or β anomers. (*45, 47*) Thus, we would expect that Jacalin should show preference for α-anomers of Gal, and WGA should show no anomeric preference but should selectively bind to GlcNAc over Gal and Lac samples.

Coupling technique	Sample	Jacalin Bound [µg mg^{-1} resin]	Jacalin Bound [nmol mg^{-1} resin]
Divinyl Sulfone (DVS)	Support-DVS	2.5 ± 0.7	0.04 ± 0.01
	Support-DVS-Gal	12.9 ± 0.6	0.20 ± 0.01
	Support-DVS-Lac	1.9 ± 0.7	0.03 ± 0.01
	Support-DVS-GlcNAc	10.5 ± 0.7	0.16 ± 0.01
Cyanuric Chloride (CC)	Support-CC-Aniline	3.3 ± 0.8	0.05 ± 0.01
	Support-CC-Aniline-Gal	2.3 ± 1.2	0.04 ± 0.02
	Support-CC-Aniline-Lac	2.5 ± 0.8	0.04 ± 0.01
	Support-CC-GlcNAc	1.9 ± 0.7	0.03 ± 0.01
p-TsH	Support	0.8 ± 0.8	0.01 ± 0.01
	Support-Gal	5.2 ± 0.9	0.08 ± 0.01
	Support-Lac	0.6 ± 0.8	0.01 ± 0.01
	Support-GlcNAc	0.6 ± 0.7	0.01 ± 0.01

Table 2.4 FITC Jacalin binding efficiency to immobilized saccharides. Three batches of each conjugate were produced and the binding capacity of each batch was measured in triplicate (N = 9). The initial concentration of the FITC-Jacalin solution was 75 µg mL^{-1} which corresponds to 15 µg per tube, and was incubated with 1 mg of resin. The results are presented as Mean ± SEM. All resin samples were 1 mg, and were prepared with 1.1 mmol of carbohydrate mL^{-1} of solid support.

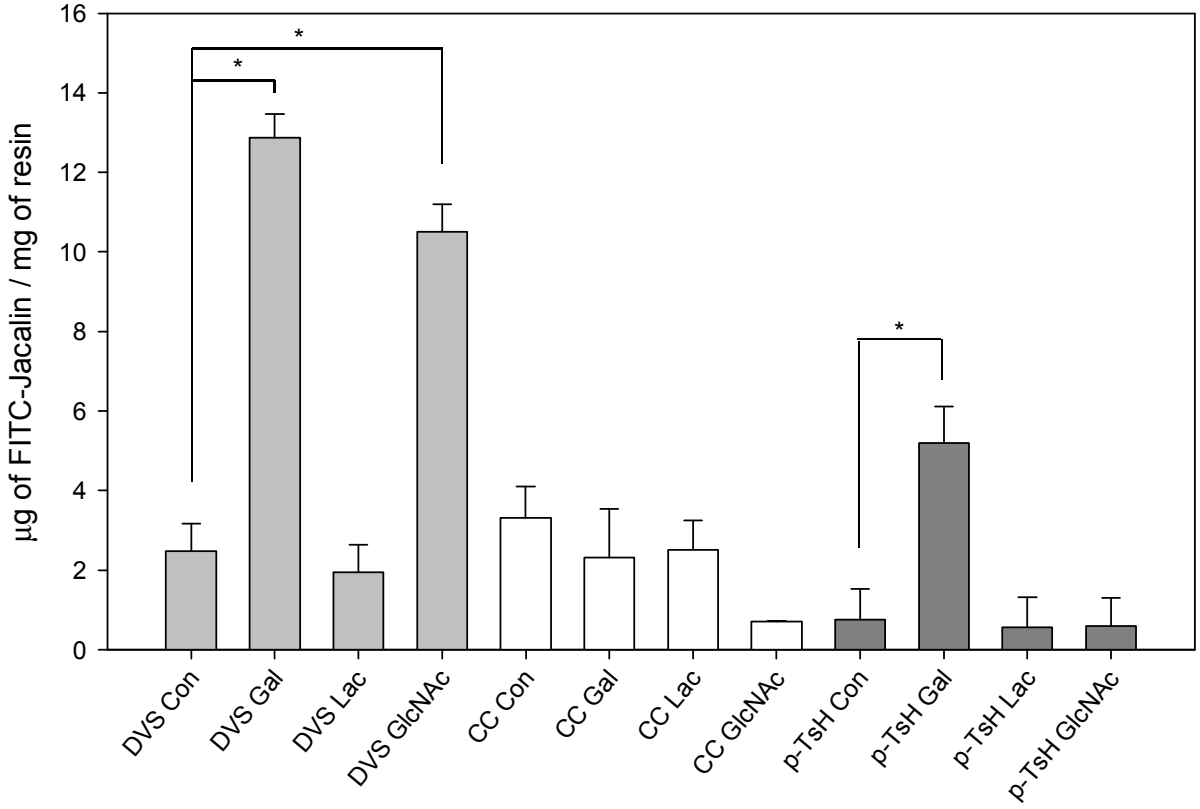


Figure 2.5 FITC-Jacalin binding to immobilized saccharides. Samples were compared against the corresponding control (DVS Con, CC Con or p-TsH Con, accordingly) using a two-tailed student's t-test (* = $p < 0.05$ with $\alpha = 0.05$, N = 9). The control samples are formed by the solid support, the linker (DVS or CC), and the remaining reactive sites were deactivated using 2-mercaptoethanol. The results are presented as Mean ± SEM. All resin samples were 1 mg, and were prepared with 1.1 mmol of carbohydrate mL^{-1} of solid support.

The results obtained from Jacalin binding experiments are shown in **Table 2.4** and **Figure 2.5**. We found that DVS-immobilized Gal had the greatest binding capacity, while CC immobilization did not give any significant binding. Interestingly, p-TsH-immobilized Gal

gave significant binding to Jacalin, but this was lower than that of DVS. We observed no binding for p-TsH-immobilized GlcNAc, yet the DVS-immobilized GlcNAc showed substantial binding capacity. These results are in contrast to the total amount of immobilized carbohydrate determined by PSA. We attribute this discrepancy to the differences in regiospecificity and stereospecificity of the DVS and p-TsH methods. First, while the DVS method can react at multiple sites (favoring primary hydroxyl groups), p-TsH immobilization occurs exclusively at the anomeric position. Additionally, the p-TsH method should favor the formation of the β-anomer, while DVS-immobilized sugars likely contain a mixture of anomers. It has been reported that Jacalin preferentially binds the α-anomer of galactose conjugates (Galα-pNP K_a = 9.3 x 10^4 Vs. Galβ-pNP K_a = no interaction), (*44, 47*) thus the reduced binding observed for p-TsH-immobilized Gal would be consistent with a higher proportion of the β-anomer being present despite the increased amount of monosaccharide on the resin. It has been reported that Jacalin behaves as a 'polyspecific' lectin, and its binding to GlcNAc has been documented (*48*) offering an explanation to the unexpected binding of this lectin to the DVS-GlcNAc support, the lack of binding to GlcNAc immbolized with p-TsH method could be attributed to anomeric specificity.

2.2.9 Determination of FITC-Wheat Germ Agglutinin (WGA) binding capacity

Although Jacalin has a known anomeric preference, we also tested WGA as a lectin that has little anomeric preference for its targets. We observed that the WGA binding capacity of the DVS- and p-TsH-conjugates were much more comparable than the corresponding binding data for Jacalin **(Figure 2.5**). These data are consistent with a preference for immobilization of β-anomer using p-TsH chemistry, but are not conclusive that this is the primary factor. It

should also be noted that the PSA data suggest a substantially larger quantity of immobilized saccharide in the p-TsH conjugates relative to DVS, but that our lectin binding data suggest comparable amounts of binding sites are found in both conjugates.

Coupling technique	Sample	WGA Bound [µg mg^{-1} resin]	WGA Bound [nmol mg^{-1} resin]
Divinyl Sulfone (DVS)	Support-DVS	0.0 ± 0.2	0.00 ± 0.01
	Support-DVS-Gal	0.0 ± 0.3	0.00 ± 0.01
	Support-DVS-Lac	0.0 ± 0.3	0.00 ± 0.01
	Support-DVS-GlcNAc	11.82 ± 0.05	0.31 ± 0.01
Cyanuric Chloride (CC)	Support-CC-Aniline	0.6 ± 0.3	0.02 ± 0.01
	Support-CC-Aniline-Gal	0.0 ± 0.2	0.00 ± 0.01
	Support-CC-Aniline-Lac	0.6 ± 0.3	0.02 ± 0.01
	Support-CC-Aniline-GlcNAc	0.8 ± 0.3	0.02 ± 0.01
p-TsH	Support	0.0 ± 0.7	0.00 ± 0.02
	Support-Gal	0.0 ± 0.1	0.00 ± 0.01
	Support-Lac	0.0 ± 0.3	0.00 ± 0.01
	Support-GlcNAc	9.8 ± 0.3	0.26 ± 0.01

Table 2.5 FITC WGA binding efficiency to immobilized saccharides. Three batches of each conjugate were produced and the binding capacity of each batch was measured in triplicate (N = 9). The initial concentration of the FITC-WGA solution was 75 µg mL^{-1} which corresponds to 15 µg per tube, and was incubated with 1 mg of resin. The results are presented as Mean ± SEM. All resin samples were 1 mg, and were prepared with 1.1 mmol of carbohydrate mL^{-1} of solid support.

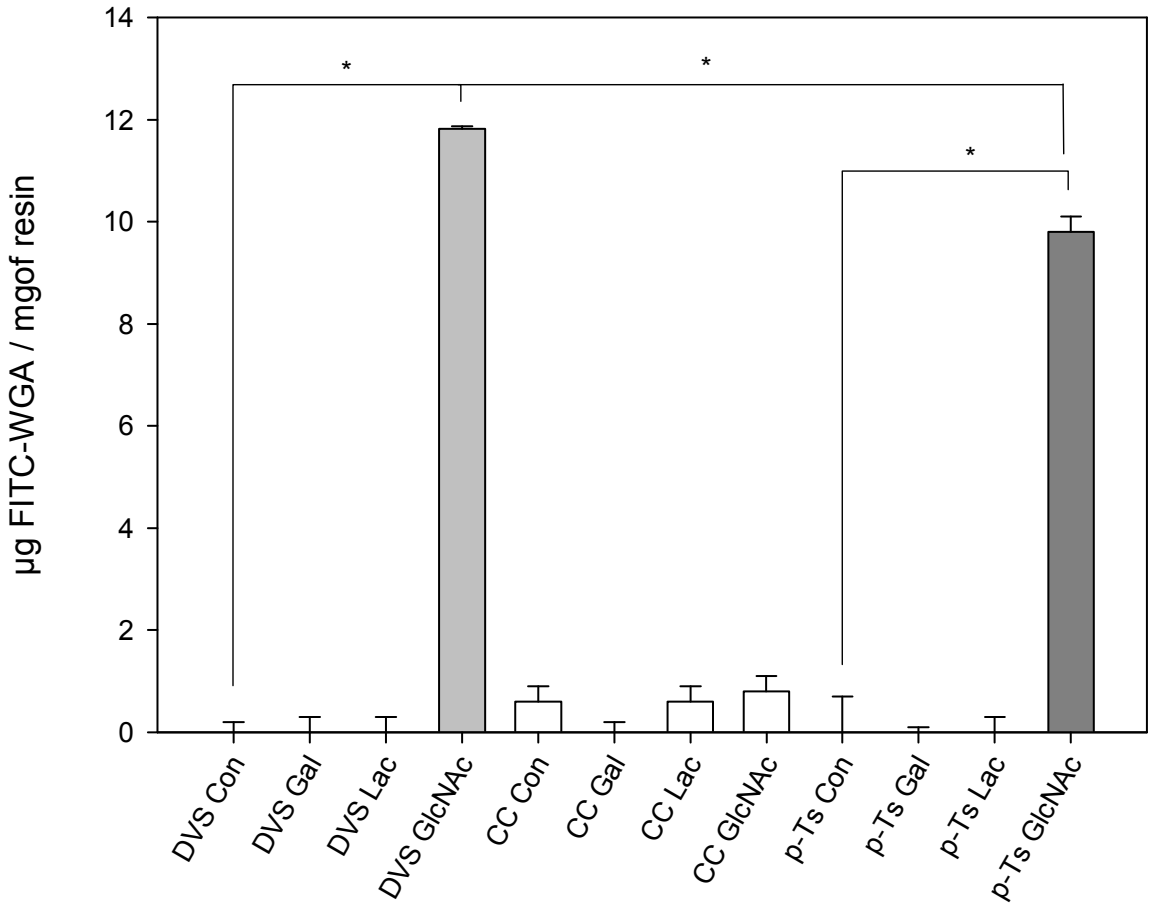


Figure 2.6 FITC-WGA binding to immobilized saccharides. Samples were compared against the corresponding control (DVS Con, CC Con or p-TsH Con, accordingly) using a two-tailed student's t-test (* = $p < 0.01$ with $\alpha = 0.05$ N = 9). The control samples are formed by the solid support, the linker (DVS or CC), and the remaining reactive sites were deactivated using 2-mercaptoethanol. The results are presented as Mean ± SEM. All resin samples were 1 mg, and were prepared with 1.1 mmol of carbohydrate mL^{-1} of solid support.

We considered that the difference in binding capacity of the DVS and p-TsH conjugates could be a result of steric crowding from overloading of the resin. To test this hypothesis we examined samples of p-TsH conjugates prepared with reduced amounts of the carbohydrate epitope. The samples used above were prepared with 1.1 mmol of carbohydrate per mL of resin, and we tested additional samples prepared with 1.11 and 0.64 mmol of saccharide mL^{-1} of resin. We obtained PSA and WGA binding data for each of these samples and compared them to the previous runs (**Table 2.6**).

Amount of carbohydrate used [mmol mL^{-1} resin]	Coupling technique	Sample	WGA Bound [µg mg^{-1} resin] Mean ± SEM	PSA [nmol Gal mg^{-1} resin] Mean ± SEM
1.11	Divinyl sulfone (DVS)	Support-DVS	0.0 ± 0.2	1740 ± 30
		Support-DVS-GlcNAc	11.82 ± 0.05	1600 ± 70
1.11	Cyanuric Chloride (CC)	Support-CC-Aniline	0.6 ± 0.3	1700 ± 20
		Support-CC-Aniline-GlcNAc	0.8 ± 0.3	1610 ± 20
1.11	p-TsH	Support	0.0 ± 0.7	1990 ± 40
		Support-GlcNAc	9.8 ± 0.3	2200 ± 100
*0.64	p-TsH	Support-GlcNAc	10.7 ± 0.4	1900 ± 160

Table 2.6 FITC WGA binding efficiency compared with carbohydrate coupling efficiency in GlcNAc samples. Three batches of each conjugate were produced and the binding capacity of each batch was measured in triplicate (N = 9). The initial concentration of FITC-WGA solution was 75 µg ml^{-1} which corresponds to 15 µg per tube. The results are presented as Mean ± SEM for the WGA and Mean ± SD for the carbohydrate loading. All resin samples were 5 mg, and were prepared with the amount of carbohydrate in the first column mL^{-1} of resin. * For this sample N = 3

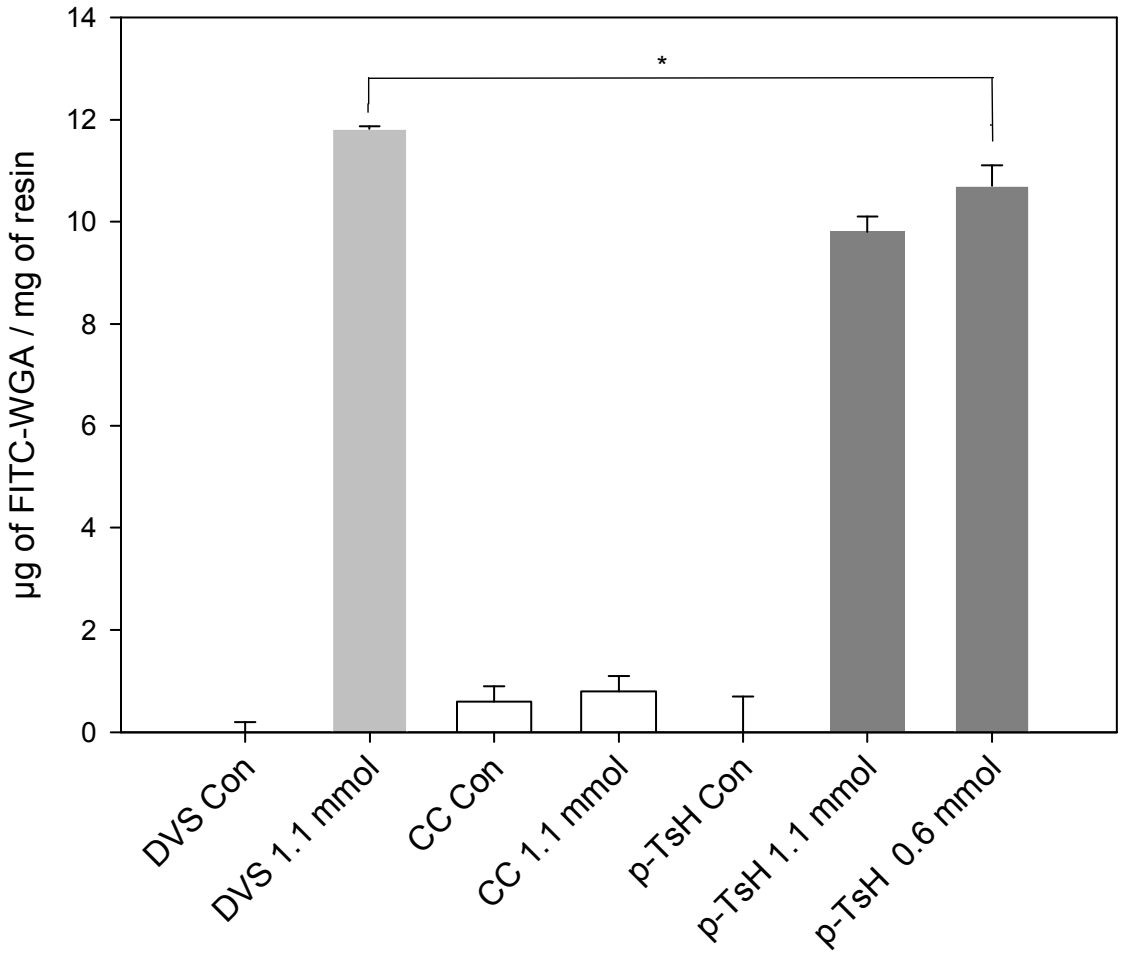


Figure 2.7 Concentration dependence of FITC-WGA binding per mg of resin with GlcNAc samples. Samples were compared using a two-tailed student's t-test. The samples produced with the p-TsH method present no statistically significant difference ($p > 0.05$, N = 9 for p-TsH 1.1 mmol, and N = 3 for p-TsH 0.6 mmol) whereas DVS 1.1 mmol and p-TsH 0.6 mmol have a statistically significant difference (* = $p < 0.00,1$ N = 9 for DVS 1.1 mmol and N = 3 for p-TsH 0.6 mmol). The results are presented as Mean ± SEM. All resin samples were 1 mg, and were prepared with the indicated mmol of carbohydrate mL^{-1} of solid support.

The results obtained from this experiment show that by *reducing* the amount of carbohydrate coupled to the solid support, it is possible to *maintain* the binding capacity of the conjugate. These data do not support our hypothesis that an excess of carbohydrate may reduce the number of active epitopes due to crowding on the matrix. However, the results highlight how efficient the p-TsH coupling step is when compared to existing methods. Based on the amount of carbohydrate required for preparation of 1 mL of settled resin (~60 mg dry weight) and the amount of protein binding to the support, we determined a ratio of active epitope to the amount of sugar used for the immobilization (**Table 2.7**). From this calculation, we concluded that DVS and p-TsH methods are competitive. However, the p-TsH method provides a more efficient immobilization chemistry at lower concentrations of carbohydrate, which may be essential if the ligand is a limiting reagent. The p-TsH method is also regio- and stereospecific, which provides a more defined affinity matrix.

$$\frac{\mu g\ WGA\ bound\ /mg}{mmol\ Carbohydrate\ used/mL}\left(\frac{60\ mg}{1\ mL}\right) = \frac{\mu g\ WGA\ bound}{mmol\ carbohydrate\ used} \qquad \textbf{(2.1)}$$

For determining the ratio of protein bound $mmol^{-1}$ of carbohydrate used, data from **Table 2.5** were analyzed with equation **2.1**. The amount of FITC-WGA bound is divided by the amount of carbohydrate used for coupling. The result is multiplied for an empirical factor (60 mg mL^{-1}) to convert 1 mL of settled solid support to the dry weight mass of solid support. The results, presented in **Table 2.6**, support the hypothesis that a ligand overcrowding was hindering the protein binding. Moreover, the results indicate that a similar or greater binding

efficiency can be achieved with the p-TsH method using almost half the amount of carbohydrate. This finding is particularly relevant in the field of glycobiology since most of the times the carbohydrate samples are available in limited amounts.

Coupling method	WGA Bound [µg mg^{-1} resin]	Amount of carbohydrate [mmol mL^{-1} resin]	Ratio [µg WGA $mmol^{-1}$ of carbohydrate]
DVS	11.82 ± 0.05	1.11	600 ± 3.0
CC	0.8 ± 0.3	1.11	40 ± 20
p-TsH activation	9.8 ± 0.3	1.11	500 ± 20
	10.7 ± 0.4	0.64	1000 ± 40

Table 2.7 Relative coupling method efficiencies. The efficiencies are expressed as a ratio of the µg of WGA bound $mmol^{-1}$ of carbohydrate used at the coupling step, and presented as Mean ± SEM. The ratio was calculated using **equation 2.1**. The means were compared using a two-tailed student's t- test for each of the six possible permutations and found to be significantly different ($p < 0.001$ $\alpha = 0.05$, $N = 9$) for all the samples with 1.11 mmol of carbohydrate, and $N = 3$ for the sample with 0.64 mmol of carbohydrate.

2.3 Conclusions

We have compared three methods for the immobilization of carbohydrate epitopes to a solid support. We drew from known methods, such as DVS and CC crosslinking, which are

well established and have been used for the immobilization of carbohydrates and other biomolecules. (*9, 31*) Additionally, we have developed an improved method based on p-TsH solution-phase protecting-group free glycosidation. (*27*) Our data provide evidence that the p-TsH method has significant advantages in its control of the regio- and stereospecificity of the immobilization, as well as improved efficiency of the method as compared to known strategies.

Using HR-MAS-^{1}HNMR we observed the appearance of NMR signals in the anomeric region of resin-carbohydrate conjugates consistent with preferential immobilization of the β-anomer using p-TsH chemistry. The chemical shift and the scalar coupling constant of these peaks match the values observed for analogs in solution. We compared the loading of each resin sample using a phenol-sulfuric acid assay as a benchmark. These data suggest that the p-TsH chemistry can immobilize twice as much glycan as the CC method, and five-times more glycan than the DVS method using similar amounts of resin and carbohydrate.

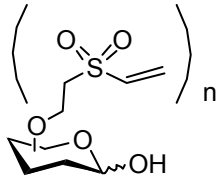
We tested the activity of the immobilized epitopes using a lectin binding assay. These studies were consistent with the anomeric preference of the p-TsH method, and found comparable amounts of active epitopes for both DVS and p-TsH methods. However, closer examination revealed that the p-TsH method has increased efficiency of immobilization at lower concentrations of glycan. Thus, we conclude that the p-TsH method is an important new alternative method for the immobilization of glycan epitopes to an affinity matrix. The p-TsH method provides a regio-specific immobilization that has improved efficiency over existing methods. We expect that this method will be of interest to glycobiologists that may be working with limited amounts of glycan, or in situations where the glycan solubility may be limited.

2.4 Experimental Methods

2.4.1 General methods

All reagents used were purchased from Sigma-Aldrich or Acros Organics, and used without further purification unless otherwise noted. All reactions were carried out under an inert atmosphere with argon at room temperature unless otherwise indicated. Reactions were monitored by analytical TLC on silica gel 60-F_{254} (0.25mm, Silicycle, Quebec, Canada) and visualized under UV light (254 nm), or stained by charring with ceric ammonium molybdate (CAM) or potassium permanganate ($KMnO_4$). Organic solvents were removed under reduced pressure, and organic products were purified by flash column chromatography on silica gel (230-400 mesh, Silicycle, Quebec, Canada). ^1H NMR spectra were acquired on Varian 400, 500 or 600 MHz instruments at room temperature as noted. ^{13}C NMR spectra were recorded at 125 or 150 MHz as noted. Electrospray-ionization mass spectra were recorded on an Agilent Technologies 6220 TOF instrument.

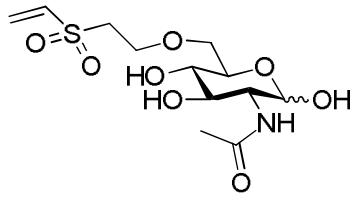
2.4.2 Synthetic methods



DVS- Galactose (2.1)

D-galactose (91 mg, 0.5 mmol) was dissolved in 1 mL of carbonate buffer (pH 11) and stirred until dissolved. In a glass vial, 295 mg (2.5 mmol) of divinyl sulfone was dissolved in 500 µL of carbonate buffer (pH 11) and then added dropwise to the solution of galactose over 5 min. The reaction was monitored by TLC, and after 70 minutes the reaction was complete.

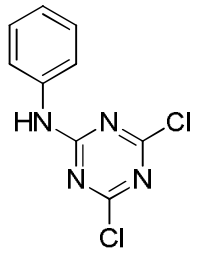
The mixture was concentrated to a final volume of approximately 0.5 mL and loaded onto a silica gel column, eluted with 25% MeOH in DCM. The fractions containing the product were concentrated *in vacuo* to afford a yellow oil in 60% yield. The product was a mixture of di-, tri-, and tetra-substituted galactose. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.9-6.76 (m, *CH*=CH_2), 6.52-6.44 (m, CH=*CH_2*), 6.23-6.18 (m, CH=*CH_2*), 5.0 (d, 3.7 Hz, H-1α) 4.45-3.28 (several m, H-1β, H2, H-3, H-4, H-5, H-6, H-6', CH_2SO_2, CH_2-O), 1.7 (s, Ac) Masses of the di-substituted ($C_{14}H_{24}O_{10}S_2Na$ M+Na^+) calcd. 439.0811 found 439.1, tri-substituted ($C_{18}H_{32}O_{13}S_3Na$ M+Na^+) calcd.575.1005 found 575.1 ($C_{22}H_{40}O_{16}S_4$ M+Na^+) calcd. 711.1199 found 711.1



6-Deoxy-6-(2-(vinylsulfonyl)ethyl)-2-*N*-Acetyl-D-glucosamine (2.2)

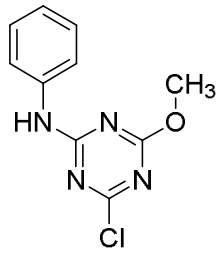
N-acetyl-D-glucosamine (40 mg, 0.18 mmol) was dissolved in 1 mL of carbonate buffer (pH 11) and stirred until dissolved. In a glass vial, 4.47 mg (0.036 mmol) of divinyl sulfone was dissolved in 500 µL of carbonate buffer and then added to the glucosamine solution drop-wise over 5 min. The reaction was monitored by TLC, and after 70 minutes the reaction was complete. The mixture was concentrated to a final volume of approximately 0.5 mL and loaded onto a Sep-Pak C18 Cartridge and eluted with 25% MeOH in H_2O. The fractions containing the product were concentrated *in vacuo* to afford an off-white solid. Isolated yield: 57% (α:β, 1:1). ^{1}H NMR (400 MHz, CD_3OD): δ = 6.91 (dd, 1H, *J* = 16.6, 9.9 Hz; *CH*=CH_2), 6.38 (d, 1H; CH=*CH_2*), 6.23 (d, 1H; CH=*CH_2*), 5.1 (d, 0.44H, *J* = 3.3 Hz; H-1α), 4.42 (d, 0.46H; *J* = 8.4 Hz, H-1β), 3.90-3.80 (m, 4H, H-3, H-6, H-6', CH_2SO_2), 3.74-3.60 (m, 3H, H-2, H-4, CH_2SO_2),

3.48-3.31 (m, 3H, H-5, CH_2-O), 2.0 (s, 3H; Ac). ESI HRMS m/z ($C_{12}H_{21}NO_8SNa$ $M+Na^+$) calcd. 362.1, found: 362.1.



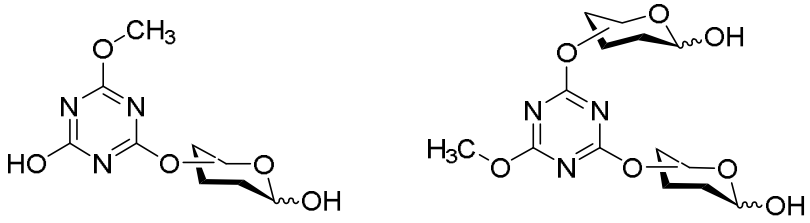
2-anilino-4,6-dichloro-[1,3,5-triazine] (2.3)

The title compound was prepared as previously reported. (*32*) Briefly, 1,3,5-trichloro-s-triazine (502 mg, 2.72 mmol) and K_2CO_3 (0.75 g, 2.98 mmol, 2 eq) were added to 1 mL of dry THF at 0 ºC and stirred. Freshly distilled aniline (278 mg, 2.98 mmol, 1.1 eq) was dissolved in 1 mL of dry DMF and added dropwise over 20 min. The solution was left stirring for 2 h, and a precipitate was formed and then filtered. The remaining solution was concentrated *in vacuo.* The product was purified by flash chromatography using 10% EtOAc in hexanes, and the fractions pooled and concentrated *in vacuo* to afford compound **2.3** as a white powder (595.5 mg, 2.47 mmol, 91.5% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.54 (d, 2H, *J* = 7.7 *Hz*; Ar*H*), 7.50 (s, 1H; N*H*), , 7.41 (t, 2H, *J* = 7.6 Hz; ArH), 7.23 (t, 1H, 7.3 Hz*;* Ar*H*) ^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.4, 170.3, 164.1, 135.7, 129.3, 125.9, 121.3 ESI-HRMS calcd. for $C_4H_3ON_3{}^{35}Cl_2$ 239.9970, found 239.9966.



2-anilino-6-chloro-4-methoxy-[1,3,5-triazine] (2.4)

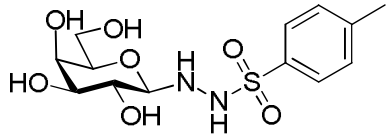
The title compound was prepared as previously reported. (*33*) Briefly, compound **2.3** (250 mg, 1.04 mmol) was dissolved in 1 mL of MeOH and stirred at 0 ºC, two portions of LiH were added (9.51 mg, 1.19 mmol) and stirred for 2.5 hours. The ice bath was removed and the reaction mixture was stirred for 14 hours. Then 20 mL of water was added and the precipitate was filtered and dried to obtain compound **2.4** in 90% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ =, 7.57 (m, 2H; Ar*H*), 7.52 (s, 1H; N*H*), 7.40 (m, 2H; Ar*H*), 7.21 (m, 1H, Ar*H*), 4.02 (s, 3H, OCH_3). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.6, 170.8, 165.3, 136.8, 129.1, 124.9, 121.0, 55.6 ppm. ESI-HRMS calcd. for $C_{10}H_9ON_4{}^{35}ClO$ 236.0465, found: 236.0461



2-galactopyranosyl-6-hydroxy-4-methoxy-[1,3,5-triazine] and 2,4-digalactopyranosyl-6methoxy-[1,3,5-triazine] (2.5a and 2.5b)

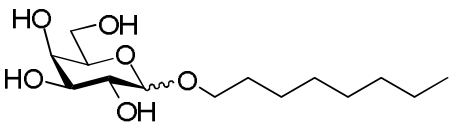
Galactose (80 mg, 0.44 mmol) was dissolved in 6 mL of water and stirred until all the solid was dissolved, followed by NaOH (26.4 mg, 0.66 mmol) until all the solid was dissolved. 4,6-dichloro-6-methoxy-[1,3,5-triazine] (80 mg, 0.45 mmol) was added drop wise to the reaction

mixture dissolved in 1 mL of acetone and left stirring for 8 h. The mixture was purified by preparative TLC using 12:1:0.4 isopropanol:MeOH:H_2O as eluent using 2 elutions. The product was recovered with MeOH and dried under reduced pressure to afford a mixture of the mono and di-substituted products. ^{1}H NMR (400 MHz, CD_3OD): δ = 6.16-6.10 (m, H-1), 4.60-4.24 (m, H-1), 4.08-3.42 (m, H-2, H-3, H-4, H-5, H-6, H-6', OCH_3). Masses of the mono-substituted ($C_{10}H_{15}O_8N_3Na^+$) calcd. 328.0757 found 328.0754, di-substituted ($C_{16}H_{25}O_{13}N_3Na^+$) calcd. 490.1285 found 490.1283



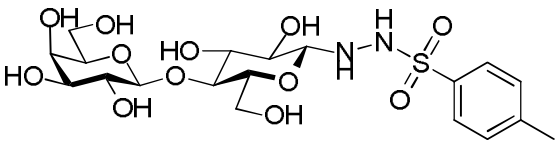
_N_'-(β-D-galactopyranosyl)-_p_-toluenesulfono-hydrazide (2.6)

Compound 2.6 (1.95 g, 5.6 mmol) was prepared as previously reported. (*27, 28*) Isolated yield was 99%. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.84 (d, 2H, J = 7.6 Hz, Ar), 7.42 (d, 2H, J = 8.1 Hz, Ar), 3.84 (d, 1H, $J_{5,6a}$ = 3.1 Hz, H-6), 3.74-3.68 (m, 3H, H-1, H-3, H-5), 3.52-3.47 (m, 3H, H-2, H-4, H-6), 2.47 (s, 3H, $PhCH_3$); An impurity starting material was detected at δ = 7.24, 7.58, and 2.4. ^{13}C NMR (125 MHz, CD_3OD): 145.4, 137.7, 130.9, 130.3, 129.4, 125.5, 92.6, 78.2, 75.5, 70.9, 69.7, 63.3, 21.8 ppm. ESI-HRMS m/z calcd. for $C_{13}H_2N_{20}O_7SNa^+$ (M+Na^+) 371.0883, found 371.0878.



Octyl-D-galactopyranoside (2.7)

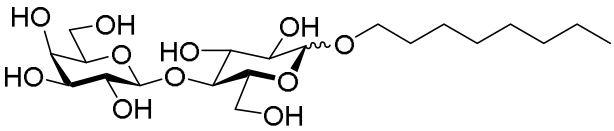
Compound 2.6 (35 mg, 0.10 mmol) was dissolved in 1 mL of dry DMF, and octanol (150 μL, 0.124 mmol) was added to the solution. *N*-Bromosuccinimide (36 mg, 0.2 mmol) was then added to the solution, and after 25 minutes, Amberlite resin (OH^-) was added to quench the reaction and the solution was stirred until the yellow colour disappeared. The resin was filtered and washed with MeOH and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (25% MeOH in CH_2Cl_2) to afford a white solid (18 mg, 0.062 mmol) in 62% isolated yield. (α:β, 1:1.4). ^{1}H NMR (500MHz, CD_3OD): δ = 4.82 (d, 0.46H, *J* = 3.3 Hz; H-1α), 4.20 (d, 0.54H, *J* = 7.5 Hz; H-1β), 3.9-3.4 (m, 7H), 1.66-1.59 (m, 2H; OCH_2), 1.38-1.31 (m, 10H; $OCH_2CH_2(CH_2)_5$), 0.90 (t, 3H, *J* = 7.0 Hz; CH_2CH_3). ^{13}C NMR (125 MHz, CD_3OD): δ = 105.3, 100.6, 76.9, 75.4, 72.9, 71.9, 71.4, 71.4, 71.1, 70.6, 69.5 63.0, 62.8, 33.3, 31.1, 30.90, 30.89, 30.7, 27.7, 27.4, 24.0, 14.7 ppm. ESI-HRMS m/z calcd. for $C_{14}H_{28}NaO_6$ ($M+Na^+$) 315.1778, found 315.1774.



***N*'-(β-D-lactopyranosyl)-*p*-toluenesulfono-hydrazide (2.8)**

The *N*'-(β-D-lactopyranosyl)-*p*-toluenesulfono-hydrazide (2.8) was prepared as previously reported, (*27, 28*) and obtained in 97% yield. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.85 (d, 2H, *J* = 8.4 Hz; Ar*H*), 7.47 (d, 2H, *J* = 7.8 Hz; Ar*H*), 4.40 (d, 1H, *J* = 7.9 Hz, H-1'),

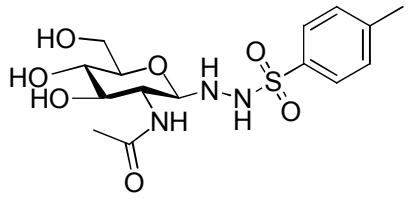
3.88 (dd, 1H, *J* = 12.0, 2.4 Hz; H-6a), 3.81-3.70 (m, 6H; H-1, H-6'a, H-4', H-6'b, H-6b), 3.65-3.51 (m, 6H; H-2, H-4, H-3, H-2', H-5, H-3', H-5'), 2.44 (s, 3H; $PhCH_3$). ^{13}C NMR (125 MHz, CD_3OD): δ = 145.4, 137.7, 130.9, 129.4, 105.4, 91.8, 81.0, 78.0, 77.4, 76.8, 75.1, 72.8, 71.7, 70.6, 62.8, 62.6, 21.8 ppm. ESI-HRMS m/z calcd. for $C_{19}H_{30}NaO_{12}S$ ($M+Na^+$) 533.1412, found 533.1401.



Octyl-D-lactopyranoside (2.9)

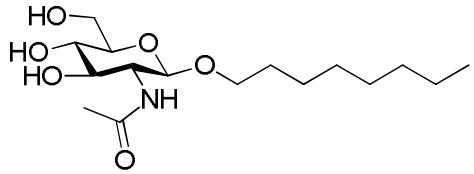
Compound 2.8 (51mg, 0.10 mmol) was dissolved in 1 mL of dry DMF, and octanol (150 µL, 0.124 mmol) was added to the solution. *N*-Bromosuccinimide (36 mg, 0.2 mmol) was added, and after 25 minutes Amberlite resin (OH^-) was added to quench the reaction and the solution was stirred until the yellow colour disappeared. The resin was filtered and washed with MeOH and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (25% MeOH in CH_2Cl_2) to afford a white solid (14 mg, 0.031 mmol) in 31% isolated yield. (α:β, 0.06:1). ^1H NMR (500MHz, CD_3OD): δ = 4.77 (d, 0.05H, *J* = 3.6 Hz; H-1α), 4.37 (d, 1H, *J* = 8.0 Hz; H-1'β), 4.28 (d, 0.95H, *J* = 8.0 Hz; H-1β), 3.90-3.68 (m, 7H; H-6a, H-6b, H-6'b, H-6'a, H-4, H-3, H-4'), 3.60-3.38 (m, 7H; H-3, H-3', H-2', H-5, H-5', OCH_2), 3.24 (under CD_3OD, 1H, *J* = 9.0 Hz; H-2), 1.66-1.59 (m, 2H; OCH_2), 1.41-1.31 (m, 10H; $OCH_2CH_2(CH_2)_5$), 0.90 (t, 3H, *J* = 6.5 Hz; CH_2CH_3) ppm. A small impurity at 7.42 and 7.83 ppm was observed corresponding to starting material. ^{13}C NMR (125 MHz, CD_3OD): δ =

103.9, 98.5, 79.6, 75.7, 73.5, 72.1, 71.8, 71.1, 70.7, 68.9, 67.9, 61.1, 31.6, 29.2, 29.0, 25.9, 22.3, 13.0 ppm. ESI-HRMS m/z calcd. for $C_{20}H_{38}NaO_{11}$ ($M+Na^+$) 477.2306, found 477.2306.



_N_-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-_p_-toluenesulfono-hydrazide (2.10)

Compound 2.10 (1.24 g, 2.4 mmol) was prepared as previously reported, (*27, 28*) with an isolated yield of 99%. Based on the coupling constant we determined that only the β anomer was present. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.73 (d, 2H, *J* = 8.2 Hz; ArH), 7.35 (d, 2H, *J* = 8.0 Hz; ArH), 3.92 (d, 1H, *J* = 9.2 Hz; H-1β), 3.87 (dd, 1H, *J* = 11.7, 1.5 Hz; H-6a), 3.60 (m, 1H; H-6b), 3.46-3.42 (m, 2H, H-2 and H-3), 3.20-3.18 (m, 2H; H-4, H-5), 2.43 (s, 3H; Ph*CH*$_3$), 2.01 (s, 3H; Ac). Two impurities (7.42 and 7.55 ppm) were observed, most likely from the tosyl hydrazide. ^{13}C NMR (125 MHz, CD_3OD): δ = 174.2, 145.4, 137.6, 130.9, 130.8, 129.43, 92.2, 79.2, 76.6, 72.7, 72.3, 63.5, 55.3, 23.0, 21.8 ppm. ESI-HRMS m/z calcd. for $C_{15}H_{23}N_3NaO_7S$ ($M+Na^+$) 412.1149, found 412.1147.



Octyl-2-acetamido-2-deoxy-β -D-glucopyranoside (2.11)

Compound 2.10 (30 mg, 0.077 mmol) was dissolved in 1 mL of dry DMF, and octanol (150 μL, 0.124 mmol) was added to the solution. *N*-Bromosuccinimide (36 mg, 0.2 mmol) was

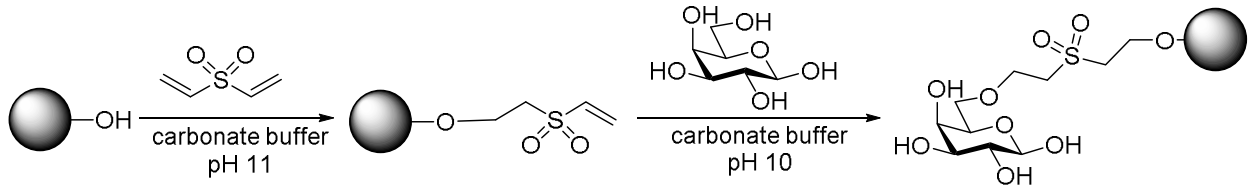
added, and after 25 minutes Amberlite resin (OH^-) was added to quench the reaction and the solution was stirred until the yellow colour disappeared. The resin was filtered and washed with MeOH and solvent was removed under reduced pressure. The residue was purified using a Sep-Pak C18 Cartridge, the product (18 mg 0.054 mmol) was eluted with 25% methanol in water and freeze-dried to afford a white solid in 70% yield. Only the β anomer was observed. ^{1}H NMR (500 MHz, CD_3OD): δ = 4.38 (d, 1H, *J* = 8.4 Hz; H-1β) 3.89-3.86 (m, 2H; H-6a, OC*Ha*HbCH_2), 3.66 (dd, 1H, *J* = 11.8, 5.7 Hz; H-6b), 3.62 (dd, 1H, *J* = 10.3, 8.5 Hz; H-2), 3.46-3.42 (m, 2H; H-3, OCHa*Hb*CH_2), 3.30 (under CD_3OD, 1H; H-4), 3.26 (ddd, 1H, *J* = 9.7, 5.7, 2.2 Hz; H-5), 1.97 (s, 3H; Ac), 1.56-1.53 (m, 2H; $OCH_2C\mathit{H_2}$), 1.38-1.30 (m, 10H; $OCH_2CH_2(CH_2)_5$), 0.90 (t, 3H *J* = 6.8 Hz; $CH_2\mathit{CH_3}$) ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 173.9, 103.0, 78.3, 76.4, 72.5, 70.9, 63.1, 57.8, 33.3, 31.0, 30.8, 27.4, 24.0, 23.3, 14.7 ppm. ESI-HRMS m/z calcd. for $C_{16}H_{31}NO_6Na$ ($M+Na^+$) 356.2044, found 356.2044.

2.4.3 Synthetic methods on solid support

2.4.3.1 Divinyl sulfone immobilization to Sepharose CL-6B

Carbohydrate modified sepharose gel was prepared as previously reported. (*9*) Settled Sepharose CL-6B (1 mL); (GE Healthcare Life Sciences, Piscataway, N.J.) was thoroughly washed with water in a sintered funnel and then re-suspended in 0.5 M carbonate buffer (pH 11) with 100 μL of divinyl sulfone. The mixture was agitated for 70 min, after which the resin was transferred to a sintered funnel and extensively washed with water. The moist cake was suspended in a 1 mL solution of the indicated carbohydrate (1.11 mmol mL^{-1} in 0.5 M carbonate buffer, pH 10) and left agitating for 18 hours. The resin was washed again with distilled water over a sintered funnel, and the moist cake re-suspended in carbonate buffer (1

mL, 0.5 M, pH 8.5) and 2-mercaptoethanol (6 µL). After 2 hours the sample was washed with distilled water and stored in 20% ethanol solution.

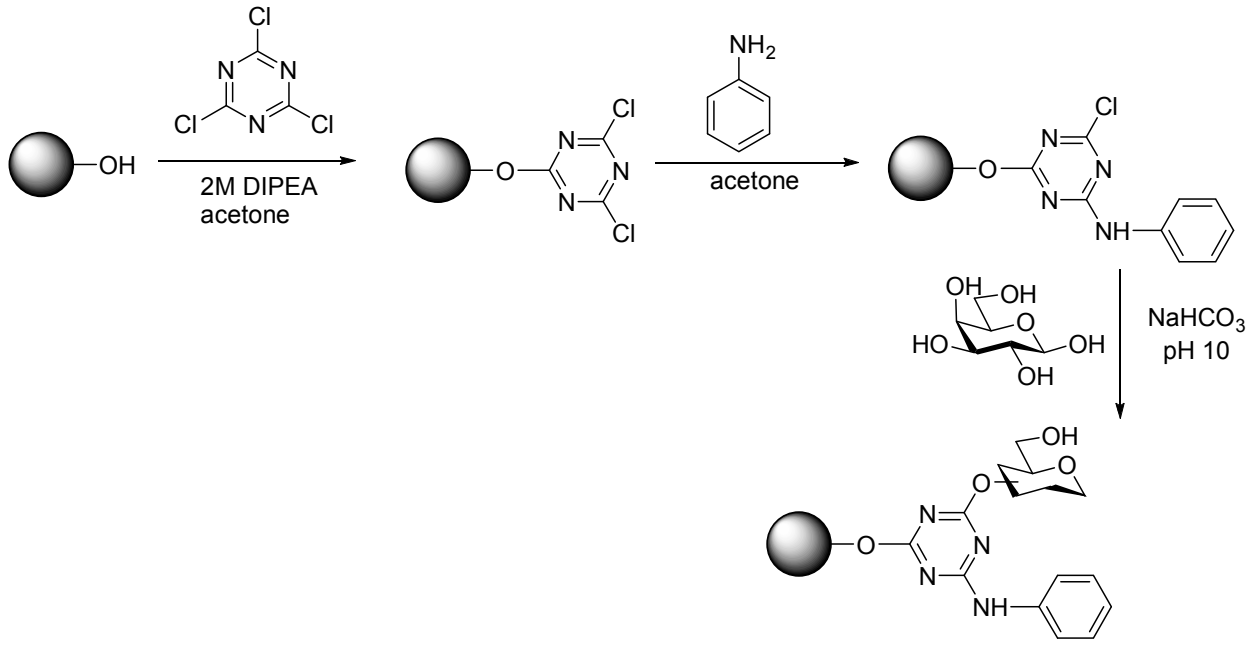


Scheme 2.8. Immobilization of galactose to Sepharose CL-6B using DVS

2.4.3.2 Cyanuric chloride immobilization to Sepharose CL-6B

The procedure of Finlay et al. was adapted (*31*). Briefly, 3 mL of settled Sepharose CL-6B was extensively washed with distilled water in a sintered glass funnel. The resin was transferred to organic phase by washing with 9 mL of increasing concentrations of acetone in water (25, 50, 75 and 100%). A final wash with 100% acetone was carried out twice. The resin was re-suspended in 3 mL of acetone and transferred to a three-necked-round-bottom flask, with a condenser attached. The mixture was heated to 50 ºC and slowly agitated while adding 600 µL of a 2 M *N,N*-diisopropylethylamine in acetone. After 30 min, a solution of trichloro-S-triazine in acetone (1 M, 600 µL) was added drop-wise and left mixing for one hour at 50 ºC. The resin was washed thoroughly with acetone in a sintered glass funnel. The resin was re-suspended in 3 mL of acetone and a solution of anline (2 M, 60 µL) in acetone was added and left mixing at room temperature. After 30 min, the resin was filtered and washed thoroughly with acetone and then transferred back to the aqueous phase by washing with decreasing concentrations of acetone in water (75, 50 and 25%), followed by two washes with distilled water. The activated resin was re-suspended in a solution containing 20 mg mL^{-1} of the

indicated carbohydrate in bicarbonate buffer (pH 10) and left mixing for 18 hours. The resin was extensively washed with distilled water.

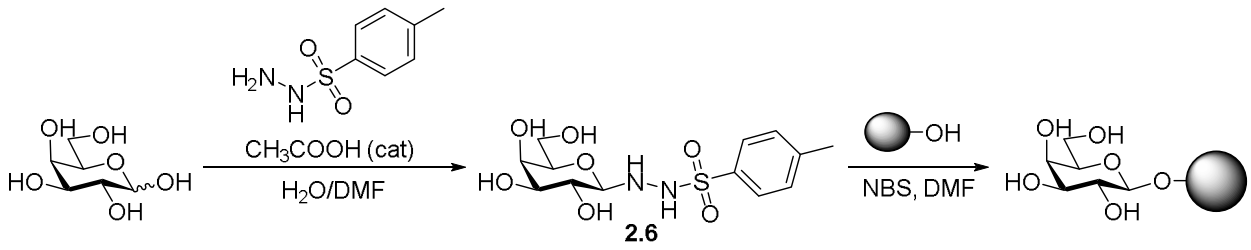


Scheme 2.9. Immobilization of galactose to Sepharose CL-6B using Cyanuric Chloride

2.4.3.3 *p*-toluenesulfonyl hydrazide immobilization to Sepharose CL-6B

Settled Sepharose CL-6B gel (1 mL) was extensively washed with distilled water in a sintered glass funnel. The resin was transferred to an organic phase by washing with 5 mL of increasing concentrations of DMF in water (25, 50, 75 and 100%). A final wash with 100% DMF was carried out twice. The resin was then re-suspended in 1 mL of DMF and transferred to a dry round bottom flask under inert atmosphere. The mixture was stirred slowly in an orbital shaker, and the indicated hydrazide was added (1.11 mmol in 1 mL DMF) and allowed to mix for five minutes. The flask was then charged with *N*-bromosuccinimide (2.5 mmol in 1mL DMF), added drop-wise to the reaction mixture. Evolution of N_2 gas was observed during the reaction. After 30 minutes the resin was transferred to a sintered glass funnel and washed

with 5 mL of DMF followed by 5 mL of decreasing concentrations of DMF in water (75, 50 and 25%), followed by two washes of distilled water.



Scheme 2.10. Immobilization of galactose to Sepharose CL-6B using p-TsH.

All resin samples were re-suspended in water and freeze-dried for storage. To prepare resin samples for HR-MAS NMR, the resin was subjected to three cycles of freeze-drying from D_2O before use in NMR experiments.

2.4.4 HR-MAS ^{1}H NMR

HR-MAS ^{1}H NMR experiments were performed using an Agilent/Varian VNMR three-channel 600 MHz spectrometer equipped with a Varian gHX nano-NMR probe. Spectra from 40 µL samples were spun at 2 kHz and recorded at 27 °C. The experiments were recorded with suppression of water signal at 4.75 ppm and a spectral width of 12000 Hz. The spectra were obtained using 2048 transients with an acquisition time of 3 seconds.

The resin was prepared as described in **Sections 2.4.3.1** to **2.4.3.3**. The resin was freeze-dried three times from D_2O before using it. Resin (3 mg, dry powder) was re-suspended in 100 µL of D_2O and mixed to form an homogeneous suspension. 40 µL were transferred to a 4 mm sample tube using gel-loading micropipette tips to prevent the sample sticking to the inner wall of the tube and blocking the space for the plug.

2.4.5 Phenol Sulfuric Acid Assay

The procedure of Masuko, et al. was adapted by changing the order of addition of the phenol solution and the sulfuric acid. (*25*) Briefly, 5 mg of dry resin was weighed in a 10 mL round bottom flask, 5 mL of 2.5 M H_2SO_4 was added to each flask and refluxed at 110 °C for 6 hours. After hydrolysis, samples were allowed to cool for 30 minutes with the condenser still attached. The flask was mixed vigorously and 1.5 mL of the suspension was transferred to a 2 mL Eppendorf tube. Tubes were centrifuged at 10,000 x *g* for 10 minutes to precipitate the resin. Taking care to not disturb the pellet, 100 µL of the supernatant was diluted in 900 µL of distilled water, in triplicate. The samples were thoroughly mixed, and 200 µL of the solution was transferred to a fresh Eppendorf tube followed by the addition of 120 µL of 5% phenol solution in water (freshly prepared), and 600 µL of concentrated H_2SO_4 was added immediately. Samples were mixed, and then incubated for 5 minutes at 95 °C in a heating-block. Samples were allowed to cool 5 minutes, then 230 µL of each tube was transferred to clear-bottomed 96-well polystyrene microplate and the A_{490} was measured in triplicates using a Spectra Max M2 plate reader.

The absorbance data collected were converted into µmol Gal mg^{-1} of resin using a previously determined calibration curve (**Figure 2.8**).

$$y = 0.0268x + 0.0179 \quad \textbf{(2.2)}$$

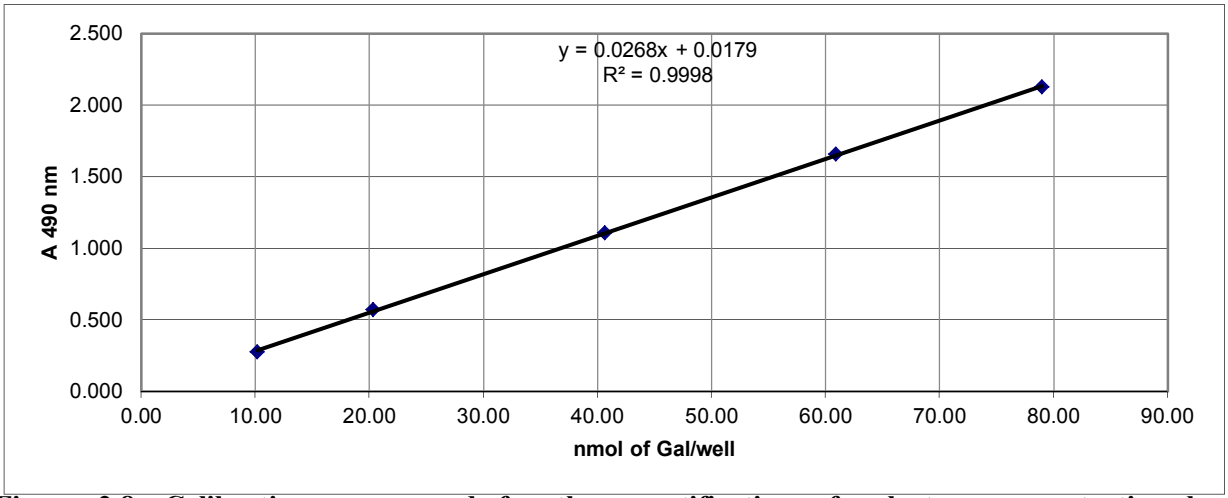


Figure 2.8. Calibration curve used for the quantification of galactose concentration by absorbance in the PSA.

2.4.6 FITC-Jacalin and FITC-WGA binding experiments

A fluorescence based assay was designed to quantify the biological activity of the immobilized carbohydrate epitopes. Briefly, 1 mg of sample as a dry powder was weighed into 0.5 mL Eppendorf tubes. Binding buffer was added to each tube (50 µL, 10 mM HEPES, 0.15 M NaCl, pH = 7.5, 0.1 mM $CaCl_2$). The samples were mixed for 30 minutes at room temperature, to allow the resin to swell and equilibrate. A solution of FITC-labeled Jacalin or FITC-labeled WGA (Vector Labs, Burlington, Ontario; 200 µL of a 75 µg/mL solution) was added to transfer 15 µg of total protein per tube. Samples were prepared in triplicate. The samples were incubated at room temperature for 3 hours and left mixing protected from light. After the incubation period, the samples were centrifuged at 10,000 x g for 10 minutes to precipitate the resin. The supernatant (40 µL) was transferred to a 384-well black-bottomed microplate. Fluorescence was measured with λ_{ex} = 490 nm, λ_{em} = 518 and a λ_{cutoff} = 515 nm using a Spectra Max M2 plate reader.

The fluorescence data were converted to µg of Jacalin or WGA bound per mg of resin using a calibration curve determined with a solution of FITC-Jacalin (**equation. 2.3, Figure 2.9**) or FITC-WGA (**equation 2.4**, **Figure 2.10**).

$$y = 81.073x - 225.84 \quad \textbf{(2.3)}$$

$$y = 286.17x + 3291.61 \quad \textbf{(2.4)}$$

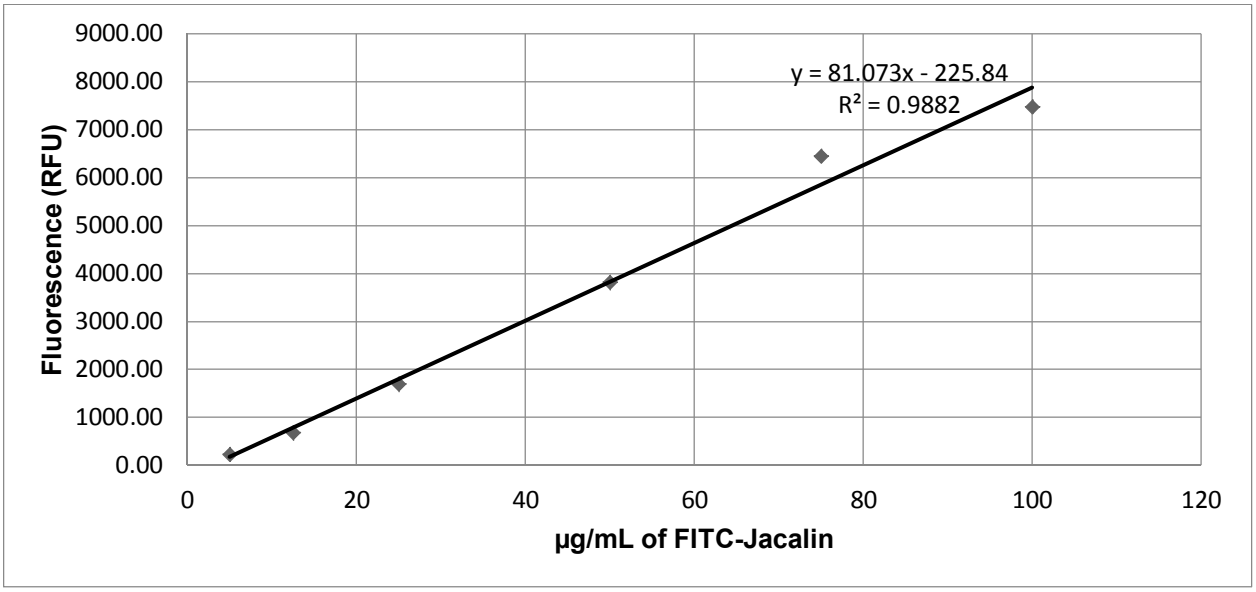


Figure 2.9. Calibration curve used for the quantification of FITC-Jacalin binding to resin samples.

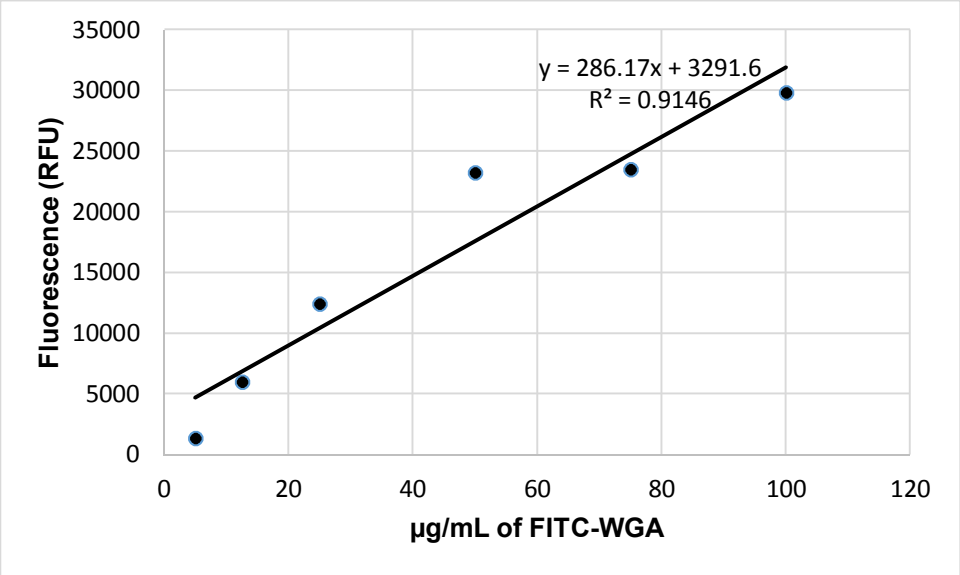


Figure 2.10. Calibration curve used for the quantification of FITC-WGA binding to resin samples.

2.5 References

1. Cuatrecasas, P. (1970) Protein Purification by Affinity Chromatography: derivatizations of agarose and polyacrylamide beads, *Journal of Biological Chemistry 245*, 3059-3065.
2. Urh, M., Simpson, D., and Zhao, K. (2009) Chapter 26 Affinity Chromatography: General Methods, In *Methods in Enzymology* (Richard, R. B., and Murray, P. D., Eds.), pp 417-438, Academic Press.
3. Wilchek, M., and Miron, T. (1999) Thirty years of affinity chromatography, *Reactive and Functional Polymers 41*, 263-268.
4. Wilchek, M., Miron, T., and Kohn, J. (1984) [1] Affinity chromatography, In *Methods in Enzymology* (William, B. J., Ed.), pp 3-55, Academic Press.
5. Ernst-Cabrera, K., and Wilchek, M. (1988) Immunoaffinity chromatography and its application for the purifiction of pharmaceutically active proteins, *Medical Science Research 16*, 305-310.
6. Wilchek, M., and Bayer, E. A. (1990) [2] Introduction to avidin-biotin technology, In *Methods in Enzymology* (Meir, W., and Edward, A. B., Eds.), pp 5-13, Academic Press.
7. Ford, C. F., Suominen, I., and Glatz, C. E. (1991) Fusion tails for the recovery and purification of recombinant proteins, *Protein Expression and Purification 2*, 95-107.
8. Nilsson, J., Ståhl, S., Lundeberg, J., Uhlén, M., and Nygren, P.-å. (1997) Affinity Fusion Strategies for Detection, Purification, and Immobilization of Recombinant Proteins, *Protein Expression and Purification 11*, 1-16.
9. Fornstedt, N., and Porath, J. (1975) Characterization studies on a new lectin found in seeds of Vicia ervilia, *FEBS Letters 57*, 187-191.

10. Nascimento, K. S., Cunha, A. I., Nascimento, K. S., Cavada, B. S., Azevedo, A. M., and Aires-Barros, M. R. (2012) An overview of lectins purification strategies, *Journal of Molecular Recognition 25*, 527-541.

11. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968) Selective enzyme purification by affinity chromatography, *Proceedings of the National Academy of Sciences of the United States of America 61*, 636-643.

12. Porath, J., Janson, J.-C., and Låås, T. (1971) Agar derivatives for chromatography, electrophoresis and gel-bound enzymes: I. Desulphated and reduced cross-linked agar and agarose in spherical bead form, *Journal of Chromatography A 60*, 167-177.

13. Porath, J., Axen, R., and Ernback, S. (1967) Chemical Coupling of Proteins to Agarose, *Nature 215*, 1491-1492.

14. Hermanson, G. T. (2008) Chapter 14 - Microparticles and Nanoparticles, In *Bioconjugate Techniques (Second Edition)* (Hermanson, G. T., Ed.), pp 582-626, Academic Press, New York.

15. MacBeath, G., and Schreiber, S. L. (2000) Printing Proteins as Microarrays for High-Throughput Function Determination, *Science 289*, 1760-1763.

16. Wong, L. S., Khan, F., and Micklefield, J. (2009) Selective Covalent Protein Immobilization: Strategies and Applications, *Chemical Reviews 109*, 4025-4053.

17. Borch, R. F., Bernstein, M. D., and Durst, H. D. (1971) Cyanohydridoborate anion as a selective reducing agent, *Journal of the American Chemical Society 93*, 2897-2904.

18. Gray, G. R. (1978) [12] Antibodies to carbohydrates: Preparation of antigens by coupling carbohydrates to proteins by reductive amination with cyanoborohydride, In *Methods in Enzymology* (Victor, G., Ed.), pp 155-160, Academic Press.

19. Gray, G. R. (1974) The direct coupling of oligosaccharides to proteins and derivatized gels, *Archives of Biochemistry and Biophysics 163*, 426-428.

20. Hermanson, G. T. (2013) Chapter 15 - Immobilization of Ligands on Chromatography Supports, In *Bioconjugate Techniques (Third edition)* (Hermanson, G. T., Ed.), pp 589-740, Academic Press, Boston.

21. Lopez-Jaramillo, F. J., Ortega-Muñoz, M., Megia-Fernandez, A., Hernandez-Mateo, F., and Santoyo-Gonzalez, F. (2012) Vinyl Sulfone Functionalization: A Feasible Approach for the Study of the Lectin–Carbohydrate Interactions, *Bioconjugate Chemistry 23*, 846-855.

22. Zarling, D. A., Watson, A., and Bach, F. H. (1980) Mapping of lymphocyte surface polypeptide antigens by chemical cross-linking with BSOCOES, *The Journal of Immunology 124*, 913-920.

23. Hermanson, G. T. (2013) Chapter 9 - Cross-Bridges and Cleavable Reagent Systems, In *Bioconjugate Techniques (Third edition)* (Hermanson, G. T., Ed.), pp 387-394, Academic Press, Boston.

24. Cheng, F., Shang, J., and Ratner, D. M. (2010) A Versatile Method for Functionalizing Surfaces with Bioactive Glycans, *Bioconjugate Chemistry 22*, 50-57.

25. Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., and Lee, Y. C. (2005) Carbohydrate analysis by a phenol–sulfuric acid method in microplate format, *Analytical Biochemistry 339*, 69-72.

26. Liang, K., and Chen, Y. (2012) Elegant Chemistry to Directly Anchor Intact Saccharides on Solid Surfaces Used for the Fabrication of Bioactivity-Conserved Saccharide Microarrays, *Bioconjugate Chemistry 23*, 1300-1308.

27. Gudmundsdottir, A. V., and Nitz, M. (2008) Protecting Group Free Glycosidations Using p-Toluenesulfonohydrazide Donors, *Organic Letters 10*, 3461-3463.

28. Edgar, L. J. G., Dasgupta, S., and Nitz, M. (2012) Protecting-Group-Free Synthesis of Glycosyl 1–Phosphates, *Organic Letters 14*, 4226-4229.

29. Dreywood, R. (1946) Qualitative Test for Carbohydrate Material, *Industrial & Engineering Chemistry Analytical Edition 18*, 499-499.

30. Salak Asghari, F., and Yoshida, H. (2006) Acid-Catalyzed Production of 5-Hydroxymethyl Furfural from d-Fructose in Subcritical Water, *Industrial & Engineering Chemistry Research 45*, 2163-2173.

31. Finlay, T. H., Troll, V., Levy, M., Johnson, A. J., and Hodgins, L. T. (1978) New methods for the preparation of biospecific adsorbents and immobilized enzymes utilizing trichloro-s-triazine, *Analytical Biochemistry 87*, 77-90.

32. Kumar, A., Srivastava, K., Raja Kumar, S., Siddiqi, M. I., Puri, S. K., Sexana, J. K., and Chauhan, P. M. S. (2011) 4-Anilinoquinoline triazines: A novel class of hybrid antimalarial agents, *European Journal of Medicinal Chemistry 46*, 676-690.

33. Belyakov, P. A., Shastin, A. V., and Strelenko, Y. A. (2001) NMR studies of the dynamic behavior of 2-anilino-6-chloro-4-methoxy-1,3,5-triazine in solution, *Russian Chemical Bulletin 50*, 2473-2474.

34. Alia, A., Ganapathy, S., and de Groot, H. M. (2009) Magic angle spinning (MAS) NMR: a new tool to study the spatial and electronic structure of photosynthetic complexes, *Photosynth Res 102*, 415-425.

35. Laws, D. D., Bitter, H.-M. L., and Jerschow, A. (2002) Solid-State NMR Spectroscopic Methods in Chemistry, *Angewandte Chemie International Edition 41*, 3096-3129.

36. García-Álvarez, I., Corrales, G., Doncel-Pérez, E., Muñoz, A., Nieto-Sampedro, M., and Fernández-Mayoralas, A. (2006) Design and Synthesis of Glycoside Inhibitors of Glioma and Melanoma Growth, *Journal of Medicinal Chemistry 50*, 364-373.

37. Albalasmeh, A. A., Berhe, A. A., and Ghezzehei, T. A. (2013) A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry, *Carbohydrate Polymers 97*, 253-261.

38. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Colorimetric method for determination of sugars and related substances, *Analytical Chemistry 28*, 350-356.

39. Englis, D., and Becker, H. (1943) Sugar Analysis by Alkaline Ferricyanide Method - Determination of Ferrocyanide by Iodometric and Other Procedures, *Industrial & Engineering Chemistry Analytical Edition 15*, 262-264.

40. Rao, P., and Pattabiraman, T. N. (1989) Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses, *Analytical Biochemistry 181*, 18-22.

41. Mecozzi, M. (2005) Estimation of total carbohydrate amount in environmental samples by the phenol–sulphuric acid method assisted by multivariate calibration, *Chemometrics and Intelligent Laboratory Systems 79*, 84-90.

42. Panagiotopoulos, C., and Sempere, R. (2005) Analytical methods for the determination of sugars in marine samples: A historial perspective and future direction, *Limnology and Oceanography: Methods 3*, 419-454.

43. Sharon, N., and Lis, H. (2003) *Lectins*, second ed., Kluwer Academic Publishers.

44. Loka, R. S., and Cairo, C. W. (2010) Immobilization of carbohydrate epitopes for surface plasmon resonance using the Staudinger ligation, *Carbohydrate Research 345*, 2641-2647.

45. Allen, A. K., Neuberger, A., and Sharon, N. (1973) The purification, composition and specificity of wheat-germ agglutinin, *Biochem. J. 131*, 155-162.

46. Nagata, Y., and Burger, M. M. (1974) Wheat Germ Agglutinin: molecular characteristics and specificity for sugar binding, *Journal of Biological Chemistry 249*, 3116-3122.

47. Mahanta, S. K., Sastry, M. V., and Surolia, A. (1990) Topography of the combining region of a Thomsen-Friedenreich-antigen-specific lectin jacalin (Artocarpus integrifolia agglutinin). A thermodynamic and circular-dichroism spectroscopic study, *Biochemical Journal 265*, 831-840.

48. Bourne, Y., Astoul, C. H., Zamboni, V., Peumans, W. J., Menu-Bouaouiche, L., Van Damme, E. J. M., Barre, A., and Rougé, P. (2002) Structural basis for the unusual carbohydrate-binding specificity of jacalin towards galactose and mannose, *Biochemical Journal 364*, 173-180.

Chapter 3

Identification of Human Milk Oligosaccharide-binding receptors from human cells using affinity chromatography.

1, 2, 3

1 Cell culture and lysate preparations were made with the help of Chunxia Zou, University of Alberta. The automated affinity chromatography protocol was performed by Jobette T. Santos, University of Alberta. ESI-MS was performed by Dr. Rambod Daneshfar, University of Alberta. Other MSexperiments were performed by the Mass Spectrometry laboratory staff, Department of Chemistry, University of Alberta

[2] CTB was a generous gift from Dr. John Klassen, University of Alberta.

[3] Recombinant galectin-3 and galectin-9 were provided by Ruixiang (Blake) Zheng, University of Alberta.

3.1 Introduction

3.1.1 Galectins

Lectins are carbohydrate binding proteins that have the capacity of recognizing carbohydrates; each lectin has its own specificity profile. Lectins play different roles that range from the mediation of cell adhesion, to facilitating cell-cell interactions, to the recognition of pathogens. (*1, 2*)

There are several types of lectins, and they are classified by their origin. Animal lectins are subdivided into several families, one of these is the galectins. Galectins are a family of proteins with the ability to recognize β-galactosides. (*3*) All galectins have conserved carbohydrate recognition domains (CRDs) composed of about 135 amino-acids. (*1*) Currently, 15 mammalian galectins have been identified, and they are further subdivided in two groups: those which contain one CRD and those containing two CRDs. (*4*)

The carbohydrate binding sites of galectins can recognize galactose and other saccharides. Each galectin may have different specificities for different oligosaccharides since they have different abilities to accommodate certain saccharides attached to galactose. (*5*) Most galectins are bivalent or multivalent with regard to their carbohydrate-binding activities: some one-CRD galectins exist as dimers; two-CRD galectins have two carbohydrate-binding sites; and galectin-3 may form oligomers. (*1, 6*) These properties allow galectins to form ordered arrays of complexes when they bind to multivalent glycoconjugates. (*7*) **Table 3.1** shows a schematic representation of the different types of galectins.

Type	Structure	Galectin
One CRD		1, 2, 5, 7, 10, 11, 13, 14, 15
Chimera		3
Two CRDs		4, 6, 8, 9, 12

Table 3.1 Different galectin structures. Galectins are divided into three groups: those containing one CRD (Galectin 1, 2, 5, 7, 10, 11, 13, 14, 15); those with two CRDs in tandem, connected by a linker of up to 70 amino-acids (Galectin 4, 6, 8, 9, 12); and Galectin 3 which contains an unusual tandem repeat proline- and glycine-rich segment fused onto the CRD. (*8*)

Galectins play very important biological functions in inflammation (cancer can be considered an extreme case of inflammation), (*9*) angiogenesis (blood supply, to both healthy and cancerous cells), immune response (there is evidence they turn off the immune recognition system), (*9*) and cancer cell migration (leading to metastasis). (*10*) Hence, it is not a surprise that galectins are an active area of research.

3.1.1.1 Galectins in tumor angiogenesis

As any other cell or tissue in the body, tumors require oxygen, nutrients, and other biologically important factors to grow. They receive all these supplies through the blood. Angiogenesis is the process of growing new capillaries from pre-existing blood vessels and involves the activation of endothelial cells, the disruption of vascular basement membranes,

and the proliferation of endothelial cells and their migration. In this complex process, galectin-1, -2, -3, -4, and -8 play important roles in mediating cell-cell and cell-matrix interactions. (*11*)

There is evidence that galectin-3 is a novel pro-angiogenic molecule and that it contributes to the plasma membrane retention and pro-angiogenic function of VEGF receptor 2. (*12, 13*) It has also been noted that galectin-3 is an important regulator of a broad range of cancer cell activities and may play important roles in cancer cell growth, transformation, apoptosis, angiogenesis, adhesion, invasion, and metastasis. The influence that galectin-3 has on cancer cells comes from its ubiquitous localization within the cell where it interacts with a range of different binding partners. (*14*)

Other galectins, such as -1, -3, -4, and -8, seem to play important roles in angiogenesis as well. These four galectins are upregulated in colorectal cancer development and metastasis. The upregulation of these galectins has been correlated with cancer cell growth, apoptosis, and angiogenesis. (*15*) A different study showed that galectin-1 is a tumor promoting protein produced by cancer cells. (*16*) In this last study the authors claim to have one of the first cases demonstrating that low-molecular weight galectin-1 blockers are potential immune system modulating anti-cancer agents.

3.1.1.2 Galectins in inflammation

Inflammation is a complex process involving vascular dilation with increased permeability and blood flow, accumulation of plasma proteins, leukocytes, and fluids. The process can be triggered by pathogens, trauma, or autoimmune activities. (*17*) Specifically for angiogenesis, inflammation can be considered as a side effect or pathology where angiogenesis is in the wrong place. When this is occurring, inflammatory responses such as the production

of cytokines (signaling molecules that will activate T cells) and chemokines are activated to potentially supress tumor growth. (*18*) Galectins are expressed by many immune and inflammatory cells and regulate some of the functions of both these types of cells. As a consequence, it is logical to believe that galectins can affect the immune and inflammatory responses of the host against the tumor. To make this scenario more complex, tumors can also release galectins that can have an impact on the immune and inflammatory responses of the host. (*8*)

Some galectins can amplify the inflammatory response, whereas others activate homeostatic signals that shut off the immune response. (*8*) It seems that the specific effect of a galectin depends on the targeted cell, its microenvironment, and the inflammatory stimulus itself. (*11*) Recent data also suggest that some galectins released by the tumor might help the tumor to evade immune surveillance. (*1*)

Galectin-3 has been identified as a galectin that promotes inflammatory responses. In a study with galectin-3-null mice, the authors observed that inflammation was less severe, in comparison to wild-type mice, when thioglycollate-elicited inflammation was induced in the peritoneal cavity. (*19*) Other studies have concluded that galectin-3 along with the presence of the allele LGALS3 +292C were responsible for a higher propensity to develop the inflammatory disease rheumatoid arthritis. (*20*) Furthermore, Forsman et al. showed that galectin-3 is present in the inflamed synovium in patients with rheumatoid arthritis, and also plays a pathogenic role in the development and progression of arthritis. They observed that galectin-3-null mice presented almost no inflammation nor bone erosion as compared with the wild type mice; the levels of pro-inflammatory cytokines were also decreased. (*21*)

3.1.1.3 Galectins in the immune response

The immune system works with several tools to defend the body from threats (**Figure 3.1**): one of these are macrophages, that engulf pathogens and other debris. Activated T cells multiply, attack, and kill threats and/or engage other type of immune cells, including B cells. B cells are responsible for producing pathogen blocking antibodies. Dendritic cells also serve to activate T cells, (*11*) and are able to take up antigen and display it for recognition by lymphocytes. (*22*)

T cells are a large family of cells with specialized functions, including: T helper cells, cytotoxic T cells, memory T cells, regulatory T cells, and natural killer T cells. When a pathogen is detected, T cells respond by releasing various cytokines, and cytolytic/cell killing molecules to destroy the threat. (*11*)

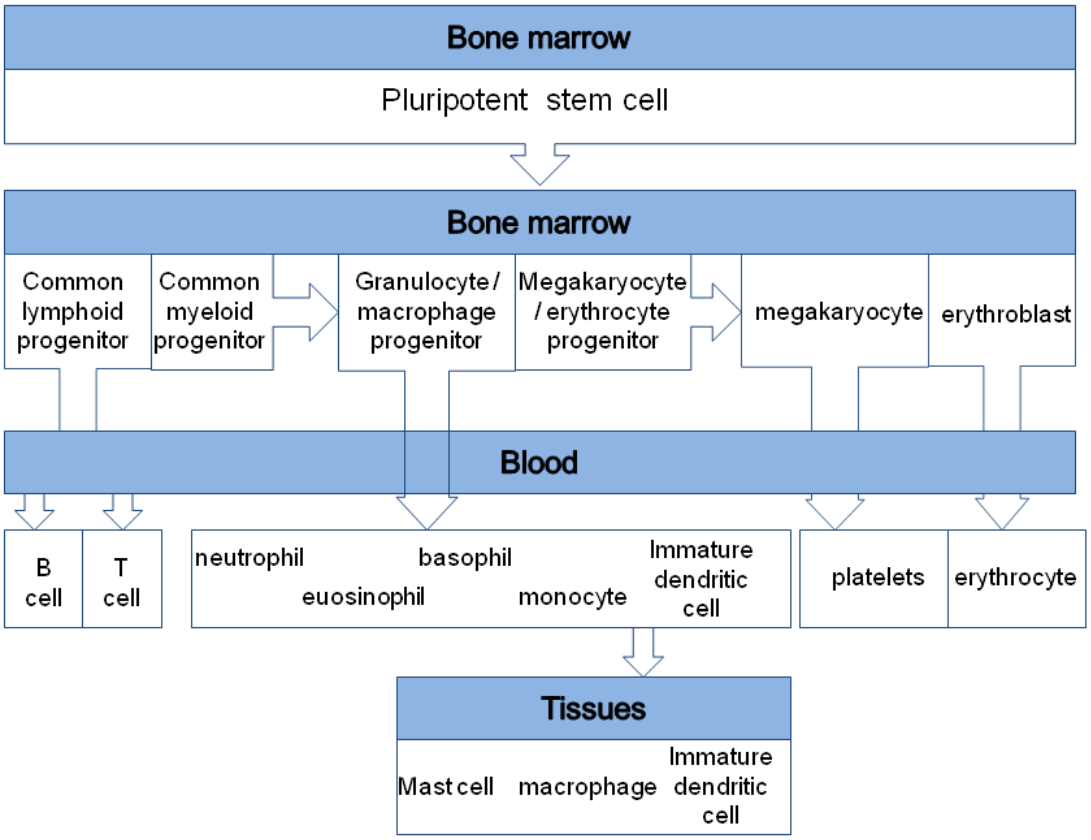


Figure 3.1 Cellular components of the immune system. Pluripotent cells divide to produce two types of specialized stem cells: a common progenitor for T and B cells that will form part of the adaptive immune system, and a myeloid progenitor that will generate different types of leukocytes (neutrophils, eusinophils, basophils, monocytes, and dendritic cells), erythrocytes, and megakaryocytes (responsible for the formation of platelets). Adapted from (*22*)

Several studies have demonstrated that galectins can disable T cells by inducing apoptosis. (*23, 24*) Galectin-1 is the primary galectin responsible for this effect, likely due to binding to T cell surface glycoproteins, such as CD7, CD43, and CD45. This may be an explanation of why several types of tumors, including colorectal, breast, melanomas, bladder,

ovarian, thyroid, gynecological, and other tumors overexpress various galectins. Similar activities have been observed with galectin-3. (*25*)

Galectin-3 may initiate signal transduction by forming multivalent complexes with glycans present on the T-cell. Complex formation should restrict the lateral mobility of the receptor complex which is crucial for the activation of the T-cells. Furthermore, galectin-3 might also reduce the immune response under certain circumstances by downregulating IL5 production. (*26*) Galectin-2 and galectin-9 have also been identified as causing T-cell apoptosis; however, less information is available about the specific pathways they follow to exert these effects. (*27, 28*)

Galectin-1, -3, and -9 are emerging as pertinent players in the modulation of acute and chronic inflammatory diseases, autoimmunity, and cancer due to their immunoregulatory bioactivities. (*9, 29, 30*) Thus, they are being recognized as molecular targets for drug discovery. A better understanding of the role of galectins in cancer and other diseases, and the interactions involved at cellular level should provide the information required for the design of galectin inhibitors or galectin therapy as required. The next section of this chapter will briefly discuss the potential use of HMO for this purpose. (*11, 31-33*)

3.1.2 Interactions of galectins with HMO

As discussed in Chapter 1, HMO play a key role in the development of the newborn infant. Among other functions, they contribute to the development of the immune system, and the intestinal flora. (*34*) There is evidence that HMO interact directly with immune cells, most likely via carbohydrate receptors. (*35, 36*) There are some examples available where HMO modulate immune response potentially via interactions with galectins. In one of these studies,

HMO interfered with the Th1/Th2 skewing in cord blood-derived mononuclear cells and to impact the Th2-type immune response of allergen-specific T cells from peanut allergic individuals. The exact mechanisms behind these effects are not fully understood. (*36*)

The affinity of galectins to HMO has been measured in several studies (**Table 3.2**). The relative affinities of galectins towards HMO, suggest that HMO are an important pool of galectin binders; however the exact physiological significance of these results remains to be explored. A deeper understanding of the molecular interactions of HMO with different receptors (including galectins), could facilitate the design of drugs to modulate biological processes like inflammation, cancer, angiogenesis, and immune response. (*11*) Considering the vast amount of carbohydrate binding receptors, a method for the high-throughput screening of these molecules, that also requires a low amount of the carbohydrate epitope would be a valuable tool for glycobiologists.

	Galectin					
	-1[a]	**-3**[b]	**-3**[c]	**-4**[c]	**-7**[d]	**-8**[e]
Lactose	1	1	1	1	1	1
N-acetyllactosamine	7.8	11.3	4.1	1	1.1	0.3
Lactulose	1.1	5.3	-	-	-	-
Lactitol	-	-	<0.01	<1	-	<0.1
GOS mucotriose	-	-	-	-	-	-
2'-Fucosyl-lactose	0.3	1.8	2.8	8.3	-	1.3
3-Fucosyl-lactose	<0.04	<0.01	<0.1	<1	-	<0.1
Lactose-*N*-tetraose	1.9	9.4	6.7	5	1.3	1.2
Lactose-*N*-neotetraose	-	-	10	4.3	1.7	4.8
Lacto-N-fucopentaose I	1.9	10	12.7	8.3	-	2.5
Lacto-N-fucopentaose II	-	-	10.8	4.3	-	6.5
Sialyl-α(2,3)lactose	0.6	1.2	1.8	<1	0.3	54
Sialyl-α(2,6)lactose	<0.03	<0.05	<0.01	<1	<0.1	<0.1

[a](*37*) [b](*38*) [c](*39*) [d](*40*) [e](*41*)

Table 3.2 Relative binding affinities of lactose-derived saccharides for Galectins. All the values were normalized within a column to lactose by the authors of each reference cited. The values have no units.

In this chapter, we describe an approach to identify HMO binding receptors, or "multiple receptors for one glycan." We used two different methods to identify HMO receptors. The first method is an immuno-precipitation assay where an HMO functionalized

solid support was incubated with cell lysate, washed, the resin heated in SDS-PAGE loading buffer, followed by SDS-PAGE, and the bands excised and analyzed by mass spectrometry to identify potential binders. The second approach involved using affinity chromatography, pooling the elution fractions, digesting the samples for mass spectrometry analysis, followed by analysis for the identification of binders. This latter approach is referred as "the shotgun approach."

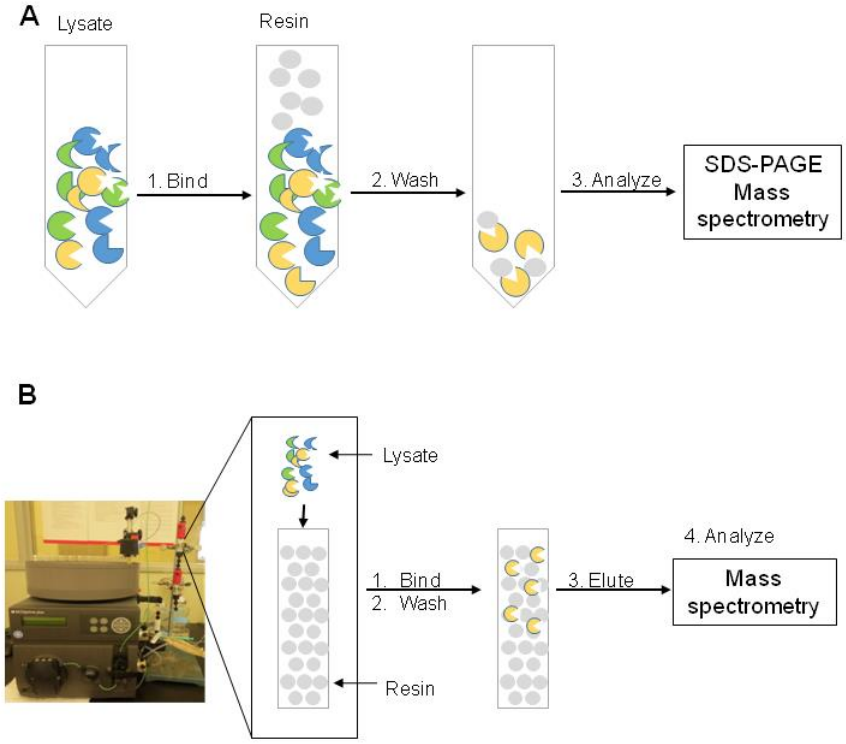


Figure 3.2 Strategies for identifying HMO receptors with solid support. HMO receptors were identified using immobilized oligosaccharides and the binding tested using either **A.** Immuno-precipitation, which involves the generation of the lysate, addition to the modified solid support, incubation for 3 hours, washing and centrifugation to precipitate the solid support followed by SDS-PAGE and digestion. **B.** The shotgun method uses an automated FPLC system. Lysate was passed

through the column, washed and eluted with a high salt and lactose buffer, the fractions concentrated and digested. Both methods were then analyzed by LC/MS-MS.

3.2 Results & Discussion

The first step for testing our approach was to immobilize HMO to a solid support. As we described in **Chapter 2**, DVS immobilization chemistry can be used to generate affinity resins for carbohydrates. Using this protocol, we proceeded to immobilize five HMO sugars (shown in **Figure 3.3**) purchased commercially. We used DVS for the immobilization of the oligosaccharides since the project presented in **Chapter 2** was run in parallel with these experiments, though p-TsH immobilization may provide improved results, the protocol was not yet developed.

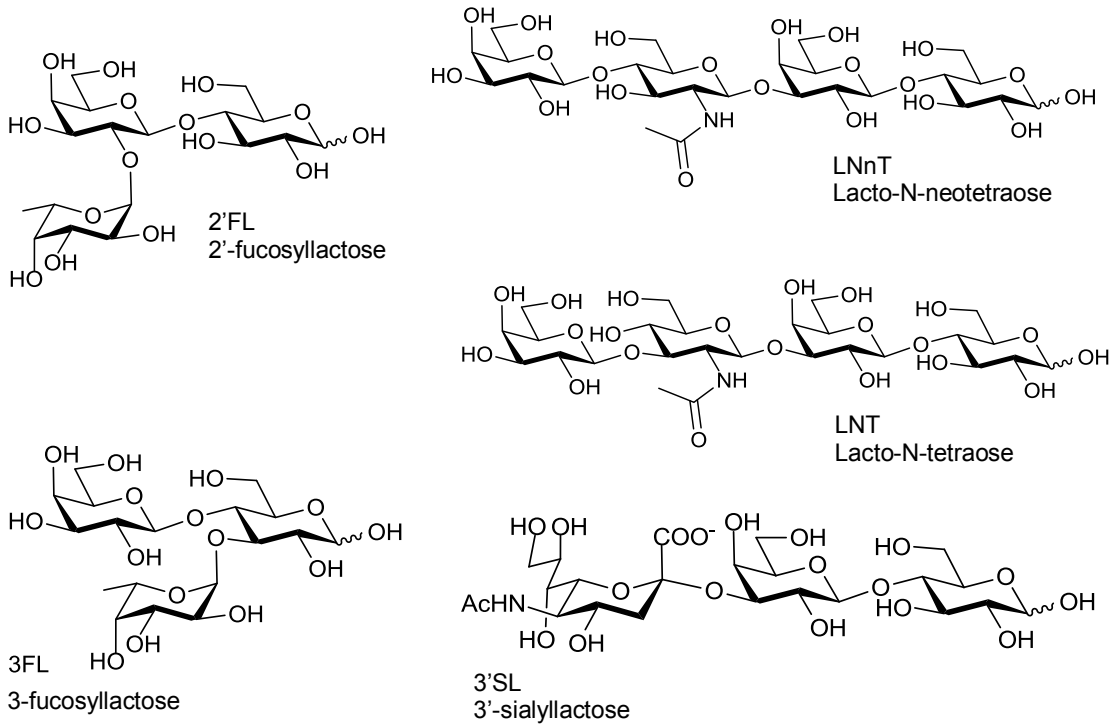


Figure 3.3 HMO immobilized to solid support for binding studies.

3.2.1 Immuno-precipitation assay

To test the immuno-precipitation approach, we decided to run a positive control. We used a pure protein that was expected to bind to one of the available HMO. We selected 2'FL and *Vibrio cholera* toxin subunit B (CTB). We chose this system since our collaborators in the Klassen group have measured the binding constant for this system, and it is relatively strong. (El-Hawiet, Kitova and Klassen, manuscript in preparation) The protein solution (1200 μL, concentration 0.5 μg mL^{-1}) was added to the solid support (20 mg as a moist cake) and incubated for three hours at room temperature. The solid support was washed with PBS (3 x 400 μL), after spinning down the resin (2000 x g for 3 min) we heated it in SDS loading buffer and loaded the solution (10 μL) in a 20% acrylamide gel. The gel was run at 150 V for approximately 1 h, and stained using Coomasie Blue stain. In order to assess non-specific binding to the solid support, we used Sepharose-CL6B as a blank and we did the same treatment as above. **Figure 3.4** shows the SDS-PAGE gel of the CTB-2'FL system. We can observe that the CTB-2'FL lane has two visible bands (~11 kDa and at ~55 kDa) whereas the CTB-Sepharose system has no evident bands.

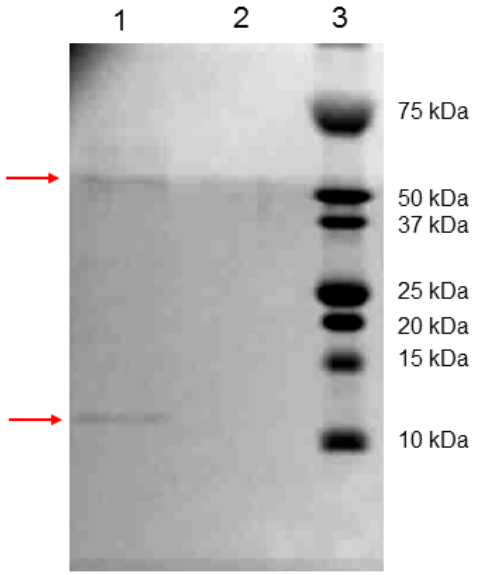


Figure 3.4 SDS-PAGE of CTB binding to Sepharose with 2'FL. Lane 1 contains Sepharose with 2'FL, and **Lane 2** shows the binding with blank Sepharose. The arrows indicate the two bands identified as CTB. The band at ~11 kDa corresponds to the CTB monomer, the band at ~55 kDa corresponds to a pentamer of CTB. **Lane** 3 shows molecular weight markers that were used to estimate the molecular weights, with protein standards. (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa) The bands corresponding to CTB monomer and pentamer were visualized using Coomasie brillian blue.

This experiment supported the idea that the HMO immobilized to Sepharose via a DVS linker could bind to HMO receptors. Moreover, the gel also shows that the Sepharose CL-6B has little non-specific interactions with the CTB since no band was observed in that lane. This data may suggest that CTB can bind to the immobilized HMO either as a monomer or as a pentamer since the two bands (~11 kDa and ~55 kDa) were observed. (*42, 43*) Having this precedent, we decided to test this approach using lysate from cells and see if the protocol was robust enough to selectively bind to HMO binding proteins in a complex mixture.

For testing the approach using a cell lysate we chose two cell lines: HeLa, (an adenocarcinoma cell line derived from cervical tissue (*44*)) and Jurkat cells (an immortilized line of T lymphocyte cells. (*45*)) These cell lines are both cancer cell lines which are well known for over-expressing galectins. It is known that Jurkat cells overexpress galectin-3, (*46*) and galectin-9. (*47*) On the other hand, some studies have revealed the presence of galectin-1 widely distributed in HeLa cells. (*48*) Based on those precedents, we decided to test these systems as they contain at least these known HMO binders in a pool of proteins. We used the immuno precipitation protocol described above.

The SDS-PAGE gel for this experiment is shown in **Figure 3.5**. Again, both cell lysates were incubated with DVS-immobilized 2'FL-Sepharose and blank Sepharose CL-6B in order to identify non-specific binders. In the case of the Jurkat cells interacting with 2'FL, we observed an intense band at ~30 kDa that it is absent in the negative control. In the case of HeLa cells there was one intense band present at ~35 kDa that was not present in the negative control, ruling out the possibility of being due to non-specific interactions.

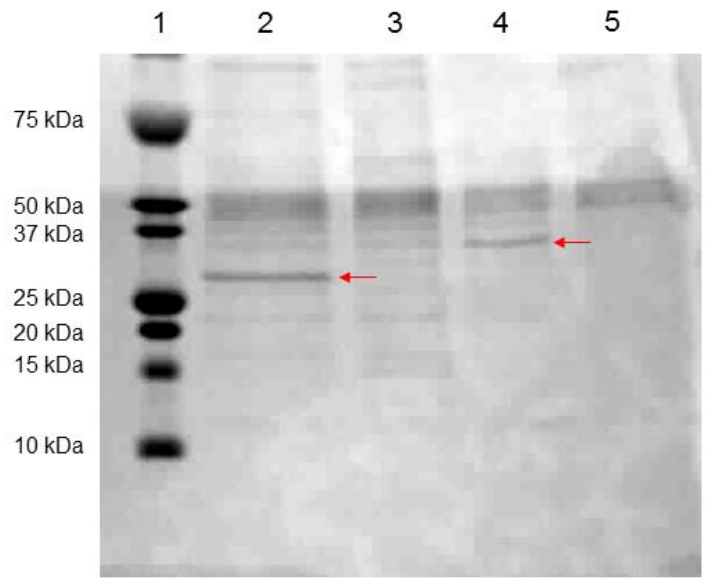


Figure 3.5 SDS-PAGE gel of Jurkat and HeLa cell lysates incubated with immobilized 2'-fucosyllactose. An SDS-PAGE gel was loaded with the eluent form the cell lysate on a column of 2'FL-Sepharose or blank Sepharose. **Lane 1.**Protein standards **Lane 2.** HeLa cell lysate with 2'FL-Sepharose. **Lane 3.** HeLa cell lysate with blank Sepharose **Lane 4.** Jurkart cell lysate with 2'FL-Sepharose. **Lane 4**. Jurkat cell lysate with blank Sepharose. In lane two we observed one strong band (~30 kDa) that was absent in lane three (negative control). That band was excised, analysed by MS and identified as galectin 1. In lane four we observed one strong band (~35 kDa) that was absent in lane four (negative control). That band was excised, analysed by MS and identified as galectin 3. The gel was a 20% acrylamide gel and all the wells were loaded with 10 μL of the protein solution. To estimate the molecular weight of the bands, protein standards (**Lane 1**) were used (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa). The bands in lanes two to five were visualized using Coomasie brilliant blue.

To identify these bands, they were excised and analyzed by MS. We found that the bands included galectin-1 and galectin-3 in HeLa and Jurkat cells, respectively. The identification of galectins as HMO binders was expected since several papers have reported

that galectins are usually overexpressed in cancer cells. (*1, 49*) Moreover, galectin-1 has been reported to be expressed in HeLa cells; (*48*) while galectin-3 is known to be overexpressed in Jurkat cells. (*46*) Moreover, **Table 3.2** shows that 2'FL has been reported to have a weak relative binding affinity (0.3 normalized to lactose) for galectin-1, and a relative binding affinity of approximately 2.3 (normalized to lactose) for galectin-3 The amino acid sequence coverage found for these two protein samples are shown in **Figure 3.6**.

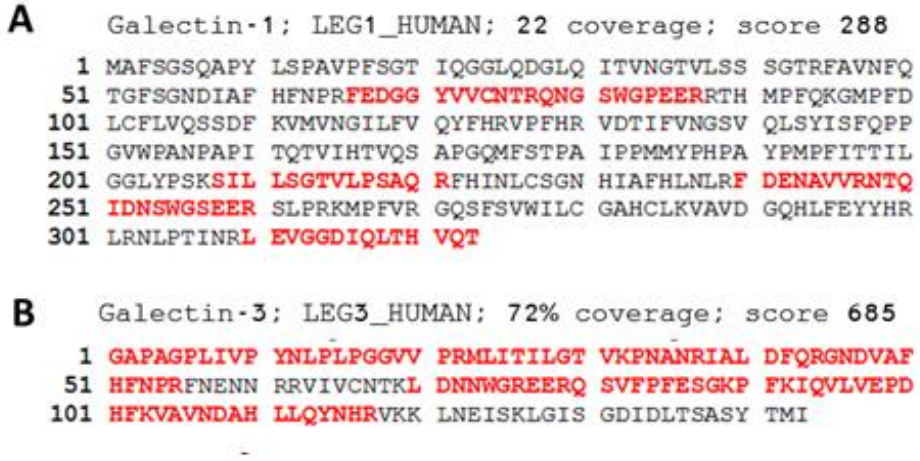


Figure 3.6 Amino acid sequence coverage of the proteins identified using 2'FL-Sepharose with HeLa and Jurkat cell lysate. The protein sequence coverage were obtained from the trypsin digestion and subsequent LC-MS/MS analysis of the bands excised from the SDS-PAGE gel (**Figure 3.5**). The MS results were compared against the NCBInr database using Mascot server. The HeLa lysate identified **A.** Galectin-1 (22% coverage; score 288); and the Jurkat lysate identified **B.** Galectin-3 (72% coverage; score 685).

These experiments again confirmed that our solid support had the ability to selectively bind HMO receptors in a large pool of binders. We next decided to test the method by packing the modified solid support in a column and running it as an affinity chromatography

experiment. For testing this method we prepared a column with lactose immobilized to Sepharose using DVS. We tested the binding of the column using a recombinant protein in a crude lysate mixture. We selected galectin-9 (also known as Ecalectin) for the optimization of our protocol since several studies have reported the interaction between galectin-9 and lactose. (*47, 50*)

The affinity chromatography method involved packing the column, equilibrating it with buffer, passing the solution containing the protein, eluting the binders, and running the SDS-PAGE gel. For the elution we decided to use a competitive elution with a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM $CaCl_2$, 50 mM 2-mercaptoethanol, and 100 mM lactose with a final pH of 7.5. We expected that lactose would be able to compete with the immobilized HMO for the binding sites of galectin-9. However, the SDS-PAGE gel (**Figure 3.7**) showed that galectin-9 was not eluted from the column (lane 4), and also showed that the galectin was still bound to the resin (lane 3). The results were encouraging, since the protein was binding to the solid support, but we needed to optimize the elution conditions for the protein to be recovered.

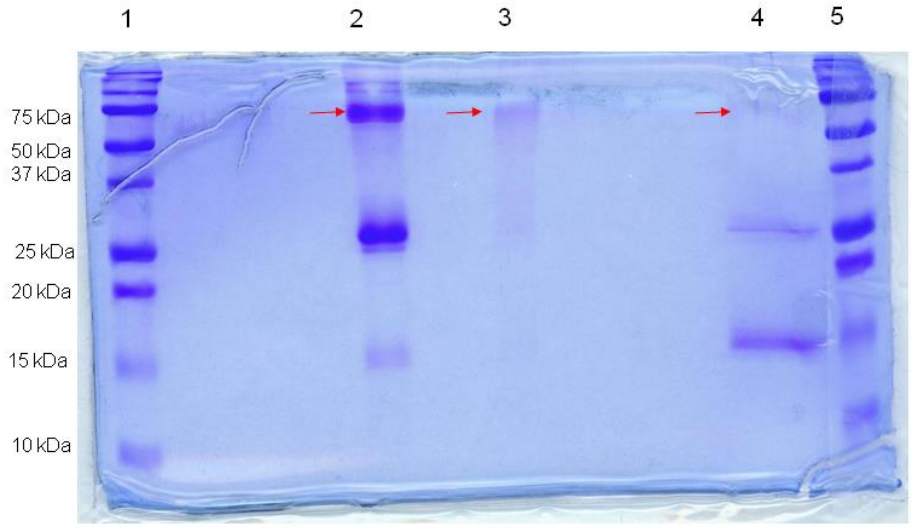


Figure 3.7 SDS-PAGE Gel of samples from affinity chromatography using a lactose-immobilized column and raw lysate mixture of recombinant galectin 9. An SDS-PAGE was loaded with samples of the different steps of the affinity chromatography method to track the binding of the recombinant-galectin-9. **Lane 1.** Protein standards. A raw lysate of **Lane 2.** Recombinant-galectin-9, 6.5 µg of protein (5 µL of a 1.3 mg mL^{-1} solution) was loaded and fractions from: **Lane 3**. Solid support from column after being heated in SDS-buffer, unknown amount of protein (30 mg of resin as moist cake in 6 µL of loading buffer), and **Lane 4.** Concentrated elution fraction 2.5 µg of protein (50 µL of a 0.05 mg mL^{-1} solution) **Lane 5** Protein standards. The gel shows that the elution of the binding protein was not achieved. On lane 3, we observed that recombinant-galectin-9 stayed bound to the resin whereas in the elution there is no evidence of galectin-9 being present. To estimate the molecular weight of the bands, protein standards (**Lane 1**) were used (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa). The bands in lanes two to four were visualized using Coomasie brilliant blue. The SDS-PAGE contained 20% acrylamide gel. The expected molecular weights are 62 kDa for the recombinant-galectin-9 and 26 kDa for Glutathione S-transferase.

The next step for the optimization of our protocol comprised finding the appropriate conditions for eluting galectin-9 from the solid support. A study by Chabot et al. performed a competitive inhibition assay of galectin-9 and found optimum results using 0.2 M of lactose. (*47*) We tested an elution buffer by only changing lactose to 0.25 M. (**Figure 3.8**)

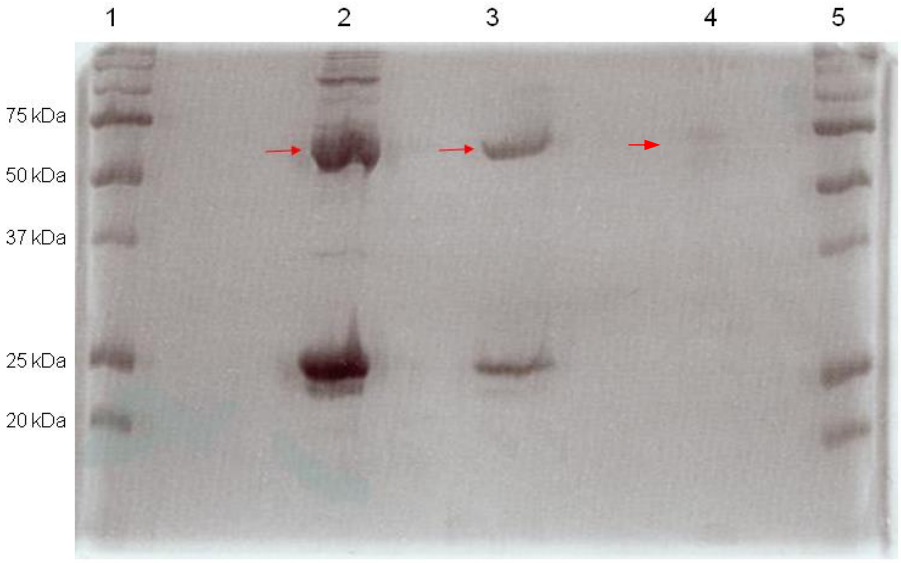


Figure 3.8 SDS-PAGE Gel of the affinity chromatography samples using a lactose column and lysate of recombinant-galectin-9 with optimized elution conditions. An SDS-PAGE was loaded with samples of the different steps of the affinity chromatography method to track the binding of the recombinant-galectin-9. **Lane 1.** Protein standards **Lane 2.** Recombinant-galectin-9, 26 µg of protein (20 µL of a 1.3 mg mL^{-1} solution) **Lane 3**. Solid support from column after being heated in SDS-buffer, unknown amount of protein (40 mg of resin as moist cake in 8 µL of loading buffer) **Lane 4.** Concentrated elution fraction ~3.0 µg of protein (50 µL of a 0.06 mg mL^{-1} solution) **Lane 5**. Protein standards. The gel shows that the elution of the binding protein was successfully achieved. On lane 3, we can observe that galectin-9 was actually found on the elution fraction whereas in the solid support there is no evidence of galectin-9 being present. To estimate the molecular weight of the bands, protein standards (**Lane 1**) were used (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa). The bands in lanes two to

four were visualized using Coomasie brilliant blue. The SDS-PAGE contained 15% acrylamide gel. The expected molecular weights are 62 kDa for the recombinant-galectin-9 and 26 kDa for Glutathione S-transferase.

The increased concentration of lactose (0.25 M) successfully disrupted the interaction between the immobilized saccharide and galectin-9. The protein was found in the elution fraction (lane 3). In order to make sure the elution had been successful, we also heated a fraction of the solid support in SDS-buffer and loaded it into the gel (lane 4), the gel shows no band corresponding to galectin-9. This experiment allowed us to conclude that the affinity chromatography protocol can capture HMO binders, and that it is possible to disrupt the interaction of galectins using 0.25 M of lactose as a competitive binder due to the common β–galactose residue. (*41*)

Having collected evidence that the method works as a way of screening for HMO binders from a large pool of proteins, we were concerned that not all examples would be so clear-cut as the galectins. We identified that the SDS-PAGE step could potentially leave certain binders out due to the limit of detection of the technique. Although detection limits may be as low as 1500 fM for visualizing certain proteins. (*51*) We chose to avoid this problem by using shotgun proteomics as described below.

3.2.2 Shotgun proteomics method

Shotgun proteomics is a method for the analysis of protein mixtures and their identification from tandem mass spectra of their proteolytic peptides. (*52*) The method offers the advantage of not needing to separate the protein by gel electrophoresis, but only to digest them enzymatically as a mixture in solution. The peptides obtained are analysed using LC-

MS/MS. The fragments obtained after MS/MS analysis are then searched against an appropriate database such as SwissProt, NCBInr, or others with the help of a search engine such as MASCOT. (*53-55*) This method offers the advantage that it can detect as little as 10 fmol of protein in solution. (*55*)

We tested the shotgun approach using a system that we were already familiar with. We used 2'FL immobilized via DVS to Sepharose and Jurkat cell lysate as we did for testing the immuno-precipitation protocol (**Figure 3.5**). We decided to modify the elution conditions by adding a high concentration of salt. We hoped the elution buffer (3 M NaCl, 0.25 M lactose, 50 mM Tris-HCl, pH 8) would be able to disrupt ionic interactions between the molecules, and also lactose would disrupt the binding as a competitive binder for HMO.

An example of the chromatogram obtained by observing the A_{280} of the Jurkat lysate solution passed through the 2'FL column is shown in **Figure 3.9**. Tracking the absorbance we could determine the point in time were the lysate has flowed through the column completely and also when proteins are being eluted off the column and need to be collected. In order to eliminate possible non-specific binders to the Sepharose we used an in-line blank Sepharose column before the 2'-FL column. The blank column was removed after all the lysate had been injected and the washing and elution steps were performed only in the 2'FL column.

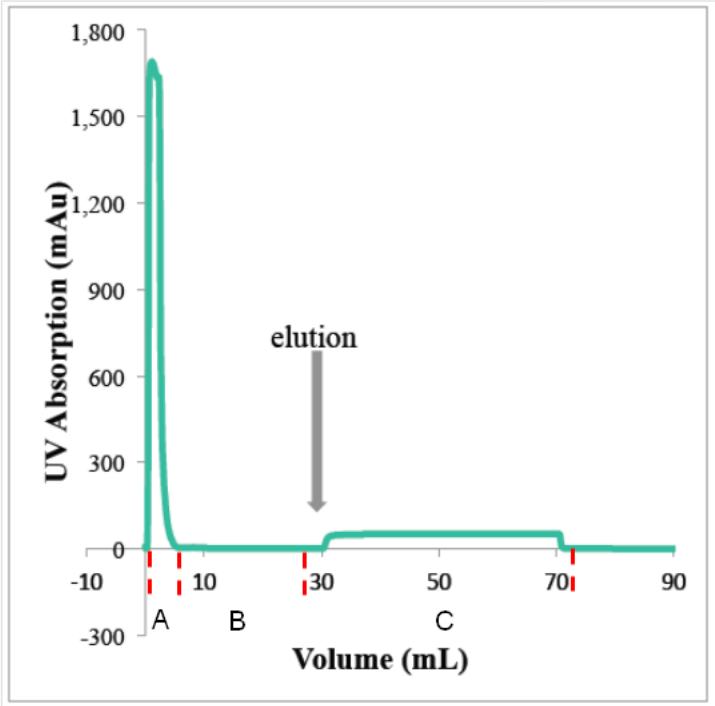


Figure 3.9 Sample FPLC chromatogram for a 2'FL column with Jurkat cell lysate. The chromatogram can be divided into 3 main sections: **A**. Injection – the lysate solution (~4.6 mg, 3 mL of a 1.54 mg mL^{-1} lysate solution) was flowed through a blank Sepharose column (1 mL of slurry), followed by the 2'FL column (1 mL of slurry of 2'FucLac functionalized Sepharose). At the end of this step the blank Sepharose column was removed; **B.** Washing – approximately 20 mL of running buffer (1 mM $CaCl_2$, 0.5 NaCl, 50 mM Tris-HCl, pH 8) was run in order to remove non-specific binders and assure the A_{280} goes to zero; **C**. Elution – ~40 mL of elution buffer (3 M NaCl, 0.25 M lactose, 50 mM Tris-HCl, pH 8) are run through the column to disrupt the binding between the proteins and the HMO. Fractions of 5 mL were collected during elution. The increase in the A_{280} serves as an indication that proteins are present in the solution, the change in A_{280} is not very big probably to the low concentration of proteins in the solution. The system was operated at flow rate of 1 mL min^{-1} and the pressure was maintained at ~0.3 MPa.

The elution fractions (8 of them, 5 mL each) were collected and pooled together, and dialyzed against running buffer using a membrane of 8000 Da (12h, two buffer changes). The resulting solution was concentrated using Amicon Ultra spin columns with a cutoff of 10,000 Da until reaching a volume of approximately 0.25 mL. The volume was further reduced to ~50 µL using evaporation with nitrogen gas, and prepared for mass spectrometry analysis using established protocols. (*53*) The proteins were extracted and detergents removed with a MeOH:$CHCl_3$:H_2O solution (1:1.2:1.8) and then precipitated from the chloroform layer via centrifugation (12,000 x g for 2 min, rt). The pellet was freeze-dried and re-suspended in urea solution (6.25 µL, 8 M, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.005% dodecyl-β-maltoside in water). The proteins were reduced using dithiotreitol solution (DTT) (0.625 µL of a 100 mM solution in water) and incubated (37 °C, 20 min). After incubation, the protein was acetylated with iodoacetamide (0.625 µL of a 200 mM solution in water) and incubated (20 min, rt, protected from light). After incubation, the proteins were digested using trypsin solution (5 µL of a solution containing 50 $ngmL^{-1}$of trypsin, 2.5 mM acetic acid in urea buffer) for 12h at 37 °C. Once the digestion is completed, formic acid (5 µL of a 10% (v/v) solution) was added to stop the enzymatic reaction. The solution was stored at -20 °C until the MS analysis was performed. (*53*) A step-by-step description of the method is available in **Section 3.4.3**.

A fraction of the concentrated elution fraction was loaded on SDS-PAGE gel for comparison (**Figure 3.10**). The gel of the lysate did not show any obvious protein bands, likely due to the low amount of protein bound to the column

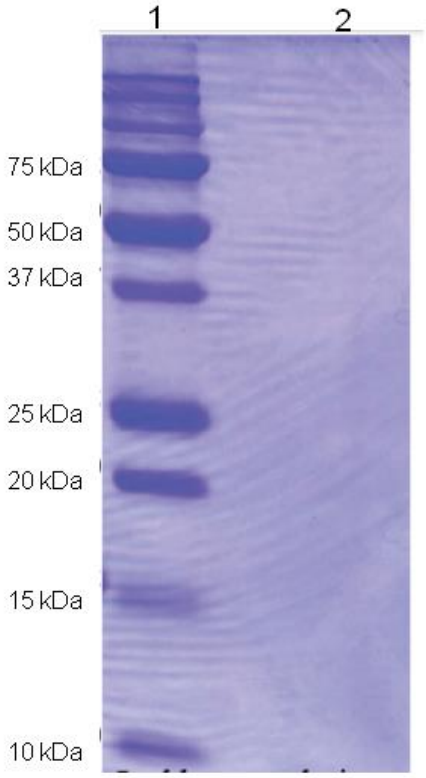


Figure 3.10 SDS-PAGE gel for the affinity chromatography method using Jurkat cell lysate run over a 2'FL column. To estimate the molecular weight of any potential bands, protein standards (**Lane 1**) were used (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa) **Lane 2.** Concentrated elution fractions (50 µL) The SDS-PAGE gel contained 15% acrylamide.

The shotgun mass spectrometry analysis of the Jurkat lysate run over a 2'FL column identified galectin-3 (21% coverage; score 334), galectin-9 (31% coverage; score 265), and galectin-9B (13% coverage; score 235) proteins. These results were encouraging, since the shoutgun approach had increased the sensitivity of our method for identifying binders to 2'FL. Moreover, the scores obtained are well above the threshold of 40 that was established, reducing the probability of the identification to be a random coincidence below 5%. The amino-acid sequences of the galectins found, the score, and coverage are shown in **Figure 3.11**.

Figure 3.11 Amino acid sequence coverage of proteins identified using affinity chromatography with DVS immobilized 2'FL column and lysate from Jurkat cells, by shotgun proteomics. The Jurkat cell lysate was run over a Sepharose column containing DVS immobilized 2'FL run on an FPLC and detected by shotgun proteomic analysis. Three proteins from the galectin family were identified: **A.** Galectin-3 (38% coverage; score 422) **B.** Galectin-9 (31% coverage; score 423), **C.** Galectin-9B (13% coverage; score 285). A complete list of protein hits can be found in **Appendix B**.

It is important to mention that we obtained several protein hits after the mass spectrometry analysis. **Figures 3.11** and **3.12** only show proteins belonging to the galectin family. We decided to only focus on this family of proteins because their binding to HMO has been characterized in the past and we are certain they bind to HMO. (*5*) The rest of the protein hits need to be analyzed in a case by

case basis, and the measurement of their binding constant might be needed to confirm them as HMO-binding receptors. A list of all the protein hits for all the experiments can be found in **Appendix B**.

The identification of galectin-3 as a binder was not surprising based on the immuno-precipitation results discussed in **Section 3.2.1** for Jurkat cells. (*46*) Furthermore, we note that the affinity galectin-3 towards 2'FL is at least 2 to 3 times higher than its affinity for lactose. (**Table 3.2**) (*38, 39*).

The observation of galectin-9 as a binder of 2'-FL is not unexpected as it has been reported to be overexpressed in immune tissues, including Jurkat cells. (*47*) Moreover, we know that galectin-9, as the other members of the galectin family, has binding specificity towards β-linked galactose, a moiety that is present in 2'-FL. (*3*) There are reports of galectin-9 binding to glycans with poly-*N*-acetyllacotsamine (LacNAc) repeats, and sulfated residues of internal LacNAc repeats. (*56*) In 2002, Hirabayashi et al. reported a K_D of 150 μM for the C-terminal CRD of galectin-9 binding to 2'FL; (*5*) to the extent of our knowledge that is the only report of 2'-FL – galectin-9 binding constant, and it was not measured for the entire protein, which contains two CRDs. The higher scores obtained for galectin-3 could be partially explained by the larger dissociation constant in comparison with the dissociation constant of galectin-3 and 2'-FL (150 and 36 μM respectively). (*5*)

To the extent of our knowledge there is not information of galectin-9B binding studies to any sugars. However, we don't think this result is unexpected since galectin-9B possess a conserved domain among the different galectins (cd00070). This domain exclusively binds β-galactosides. (*57*)

At this point, we had established that our strategy could identify HMO receptors from cell lysate using shotgun proteomics. We had tested a single HMO, 2'-FL, so we set out to test

additional HMO columns to see if a different set of receptors could be identified. For this experiment we immobilized Lactose-*N*-tetraose (LNT) using DVS as a linker, and again we used Jurkat cell lysate.

In this experiment, we identified four binders: galectin-3 (33% coverage; score 493), galectin-9 (27% coverage; score 565), galectin-9b (15% coverage; score 390), and galectin-8 (9% coverage; score 62). The corresponding amino-acid sequences are shown below (**Figure 3.12**). The presence of galectin-3 and -9 again is not surprising, considering that both of them are overexpressed in the Jurkat cells. Galectin-8 is known to play an important role in inhibition of Jurkat cell adhesion to integrins. (*58*) Galectin-8 has also been identified as a potent pro-apoptotic agent in this cell line. (*59*). The dissociation constants for galectin-3, -8, and -9 to 2'FL ranges in the μM range (36, 120, and 150 μM, respectively). (*5*) These data further support that the binding of these proteins is not random coincidence or due to non-specific binding.

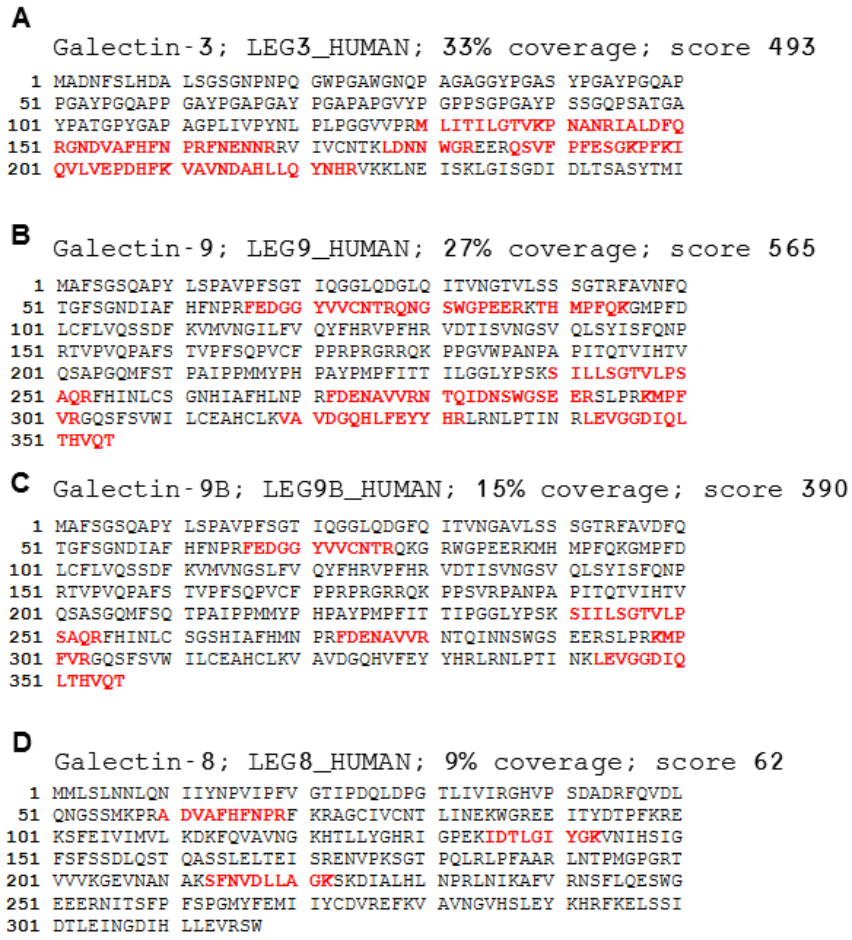


Figure 3.12 Amino acid sequence coverage of proteins identified using affinity chromatography with DVS immobilized LNT column and lysate from Jurkat cells, by shotgun proteomics. The Jurkat cell lysate was run over a Sepharose column containing DVS immobilized LNT run on an FPLC and detected by shotgun proteomic analysis. Four proteins from the galectin family were identified **A.** Galectin-3 (33% coverage; score 493) **B.** Galectin-9 (27% coverage; score 565), **C.** Galectin-9B (15% coverage; score 390), **D.** Galectin-8 (9% coverage; score 62). A complete list of protein hits can be found in **Appendix B**.

A summary of the results obtained with the affinity chromatography method followed by the shotgun proteomics analysis is presented below (**Table 3.3**)

	Galectin-3		Galectin-9		Galectin-9B		Galectin-8	
	Score	**Coverage (%)**	**Score**	**Coverage (%)**	**Score**	**Coverage (%)**	**Score**	**Coverage (%)**
2'FL	422	38	423	31	285	13	-	-
LNT	493	33	565	27	390	15	62	9

Table 3.3 Summary of galectin coverage from affinity chromatography of Jurkat cell lysate with 2'FL- and LNT-Sepharose. The proteins were separated using affinity chromatography and identified by mass spectrometry using the shotgun approach

The scores we obtained could potentially be used as an indicator of the relative affinity of the galectin towards the HMO. These values are in accordance with results published in other studies. The first point that can be made is that galectins have the highest affinity towards saccharides with a Gal-β1,3-GlcNAc structure, which is found in LNT but absent in 2'FL. (*5, 60, 61*)

Another important point to highlight about these results is the fact that the method was able to obtain a whole-cell profile of HMO receptors from lysate in a single experiment. The method provides specificity. For example, we found that galectin-8 does not bind to 2'FL, but it does to LNT, in accordance with other results in literature. The K_D's for galectin-3, -8, and -9 to 2'FL are 36, 120 and 150 μM, respectively. (*5*) On the other hand the same corresponding K_D's for these galectins to LNT are 2.6, 43, and 12 μM, respectively. The smaller dissociation constants of the galectins to LNT show that they bind more strongly to LNT than to 2'FL,

providing an explanation for why we got higher scores for LNT. It is important to remember that we are only comparing the binding profile of galectins; however, other proteins were detected (See **Apprendix B**) which we are currently investigating.

3.3 Conclusions

HMO are an active area of research due to the potential beneficial effects that they offer for the newborn infant and the lactating mother. (*62*) They have become of scientific interest for the therapeutic activity they could exert for the treatment of diseases like cancer (*63*), arthritis, and allergies. (*36*) Unfortunately, very little is known about the molecular mechanisms or interactions underlying all these potential beneficial effects.

Several groups are working in developing methods for the quick screening of HMO binding partners using different techniques. One of the most popular approaches is microarrays, (*64-66*) where multiple carbohydrates are immobilized in a small surface and exposed to a single protein. In this chapter we have presented an approach were one glycan is exposed to several receptors and those receptors are later identified by mass spectrometry (one glycan, many receptors).

Through a series of binding experiments we were able to optimize a protocol that can be automated using FPLC and allows the screening of thousands of receptors in a very short period of time. We started by gathering evidence that our modified solid support could capture binders, then we moved on to collecting data that could show that the modified solid support maintained its binding activity even when the contact time between the carbohydrate and the receptor is reduced to the contact time inside a column. We also confirmed that the shotgun

approach can be used for the identification of receptors without requiring previous separation or large quantities of material.

The results of our affinity strategy showed the potential of the method for identifying galectins in mammalian cells that bind HMO. Moreover the results obtained, as mass spectrometry scores, show certain relation with the dissociation constants reported in the literature. However; for measuring relative affinities, more data needs to be collected and an in-depth statistical analysis would also be required.

We have established protocols for the high throughput screening of HMO binding proteins. From a lysate sample of Jurkat cells, we identified galectin-3, galectin-9, and galectin-9B as binders to 2'FL. The same three galectins, plus galectin-8, were identified as binders of LNT when present in a lysate sample from Jurkat cells.

3.4 Experimental Methods

3.4.1 Lysate preparation

Jurkat cells were obtained as pellets and resuspended in lysis buffer (1 mM $CaCl_2$, 0.5 NaCl, 50 mM Tris-HCl 0.5% Triton X-100, pH 8) and the cells were disrupted using an ultrasonic cell disruptor. Protease inhibitor cocktail (Roche, Germany) was added to the solution and left mixing overnight at 4 ºC. The solution was centrifuged for 50 minutes at 12,000 x g at 4 ºC. The supernatant was collected and diluted using lysis buffer to obtain A_{280} of approximately 2 (1.54 mg mL^{-1} assuming that a 1 mg mL^{-1} solution of protein will have an absorbance of 1.3 (*67*)).

3.4.2 Immuno-precipitation method

An aliquot of the modified or unmodified solid support was washed first with 5 volumes of MiliQ water followed by five volumes of phosphate buffered saline (PBS). The solid was filtered to obtain a moist cake. The moist cake (30 mg) was weighed into an Eppendorf tube. The protein-containing solution (1.35 mL) was added to each tube and left mixing for 3 hours at room temperature. Subsequently the tubes were centrifuged at 2,000 x g for 3 minutes to precipitate the solid support and the supernatant was removed without disturbing the pellet. PBS (1 mL) was added to each tube and mixed for five minutes to wash the solid support and remove non-specific binders. The tubes were centrifuged again at 2,000 x g for 3 minutes, and the washing step was performed twice more for a total of 3 washing cycles.

The SDS-PAGE gel was prepared and run as per standard protocol. (*68*) The solid support samples were re-suspended in 6 μL of loading buffer, and heated at 95 ºC for five

minutes, followed by cooling in an ice-bath and centrifugation. The supernatant was loaded into the wells of the gel. The gel was run at 150 V for approximately 90 minutes. Finally it was stained using Coomasie-blue using standard procedure.

3.4.3 Shotgun proteomics method

The solid support (1 mL slurry) with the immobilized HMO was packed in a Tricorn 5/50 column, and attached to an ÄKTAprime Plus FPLC system (GE Healthcare, Uppsala, Sweden) with an in-line column filled with unmodified Sepharose CL-6B. The columns were equilibrated with 10 column volumes of running buffer (1 mM $CaCl_2$, 0.5 NaCl, 50 mM Tris-HCl, pH 8) at a flow of 1 mL min^{-1}. The protein solution (3 mL) was injected. The unmodified Sepharose CL-6B column was removed and the column with the immobilized HMO was washed with approximately 10 column volumes of running buffer, followed by elution with approximately 20 column volumes of elution buffer (3 M NaCl, 0.2 M lactose, 50 mM Tris-HCl, pH 8). Fractions of 5 mL were collected. The column was then eluted with an equal volume of Glycine HCl, pH 4.5, collected in 5 mL fractions. The column was washed with an equal volume of running buffer containing 0.02% (w/v) of NaN_3 for storage.

The elution fractions collected were pooled together and dialyzed against running buffer using a membrane of 8000 Da (12 h, two buffer changes). The resulting solution was concentrated using Amicon Ultra spin columns with a cutoff of 10,000 Da until reaching a volume of approximately 0.25 mL. The volume was further reduced to ~50 µL using evaporation with nitrogen gas, and prepared for mass spectrometry analysis using the protocol developed by Kubota et al. The protein solution (~50 µL) was transferred to a low-binding 500 µL tube and mixed with MeOH (200 µL), chloroform (50 µL) was added and mixed, finally

MiliQ water (150 μL) was added and the sample was mixed vigorously. The solution was centrifuged (10,000 x g for 2 min, rt), forming two layers. The top layer was removed and discarded. MeOH (200 μL) was added to the bottom layer and mixed. The tube was centrifuged (12,000 x g for 2 min, rt), after centrifugation the supernatant was removed, taking care of not disturbing the pellet. The pellet was freeze-dried. Once the pellet was dry, an urea solution was added (6.25 μL, 8 M, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.005% dodecyl-β-maltoside in water). Dithiotreitol (DTT) was added to the mixture (0.625 μL of a 100 mM solution in water). The solution was centrifuged for 5 seconds and incubated (37 °C, 20 min). After the incubation, the solution was allowed to cool down to room temperature and iodoacetamide was added (0.625 μL of a 200 mM solution in water). The solution was mixed and centrifuged for 5 seconds and incubated (20 min, rt, protected from light). After incubation, dilute urea buffer was added (50 μL, 42.5 μL of urea solution plus 7.5 μL of MiliQ water), the solution was mixed and centrifuged (2000 x g for 5 seconds). Trypsin (Trypsin gold, mass spectrometry grade, Promega. Madison, WI) solution was added (5 μL of a solution containing 50 ng $mL^{-1}$of trypsin, 2.5 mM acetic acid in urea buffer) mixed and centrifuged (2000 x g for 5 seconds). The proteins were digested (12h at 37 °C). Once the digestion was completed, formic acid (5 μL of a 10% (v/v) solution) was added to stop the enzymatic reaction. The solution was stored at -20 °C until the MS analysis was performed. (*53*)

3.4.4 Mass spectrometry analysis.

All experiments were carried out in positive ion mode using a Synapt G2 mass spectrometer (Waters, Manchester, UK) equipped with a nanoACQUITY UPLC (Waters, Manchester, UK). Peptide products of trypsin-digested proteins were analyzed by LC-MS/MS

using a data dependent analysis method. Tryptic peptides, (15 µL injection), were separated on a BEH130 C18 (1.7 µm), 75 µm x 100 mm analytical RP column (Waters, Manchester, UK). The flow rate was 0.3 µL min^{-1} and the column temperature was maintained at 35 °C. The mobile phase comprised solvent A (0.1% formic acid in wáter) and solvent B (0.1% formic acid in acetonitrile). The eluted peptides were separated with a gradient of 1-50% solvent B over 45 min, followed by a rinse with solvent B at 95% for 8 min. MS/MS spectra were acquired for up to six of the most intense precursor ions with charge states of +1, +2, or +3 after each MS survey scan. The data analysis was carried out using MassLynx (Waters; MassLynx, version 4.1). The search engine Mascot (Matrix Science) was used against SwissProt database for processing the MS data and exported as a CSV file (See **Appendix B**).

3.4.5 Cell culture

3.4.5.1 Jurkat cells

Jurkat cells (clone E61) were obtained from ATCC (Mannassas, VA) and cultured in RPMI 1640 media supplemented with fetal bovine serum (FBS, 10% v/v), penicillin (10 units mL^{-1}), and streptomycin (10 mg mL^{-1}) in a humidified incubator (5% CO_2) at 37 °C.

3.4.5.2 HeLa cells

HeLa cells (CCL-2) were obtained from ATCC (Mannassas, VA) and cultured in Eagle's Minimum Essential Medium supplemented with fetal bovine serum (FBS, 10% v/v), penicillin (10 units mL^{-1}), and streptomycin (10 mg mL^{-1}) in a humidified incubator (5% CO_2) at 37 °C.

3.4.5.3 HT-29 cells

HT-29 cells were obtained from ATCC (Mannassas, VA) and cultured in McCoy's 5a medium supplemented with fetal bovine serum (FBS, 10% v/v), penicillin (10 units mL^{-1}), and streptomycin (10 mg mL^{-1}) in a humidified incubator (5% CO_2) at 37 °C.

3.4.5.4 T84 cells

T84 cells were obtained from ATCC (Mannassas, VA) and cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium with 2.5 mM L-glutamine, 95%; supplemented with fetal bovine serum (FBS, 10% v/v), penicillin (10 units mL^{-1}), and streptomycin (10 mg mL^{-1}) in a humidified incubator (5% CO_2) at 37 °C.

3.4.6 Column preparation

Carbohydrate modified sepharose gel was prepared as previously reported. (*69*) One mL of settled Sepharose CL-6B (GE Healthcare Life Sciences, Piscataway, N.J.) was thoroughly washed with water in a sintered funnel and then re-suspended in 0.5 M carbonate buffer (pH 11) with 100 µL of divinyl sulfone (Sigma Aldrich, Milwaukee, WI). The mixture was agitated for 70 min, after which the resin was transferred to a sintered funnel and extensively washed with water. The moist cake was suspended in a 1 mL solution of the indicated carbohydrate (Elicityl, Crolles, France) (1.11 mmol mL^{-1} in 0.5 M carbonate buffer, pH 10) and left agitating for 18 hours. The resin was washed again with distilled water over a sintered funnel, and the moist cake re-suspended in carbonate buffer (1 mL, 0.5 M, pH 8.5) and of 2-mercaptoethanol (6 µL). After 2 hours the sample was washed with distilled water and stored in 20% ethanol solution.

3.5 References

1. Liu, F.-T., and Rabinovich, G. A. (2005) Galectins as modulators of tumour progression, *Nature Reviews Cancer 5*, 29-41.
2. Hirabayashi, J. (2008) Concept, Strategy and Realization of Lectin-based Glycan Profiling, *Journal of Biochemistry 144*, 139-147.
3. Barondes, S. H., Castronovo, V., Cooper, D. N. W., Cummings, R. D., Drickamer, K., Felzi, T., Gitt, M. A., Hirabayashi, J., Hughes, C., Kasai, K.-i., Leffler, H., Liu, F.-T., Lotan, R., Mercurio, A. M., Monsigny, M., Pillai, S., Poirer, F., Raz, A., Rigby, P. W. J., Rini, J. M., and Wang, J. L. (1994) Galectins: A family of animal β-galactoside-binding lectins, *Cell 76*, 597-598.
4. Leffler, H., Carlsson, S., Hedlund, M., Qian, Y., and Poirier, F. (2002) Introduction to galectins, *Glycoconj J 19*, 433-440.
5. Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W. E. G., Yagi, F., and Kasai, K.-i. (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography, *Biochimica et Biophysica Acta (BBA) - General Subjects 1572*, 232-254.
6. Ahmad, N., Gabius, H.-J., André, S., Kaltner, H., Sabesan, S., Roy, R., Liu, B., Macaluso, F., and Brewer, C. F. (2004) Galectin-3 Precipitates as a Pentamer with Synthetic Multivalent Carbohydrates and Forms Heterogeneous Cross-linked Complexes, *Journal of Biological Chemistry 279*, 10841-10847.
7. Brewer, C. F., Miceli, M. C., and Baum, L. G. (2002) Clusters, bundles, arrays and lattices: novel mechanisms for lectin–saccharide-mediated cellular interactions, *Current Opinion in Structural Biology 12*, 616-623.

8. Rabinovich, G. A., Baum, L. G., Tinari, N., Paganelli, R., Natoli, C., Liu, F.-T., and Iacobelli, S. (2002) Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response?, *Trends in Immunology 23*, 313-320.

9. Rabinovich, G. A., Rubinstein, N., and Toscano, M. A. (2002) Role of galectins in inflammatory and immunomodulatory processes, *Biochimica et Biophysica Acta (BBA) - General Subjects 1572*, 274-284.

10. Kuwabara, I., Sano, H., and Liu, F.-T. (2003) Functions of Galectins in Cell Adhesion and Chemotaxis, In *Methods in Enzymology* (Yuan, C. L., and Reiko, T. L., Eds.), pp 532-552, Academic Press.

11. Anatole, A. K. (2012) Galectin-Targeted Drug Design, In *Glycobiology and Drug Design*, pp 25-66, American Chemical Society.

12. Markowska, A. I., Liu, F.-T., and Panjwani, N. (2010) Galectin-3 is an important mediator of VEGF- and bFGF-mediated angiogenic response, *The Journal of Experimental Medicine 207*, 1981-1993.

13. Markowska, A. I., Jefferies, K. C., and Panjwani, N. (2011) Galectin-3 Protein Modulates Cell Surface Expression and Activation of Vascular Endothelial Growth Factor Receptor 2 in Human Endothelial Cells, *Journal of Biological Chemistry 286*, 29913-29921.

14. Newlaczyl, A. U., and Yu, L.-G. (2011) Galectin-3 – A jack-of-all-trades in cancer, *Cancer Letters 313*, 123-128.

15. Barrow, H., Rhodes, J. M., and Yu, L.-G. (2011) The role of galectins in colorectal cancer progression, *International Journal of Cancer 129*, 1-8.

16. Ito, K., Scott, S., Cutler, S., Dong, L.-F., Neuzil, J., Blanchard, H., and Ralph, S. (2011) Thiodigalactoside inhibits murine cancers by concurrently blocking effects of galectin-1 on immune dysregulation, angiogenesis and protection against oxidative stress, *Angiogenesis 14*, 293-307.

17. Almkvist, J., and Karlsson, A. (2002) Galectins as inflammatory mediators, *Glycoconj J 19*, 575-581.

18. Balkwill, F. (2004) Cancer and the chemokine network, *Nature Reviews Cancer 4*, 540-550.

19. Liu, F.-T., Hsu, D. K., Yang, R.-Y., Chen, H.-Y., and Saegusa, J. (2008) Galectins in Regulation of Inflammation and Immunity, In *Galectins*, pp 97-113, John Wiley & Sons, Inc.

20. Hu, C.-Y., Chang, S.-K., Wu, C.-S., Tsai, W.-I., and Hsu, P.-N. (2011) Galectin-3 gene (LGALS3) +292C allele is a genetic predisposition factor for rheumatoid arthritis in Taiwan, *Clin Rheumatol 30*, 1227-1233.

21. Forsman, H., Islander, U., Andréasson, E., Andersson, A., Önnheim, K., Karlström, A., Sävman, K., Magnusson, M., Brown, K. L., and Karlsson, A. (2011) Galectin 3 aggravates joint inflammation and destruction in antigen-induced arthritis, *Arthritis & Rheumatism 63*, 445-454.

22. Janeway, C. A., Travers, P., and Walport, M. (2001) The components of the immune system, In *Immunobiology: The Immune System in Health and Disease.* 5th edition ed., Garland Science, New York.

23. Perillo, N. L., Pace, K. E., Seilhamer, J. J., and Baum, L. G. (1995) Apoptosis of T cells mediated by galectin-1, *Nature 378*, 736-739.

24. Rabinovich, G. A., Iglesias, M. M., Modesti, N. M., Castagna, L. F., Wolfenstein-Todel, C., Riera, C. M., and Sotomayor, C. E. (1998) Activated Rat Macrophages Produce a Galectin-1-Like Protein That Induces Apoptosis of T Cells: Biochemical and Functional Characterization, *The Journal of Immunology 160*, 4831-4840.

25. Fukumori, T., Takenaka, Y., Yoshii, T., Kim, H.-R. C., Hogan, V., Inohara, H., Kagawa, S., and Raz, A. (2003) CD29 and CD7 Mediate Galectin-3-Induced Type II T-Cell Apoptosis, *Cancer Research 63*, 8302-8311.

26. Cortejano, I., del Pozo, V., Cardaba, B., de Andres, B., Gallardo, S., del Amo, A., Arrieta, I., Jurado, A., Palomino, P., Liu, F.-T., and Lahoz, C. (1998) Galectin-3 down-regulates IL-5 gene expression on different cell types., *The Journal of Immunology 161*, 385-389.

27. Sturm, A., Lensch, M., André, S., Kaltner, H., Wiedenmann, B., Rosewicz, S., Dignass, A. U., and Gabius, H.-J. (2004) Human Galectin-2: Novel Inducer of T Cell Apoptosis with Distinct Profile of Caspase Activation, *The Journal of Immunology 173*, 3825-3837.

28. Wada, J., Ota, K., Kumar, A., Wallner, E. I., and Kanwar, Y. S. (1997) Developmental regulation, expression, and apoptotic potential of galectin-9, a beta-galactoside binding lectin, *The Journal of Clinical Investigation 99*, 2452-2461.

29. Dunn, G. P., Old, L. J., and Schreiber, R. D. (2004) The Immunobiology of Cancer Immunosurveillance and Immunoediting, *Immunity 21*, 137-148.

30. Ilarregui, J. M., Bianco, G. A., Toscano, M. A., and Rabinovich, G. A. (2005) The coming of age of galectins as immunomodulatory agents: impact of these carbohydrate

binding proteins in T cell physiology and chronic inflammatory disorders, *Annals of the Rheumatic Diseases 64*, iv96-iv103.

31. Camby, I., Le Mercier, M., Mathiey, V., Ingrassia, L., Lefranc, F., and Kiss, R. (2008) Galectin-1 as a potential therapeutic target for cancer progression, *Drugs of the Future 33*, 1057-1069.

32. André, S., Pieters, R. J., Vrasidas, I., Kaltner, H., Kuwabara, I., Liu, F.-T., Liskamp, R. M. J., and Gabius, H.-J. (2001) Wedgelike Glycodendrimers as Inhibitors of Binding of Mammalian Galectins to Glycoproteins, Lactose Maxiclusters, and Cell Surface Glycoconjugates, *ChemBioChem 2*, 822-830.

33. Sörme, P., Qian, Y., Nyholm, P., Leffler, H., and Nilsson, U. (2002) Low micromolar inhibitors of galectin-3 based on 3'-derivatization of N-acetyllactosamine, *Chembiochem: a European journal of chemical biology 3*, 183-189.

34. Rijnierse, A., Jeurink, P. V., van Esch, B. C. A. M., Garssen, J., and Knippels, L. M. J. (2011) Food-derived oligosaccharides exhibit pharmaceutical properties, *European Journal of Pharmacology 668, Supplement 1*, S117-S123.

35. Eiwegger, T., Stahl, B., Haidl, P., Schmitt, J., Boehm, G., Dehlink, E., Urbanek, R., and Szépfalusi, Z. (2010) Prebiotic oligosaccharides: In vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties, *Pediatric Allergy and Immunology 21*, 1179-1188.

36. Eiwegger, T., Stahl, B., Schmitt, J., Boehm, G., Gerstmayr, M., Pichler, J., Dehlink, E., Loibichler, C., Urbanek, R., and Szepfalusi, Z. (2004) Human Milk-Derived Oligosaccharides and Plant-Derived Oligosaccharides Stimulate Cytokine Production of Cord Blood T-Cells In Vitro, *Pediatric Research 56*, 536-540.

37. Sparrow, C. P., Leffler, H., and Barondes, S. H. (1987) Multiple soluble beta-galactoside-binding lectins from human lung, *Journal of Biological Chemistry 262*, 7383-7390.

38. Leffler, H., and Barondes, S. H. (1986) Specificity of binding of three soluble rat lung lectins to substituted and unsubstituted mammalian beta-galactosides, *Journal of Biological Chemistry 261*, 10119-10126.

39. Ideo, H., Seko, A., Ohkura, T., Matta, K. L., and Yamashita, K. (2002) High-affinity binding of recombinant human galectin-4 to SO3−→3Galβ1→3GalNAc pyranoside, *Glycobiology 12*, 199-208.

40. Ahmad, N., Gabius, H. J., Kaltner, H., André, S., Kuwabara, I., Liu, F.-T., Oscarson, S., Norberg, T., and Brewer, C. F. (2002) Thermodynamic binding studies of cell surface carbohydrate epitopes to galectins-1, -3, and -7: Evidence for differential binding specificities, *Canadian Journal of Chemistry 80*, 1096-1104.

41. Ideo, H., Seko, A., Ishizuka, I., and Yamashita, K. (2003) The N-terminal carbohydrate recognition domain of galectin-8 recognizes specific glycosphingolipids with high affinity, *Glycobiology 13*, 713-723.

42. Zrimi, J., Ng Ling, A., Giri-Rachman Arifin, E., Feverati, G., and Lesieur, C. (2010) Cholera Toxin B Subunits Assemble into Pentamers - Proposition of a Fly-Casting Mechanism, *PLoS ONE 5*, e15347.

43. Jobling, M. G., and Holmes, R. K. (2002) Mutational Analysis of Ganglioside GM1-Binding Ability, Pentamer Formation, and Epitopes of Cholera Toxin B (CTB) Subunits and CTB/Heat-Labile Enterotoxin B Subunit Chimeras, *Infection and Immunity 70*, 1260-1271.

44. Masters, J. R. (2002) HeLa cells 50 years on: the good, the bad and the ugly, *Nature Reviews Cancer 2*, 315-319.

45. Abraham, R. T., and Weiss, A. (2004) Jurkat T cells and development of the T-cell receptor signalling paradigm, *Nature Reviews Immunology 4*, 301-308.

46. Yang, R. Y., Hsu, D. K., and Liu, F. T. (1996) Expression of galectin-3 modulates T-cell growth and apoptosis, *Proceedings of the National Academy of Sciences of the United States of America 93*, 6737-6742.

47. Chabot, S., Kashio, Y., Seki, M., Shirato, Y., Nakamura, K., Nishi, N., Nakamura, T., Matsumoto, R., and Hirashima, M. (2002) Regulation of galectin-9 expression and release in Jurkat T cell line cells, *Glycobiology 12*, 111-118.

48. Vyakarnam, A., Lenneman, A. J., Lakkides, K. M., Patterson, R. J., and Wang, J. L. (1998) A Comparative Nuclear Localization Study of Galectin-1 with Other Splicing Components, *Experimental Cell Research 242*, 419-428.

49. Hasan, S. S., Ashraf, G. M., and Banu, N. (2007) Galectins – Potential targets for cancer therapy, *Cancer Letters 253*, 25-33.

50. Sehrawat, S., Reddy, P. B. J., Rajasagi, N., Suryawanshi, A., Hirashima, M., and Rouse, B. T. (2010) Galectin-9/TIM-3 Interaction Regulates Virus-Specific Primary and Memory CD8+ T Cell Response, *PLoS Pathogens 6*, e1000882.

51. Winkler, C., Denker, K., Wortelkamp, S., and Sickmann, A. (2007) Silver- and Coomassie-staining protocols: Detection limits and compatibility with ESI MS, *Electrophoresis 28*, 2095-2099.

52. Marcotte, E. M. (2007) How do shotgun proteomics algorithms identify proteins?, *Nature Biotechnology 25*, 755-757.

53. Kubota, K., Kosaka, T., and Ichikawa, K. (2009) Shotgun Protein Analysis by Liquid Chromatography-Tandem Mass Spectrometry, In *Two-Dimensional Electrophoresis Protocols* (Tyther, R., and Sheehan, D., Eds.), pp 483-494, Humana Press.

54. Meissner, F., and Mann, M. (2014) Quantitative shotgun proteomics: considerations for a high-quality workflow in immunology, *Nature Immunology 15*, 112-117.

55. Old, W. M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K. G., Mendoza, A., Sevinsky, J. R., Resing, K. A., and Ahn, N. G. (2005) Comparison of Label-free Methods for Quantifying Human Proteins by Shotgun Proteomics, *Molecular & Cellular Proteomics 4*, 1487-1502.

56. Nagae, M., Nishi, N., Nakamura-Tsuruta, S., Hirabayashi, J., Wakatsuki, S., and Kato, R. (2008) Structural Analysis of the Human Galectin-9 N-terminal Carbohydrate Recognition Domain Reveals Unexpected Properties that Differ from the Mouse Orthologue, *Journal of Molecular Biology 375*, 119-135.

57. Marchler-Bauer, A., Zheng, C., Chitsaz, F., Derbyshire, M. K., Geer, L. Y., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., and Lanczycki, C. J. (2012) CDD: conserved domains and protein three-dimensional structure, *Nucleic acids research*, gks1243.

58. Hadari, Y. R., Arbel-Goren, R., Levy, Y., Amsterdam, A., Alon, R., Zakut, R., and Zick, Y. (2000) Galectin-8 binding to integrins inhibits cell adhesion and induces apoptosis, *Journal of Cell Science 113*, 2385-2397.

59. Norambuena, A., Metz, C., Vicuña, L., Silva, A., Pardo, E., Oyanadel, C., Massardo, L., González, A., and Soza, A. (2009) Galectin-8 Induces Apoptosis in Jurkat T Cells

by Phosphatidic Acid-mediated ERK1/2 Activation Supported by Protein Kinase A Down-regulation, *Journal of Biological Chemistry 284*, 12670-12679.

60. Hirabayashi, J., and Kasai, K.-i. (2002) Separation technologies for glycomics, *Journal of Chromatography B 771*, 67-87.

61. Arata, Y., Hirabayashi, J., and Kasai, K.-i. (2001) Sugar Binding Properties of the Two Lectin Domains of the Tandem Repeat-type Galectin LEC-1 (N32) of Caenorhabditis elegans : Detailed Analysis by an Improved Frontal Affinity Chromatography Method, *Journal of Biological Chemistry 276*, 3068-3077.

62. Bode, L. (2012) Human milk oligosaccharides: Every baby needs a sugar mama, *Glycobiology 22*, 1147-1162.

63. Rabinovich, G. A. (1999) Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy, *Cell Death Differ 6*, 711-721.

64. Yu, Y., Mishra, S., Song, X., Lasanajak, Y., Bradley, K. C., Tappert, M. M., Air, G. M., Steinhauer, D. A., Halder, S., Cotmore, S., Tattersall, P., Agbandje-McKenna, M., Cummings, R. D., and Smith, D. F. (2012) Functional Glycomic Analysis of Human Milk Glycans Reveals the Presence of Virus Receptors and Embryonic Stem Cell Biomarkers, *Journal of Biological Chemistry 287*, 44784-44799.

65. Adams, E. W., Ratner, D. M., Bokesch, H. R., McMahon, J. B., O'Keefe, B. R., and Seeberger, P. H. (2004) Oligosaccharide and Glycoprotein Microarrays as Tools in HIV Glycobiology: Glycan-Dependent gp120/Protein Interactions, *Chemistry & Biology 11*, 875-881.

66. Shang, J., Piskarev, V. E., Xia, M., Huang, P., Jiang, X., Likhosherstov, L. M., Novikova, O. S., Newburg, D. S., and Ratner, D. M. (2013) Identifying human milk glycans that inhibit norovirus binding using surface plasmon resonance, *Glycobiology 23*, 1491-1498.

67. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein, *Protein Science 4*, 2411-2423.

68. Gallagher, S. R. (2001) One-Dimensional SDS Gel Electrophoresis of Proteins, In *Current Protocols in Protein Science*, John Wiley & Sons, Inc.

69. Fornstedt, N., and Porath, J. (1975) Characterization studies on a new lectin found in seeds of Vicia ervilia, *FEBS Letters 57*, 187-191.

Chapter 4

Conclusions and future directions

4.1 Conclusions

Human milk oligosaccharides are a growing area of research due to the many potential health benefits they offer. Among the benefits postulated for these molecules, they are thought to act as prebiotics, antimicrobials, modulators of immune response, nutrients for brain development, and may provide other health benefits for the lactating mother. (*1*) Several studies have been conducted to uncover the beneficial effects that HMO offer; however, very little is known about the molecular mechanisms underlying these benefits. (*2-4*)

In this thesis, we have detailed work performed towards developing a method that will allow the high-throughput screening of potential HMO binders using affinity chromatography and mass spectrometry. The goal of this is to identify the molecular targets of these oligosaccharides; which will provide future leads for exploring the molecular mechanisms and pathways involved in their biological activity. For achieving this objective, we first envisioned developing a new method for immobilizing carbohydrates into a solid support, which was specific for the reducing end of the carbohydrate, and preserved the saccharide structure. The work performed for achieving this is described in **Chapter 2**. The testing of the affinity chromatography – mass spectrometry method for the screening of HMO binders with some preliminary results is presented in **Chapter 3**. A detailed discussion about the importance of studying protein-carbohydrate interactions, highlighting the importance of studying HMO, along with the methods currently available for studying these type of interactions can be found in **Chapter 1**.

The p-TsH method that we implemented for the immobilization of carbohydrate epitopes to an affinity matrix proved to be the most efficient at low concentrations of glycan when compared to the DVS and CC approaches. We were also able to observe that the p-TsH

method can immobilize from two to five times more glycan when compared to the CC and DVS methods. In terms of the binding activity of the immobilized epitopes, we conducted a lectin-binding assay that showed that p-TsH has comparable amounts of active epitopes as DVS and it requires at most half the amount of glycan. The method that we have developed offers the advantage of being regio-selective and highly efficient approach for the immobilization of glycans to an affinity matrix. This method can potentially be of interest for testing samples that are available in limited amounts.

The affinity chromatography protocol that we developed for the screening of HMO binders has been useful for the identification of galectins present in Jurkat cells. We tested the specificity of different columns by analysing the binders from Jurkat cell lysate and we were able to observe that different proteins in fact bind to different HMO. This observation further supports the idea that the binders found are indeed HMO binders and they are not due to non-specific interactions with the solid support. Analysing our results against dissociation constants reported in the literature, we were able to observe that the mass spectrometry scores correlate with the magnitude of the reported binding constants. This approach for the screening of HMO binders may be of interest when the quantity of HMO is very limited and the amount of potential binders is very large.

The results obtained in **Chapter 2** open up the possibility of greatly improving the affinity chromatography method developed in **Chapter 3**. We envision that if we utilise the columns with p-TsH immobilized HMO, we should be able to identify a larger number of receptors since, as we discussed, these columns have more carbohydrate receptor available per mg of resin, and also a higher protein binding capacity. Moreover, the immobilization occurring at the reducing end decreases the possibility of blocking the moiety of the HMO that

is recognized by proteins. Furthermore, the utilisation of p-TsH-immobilized HMO could facilitate the screening of other oligosaccharides whose availability is limited, besides the five we currently have, since a smaller amount of them would be required.

Overall we have developed a simple method for the stereo- and region-selective immobilization of carbohydrates into Sepharose CL-6B that requires minimal chemical transformations. The carbohydrate immobilization method developed, coupled with the affinity chromatography protocol established, allows the possibility of high throughput screening of HMO binders and their identification using shotgun proteomics. The results obtained in **Chapter 3** demonstrate the sensitivity and specificity of the method developed, and its potential to identify binders present even at low concentrations.

In addition to the accomplishments achieved and the progress made towards the establishment of our protocols, a number of challenges emerged and some areas of opportunity were identified. The different challenges that appeared present opportunities for future work to improve the method as discussed below.

4.2 Future directions

4.2.1 Tosyl Hydrazide method development.

For developing the method we took advantage of the nucleophilicity of the hydroxyl groups present in Sepharose CL-6B. If we observe the structure (**Figure 4.1**), we will realize that the most nucleophilic site is the primary hydroxyl group of the D-Galactose residue.

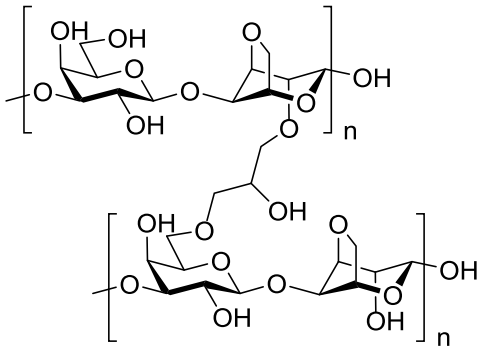
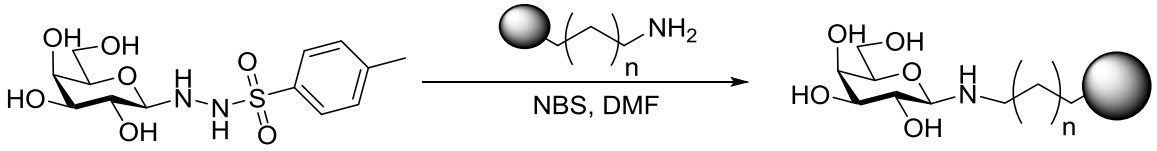


Figure 4.1 Chemical structure of Sepharose CL Sepharose is a cross-linked agarose (D-galactose and 3,6-anhydro-L-galactose) polymer where most of the hydroxyl groups are blocked or are used for cross-linking the repeating units.

One of the alternatives that we have envisioned which could improve coupling, was to use amino functionalised beads in order to get a stronger nucleophile that could potentially attack the reducing end. **Scheme 4.1** presents a general overview of the proposed method. One potential advantage of this approach is that amino-modified beads have a hydrocarbon chain that could work as a spacer between the bead surface and the carbohydrate, which could potentially increase the protein binding capacity of the method since that would reduce the possibility of steric crowding, as previous studies have reported. (*5*) The main disadvantage of this approach is that the length of the spacer has to be carefully optimized otherwise the effect of the spacer could be detrimental and reduce the binding capacity of the support.



Scheme 4.1. Immobilization of hydrazide-activated galactose to a solid support using an amino-functionalized solid support.

One more option that could potentially increase the binding capacity of our immobilization strategy is to include a polyethylene glycol spacer arm between the Sepharose and the activated carbohydrate. The spacer would help to reduce the steric crowding. The main disadvantages of this approach are that one extra step is required to do the coupling, and that the optimization of the spacer is also required.

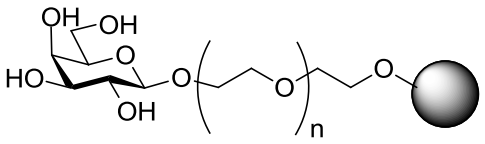


Figure 4.2 Representation of galactose immobilized to Sepharose using PEG linker.

4.2.2 Affinity chromatography method.

As we mentioned in **Chapter 3**, the galectins that we identified from Jurkat cells were isolated using columns where the HMO was immobilized with DVS; the main reason we did not use carbohydrate immobilized using p-TsH is because both projects were run in parallel. The first thing that we needed to finish was the screening of Jurkat cells using 3-FucLac, LNnT, 3'-SiaLac, and L-Fuc in order to obtain a broader binding profile for this cell line. The next logical step would be to compare the binding profile obtained using the DVS-immobilized carbohydrates with the p-TsH immobilized ones, and look for differences that could potentially be attributed to the anomeric configurations or which are the result of change to the binding capacity of the support.

We also have in mind to screen other mammalian cell lines, among them: HeLa cells, since this is a well-known system of cervix adenocarcinoma; HT29, a colorectal adenocarcinoma cell line; T84, also a colorectal adenocarcinoma cell line. All these will serve

to generate data to further validate the capacity of the method developed to selectively bind to HMO binders. Moreover, they serve as positive controls, since it is already known that cancer cell lines will overexpress certain galectins, and we have already demonstrated that galectins bind to our columns.

We believe that the screening of healthy human epithelial cell lines could open up the possibility to discovering whether HMO act as soluble decoys or they bind to epithelial cells in the intestine and block the receptors that certain pathogens as enteropathogenic *E. coli* recognize and bind to before colonizing and causing the disease. (*6*) Possible cell lines could include FHs 74 and IEC-6, both from the small intestine of healthy individuals. (*7*) Another very interesting system and of high medical relevance that could be screened using our method is necrotizing enterocolitis. It has been reported that DSLNT helps to reduce the incidence of this disease; (*3*) however, little is known about how this oligosaccharide works at molecular level. Screening of intestinal tissue against this HMO could open up the door to a deeper understanding on the causes behind this disease, and its treatment and prevention.

It is also part of our future plans to screen a series of bacterial samples. *Campylobacter jejuni* is one of the pathogens that has been extensively studied due high incidence and morbidity of the disease, especially in developing countries. (*8*) Ruiz-Palacios et al. identified the antigen responsible for the disease in a group of children in Mexico city (*9*) this system could help to validate the utility of our method for screening bacterial cells. Moreover, finding HMO with the ability to bind to this pathogen could open the possibility of developing a formula component that could prevent the occurrence of enteritis.

There are also reports of different viruses whose pathogenicity is inhibited when HMO are present in the environment. One of these viruses is norovirus (*10*), the study of the HMO-

binding receptors present in the viral caspid could also open up the possibility of developing a drug for preventing the onset of the disease. One virus of high relevance for the medical community is HIV. There is evidence that HMO can potentially inhibit the pathogenicity of this microorganism when present in the environment, again the interactions behind these observations are still to be revealed. (*11-13*)

There are studies where they have taken advantage of the specificity of galectin-3 towards 2'FL. For example, Wang et al designed a series of galectin inhibitors, they noted that when adding a 2'FL moiety to their inhibitors they gained an increase in selectivity towards galectin-3. (*14*) This example shows how having a better understanding of the molecular interaction of HMO with different binders in the body could address certain illnesses, as the ones described in **Chaper 1** or in the introduction of **Chapter 3.**

An interesting avenue of research would be to study the strength of the interactions occurring between HMO and their binders. Our collaborators in the Klassen lab have used ESI-MS to determine the affinity of HMO-toxin interactions. (*15, 16*) Understanding the strength of these interactions is an important feature to consider when designing drugs that work by disrupting binding or by competition.

Discovering HMO binders in different cellular systems will uncover the tip of the ice-berg when it comes to developing more research from the present findings. The possibilities are vast and will need to be analyzed in a case by case basis. We believe that with these two projects we can contribute in a significant way to better understand how HMO interact in the human body, and to further unveil the potential they have to improving human health.

4.3 References

1. Bode, L. (2012) Human milk oligosaccharides: Every baby needs a sugar mama, *Glycobiology 22*, 1147-1162.
2. Ashkenazi, S. (1994) Review of the Effect of Human-Milk Fractions on the Adherence of Diarrheogenic Escherichia coli to the Gut in an Animal-Model., *Israel Journal of Medical Sciences 30*, 335-338.
3. Jantscher-Krenn, E., Zherebtsov, M., Nissan, C., Goth, K., Guner, Y. S., Naidu, N., Choudhury, B., Grishin, A. V., Ford, H. R., and Bode, L. (2012) The human milk oligosaccharide disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats, *Gut 61*, 1417-1425.
4. Jantscher-Krenn, E., Lauwaet, T., Bliss, L. A., Reed, S. L., Gillin, F. D., and Bode, L. (2012) Human milk oligosaccharides reduce Entamoeba histolytica attachment and cytotoxicity in vitro, *British Journal of Nutrition 108*, 1839-1846.
5. Zheng, H., and Du, X. (2013) Reduced steric hindrance and optimized spatial arrangement of carbohydrate ligands in imprinted monolayers for enhanced protein binding, *Biochimica et Biophysica Acta (BBA) - Biomembranes 1828*, 792-800.
6. Manthey, C. F., Autran, C. A., Eckmann, L., and Bode, L. (2014) Human Milk Oligosaccharides Protect Against Enteropathogenic Escherichia coli Attachment In Vitro and EPEC Colonization in Suckling Mice, *Journal of Pediatric Gastroenterology and Nutrition 58*, 165-168 110.1097/MPG.0000000000000172.
7. Attene-Ramos, M. S., Nava, G. M., Muellner, M. G., Wagner, E. D., Plewa, M. J., and Gaskins, H. R. (2010) DNA damage and toxicogenomic analyses of hydrogen sulfide in

human intestinal epithelial FHs 74 Int cells, *Environmental and Molecular Mutagenesis 51*, 304-314.

8. Altekruse, S. F., Sternt, N. J., Fields, P. I., and Swerdlow, D., L. (1999) Campylobacter jejuni—An Emerging Foodborne Pathogen, In *Emerging Infectious Diseases*, Center for Disease Control and Prevention Atlanta.

9. Ruiz-Palacios, G. M., Cervantes, L. E., Ramos, P., Chavez-Munguia, B., and Newburg, D. S. (2003) Campylobacter jejuni Binds Intestinal H(O) Antigen (Fucα1, 2Galβ1, 4GlcNAc), and Fucosyloligosaccharides of Human Milk Inhibit Its Binding and Infection, *Journal of Biological Chemistry 278*, 14112-14120.

10. Shang, J., Piskarev, V. E., Xia, M., Huang, P., Jiang, X., Likhosherstov, L. M., Novikova, O. S., Newburg, D. S., and Ratner, D. M. (2013) Identifying human milk glycans that inhibit norovirus binding using surface plasmon resonance, *Glycobiology 23*, 1491-1498.

11. Hong, P., Ninonuevo, M. R., Lee, B., Lebrilla, C., and Bode, L. (2009) Human milk oligosaccharides reduce HIV-1-gp120 binding to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), *British Journal of Nutrition 101*, 482-486.

12. Yu, Y., Mishra, S., Song, X., Lasanajak, Y., Bradley, K. C., Tappert, M. M., Air, G. M., Steinhauer, D. A., Halder, S., Cotmore, S., Tattersall, P., Agbandje-McKenna, M., Cummings, R. D., and Smith, D. F. (2012) Functional Glycomic Analysis of Human Milk Glycans Reveals the Presence of Virus Receptors and Embryonic Stem Cell Biomarkers, *Journal of Biological Chemistry 287*, 44784-44799.

13. Adams, E. W., Ratner, D. M., Bokesch, H. R., McMahon, J. B., O'Keefe, B. R., and Seeberger, P. H. (2004) Oligosaccharide and Glycoprotein Microarrays as Tools in HIV

Glycobiology: Glycan-Dependent gp120/Protein Interactions, *Chemistry & Biology 11*, 875-881.

14. Wang, G.-N., Andre, S., Gabius, H.-J., and Murphy, P. V. (2012) Bi- to tetravalent glycoclusters: synthesis, structure-activity profiles as lectin inhibitors and impact of combining both valency and headgroup tailoring on selectivity, *Organic & Biomolecular Chemistry 10*, 6893-6907.

15. Kitova, E. N., Kitov, P. I., Paszkiewicz, E., Kim, J., Mulvey, G. L., Armstrong, G. D., Bundle, D. R., and Klassen, J. S. (2007) Affinities of Shiga toxins 1 and 2 for univalent and oligovalent Pk-trisaccharide analogs measured by electrospray ionization mass spectrometry, *Glycobiology 17*, 1127-1137.

16. Kitova, E., El-Hawiet, A., and Klassen, J. (2014) Screening Carbohydrate Libraries for Protein Interactions Using the Direct ESI-MS Assay. Applications to Libraries of Unknown Concentration, *J. Am. Soc. Mass Spectrom.*, 1-9.

Bibiography

(in alphabetical order)

1. Abraham, R. T., and Weiss, A. (2004) Jurkat T cells and development of the T-cell receptor signalling paradigm, *Nature Reviews Immunology 4*, 301-308.
2. Adams, E. W., Ratner, D. M., Bokesch, H. R., McMahon, J. B., O'Keefe, B. R., and Seeberger, P. H. (2004) Oligosaccharide and Glycoprotein Microarrays as Tools in HIV Glycobiology: Glycan-Dependent gp120/Protein Interactions, *Chemistry & Biology 11*, 875-881.
3. Ahmad, N., Gabius, H. J., Kaltner, H., André, S., Kuwabara, I., Liu, F.-T., Oscarson, S., Norberg, T., and Brewer, C. F. (2002) Thermodynamic binding studies of cell surface carbohydrate epitopes to galectins-1, -3, and -7: Evidence for differential binding specificities, *Canadian Journal of Chemistry 80*, 1096-1104.
4. Ahmad, N., Gabius, H.-J., André, S., Kaltner, H., Sabesan, S., Roy, R., Liu, B., Macaluso, F., and Brewer, C. F. (2004) Galectin-3 Precipitates as a Pentamer with Synthetic Multivalent Carbohydrates and Forms Heterogeneous Cross-linked Complexes, *Journal of Biological Chemistry 279*, 10841-10847.
5. Ahmed, H., Fink, N. E., Pohl, J., and Vasta, G. R. (1996) Galectin-1 from Bovine Spleen: Biochemical Characterization, Carbohydrate Specificity and Tissue-Specific Isoform Profiles, *Journal of Biochemistry 120*, 1007-1019.
6. Albalasmeh, A. A., Berhe, A. A., and Ghezzehei, T. A. (2013) A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry, *Carbohydrate Polymers 97*, 253-261.
7. Albrecht, S., Schols, H. A., van den Heuvel, E. G. H. M., Voragen, A. G. J., and Gruppen, H. (2011) Occurrence of oligosaccharides in feces of breast-fed babies in

their first six months of life and the corresponding breast milk, *Carbohydrate Research 346*, 2540-2550.

8. Alia, A., Ganapathy, S., and de Groot, H. M. (2009) Magic angle spinning (MAS) NMR: a new tool to study the spatial and electronic structure of photosynthetic complexes, *Photosynth Res 102*, 415-425.

9. Allen, A. K., Neuberger, A., and Sharon, N. (1973) The purification, composition and specificity of wheat-germ agglutinin, *Biochem. J. 131*, 155-162.

10. Almkvist, J., and Karlsson, A. (2002) Galectins as inflammatory mediators, *Glycoconj J 19*, 575-581.

11. Altekruse, S. F., Sternt, N. J., Fields, P. I., and Swerdlow, D., L. (1999) Campylobacter jejuni—An Emerging Foodborne Pathogen, In *Emerging Infectious Diseases*, Center for Disease Control and Prevention Atlanta.

12. Ambrosi, M., Cameron, N. R., and Davis, B. G. (2005) Lectins: tools for the molecular understanding of the glycocode, *Organic & Biomolecular Chemistry 3*, 1593-1608.

13. Anatole, A. K. (2012) Galectin-Targeted Drug Design, In *Glycobiology and Drug Design*, pp 25-66, American Chemical Society.

14. Andersson, B., Porras, O., Hanson, L. Å., Lagergård, T., and Svanborg-Edén, C. (1986) Inhibition of Attachment of Streptococcus pneumoniae and Haemophilus influenzae by Human Milk and Receptor Oligosaccharides, *Journal of Infectious Diseases 153*, 232-237.

15. André, S., Pieters, R. J., Vrasidas, I., Kaltner, H., Kuwabara, I., Liu, F.-T., Liskamp, R. M. J., and Gabius, H.-J. (2001) Wedgelike Glycodendrimers as Inhibitors of Binding of

Mammalian Galectins to Glycoproteins, Lactose Maxiclusters, and Cell Surface Glycoconjugates, *ChemBioChem 2*, 822-830.

16. Arata, Y., Hirabayashi, J., and Kasai, K.-i. (2001) Sugar Binding Properties of the Two Lectin Domains of the Tandem Repeat-type Galectin LEC-1 (N32) of Caenorhabditis elegans : Detailed Analysis by an Improved Frontal Affinity Chromatography Method, *Journal of Biological Chemistry 276*, 3068-3077.

17. Ashkenazi, S. (1994) Review of the Effect of Human-Milk Fractions on the Adherence of Diarrheogenic Escherichia coli to the Gut in an Animal-Model., *Israel Journal of Medical Sciences 30*, 335-338.

18. Attene-Ramos, M. S., Nava, G. M., Muellner, M. G., Wagner, E. D., Plewa, M. J., and Gaskins, H. R. (2010) DNA damage and toxicogenomic analyses of hydrogen sulfide in human intestinal epithelial FHs 74 Int cells, *Environmental and Molecular Mutagenesis 51*, 304-314.

19. Balkwill, F. (2004) Cancer and the chemokine network, *Nature Reviews Cancer 4*, 540-550.

20. Bamdad, C. (1998) The Use of Variable Density Self-Assembled Monolayers to Probe the Structure of a Target Molecule, *Biophysical Journal 75*, 1989-1996.

21. Barondes, S. H., Castronovo, V., Cooper, D. N. W., Cummings, R. D., Drickamer, K., Felzi, T., Gitt, M. A., Hirabayashi, J., Hughes, C., Kasai, K.-i., Leffler, H., Liu, F.-T., Lotan, R., Mercurio, A. M., Monsigny, M., Pillai, S., Poirer, F., Raz, A., Rigby, P. W. J., Rini, J. M., and Wang, J. L. (1994) Galectins: A family of animal β-galactoside-binding lectins, *Cell 76*, 597-598.

22. Barrow, H., Rhodes, J. M., and Yu, L.-G. (2011) The role of galectins in colorectal cancer progression, *International Journal of Cancer 129*, 1-8.

23. Belyakov, P. A., Shastin, A. V., and Strelenko, Y. A. (2001) NMR studies of the dynamic behavior of 2-anilino-6-chloro-4-methoxy-1,3,5-triazine in solution, *Russian Chemical Bulletin 50*, 2473-2474.

24. Benhamou, N., Chamberland, H., and Pauzé, F. J. (1990) Implication of Pectic Components in Cell Surface Interactions between Tomato Root Cells and Fusarium oxysporum f. sp. radicis-lycopersici: A Cytochemical Study by Means of a Lectin with Polygalacturonic Acid-Binding Specificity, *Plant Physiology 92*, 995-1003.

25. Bénitez, J. A., Spelbrink, R. G., Silva, A., Phillips, T. E., Stanley, C. M., Boesman-Finkelstein, M., and Finkelstein, R. A. (1997) Adherence of Vibrio cholerae to cultured differentiated human intestinal cells: an in vitro colonization model, *Infection and Immunity 65*, 3474-3477.

26. Bode, L. (2006) Recent Advances on Structure, Metabolism, and Function of Human Milk Oligosaccharides, *The Journal of Nutrition 136*, 2127-2130.

27. Bode, L. (2012) Human milk oligosaccharides: Every baby needs a sugar mama, *Glycobiology 22*, 1147-1162.

28. Bode, L., Rudloff, S., Kunz, C., Strobel, S., and Klein, N. (2004) Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a decrease in neutrophil β 2 integrin expression, *Journal of Leukocyte Biology 76*, 820-826.

29. Boggs, J. M., Wang, H., Gao, W., Arvanitis, D. N., Gong, Y., and Min, W. (2004) A glycosynapse in myelin?, *Glycoconj J 21*, 97-110.

30. Borch, R. F., Bernstein, M. D., and Durst, H. D. (1971) Cyanohydridoborate anion as a selective reducing agent, *Journal of the American Chemical Society 93*, 2897-2904.

31. Bourne, Y., Astoul, C. H., Zamboni, V., Peumans, W. J., Menu-Bouaouiche, L., Van Damme, E. J. M., Barre, A., and Rougé, P. (2002) Structural basis for the unusual carbohydrate-binding specificity of jacalin towards galactose and mannose, *Biochemical Journal 364*, 173-180.

32. Brandley, B. K., and Schnaar, R. L. (1986) Cell-surface carbohydrates in cell recognition and response, *Journal of Leukocyte Biology 40*, 97-111.

33. Brewer, C. F., Miceli, M. C., and Baum, L. G. (2002) Clusters, bundles, arrays and lattices: novel mechanisms for lectin–saccharide-mediated cellular interactions, *Current Opinion in Structural Biology 12*, 616-623.

34. Buts, L., Bouckaert, J., De Genst, E., Loris, R., Oscarson, S., Lahmann, M., Messens, J., Brosens, E., Wyns, L., and De Greve, H. (2003) The fimbrial adhesin F17-G of enterotoxigenic Escherichia coli has an immunoglobulin-like lectin domain that binds N-acetylglucosamine, *Molecular Microbiology 49*, 705-715.

35. Camby, I., Le Mercier, M., Mathiey, V., Ingrassia, L., Lefranc, F., and Kiss, R. (2008) Galectin-1 as a potential therapeutic target for cancer progression, *Drugs of the Future 33*, 1057-1069.

36. Chabot, S., Kashio, Y., Seki, M., Shirato, Y., Nakamura, K., Nishi, N., Nakamura, T., Matsumoto, R., and Hirashima, M. (2002) Regulation of galectin-9 expression and release in Jurkat T cell line cells, *Glycobiology 12*, 111-118.

37. Charville, H., Jin, J., Evans, C. W., Brimble, M. A., and Williams, D. E. (2013) The synthesis and lectin-binding properties of novel mannose-functionalised polymers, *RSC Advances 3*, 15435-15441.

38. Chaturvedi, P., Warren, C., Buescher, C., Pickering, L., and Newburg, D. (2001) Survival of Human Milk Oligosaccharides in the Intestine of Infants, In *Bioactive Components of Human Milk* (Newburg, D., Ed.), pp 315-323, Springer US.

39. Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J., and Marks, R. M. (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate, *Nature Medicine 3*, 866-871.

40. Cheng, F., Shang, J., and Ratner, D. M. (2010) A Versatile Method for Functionalizing Surfaces with Bioactive Glycans, *Bioconjugate Chemistry 22*, 50-57.

41. Coppa, G. V., Gabrielli, O., Pierani, P., Catassi, C., Carlucci, A., and Giorgi, P. L. (1993) Changes in Carbohydrate Composition in Human Milk Over 4 Months of Lactation, *Pediatrics 91*, 637-641.

42. Coppa, G. V., Pierani, P., Zampini, L., Bruni, S., Carloni, I., and Gabrielli, O. (2001) Characterization of Oligosaccharides in Milk and Feces of Breast-Fed Infants by High-Performance Anion-Exchange Chromatography, In *Bioactive Components of Human Milk* (Newburg, D., Ed.), pp 307-314, Springer US.

43. Cortejano, I., del Pozo, V., Cardaba, B., de Andres, B., Gallardo, S., del Amo, A., Arrieta, I., Jurado, A., Palomino, P., Liu, F.-T., and Lahoz, C. (1998) Galectin-3 down-regulates IL-5 gene expression on different cell types., *The Journal of Immunology 161*, 385-389.

44. Costerton, J. W., Irvin, R. T., and Cheng, K. J. (1981) The Bacterial Glycocalyx in Nature and Disease, *Annual Review of Microbiology 35*, 299-324.

45. Crocker, P. R., Paulson, J. C., and Varki, A. (2007) Siglecs and their roles in the immune system, *Nature Reviews Immunology 7*, 255-266.

46. Cuatrecasas, P. (1970) Protein Purification by Affinity Chromatography: derivatizations of agarose and polyacrylamide beads, *Journal of Biological Chemistry 245*, 3059-3065.

47. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968) Selective enzyme purification by affinity chromatography, *Proceedings of the National Academy of Sciences of the United States of America 61*, 636-643.

48. Dai, D., Nanthkumar, N. N., Newburg, D. S., and Walker, W. A. (2000) Role of Oligosaccharides and Glycoconjugates in Intestinal Host Defense, *Journal of Pediatric Gastroenterology and Nutrition 30*, S23-S33.

49. Dam, T. K., and Brewer, C. F. (2002) Thermodynamic Studies of Lectin−Carbohydrate Interactions by Isothermal Titration Calorimetry, *Chemical Reviews 102*, 387-430.

50. Dinglasan, R. R., and Jacobs-Lorena, M. (2005) Insight into a Conserved Lifestyle: Protein-Carbohydrate Adhesion Strategies of Vector-Borne Pathogens, *Infection and Immunity 73*, 7797-7807.

51. Disney, M. D., and Seeberger, P. H. (2004) Carbohydrate arrays as tools for the glycomics revolution, *Drug Discovery Today: TARGETS 3*, 151-158.

52. Dotz, V., Rudloff, S., Blank, D., Lochnit, G., Geyer, R., and Kunz, C. (2014) 13C-labeled oligosaccharides in breastfed infants' urine: Individual-, structure- and time-dependent differences in the excretion, *Glycobiology 24*, 185-194.

53. Dreywood, R. (1946) Qualitative Test for Carbohydrate Material, *Industrial & Engineering Chemistry Analytical Edition 18*, 499-499.

54. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) Colorimetric method for determination of sugars and related substances, *Analytical Chemistry 28*, 350-356.

55. Dunn, G. P., Old, L. J., and Schreiber, R. D. (2004) The Immunobiology of Cancer Immunosurveillance and Immunoediting, *Immunity 21*, 137-148.

56. Edgar, L. J. G., Dasgupta, S., and Nitz, M. (2012) Protecting-Group-Free Synthesis of Glycosyl 1–Phosphates, *Organic Letters 14*, 4226-4229.

57. Eiwegger, T., Stahl, B., Haidl, P., Schmitt, J., Boehm, G., Dehlink, E., Urbanek, R., and Szépfalusi, Z. (2010) Prebiotic oligosaccharides: In vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties, *Pediatric Allergy and Immunology 21*, 1179-1188.

58. Eiwegger, T., Stahl, B., Schmitt, J., Boehm, G., Gerstmayr, M., Pichler, J., Dehlink, E., Loibichler, C., Urbanek, R., and Szepfalusi, Z. (2004) Human Milk-Derived Oligosaccharides and Plant-Derived Oligosaccharides Stimulate Cytokine Production of Cord Blood T-Cells In Vitro, *Pediatric Research 56*, 536-540.

59. El-Hawiet, A., Kitova, E. N., and Klassen, J. S. (2013) Quantifying Protein Interactions with Isomeric Carbohydrate Ligands Using a Catch and Release Electrospray Ionization-Mass Spectrometry Assay, *Analytical Chemistry 85*, 7637-7644.

60. El-Hawiet, A., Shoemaker, G. K., Daneshfar, R., Kitova, E. N., and Klassen, J. S. (2011) Applications of a Catch and Release Electrospray Ionization Mass Spectrometry Assay for Carbohydrate Library Screening, *Analytical Chemistry 84*, 50-58.

61. Engfer, M. B., Stahl, B., Finke, B., Sawatzki, G., and Daniel, H. (2000) Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract, *The American Journal of Clinical Nutrition 71*, 1589-1596.

62. Englis, D., and Becker, H. (1943) Sugar Analysis by Alkaline Ferricyanide Method - Determination of Ferrocyanide by Iodometric and Other Procedures, *Industrial & Engineering Chemistry Analytical Edition 15*, 262-264.

63. Ernst-Cabrera, K., and Wilchek, M. (1988) Immunoaffinity chromatography and its application for the purifiction of pharmaceutically active proteins, *Medical Science Research 16*, 305-310.

64. Esko, J. D. (1991) Genetic analysis of proteoglycan structure, function and metabolism, *Current Opinion in Cell Biology 3*, 805-816.

65. Esko, J. D., Kimata, K., and Lindahl, U. (2009) Chapter 16 Proteoglycans and Sulfated Glycosaminoglycans, In *Essentials of Glycobiology* (Varki, A., Cummings, R., Esko, J. D., Freeze, H. H., Stanley, C. M., Bertozzi, C. R., Hart, G. W., and Etzler, M. E., Eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

66. Finlay, T. H., Troll, V., Levy, M., Johnson, A. J., and Hodgins, L. T. (1978) New methods for the preparation of biospecific adsorbents and immobilized enzymes utilizing trichloro-s-triazine, *Analytical Biochemistry 87*, 77-90.

67. Ford, C. F., Suominen, I., and Glatz, C. E. (1991) Fusion tails for the recovery and purification of recombinant proteins, *Protein Expression and Purification 2*, 95-107.

68. Fornstedt, N., and Porath, J. (1975) Characterization studies on a new lectin found in seeds of Vicia ervilia, *FEBS Letters 57*, 187-191.

69. Forsman, H., Islander, U., Andréasson, E., Andersson, A., Önnheim, K., Karlström, A., Sävman, K., Magnusson, M., Brown, K. L., and Karlsson, A. (2011) Galectin 3 aggravates joint inflammation and destruction in antigen-induced arthritis, *Arthritis & Rheumatism 63*, 445-454.

70. Fukumori, T., Takenaka, Y., Yoshii, T., Kim, H.-R. C., Hogan, V., Inohara, H., Kagawa, S., and Raz, A. (2003) CD29 and CD7 Mediate Galectin-3-Induced Type II T-Cell Apoptosis, *Cancer Research 63*, 8302-8311.

71. Gagneux, P., and Varki, A. (1999) Evolutionary considerations in relating oligosaccharide diversity to biological function, *Glycobiology 9*, 747-755.

72. Gahmberg, C. G., and Tolvanen, M. (1996) Why mammalian cell surface proteins are glycoproteins, *Trends in Biochemical Sciences 21*, 308-311.

73. Gallagher, S. R. (2001) One-Dimensional SDS Gel Electrophoresis of Proteins, In *Current Protocols in Protein Science*, John Wiley & Sons, Inc.

74. German, J. B., Freeman, S. L., Lebrilla, C. B., and Mills, D. A. (2007) Human Milk Oligosaccharides: evolution, structures and bioselectivity as substrates for intestinal bacteria, In *62nd Nestle Nutrition Workshop* (Bier, D. M., German, J. B., and B., L., Eds.), pp 205-222, Nestec Ltd, Helsinki.

75. Gibson, G., and Wang, X. (1994) Regulatory effects of bifidobacteria on the growth of other colonic bacteria, *Journal of Applied Bacteriology (United Kingdom)*.

76. Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A., and Roberfroid, M. B. (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics, *Nutrition Research Reviews 17*, 259-275.

77. Gibson, G. R., and Wang, X. (1994) Regulatory effects of bifidobacteria on the growth of other colonic bacteria, *Journal of Applied Bacteriology 77*, 412-420.

78. Ginsberg, C., Brown, S., and Walker, S. (2008) Bacterial Cell Wall Components, In *Glycoscience* (Fraser-Reid, B., Tatsuta, K., and Thiem, J., Eds.), pp 1535-1600, Springer Berlin Heidelberg.

79. Glinsky, V. V., Glinsky, G. V., Glinskii, O. V., Huxley, V. H., Turk, J. R., Mossine, V. V., Deutscher, S. L., Pienta, K. J., and Quinn, T. P. (2003) Intravascular Metastatic Cancer Cell Homotypic Aggregation at the Sites of Primary Attachment to the Endothelium, *Cancer Research 63*, 3805-3811.

80. Goldstein, I. J., Reichert, C. M., and Misaki, A. (1974) Interaction of Concavalin A with model substrates, *Annals of the New York Academy of Sciences 234*, 283-296.

81. Grant, O. C., Smith, H. M., Firsova, D., Fadda, E., and Woods, R. J. (2014) Presentation, presentation, presentation! Molecular-level insight into linker effects on glycan array screening data, *Glycobiology 24*, 17-25.

82. Gray, G. R. (1974) The direct coupling of oligosaccharides to proteins and derivatized gels, *Archives of Biochemistry and Biophysics 163*, 426-428.

83. Gray, G. R. (1978) [12] Antibodies to carbohydrates: Preparation of antigens by coupling carbohydrates to proteins by reductive amination with cyanoborohydride, In *Methods in Enzymology* (Victor, G., Ed.), pp 155-160, Academic Press.

84. Gudmundsdottir, A. V., and Nitz, M. (2008) Protecting Group Free Glycosidations Using p-Toluenesulfonohydrazide Donors, *Organic Letters 10*, 3461-3463.

85. Gustafsson, A., Hultberg, A., Sjöström, R., Kacskovics, I., Breimer, M. E., Borén, T., Hammarström, L., and Holgersson, J. (2006) Carbohydrate-dependent inhibition of Helicobacter pylori colonization using porcine milk, *Glycobiology 16*, 1-10.

86. Hadari, Y. R., Arbel-Goren, R., Levy, Y., Amsterdam, A., Alon, R., Zakut, R., and Zick, Y. (2000) Galectin-8 binding to integrins inhibits cell adhesion and induces apoptosis, *Journal of Cell Science 113*, 2385-2397.

87. Hallgren, P., Lindberg, B. S., and Lundblad, A. (1977) Quantitation of some urinary oligosaccharides during pregnancy and lactation, *Journal of Biological Chemistry 252*, 1034-1040.

88. Harmsen, H. J. M., Wildeboer–Veloo, A. C. M., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G., and Welling, G. W. (2000) Analysis of Intestinal Flora Development in Breast-Fed and Formula-Fed Infants by Using Molecular Identification and Detection Methods, *Journal of Pediatric Gastroenterology and Nutrition 30*, 61-67.

89. Harrington, P. R., Lindesmith, L., Yount, B., Moe, C. L., and Baric, R. S. (2002) Binding of Norwalk Virus-Like Particles to ABH Histo-Blood Group Antigens Is Blocked by Antisera from Infected Human Volunteers or Experimentally Vaccinated Mice, *Journal of Virology 76*, 12335-12343.

90. Hart, G. W. (1992) Glycosylation, *Current Opinion in Cell Biology 4*, 1017-1023.

91. Hasan, S. S., Ashraf, G. M., and Banu, N. (2007) Galectins – Potential targets for cancer therapy, *Cancer Letters 253*, 25-33.

92. He, X. G., Gerona-Navarro, G., and Jaffrey, S. R. (2005) Ligand Discovery Using Small Molecule Microarrays, *Journal of Pharmacology and Experimental Therapeutics 313*, 1-7.

93. Hermanson, G. T. (2008) Chapter 14 - Microparticles and Nanoparticles, In *Bioconjugate Techniques (Second Edition)* (Hermanson, G. T., Ed.), pp 582-626, Academic Press, New York.

94. Hermanson, G. T. (2013) Chapter 15 - Immobilization of Ligands on Chromatography Supports, In *Bioconjugate Techniques (Third edition)* (Hermanson, G. T., Ed.), pp 589-740, Academic Press, Boston.

95. Hermanson, G. T. (2013) Chapter 9 - Cross-Bridges and Cleavable Reagent Systems, In *Bioconjugate Techniques (Third edition)* (Hermanson, G. T., Ed.), pp 387-394, Academic Press, Boston.

96. Herrera-Insua, I., Gomez, H., Diaz-Gonzalez, V., Chaturvedi, P., Newburg, D., and Cleary, T. (2001) Human Milk Lipids Bind Shiga Toxin, In *Bioactive Components of Human Milk* (Newburg, D., Ed.), pp 333-339, Springer US.

97. Hirabayashi, J. (2008) Concept, Strategy and Realization of Lectin-based Glycan Profiling, *Journal of Biochemistry 144*, 139-147.

98. Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W. E. G., Yagi, F., and Kasai, K.-i. (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography, *Biochimica et Biophysica Acta (BBA) - General Subjects 1572*, 232-254.

99. Hirabayashi, J., and Kasai, K.-i. (2002) Separation technologies for glycomics, *Journal of Chromatography B 771*, 67-87.

100. Hofstadler, S. A., and Sannes-Lowery, K. A. (2006) Applications of ESI-MS in drug discovery: interrogation of noncovalent complexes, *Nature Reviewas Drug Discovery 5*, 585-595.

101. Holmgren, J., Svennerholm, A. M., and Lindblad, M. (1983) Receptor-like glycocompounds in human milk that inhibit classical and El Tor Vibrio cholerae cell adherence (hemagglutination), *Infection and Immunity 39*, 147-154.

102. Hong, P., Ninonuevo, M. R., Lee, B., Lebrilla, C., and Bode, L. (2009) Human milk oligosaccharides reduce HIV-1-gp120 binding to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), *British Journal of Nutrition 101*, 482-486.

103. Hook, M., Kjellen, L., Johansson, S., and Robinson, J. (1984) Cell-Surface Glycosaminoglycans, *Annual Review of Biochemistry 53*, 847-869.

104. Horlacher, T., and Seeberger, P. H. (2008) Carbohydrate arrays as tools for research and diagnostics, *Chemical Society Reviews 37*, 1414-1422.

105. Hsu, K.-L., Gildersleeve, J. C., and Mahal, L. K. (2008) A simple strategy for the creation of a recombinant lectin microarray, *Molecular BioSystems 4*, 654-662.

106. Hu, C.-Y., Chang, S.-K., Wu, C.-S., Tsai, W.-I., and Hsu, P.-N. (2011) Galectin-3 gene (LGALS3) +292C allele is a genetic predisposition factor for rheumatoid arthritis in Taiwan, *Clin Rheumatol 30*, 1227-1233.

107. Ideo, H., Seko, A., Ishizuka, I., and Yamashita, K. (2003) The N-terminal carbohydrate recognition domain of galectin-8 recognizes specific glycosphingolipids with high affinity, *Glycobiology 13*, 713-723.

108. Ideo, H., Seko, A., Ohkura, T., Matta, K. L., and Yamashita, K. (2002) High-affinity binding of recombinant human galectin-4 to SO3⁻→3Galβ1→3GalNAc pyranoside, *Glycobiology 12*, 199-208.

109. Ilarregui, J. M., Bianco, G. A., Toscano, M. A., and Rabinovich, G. A. (2005) The coming of age of galectins as immunomodulatory agents: impact of these carbohydrate binding proteins in T cell physiology and chronic inflammatory disorders, *Annals of the Rheumatic Diseases 64*, iv96-iv103.

110. Imberty, A., Wimmerová, M., Mitchell, E. P., and Gilboa-Garber, N. (2004) Structures of the lectins from Pseudomonas aeruginosa: insights into the molecular basis for host glycan recognition, *Microbes and Infection 6*, 221-228.

111. Ito, K., Scott, S., Cutler, S., Dong, L.-F., Neuzil, J., Blanchard, H., and Ralph, S. (2011) Thiodigalactoside inhibits murine cancers by concurrently blocking effects of galectin-1 on immune dysregulation, angiogenesis and protection against oxidative stress, *Angiogenesis 14*, 293-307.

112. Iwamori, M., Takamizawa, K., Momoeda, M., Iwamori, Y., and Taketani, Y. (2008) Gangliosides in human, cow and goat milk, and their abilities as to neutralization of cholera toxin and botulinum type A neurotoxin, *Glycoconj J 25*, 675-683.

113. Janeway, C. A., Travers, P., and Walport, M. (2001) The components of the immune system, In *Immunobiology: The Immune System in Health and Disease.* 5th edition ed., Garland Science, New York.

114. Jantscher-Krenn, E., Lauwaet, T., Bliss, L. A., Reed, S. L., Gillin, F. D., and Bode, L. (2012) Human milk oligosaccharides reduce Entamoeba histolytica attachment and cytotoxicity in vitro, *British Journal of Nutrition 108*, 1839-1846.

115. Jantscher-Krenn, E., Zherebtsov, M., Nissan, C., Goth, K., Guner, Y. S., Naidu, N., Choudhury, B., Grishin, A. V., Ford, H. R., and Bode, L. (2012) The human milk oligosaccharide disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats, *Gut 61*, 1417-1425.

116. Jobling, M. G., and Holmes, R. K. (2002) Mutational Analysis of Ganglioside GM1-Binding Ability, Pentamer Formation, and Epitopes of Cholera Toxin B (CTB) Subunits and CTB/Heat-Labile Enterotoxin B Subunit Chimeras, *Infection and Immunity 70*, 1260-1271.

117. Kamio, Y., and Nikaido, H. (1976) Outer membrane of Salmonella typhimurium: accessibility of phospholipid head groups to phospholipase C and cyanogen bromide activated dextran in the external medium, *Biochemistry 15*, 2561-2570.

118. Khan, A. S., Kniep, B., Oelschlaeger, T. A., Van Die, I., Korhonen, T., and Hacker, J. (2000) Receptor Structure for F1C Fimbriae of Uropathogenic Escherichia coli, *Infection and Immunity 68*, 3541-3547.

119. Kitova, E., El-Hawiet, A., and Klassen, J. (2014) Screening Carbohydrate Libraries for Protein Interactions Using the Direct ESI-MS Assay. Applications to Libraries of Unknown Concentration, *J. Am. Soc. Mass Spectrom.*, 1-9.

120. Kitova, E. N., Kitov, P. I., Paszkiewicz, E., Kim, J., Mulvey, G. L., Armstrong, G. D., Bundle, D. R., and Klassen, J. S. (2007) Affinities of Shiga toxins 1 and 2 for univalent and oligovalent Pk-trisaccharide analogs measured by electrospray ionization mass spectrometry, *Glycobiology 17*, 1127-1137.

121. Klein, N., Schwertmann, A., Peters, M., Kunz, C., and Strobe, S. (2002) Immunomodulatory Effects of Breast Milk Oligosaccharides, In *Short and Long Term*

Effects of Breast Feeding on Child Health (Koletzko, B., Michaelsen, K., and Hernell, O., Eds.), pp 251-259, Springer US.

122. Ko, J., and Splitter, G. A. (2003) Molecular Host-Pathogen Interaction in Brucellosis: Current Understanding and Future Approaches to Vaccine Development for Mice and Humans, *Clinical Microbiology Reviews 16*, 65-78.

123. Koliwer-Brandl, H., Siegert, N., Umnus, K., Kelm, A., Tolkach, A., Kulozik, U., Kuballa, J., Cartellieri, S., and Kelm, S. (2011) Lectin inhibition assays for the analysis of bioactive milk sialoglycoconjugates, *International Dairy Journal 21*, 413-420.

124. Kubota, K., Kosaka, T., and Ichikawa, K. (2009) Shotgun Protein Analysis by Liquid Chromatography-Tandem Mass Spectrometry, In *Two-Dimensional Electrophoresis Protocols* (Tyther, R., and Sheehan, D., Eds.), pp 483-494, Humana Press.

125. Kumar, A., Srivastava, K., Raja Kumar, S., Siddiqi, M. I., Puri, S. K., Sexana, J. K., and Chauhan, P. M. S. (2011) 4-Anilinoquinoline triazines: A novel class of hybrid antimalarial agents, *European Journal of Medicinal Chemistry 46*, 676-690.

126. Kumari, K., Gulati, S., Smith, D., Gulati, U., Cummings, R., and Air, G. (2007) Receptor binding specificity of recent human H3N2 influenza viruses, *Virology Journal 4*, 42-53.

127. Kunz, C., Rudloff, S., Baier, W., Klein, N., and Strobel, S. (2000) OLIGOSACCHARIDES IN HUMAN MILK: Structural, Functional, and Metabolic Aspects, *Annual Review of Nutrition 20*, 699-722.

128. Kunz, C., Rudloff, S., Schad, W., and Braun, D. (1999) Lactose-derived oligosaccharides in the milk of elephants: comparison with human milk, *British Journal of Nutrition 82*, 391-399.

129. Kuwabara, I., Sano, H., and Liu, F.-T. (2003) Functions of Galectins in Cell Adhesion and Chemotaxis, In *Methods in Enzymology* (Yuan, C. L., and Reiko, T. L., Eds.), pp 532-552, Academic Press.

130. Lacy, D. B., and Stevens, R. C. (1999) Sequence homology and structural analysis of the clostridial neurotoxins, *Journal of Molecular Biology 291*, 1091-1104.

131. Lægreid, A., and Kolstø Otnæss, A.-B. (1987) Trace amounts of ganglioside GM1 in human milk inhibit enterotoxins from Vibrio cholerae and Escherichia coli, *Life Sciences 40*, 55-62.

132. Lane, J. A., Mehra, R. K., Carrington, S. D., and Hickey, R. M. (2011) Development of biosensor-based assays to identify anti-infective oligosaccharides, *Analytical Biochemistry 410*, 200-205.

133. Laws, D. D., Bitter, H.-M. L., and Jerschow, A. (2002) Solid-State NMR Spectroscopic Methods in Chemistry, *Angewandte Chemie International Edition 41*, 3096-3129.

134. Leavitt, S., and Freire, E. (2001) Direct measurement of protein binding energetics by isothermal titration calorimetry, *Current Opinion in Structural Biology 11*, 560-566.

135. Lee, Y. C., and Lee, R. T. (1995) Carbohydrate-Protein Interactions: Basis of Glycobiology, *Accounts of Chemical Research 28*, 321-327.

136. Leffler, H., and Barondes, S. H. (1986) Specificity of binding of three soluble rat lung lectins to substituted and unsubstituted mammalian beta-galactosides, *Journal of Biological Chemistry 261*, 10119-10126.

137. Leffler, H., Carlsson, S., Hedlund, M., Qian, Y., and Poirier, F. (2002) Introduction to galectins, *Glycoconj J 19*, 433-440.

138. Ley, K. (2003) The role of selectins in inflammation and disease, *Trends in Molecular Medicine 9*, 263-268.

139. Liang, K., and Chen, Y. (2012) Elegant Chemistry to Directly Anchor Intact Saccharides on Solid Surfaces Used for the Fabrication of Bioactivity-Conserved Saccharide Microarrays, *Bioconjugate Chemistry 23*, 1300-1308.

140. Lin, H., Kitova, E., and Klassen, J. (2014) Measuring Positive Cooperativity Using the Direct ESI-MS Assay. Cholera Toxin B Subunit Homopentamer Binding to GM1 Pentasaccharide, *J. Am. Soc. Mass Spectrom. 25*, 104-110.

141. Linman, M. J., Taylor, J. D., Yu, H., Chen, X., and Cheng, Q. (2008) Surface Plasmon Resonance Study of Protein−Carbohydrate Interactions Using Biotinylated Sialosides, *Analytical Chemistry 80*, 4007-4013.

142. Liu, F.-T., Hsu, D. K., Yang, R.-Y., Chen, H.-Y., and Saegusa, J. (2008) Galectins in Regulation of Inflammation and Immunity, In *Galectins*, pp 97-113, John Wiley & Sons, Inc.

143. Liu, F.-T., and Rabinovich, G. A. (2005) Galectins as modulators of tumour progression, *Nature Reviews Cancer 5*, 29-41.

144. Liu, Y., Palma, A. S., and Feizi, T. (2009) Carbohydrate microarrays: key developments in glycobiology, *Biological Chemistry 390*, 647-656.

145. LoCascio, R. G., Ninonuevo, M. R., Freeman, S. L., Sela, D. A., Grimm, R., Lebrilla, C. B., Mills, D. A., and German, J. B. (2007) Glycoprofiling of Bifidobacterial Consumption of Human Milk Oligosaccharides Demonstrates Strain Specific, Preferential Consumption of Small Chain Glycans Secreted in Early Human Lactation, *Journal of Agricultural and Food Chemistry 55*, 8914-8919.

146. Loka, R. S., and Cairo, C. W. (2010) Immobilization of carbohydrate epitopes for surface plasmon resonance using the Staudinger ligation, *Carbohydrate Research 345*, 2641-2647.

147. Lopez-Jaramillo, F. J., Ortega-Muñoz, M., Megia-Fernandez, A., Hernandez-Mateo, F., and Santoyo-Gonzalez, F. (2012) Vinyl Sulfone Functionalization: A Feasible Approach for the Study of the Lectin–Carbohydrate Interactions, *Bioconjugate Chemistry 23*, 846-855.

148. Lucas, A., and Cole, T. J. (1990) Breast milk and neonatal necrotising enterocolitis, *The Lancet 336*, 1519-1523.

149. Lucas, A., Morley, R., Cole, T. J., Lucas, P. J., Gore, S. M., Crowle, P., Pearse, R., Boon, A. J., and Powell, R. (1990) Early diet in preterm babies and developmental status at 18 months, *The Lancet 335*, 1477-1481.

150. Luft, J. H. (1966) Fine structures of capillary and endocapillary layer as revealed by ruthenium red, *Federation proceedings 25*.

151. Luhn, K., Wild, M. K., Eckhardt, M., Gerardy-Schahn, R., and Vestweber, D. (2001) The gene defective in leukocyte adhesion deficiency II encodes a putative GDP-fucose transporter, *Nature Genetics 28*, 69-72.

152. MacBeath, G., and Schreiber, S. L. (2000) Printing Proteins as Microarrays for High-Throughput Function Determination, *Science 289*, 1760-1763.

153. Mahanta, S. K., Sastry, M. V., and Surolia, A. (1990) Topography of the combining region of a Thomsen-Friedenreich-antigen-specific lectin jacalin (Artocarpus integrifolia agglutinin). A thermodynamic and circular-dichroism spectroscopic study, *Biochemical Journal 265*, 831-840.

154. Mammen, M., Choi, S.-K., and Whitesides, G. M. (1998) Polyvalent Interactions in Biological Systems: Implications for Design and Use of Multivalent Ligands and Inhibitors, *Angewandte Chemie International Edition 37*, 2754-2794.

155. Mandrell, R. E., Griffiss, J. M., and Macher, B. A. (1988) Lipooligosaccharides (LOS) of Neisseria gonorrhoeae and Neisseria meningitidis have components that are immunochemically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes, *The Journal of Experimental Medicine 168*, 107-126.

156. Manthey, C. F., Autran, C. A., Eckmann, L., and Bode, L. (2014) Human Milk Oligosaccharides Protect Against Enteropathogenic Escherichia coli Attachment In Vitro and EPEC Colonization in Suckling Mice, *Journal of Pediatric Gastroenterology and Nutrition 58*, 165-168 110.1097/MPG.0000000000000172.

157. Marchler-Bauer, A., Zheng, C., Chitsaz, F., Derbyshire, M. K., Geer, L. Y., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., and Lanczycki, C. J. (2012) CDD: conserved domains and protein three-dimensional structure, *Nucleic acids research*, gks1243.

158. Marcotte, E. M. (2007) How do shotgun proteomics algorithms identify proteins?, *Nature Biotechnology 25*, 755-757.

159. Marjeta Urh, D. S., Kate Zhao. (2009) Affinity Chromatography: General Methods, In *Guide to Protein Purification* (Richard R. Burgess, M. P. D., Ed.).

160. Markowska, A. I., Jefferies, K. C., and Panjwani, N. (2011) Galectin-3 Protein Modulates Cell Surface Expression and Activation of Vascular Endothelial Growth

Factor Receptor 2 in Human Endothelial Cells, *Journal of Biological Chemistry 286*, 29913-29921.

161. Markowska, A. I., Liu, F.-T., and Panjwani, N. (2010) Galectin-3 is an important mediator of VEGF- and bFGF-mediated angiogenic response, *The Journal of Experimental Medicine 207*, 1981-1993.

162. Martín, R., Heilig, H. G. H. J., Zoetendal, E. G., Jiménez, E., Fernández, L., Smidt, H., and Rodríguez, J. M. (2007) Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women, *Research in Microbiology 158*, 31-37.

163. Masters, J. R. (2002) HeLa cells 50 years on: the good, the bad and the ugly, *Nature Reviews Cancer 2*, 315-319.

164. Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., and Lee, Y. C. (2005) Carbohydrate analysis by a phenol–sulfuric acid method in microplate format, *Analytical Biochemistry 339*, 69-72.

165. Matzuk, M. M., Krieger, M., Corless, C. L., and Boime, I. (1987) Effects of preventing O-glycosylation on the secretion of human chorionic gonadotropin in Chinese hamster ovary cells, *Proceedings of the National Academy of Sciences of the United States of America 84*, 6354-6358.

166. McMillan, S. J., and Crocker, P. R. (2008) CD33-related sialic-acid-binding immunoglobulin-like lectins in health and disease, *Carbohydrate Research 343*, 2050-2056.

167. Mecozzi, M. (2005) Estimation of total carbohydrate amount in environmental samples by the phenol–sulphuric acid method assisted by multivariate calibration, *Chemometrics and Intelligent Laboratory Systems 79*, 84-90.

168. Meissner, F., and Mann, M. (2014) Quantitative shotgun proteomics: considerations for a high-quality workflow in immunology, *Nature Immunology 15*, 112-117.

169. Moll, D., Schweinsberg, S., Hammann, C., and Herberg, F. W. (2007) Comparative thermodynamic analysis of cyclic nucleotide binding to protein kinase A, *Biological Chemistry 388*, 163-172.

170. Morales-Sanfrutos, J., Lopez-Jaramillo, J., Ortega-Munoz, M., Megia-Fernandez, A., Perez-Balderas, F., Hernandez-Mateo, F., and Santoyo-Gonzalez, F. (2010) Vinyl sulfone: a versatile function for simple bioconjugation and immobilization, *Organic & Biomolecular Chemistry 8*, 667-675.

171. Morrow, A. L., Ruiz-Palacios, G. M., Altaye, M., Jiang, X., Lourdes Guerrero, M., Meinzen-Derr, J. K., Farkas, T., Chaturvedi, P., Pickering, L. K., and Newburg, D. S. (2004) Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants, *The Journal of Pediatrics 145*, 297-303.

172. Mysore, J. V., Wigginton, T., Simon, P. M., Zopf, D., Heman-Ackah, L. M., and Dubois, A. Treatment of Helicobacter pylori infection in rhesus monkeys using a novel antiadhesion compound, *Gastroenterology 117*, 1316-1325.

173. Naarding, M. A., Ludwig, I. S., Groot, F., Berkhout, B., Geijtenbeek, T. B. H., Pollakis, G., and Paxton, W. A. (2005) Lewis X component in human milk binds DC-SIGN and inhibits HIV-1 transfer to CD4+ T lymphocytes, *The Journal of Clinical Investigation 115*, 3256-3264.

174. Nagae, M., Nishi, N., Nakamura-Tsuruta, S., Hirabayashi, J., Wakatsuki, S., and Kato, R. (2008) Structural Analysis of the Human Galectin-9 N-terminal Carbohydrate

Recognition Domain Reveals Unexpected Properties that Differ from the Mouse Orthologue, *Journal of Molecular Biology 375*, 119-135.

175. Nagata, Y., and Burger, M. M. (1974) Wheat Germ Agglutinin: molecular characteristics and specificity for sugar binding, *Journal of Biological Chemistry 249*, 3116-3122.

176. Nascimento, K. S., Cunha, A. I., Nascimento, K. S., Cavada, B. S., Azevedo, A. M., and Aires-Barros, M. R. (2012) An overview of lectins purification strategies, *Journal of Molecular Recognition 25*, 527-541.

177. Newburg, D. S., Ashkenazi, S., and Cleary, T. G. (1992) Human Milk Contains the Shiga Toxin and Shiga-like Toxin Receptor Glycolipid Gb3, *The Journal of Infectious Diseases 166*, 832-836.

178. Newlaczyl, A. U., and Yu, L.-G. (2011) Galectin-3 – A jack-of-all-trades in cancer, *Cancer Letters 313*, 123-128.

179. Ng, E. S. M., Chan, N. W. C., Lewis, D. F., Hindsgaul, O., and Schriemer, D. C. (2007) Frontal affinity chromatography[mdash]mass spectrometry, *Nature Protocols 2*, 1907-1917.

180. Nilsson, J., Ståhl, S., Lundeberg, J., Uhlén, M., and Nygren, P.-å. (1997) Affinity Fusion Strategies for Detection, Purification, and Immobilization of Recombinant Proteins, *Protein Expression and Purification 11*, 1-16.

181. Norambuena, A., Metz, C., Vicuña, L., Silva, A., Pardo, E., Oyanadel, C., Massardo, L., González, A., and Soza, A. (2009) Galectin-8 Induces Apoptosis in Jurkat T Cells by Phosphatidic Acid-mediated ERK1/2 Activation Supported by Protein Kinase A Down-regulation, *Journal of Biological Chemistry 284*, 12670-12679.

182. Old, W. M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K. G., Mendoza, A., Sevinsky, J. R., Resing, K. A., and Ahn, N. G. (2005) Comparison of Label-free Methods for Quantifying Human Proteins by Shotgun Proteomics, *Molecular & Cellular Proteomics 4*, 1487-1502.

183. O'Shannessy, D. J., Brighamburke, M., Soneson, K. K., Hensley, P., and Brooks, I. (1993) Determination of Rate and Equilibrium Binding Constants for Macromolecular Interactions Using Surface Plasmon Resonance: Use of Nonlinear Least Squares Analysis Methods, *Analytical Biochemistry 212*, 457-468.

184. Otnaess, A. B., Laegreid, A., and Ertresvåg, K. (1983) Inhibition of enterotoxin from Escherichia coli and Vibrio cholerae by gangliosides from human milk, *Infection and Immunity 40*, 563-569.

185. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein, *Protein Science 4*, 2411-2423.

186. Panagiotopoulos, C., and Sempere, R. (2005) Analytical methods for the determination of sugars in marine samples: A historial perspective and future direction, *Limnology and Oceanography: Methods 3*, 419-454.

187. Paulson, J. C. (1989) Glycoproteins: what are the sugar chains for?, *Trends in Biochemical Sciences 14*, 272-276.

188. Perillo, N. L., Pace, K. E., Seilhamer, J. J., and Baum, L. G. (1995) Apoptosis of T cells mediated by galectin-1, *Nature 378*, 736-739.

189. Platt, D., Witczak, Z. J., and Klesov, A. A. (2008) *Galectins*, Wiley, Hoboken, N.J.

190. Pohl, N. L., and Kiessling, L. L. (1999) Scope of multivalent ligand function: lactose-bearing neoglycopolymers by ring-opening metathesis polymerization, *Synthesis*, 1515-1519.

191. Porath, J., Axen, R., and Ernback, S. (1967) Chemical Coupling of Proteins to Agarose, *Nature 215*, 1491-1492.

192. Porath, J., Janson, J.-C., and Låås, T. (1971) Agar derivatives for chromatography, electrophoresis and gel-bound enzymes: I. Desulphated and reduced cross-linked agar and agarose in spherical bead form, *Journal of Chromatography A 60*, 167-177.

193. Rabinovich, G. A. (1999) Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy, *Cell Death Differ 6*, 711-721.

194. Rabinovich, G. A., Baum, L. G., Tinari, N., Paganelli, R., Natoli, C., Liu, F.-T., and Iacobelli, S. (2002) Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response?, *Trends in Immunology 23*, 313-320.

195. Rabinovich, G. A., Iglesias, M. M., Modesti, N. M., Castagna, L. F., Wolfenstein-Todel, C., Riera, C. M., and Sotomayor, C. E. (1998) Activated Rat Macrophages Produce a Galectin-1-Like Protein That Induces Apoptosis of T Cells: Biochemical and Functional Characterization, *The Journal of Immunology 160*, 4831-4840.

196. Rabinovich, G. A., Rubinstein, N., and Toscano, M. A. (2002) Role of galectins in inflammatory and immunomodulatory processes, *Biochimica et Biophysica Acta (BBA) - General Subjects 1572*, 274-284.

197. Rademacher, T. W., Parekh, R. B., and Dwek, R. A. (1988) Glycobiology, *Annual Review of Biochemistry 57*, 785-838.

198. Raetz, C. R. H., and Whitfield, C. (2002) Lipopolysaccharide Endotoxins, *Annual Review of Biochemistry 71*, 635-700.

199. Rao, P., and Pattabiraman, T. N. (1989) Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses, *Analytical Biochemistry 181*, 18-22.

200. Reitsma, S., Slaaf, D., Vink, H., van Zandvoort, M. M. J., and oude Egbrink, M. A. (2007) The endothelial glycocalyx: composition, functions, and visualization, *Pflugers Arch - Eur J Physiol 454*, 345-359.

201. Rijnierse, A., Jeurink, P. V., van Esch, B. C. A. M., Garssen, J., and Knippels, L. M. J. (2011) Food-derived oligosaccharides exhibit pharmaceutical properties, *European Journal of Pharmacology 668, Supplement 1*, S117-S123.

202. Rudloff, S., Pohlentz, G., Borsch, C., Lentze, M. J., and Kunz, C. (2012) Urinary excretion of in vivo 13C-labelled milk oligosaccharides in breastfed infants, *British Journal of Nutrition 107*, 957-963.

203. Rudloff, S., Pohlentz, G., Diekmann, L., Egge, H., and Kunz, C. (1996) Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula, *Acta Pædiatrica 85*, 598-603.

204. Rudloff, S., Stefan, C., Pohlentz, G., and Kunz, C. (2002) Detection of ligands for selectins in the oligosaccharide fraction of human milk, *Eur J Nutr 41*, 85-92.

205. Ruiz-Palacios, G. M., Cervantes, L. E., Ramos, P., Chavez-Munguia, B., and Newburg, D. S. (2003) Campylobacter jejuni Binds Intestinal H(O) Antigen (Fucα1, 2Galβ1, 4GlcNAc), and Fucosyloligosaccharides of Human Milk Inhibit Its Binding and Infection, *Journal of Biological Chemistry 278*, 14112-14120.

206. Ryan, C. A., and Farmer, E. E. (1991) Oligosaccharide Signals in Plants: A Current Assessment, *Annual Review of Plant Physiology and Plant Molecular Biology 42*, 651-674.

207. Sacchettini, J. C., Baum, L. G., and Brewer, C. F. (2001) Multivalent Protein−Carbohydrate Interactions. A New Paradigm for Supermolecular Assembly and Signal Transduction, *Biochemistry 40*, 3009-3015.

208. Salak Asghari, F., and Yoshida, H. (2006) Acid-Catalyzed Production of 5-Hydroxymethyl Furfural from d-Fructose in Subcritical Water, *Industrial & Engineering Chemistry Research 45*, 2163-2173.

209. Sanchez-Juanes, F., Alonso, J. M., Zancada, L., and Hueso, P. (2009) Glycosphingolipids from bovine milk and milk fat globule membranes: a comparative study. Adhesion to enterotoxigenic Escherichia coli strain, *Biological Chemistry 390*, 31-40.

210. Schroten, H., Hanisch, F. G., Plogmann, R., Hacker, J., Uhlenbruck, G., Nobis-Bosch, R., and Wahn, V. (1992) Inhibition of adhesion of S-fimbriated Escherichia coli to buccal epithelial cells by human milk fat globule membrane components: a novel aspect of the protective function of mucins in the nonimmunoglobulin fraction, *Infection and Immunity 60*, 2893-2899.

211. Sehrawat, S., Reddy, P. B. J., Rajasagi, N., Suryawanshi, A., Hirashima, M., and Rouse, B. T. (2010) Galectin-9/TIM-3 Interaction Regulates Virus-Specific Primary and Memory CD8+ T Cell Response, *PLoS Pathogens 6*, e1000882.

212. Seo, J. H., Adachi, K., Lee, B. K., Kang, D. G., Kim, Y. K., Kim, K. R., Lee, H. Y., Kawai, T., and Cha, H. J. (2007) Facile and Rapid Direct Gold Surface Immobilization with Controlled Orientation for Carbohydrates, *Bioconjugate Chemistry 18*, 2197-2201.

213. Sequeira, L. (1978) Lectins and their role in host-pathogen specificity, *Annual Review of Phytopathology 16*, 453-481.

214. Shang, J., Piskarev, V. E., Xia, M., Huang, P., Jiang, X., Likhosherstov, L. M., Novikova, O. S., Newburg, D. S., and Ratner, D. M. (2013) Identifying human milk glycans that inhibit norovirus binding using surface plasmon resonance, *Glycobiology 23*, 1491-1498.

215. Sharon, N. (2006) Carbohydrates as future anti-adhesion drugs for infectious diseases, *Biochimica et Biophysica Acta (BBA) - General Subjects 1760*, 527-537.

216. Sharon, N., and Lis, H. (1989) Lectins as cell recognition molecules, *Science 246*, 227-234.

217. Sharon, N., and Lis, H. (2003) *Lectins*, second ed., Kluwer Academic Publishers.

218. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2007) In-gel digestion for mass spectrometric characterization of proteins and proteomes, *Nat. Protocols 1*, 2856-2860.

219. Shinohara, Y., Hasegawa, Y., Kaku, H., and Shibuya, N. (1997) Elucidation of the mechanism enhancing the avidity of lectin with oligosaccharides on the solid phase surface, *Glycobiology 7*, 1201-1208.

220. Shoemaker, G. K., Soya, N., Palcic, M. M., and Klassen, J. S. (2008) Temperature-dependent cooperativity in donor–acceptor substrate binding to the human blood group glycosyltransferases, *Glycobiology 18*, 587-592.

221. Sigma-Aldrich. (2003) Sepharose and Sepharose CL gel filtration media, (Sigma-Aldrich, Ed.), Saint Louis.

222. Silhavy, T. J., Kahne, D., and Walker, S. (2010) The Bacterial Cell Envelope, *Cold Spring Harbor Perspectives in Biology 2*.

223. Simon, P. M., Goode, P. L., Mobasseri, A., and Zopf, D. (1997) Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides, *Infection and Immunity 65*, 750-757.

224. Slon-Usakiewicz, J. J., Ng, W., Dai, J.-R., Pasternak, A., and Redden, P. R. (2005) Frontal affinity chromatography with MS detection (FAC-MS) in drug discovery, *Drug Discovery Today 10*, 409-416.

225. Sörme, P., Qian, Y., Nyholm, P., Leffler, H., and Nilsson, U. (2002) Low micromolar inhibitors of galectin-3 based on 3'-derivatization of N-acetyllactosamine, *Chembiochem: a European journal of chemical biology 3*, 183-189.

226. Soya, N., Shoemaker, G. K., Palcic, M. M., and Klassen, J. S. (2009) Comparative study of substrate and product binding to the human ABO(H) blood group glycosyltransferases, *Glycobiology 19*, 1224-1234.

227. Sparrow, C. P., Leffler, H., and Barondes, S. H. (1987) Multiple soluble beta-galactoside-binding lectins from human lung, *Journal of Biological Chemistry 262*, 7383-7390.

228. Spurlino, J. C., Lu, G. Y., and Quiocho, F. A. (1991) The 2.3-A resolution structure of the maltose- or maltodextrin-binding protein, a primary receptor of bacterial active transport and chemotaxis, *Journal of Biological Chemistry 266*, 5202-5219.

229. Stefanutti, G., Lister, P., Smith, V. V., Peters, M. J., Klein, N. J., Pierro, A., and Eaton, S. (2005) P-selectin expression, neutrophil infiltration, and histologic injury in neonates with necrotizing enterocolitis, *Journal of Pediatric Surgery 40*, 942-948.

230. Sturm, A., Lensch, M., André, S., Kaltner, H., Wiedenmann, B., Rosewicz, S., Dignass, A. U., and Gabius, H.-J. (2004) Human Galectin-2: Novel Inducer of T Cell Apoptosis with Distinct Profile of Caspase Activation, *The Journal of Immunology 173*, 3825-3837.

231. Su, S. V., Gurney, K. B., and Benhur, L. (2003) Sugar and Spice: Viral Envelope-DC-SIGN Interactions in HIV Pathogenesis, *Current HIV Research 1*, 87-99.

232. Trauger, S. A., Webb, W., and Siuzdak, G. (2002) Peptide and protein analysis with mass spectrometry, *Spectroscopy: An International Journal 16*, 15-28.

233. Ukkonen, P., Varis, K., Jernfors, M., Herva, E., Jokinen, J., Ruokokoski, E., Zopf, D., and Kilpi, T. (2000) Treatment of acute otitis media with an antiadhesive oligosaccharide: a randomised, double-blind, placebo-controlled trial, *The Lancet 356*, 1398-1402.

234. Urh, M., Simpson, D., and Zhao, K. (2009) Chapter 26 Affinity Chromatography: General Methods, In *Methods in Enzymology* (Richard, R. B., and Murray, P. D., Eds.), pp 417-438, Academic Press.

235. Utsuno, K., and Uludağ, H. (2010) Thermodynamics of Polyethylenimine-DNA Binding and DNA Condensation, *Biophysical Journal 99*, 201-207.

236. van Haaren, P. M. A., VanBavel, E., Vink, H., and Spaan, J. A. E. (2003) Localization of the permeability barrier to solutes in isolated arteries by confocal microscopy,

American Journal of Physiology - Heart and Circulatory Physiology 285, H2848-H2856.

237. Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct, *Glycobiology 3*, 97-130.

238. Varki, A., and Lowe, J. B. (2009) Biological Roles of Glycans, In *Essentials of Glycobiology* (Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hart, G. W., and Etzler, M. E., Eds.) 2nd ed., pp 75-88, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).

239. Vink, H., and Duling, B. R. (1996) Identification of Distinct Luminal Domains for Macromolecules, Erythrocytes, and Leukocytes Within Mammalian Capillaries, *Circulation Research 79*, 581-589.

240. Vollmer, W. (2008) Structural variation in the glycan strands of bacterial peptidoglycan, *FEMS Microbiology Reviews 32*, 287-306.

241. Vyakarnam, A., Lenneman, A. J., Lakkides, K. M., Patterson, R. J., and Wang, J. L. (1998) A Comparative Nuclear Localization Study of Galectin-1 with Other Splicing Components, *Experimental Cell Research 242*, 419-428.

242. Wada, J., Ota, K., Kumar, A., Wallner, E. I., and Kanwar, Y. S. (1997) Developmental regulation, expression, and apoptotic potential of galectin-9, a beta-galactoside binding lectin, *The Journal of Clinical Investigation 99*, 2452-2461.

243. Wang, B. (2009) Sialic Acid Is an Essential Nutrient for Brain Development and Cognition, *Annual Review of Nutrition 29*, 177-222.

244. Wang, B., McVeagh, P., Petocz, P., and Brand-Miller, J. (2003) Brain ganglioside and glycoprotein sialic acid in breastfed compared with formula-fed infants, *The American Journal of Clinical Nutrition 78*, 1024-1029.

245. Wang, G.-N., Andre, S., Gabius, H.-J., and Murphy, P. V. (2012) Bi- to tetravalent glycoclusters: synthesis, structure-activity profiles as lectin inhibitors and impact of combining both valency and headgroup tailoring on selectivity, *Organic & Biomolecular Chemistry 10*, 6893-6907.

246. Wang, L., Cummings, R. D., Smith, D. F., Huflejt, M., Campbell, C. T., Gildersleeve, J. C., Gerlach, J. Q., Kilcoyne, M., Joshi, L., Serna, S., Reichardt, N.-C., Parera Pera, N., Pieters, R. J., Eng, W., and Mahal, L. K. (2014) Cross-platform comparison of glycan microarray formats, *Glycobiology 24*, 507-517.

247. Wang, P., Lee, H., Fukuda, M., and Seeberger, P. H. (2007) One-pot synthesis of a pentasaccharide with antibiotic activity against Helicobacter pylori, *Chemical Communications*, 1963-1965.

248. Weinstein, D. L., O'Neill, B. L., Hone, D. M., and Metcalf, E. S. (1998) Differential Early Interactions between Salmonella enterica Serovar Typhi and Two Other Pathogenic Salmonella Serovars with Intestinal Epithelial Cells, *Infection and Immunity 66*, 2310-2318.

249. Wilchek, M., and Bayer, E. A. (1990) [2] Introduction to avidin-biotin technology, In *Methods in Enzymology* (Meir, W., and Edward, A. B., Eds.), pp 5-13, Academic Press.

250. Wilchek, M., and Miron, T. (1999) Thirty years of affinity chromatography, *Reactive and Functional Polymers 41*, 263-268.

251. Wilchek, M., Miron, T., and Kohn, J. (1984) [1] Affinity chromatography, In *Methods in Enzymology* (William, B. J., Ed.), pp 3-55, Academic Press.

252. Winkler, C., Denker, K., Wortelkamp, S., and Sickmann, A. (2007) Silver- and Coomassie-staining protocols: Detection limits and compatibility with ESI MS, *Electrophoresis 28*, 2095-2099.

253. Wong, L. S., Khan, F., and Micklefield, J. (2009) Selective Covalent Protein Immobilization: Strategies and Applications, *Chemical Reviews 109*, 4025-4053.

254. Yang, R. Y., Hsu, D. K., and Liu, F. T. (1996) Expression of galectin-3 modulates T-cell growth and apoptosis, *Proceedings of the National Academy of Sciences of the United States of America 93*, 6737-6742.

255. Yu, H., Lau, K., Thon, V., Autran, C. A., Jantscher-Krenn, E., Xue, M., Li, Y., Sugiarto, G., Qu, J., Mu, S., Ding, L., Bode, L., and Chen, X. (2014) Synthetic Disialyl Hexasaccharides Protect Neonatal Rats from Necrotizing Enterocolitis, *Angewandte Chemie 126*, 6805-6809.

256. Yu, Y., Mishra, S., Song, X., Lasanajak, Y., Bradley, K. C., Tappert, M. M., Air, G. M., Steinhauer, D. A., Halder, S., Cotmore, S., Tattersall, P., Agbandje-McKenna, M., Cummings, R. D., and Smith, D. F. (2012) Functional Glycomic Analysis of Human Milk Glycans Reveals the Presence of Virus Receptors and Embryonic Stem Cell Biomarkers, *Journal of Biological Chemistry 287*, 44784-44799.

257. Zarling, D. A., Watson, A., and Bach, F. H. (1980) Mapping of lymphocyte surface polypeptide antigens by chemical cross-linking with BSOCOES, *The Journal of Immunology 124*, 913-920.

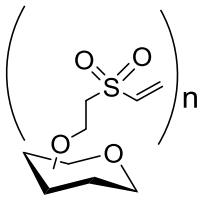
258. Zheng, H., and Du, X. (2013) Reduced steric hindrance and optimized spatial arrangement of carbohydrate ligands in imprinted monolayers for enhanced protein binding, *Biochimica et Biophysica Acta (BBA) - Biomembranes 1828*, 792-800.

259. Zrimi, J., Ng Ling, A., Giri-Rachman Arifin, E., Feverati, G., and Lesieur, C. (2010) Cholera Toxin B Subunits Assemble into Pentamers - Proposition of a Fly-Casting Mechanism, *PLoS ONE 5*, e15347.

Appendix A

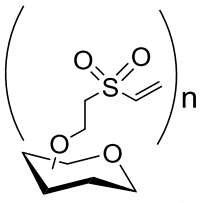
NMR Spectra

^{1}H NMR, $CDCl_3$, 500 MHz

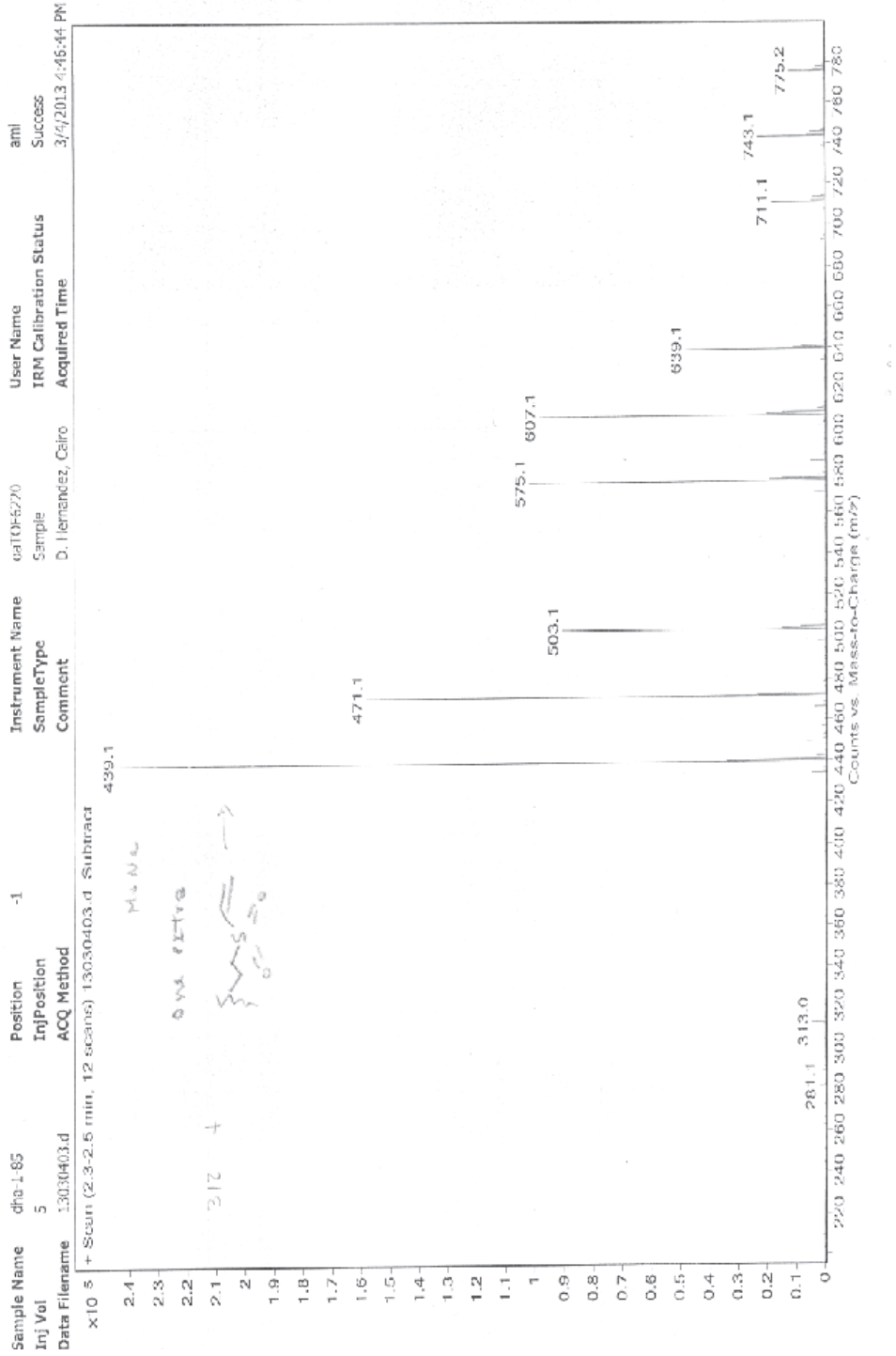


2.1

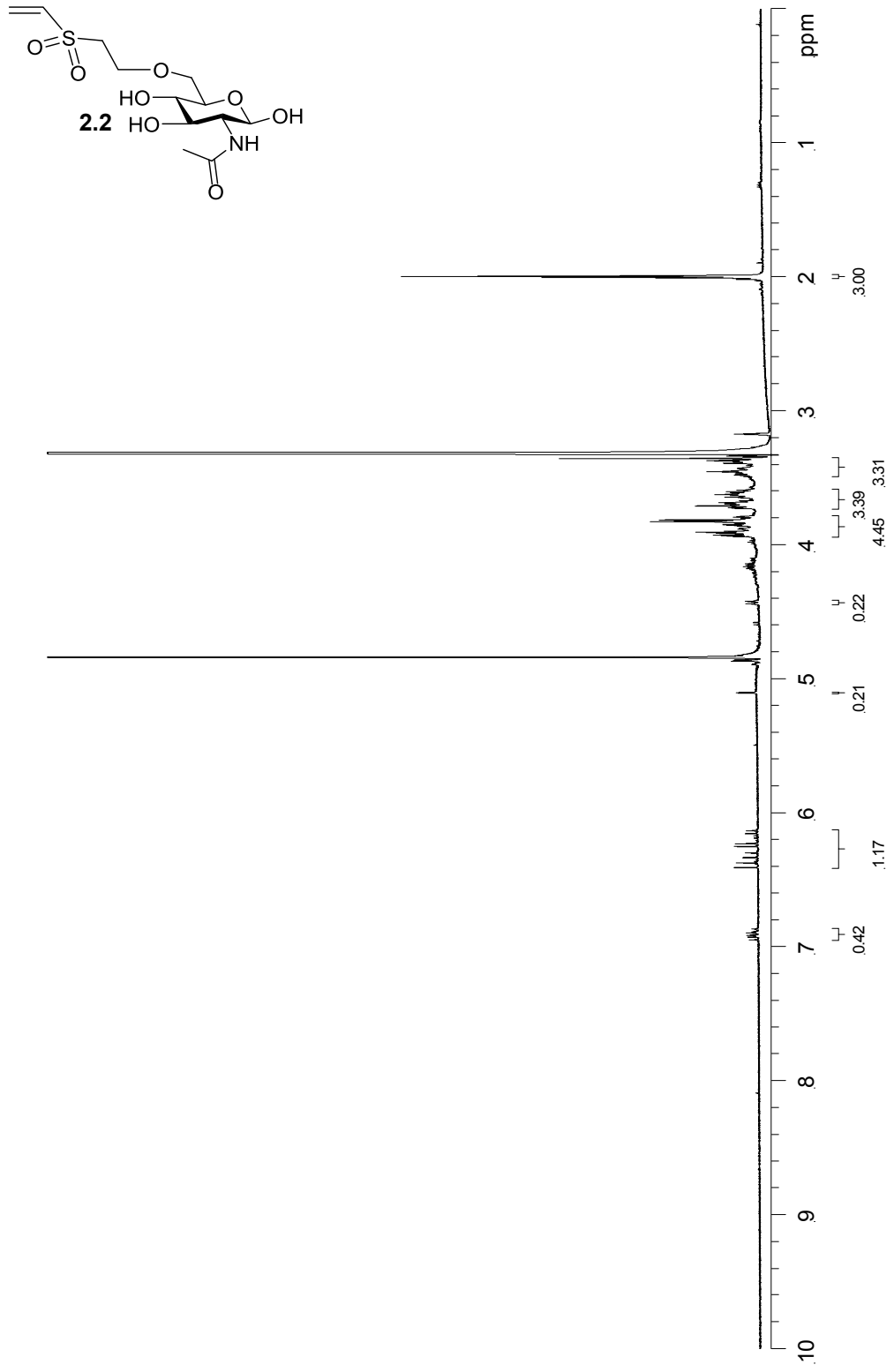
ESI MS



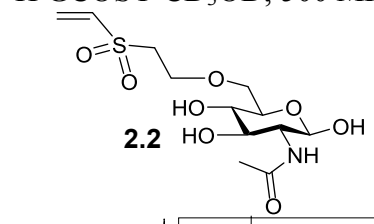
2.1


Sample Name dha-1-85
Inj Vol 5
Data Filename 130304O3.d
Position -1
InjPosition
ACQ Method
Instrument Name
SampleType
Comment
Sample
D. Hernandez, Cairo
User Name
IRM Calibration Status
Acquired Time
aml
Success
3/4/2013 4:46:44 PM
+ Scan (2.3-2.5 min, 12 scans) 130304O3.d Subtract
439.1
471.1
503.1
575.1
607.1
639.1
711.1
743.1
775.2
313.0
261.1
Counts vs. Mass-to-Charge (m/z)

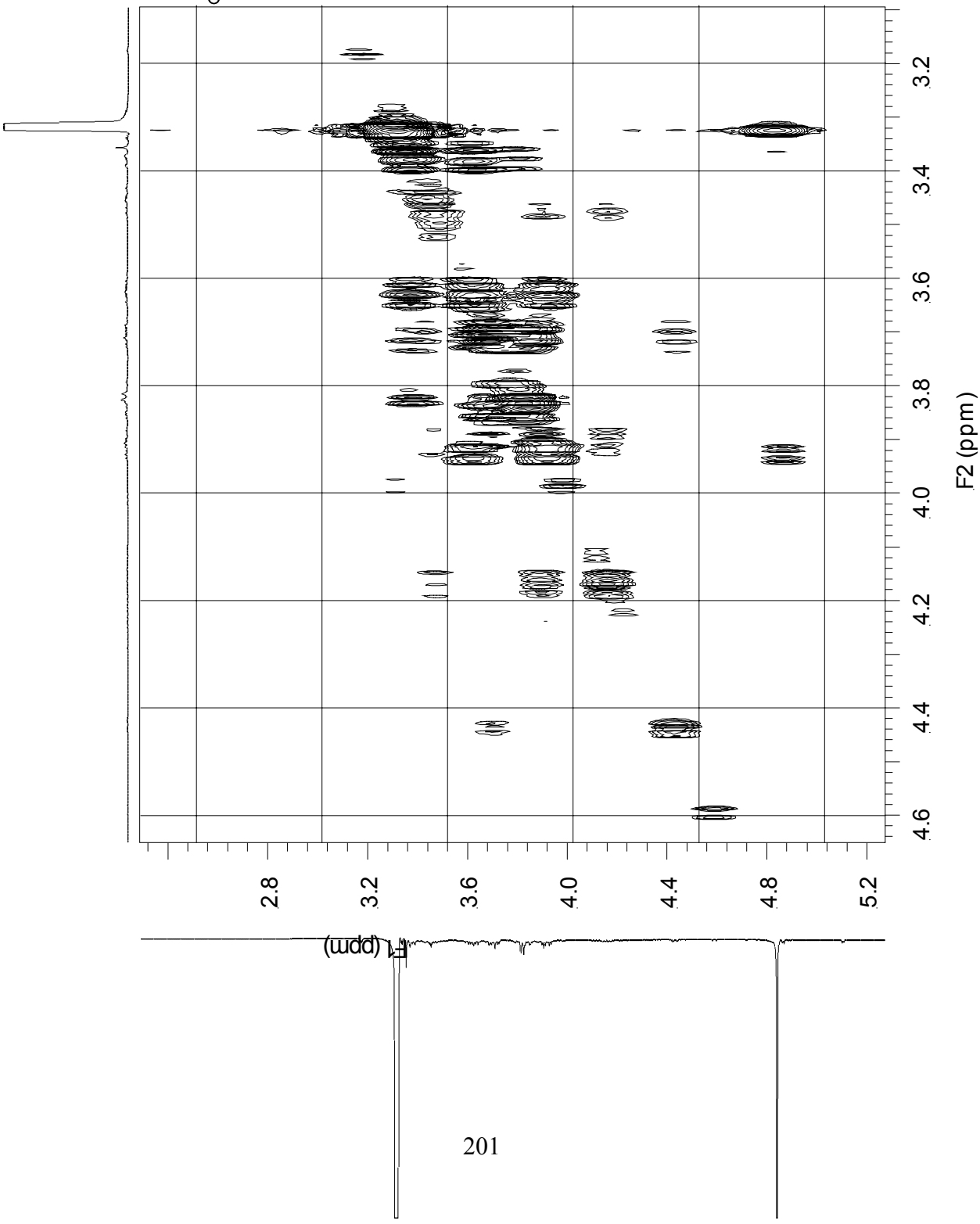
^{1}H NMR, CD_3OD, 500 MHz



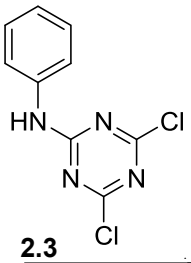
^{1}H GCOSY CD_3OD, 500 MHz



2.2

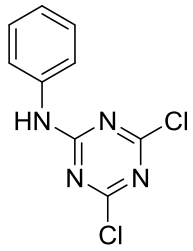


^{1}H NMR, $CDCl_3$, 500 MHz



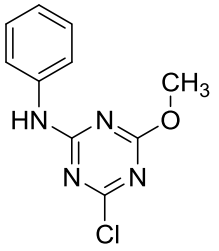
2.3

^{13}C-APT NMR, $CDCl_3$, 125 MHz



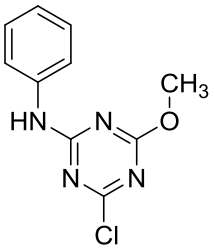
2.3

^{1}H NMR, $CDCl_3$, 500 MHz



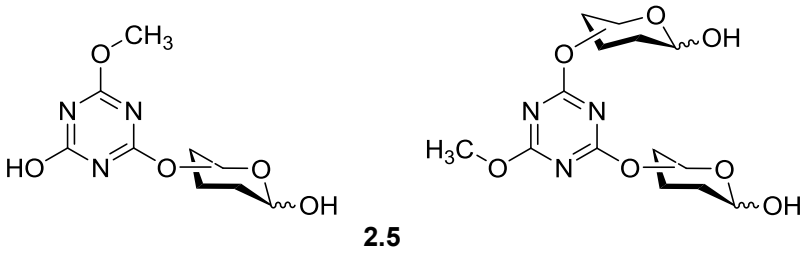
2.4

^{13}C NMR, $CDCl_3$, 125 MHz



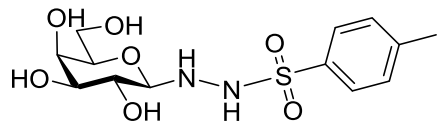
2.4

^{1}H NMR, CD_3OD, 400 MHz



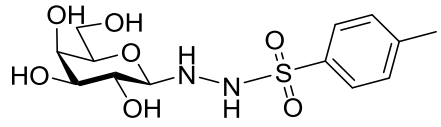
2.5

^{1}H NMR, CD_3OD, 500 MHz

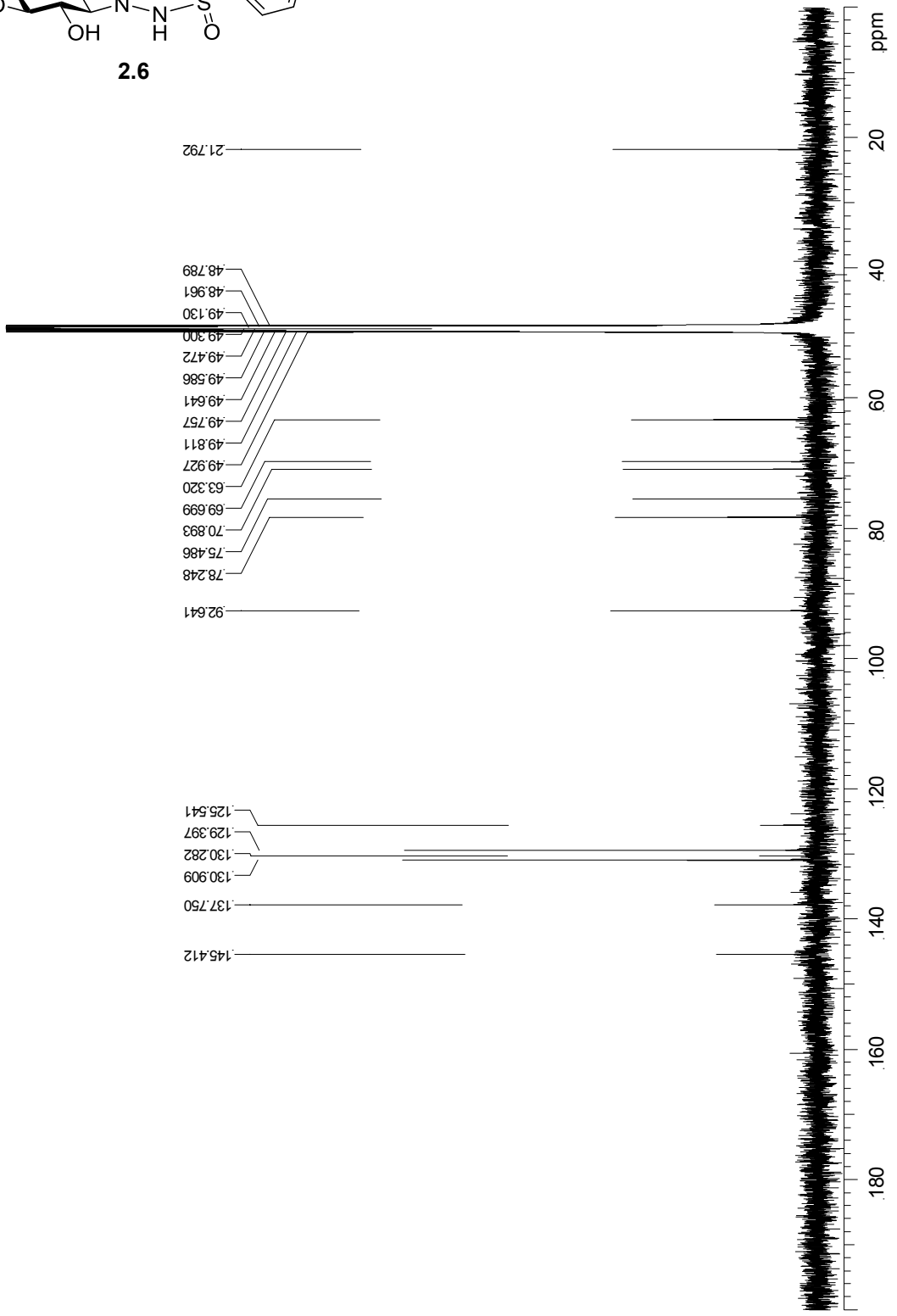


2.6

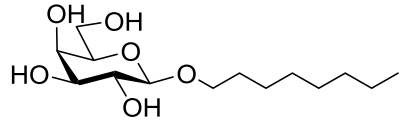
^{13}C NMR, CD_3OD, 125 MHz



2.6

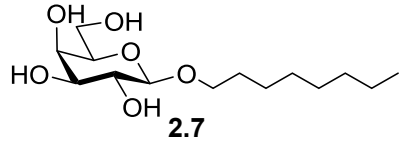


^{1}H NMR, CD_3OD, 500 MHz

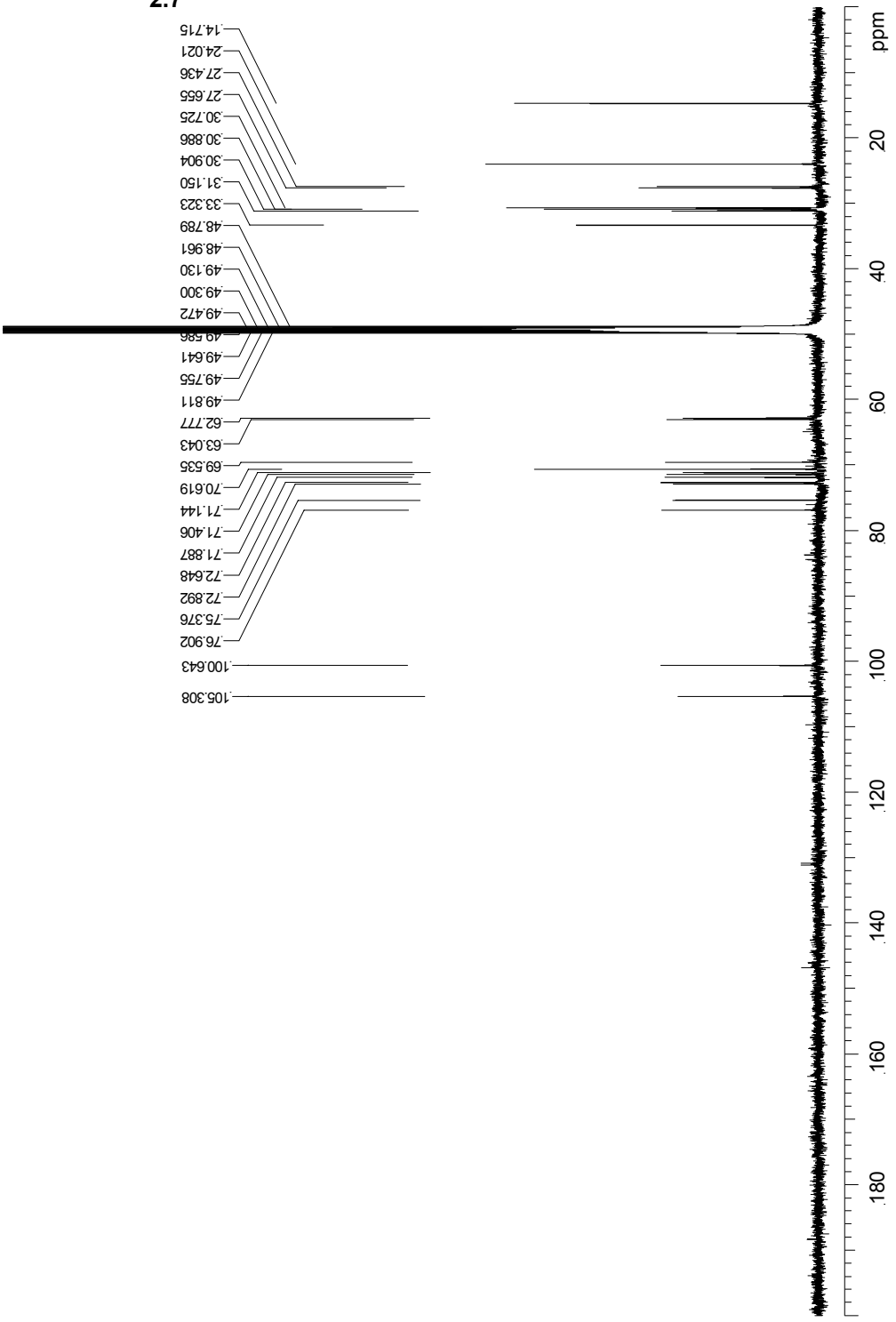


2.7

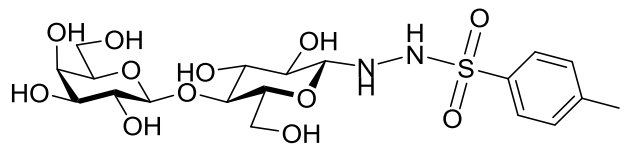
^{13}C NMR, CD_3OD, 125 MHz



2.7

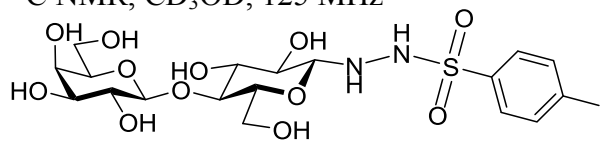


^{1}H NMR, CD_3OD, 500 MHz



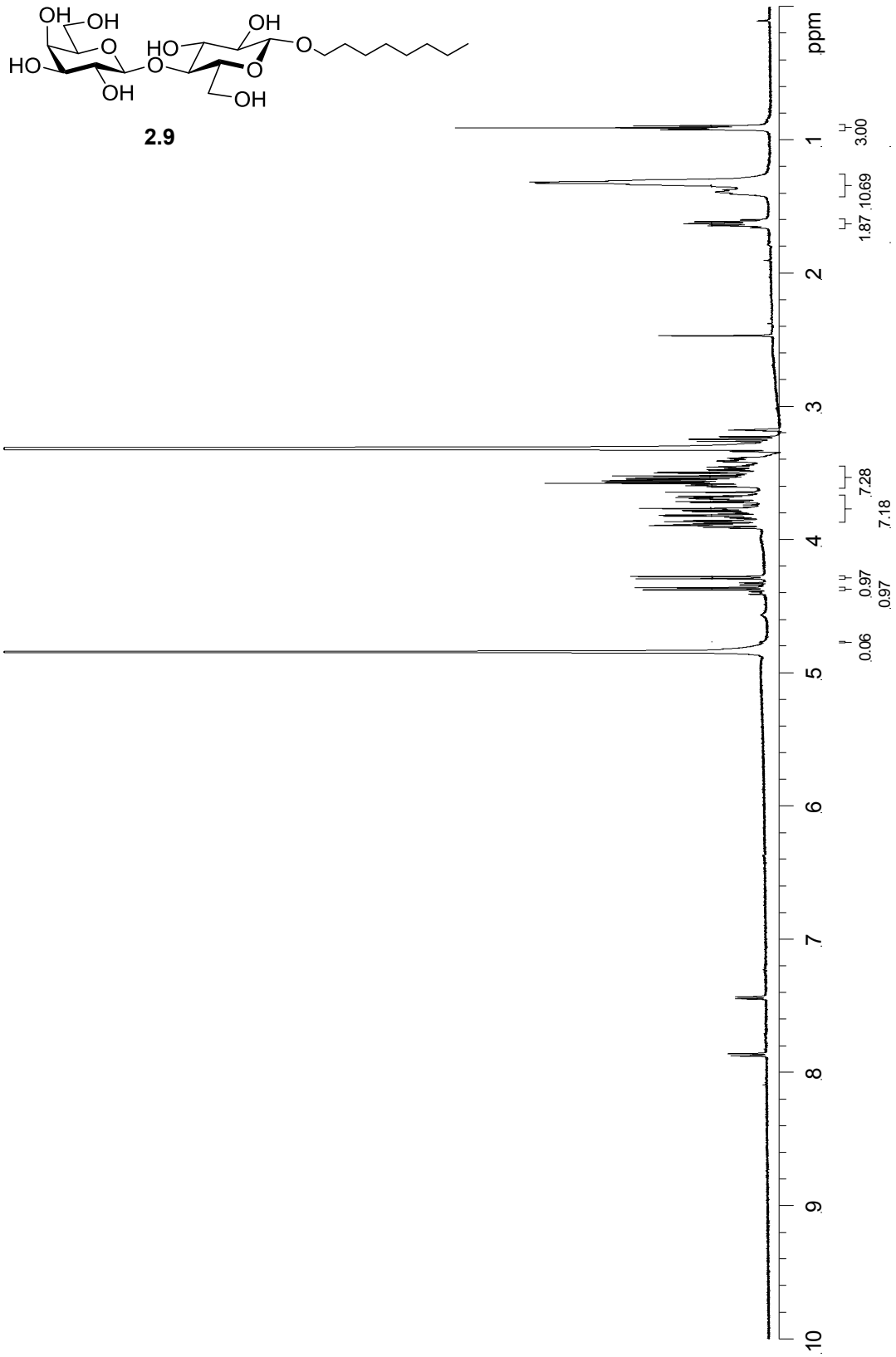
2.8

^{13}C NMR, CD_3OD, 125 MHz



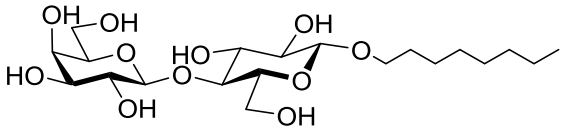
2.8

^{1}H NMR, CD_3OD, 500 MHz

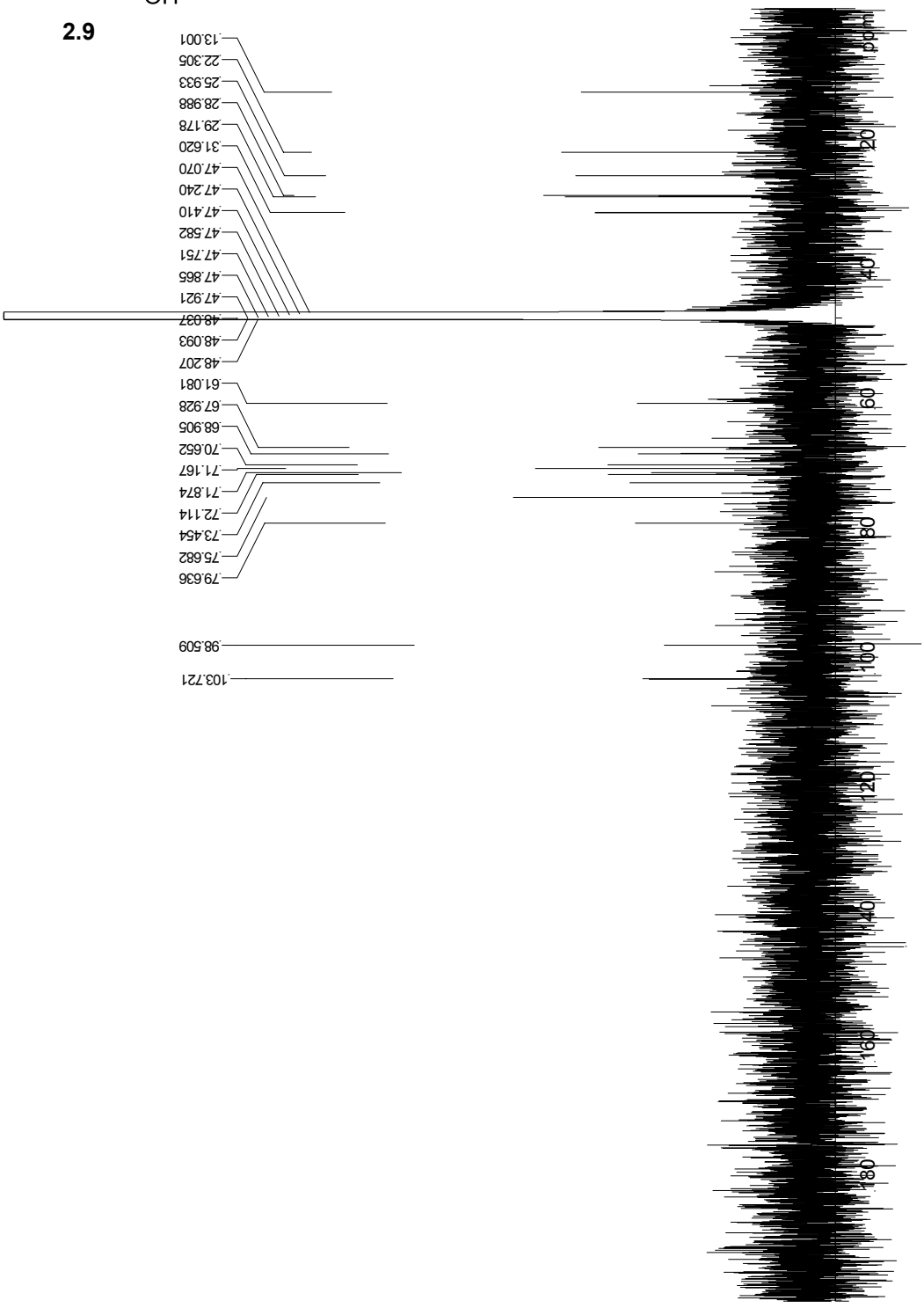


2.9

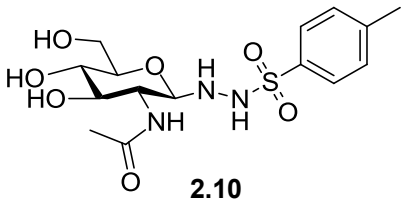
^{13}C NMR, CD_3OD, 125 MHz



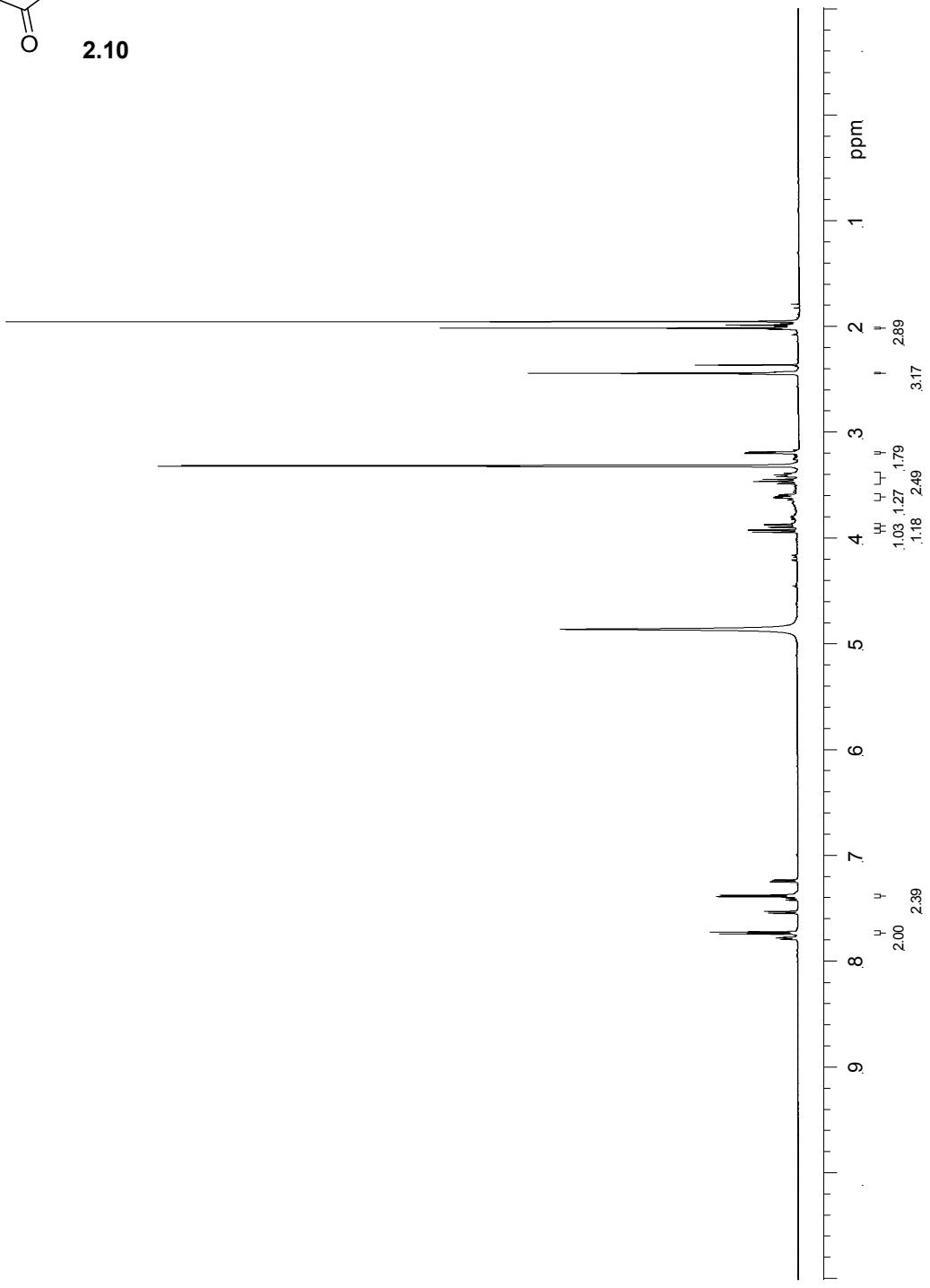
2.9



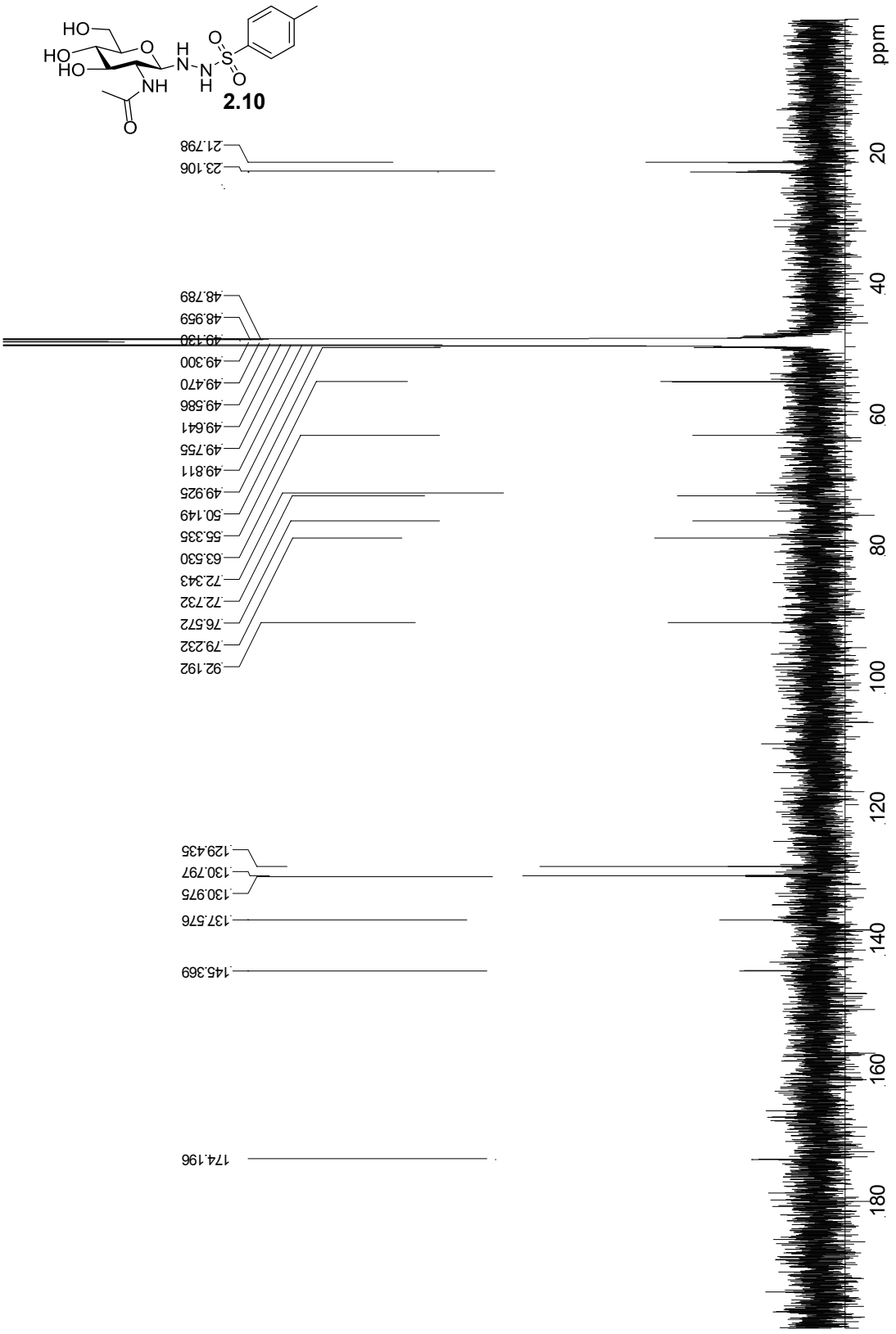
^{1}H NMR, CD_3OD, 500 MHz



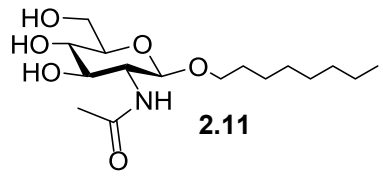
2.10



^{13}C NMR, CD_3OD, 125 MHz

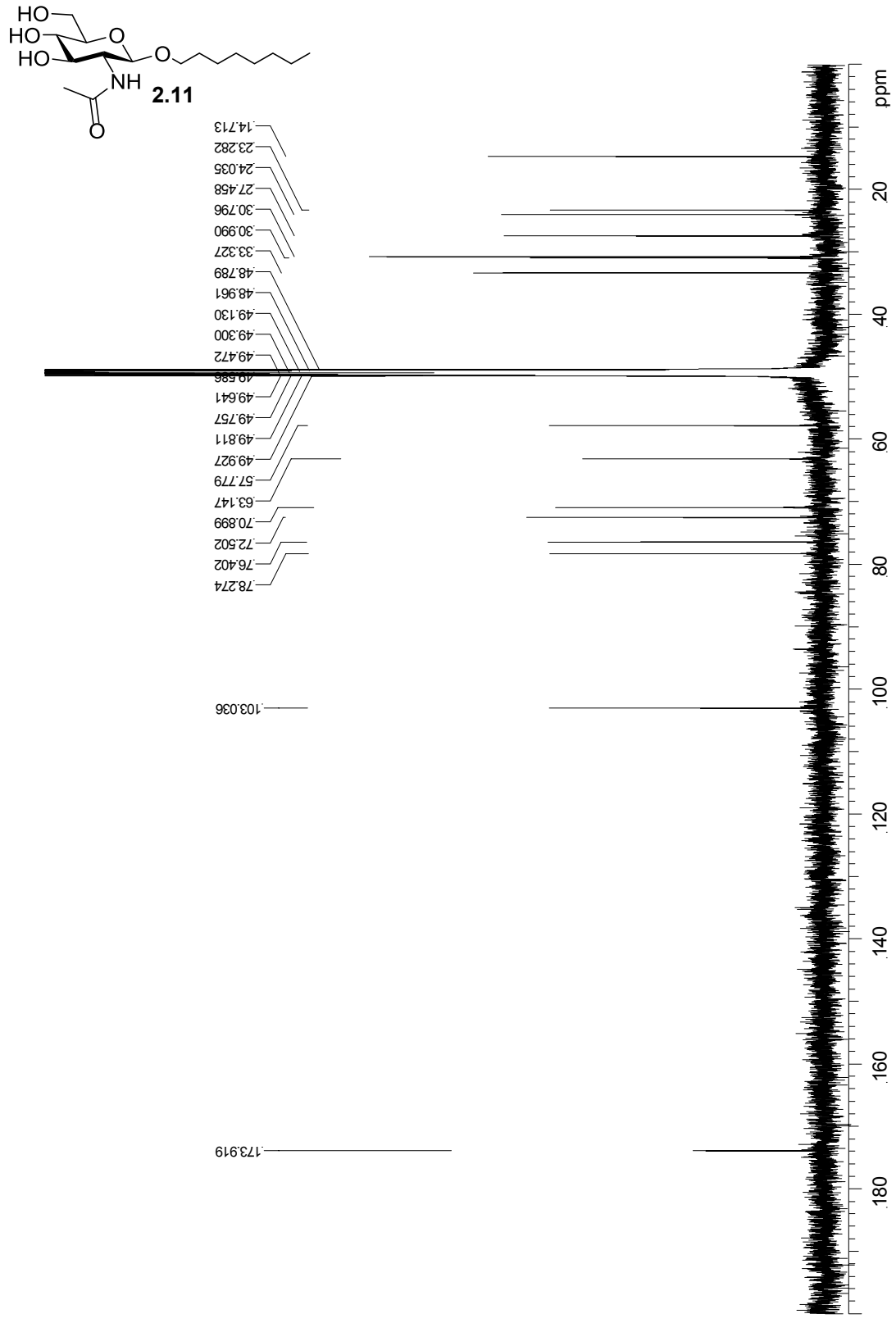


^{1}H NMR, CD_3OD, 500 MHz



2.11

^{13}C NMR, CD_3OD, 125 MHz



Appendix B

Complete list of protein hits identified using

Mass Spectrometry

Table B.1 List of proteins identified using 2'FL immobilized with DVS and lysate from HeLa cells

Entry	Accession number	Protein description	Score	Mass (Da)	Coverage (%)
1	LEG1_HUMAN	Galectin 1 OS=Homo sapiens GN=LGALS1	288	36182	22
2	LEG9B_HUMAN	Galectin-9B OS=Homo sapiens GN=LGALS9B PE=2 SV=3	193	39977	13.5
3	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	175	66170	5.1
4	PHB2_HUMAN	Prohibitin-2 OS=Homo sapiens GN=PHB2 PE=1 SV=2	69	33276	4
5	PCNA_HUMAN	Proliferating cell nuclear antigen OS=Homo sapiens GN=PCNA PE=1 SV=1	55	29092	4.2
6	VDAC1_HUMAN	Voltage-dependent anion-selective channel protein 1 OS=Homo sapiens GN=VDAC1 PE=1 SV=2	37	30868	3.9
7	CC049_HUMAN	Putative uncharacterized protein C3orf49 OS=Homo sapiens GN=C3orf49 PE=2 SV=1	33	33611	4.5

A description of the experiment performed to obtain these data is described in page 117

Table B.2 List of proteins identified using 2'FL immobilized with DVS and lysate from Jurkat cells

Entry	Accession number	Protein description	Score	Mass (Da)	Coverage (%)
1	LEG3_HUMAN	Galectin-3 OS=Homo sapiens GN=LGALS3 PE=1 SV=5	685	26193	72
2	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	132	66170	7.5
3	1433T_HUMAN	14-3-3 protein theta OS=Homo sapiens GN=YWHAQ PE=1 SV=1	97	28032	5.7
4	RL13_HUMAN	60S ribosomal protein L13 OS=Homo sapiens GN=RPL13 PE=1 SV=4	93	24304	11.4
5	ADT1_HUMAN	ADP/ATP translocase 1 OS=Homo sapiens GN=SLC25A4 PE=1 SV=4	89	33271	7.4
6	ADT2_HUMAN	ADP/ATP translocase 2 OS=Homo sapiens GN=SLC25A5 PE=1 SV=7	87	33059	11.1
7	K1H1_HUMAN	Keratin, type I cuticular Ha1 OS=Homo sapiens GN=KRT31 PE=2 SV=3	74	48633	2.9
8	K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	72	65678	3.8
9	KRT81_HUMAN	Keratin, type II cuticular Hb1 OS=Homo sapiens GN=KRT81 PE=1 SV=3	69	56832	2
10	SDHB_HUMAN	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial OS=Homo sapiens GN=SDHB PE=1 SV=3	56	32407	3.6
11	PGRC2_HUMAN	Membrane-associated progesterone receptor component 2 OS=Homo sapiens GN=PGRMC2 PE=1 SV=1	52	23861	8.5
12	DDX27_HUMAN	Probable ATP-dependent RNA helicase DDX27 OS=Homo sapiens GN=DDX27 PE=1 SV=2	50	90292	1.6

Table B.2 List of proteins identified using 2'FL immobilized with DVS and lysate from Jurkat cells (continued)

Entry	Accession number	Protein description	Score	Mass (Da)	Coverage (%)
13	RBP2_HUMAN	E3 SUMO-protein ligase RanBP2 OS=Homo sapiens GN=RANBP2 PE=1 SV=2	41	362365	0.4
14	RGPD3_HUMAN	RanBP2-like and GRIP domain-containing protein 3 OS=Homo sapiens GN=RGPD3 PE=2 SV=2	41	198732	1.2
15	HDAC4_HUMAN	Histone deacetylase 4 OS=Homo sapiens GN=HDAC4 PE=1 SV=3	38	119764	8.5
16	MADD_HUMAN	MAP kinase-activating death domain protein OS=Homo sapiens GN=MADD PE=1 SV=2	34	184500	4.5
17	BEND6_HUMAN	BEN domain-containing protein 6 OS=Homo sapiens GN=BEND6 PE=2 SV=2	33	31431	0.4
18	ACSF2_HUMAN	Acyl-CoA synthetase family member 2, mitochondrial OS=Homo sapiens GN=ACSF2 PE=1 SV=2	32	68993	2.6
19	ATRX_HUMAN	Transcriptional regulator ATRX OS=Homo sapiens GN=ATRX PE=1 SV=5	32	284863	9.7
20	PCLO_HUMAN	Protein piccolo OS=Homo sapiens GN=PCLO PE=1 SV=4	32	554704	5.4
21	KLHL5_HUMAN	Kelch-like protein 5 OS=Homo sapiens GN=KLHL5 PE=2 SV=3	32	85771	0.7
22	ABI2_HUMAN	Abl interactor 2 OS=Homo sapiens GN=ABI2 PE=1 SV=1	32	55686	1
23	IQCH_HUMAN	IQ domain-containing protein H OS=Homo sapiens GN=IQCH PE=2 SV=2	30	118016	1.6
24	EPHA8_HUMAN	Ephrin type-A receptor 8 OS=Homo sapiens GN=EPHA8 PE=1 SV=2	30	112699	1.8
25	KALRN_HUMAN	Kalirin OS=Homo sapiens GN=KALRN PE=1 SV=2	30	343097	0.4

Table B.2 List of proteins identified using 2'FL immobilized with DVS and lysate from Jurkat cells (continued)

Entry	Accession number	Protein description	Score	Mass (Da)	Coverage (%)
26	LGMN_HUMAN	Legumain OS=Homo sapiens GN=LGMN PE=1 SV=1	30	49779	1.2
27	NHS_HUMAN	Nance-Horan syndrome protein OS=Homo sapiens GN=NHS PE=1 SV=2	30	180451	0.3
28	SH3R2_HUMAN	Putative E3 ubiquitin-protein ligase SH3RF2 OS=Homo sapiens GN=SH3RF2 PE=1 SV=3	30	80068	0.7
29	SOX6_HUMAN	Transcription factor SOX-6 OS=Homo sapiens GN=SOX6 PE=1 SV=3	30	92263	0.6
30	CSAD_HUMAN	Cysteine sulfinic acid decarboxylase OS=Homo sapiens GN=CSAD PE=1 SV=2	28	55672	1.8
31	H4_HUMAN	Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2	26	11360	9.7

A description of the experiment performed to obtain these data is described in page 117

Table B.3 List of proteins identified using 2'FL immobilized with DVS and lysate from Jurkat cells shotgun approach

Entry	Accesion number	Protein description	Score	Mass (Da)	Coverage (%)
1	K1C10_HUMAN	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	909	59020	24
2	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	855	66170	28.1
3	K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	642	65678	21.1
4	K1C9_HUMAN	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	627	62255	20.4
5	LEG9_HUMAN	Galectin-9 OS=Homo sapiens GN=LGALS9 PE=1 SV=2	423	39835	22
6	LEG3_HUMAN	Galectin-3 OS=Homo sapiens GN=LGALS3 PE=1 SV=5	422	26193	38
7	H4_HUMAN	Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2	303	11360	57.3
8	LEG9B_HUMAN	Galectin-9B OS=Homo sapiens GN=LGALS9B PE=2 SV=3	285	39977	13.5
9	K1C13_HUMAN	Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13 PE=1 SV=4	215	49900	8.7
10	K1C14_HUMAN	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4	195	51872	18
11	K1C16_HUMAN	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4	191	51578	13.5
12	K1C15_HUMAN	Keratin, type I cytoskeletal 15 OS=Homo sapiens GN=KRT15 PE=1 SV=3	135	49409	9.6
13	K2C6B_HUMAN	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5	106	60315	3.4
14	H12_HUMAN	Histone H1.2 OS=Homo sapiens GN=HIST1H1C PE=1 SV=2	98	21352	14.1
15	H15_HUMAN	Histone H1.5 OS=Homo sapiens GN=HIST1H1B PE=1 SV=3	96	22566	18.1
16	H2B1C_HUMAN	Histone H2B type 1-C/E/F/G/I OS=Homo sapiens GN=HIST1H2BC PE=1 SV=4	83	13898	34.9

Table B.3 List of proteins identified using 2'FL immobilized with DVS and lysate from Jurkat cells shotgun approach (continued)

Entry	Accession number	Protein description	Score	Mass (Da)	Coverage (%)
17	K2C5_HUMAN	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3	78	62568	5.4
18	K2C4_HUMAN	Keratin, type II cytoskeletal 4 OS=Homo sapiens GN=KRT4 PE=1 SV=4	72	57649	3.9
19	H2A1A_HUMAN	Histone H2A type 1-A OS=Homo sapiens GN=HIST1H2AA PE=1 SV=3	59	14225	12.2
20	H31T_HUMAN	Histone H3.1t OS=Homo sapiens GN=HIST3H3 PE=1 SV=3	52	15613	19.1
21	K2C73_HUMAN	Keratin, type II cytoskeletal 73 OS=Homo sapiens GN=KRT73 PE=1 SV=1	51	59457	3.9
22	DCD_HUMAN	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2	38	11391	20
23	VOPP1_HUMAN	Vesicular, overexpressed in cancer, prosurvival protein 1 OS=Homo sapiens GN=VOPP1 PE=2 SV=1	37	19839	2.9
24	ARI5A_HUMAN	AT-rich interactive domain-containing protein 5A OS=Homo sapiens GN=ARID5A PE=1 SV=2	35	64718	3.4
25	XYLB_HUMAN	Xylulose kinase OS=Homo sapiens GN=XYLB PE=1 SV=3	34	59086	2.4
26	ASAP2_HUMAN	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 2 OS=Homo sapiens GN=ASAP2 PE=1 SV=3	32	112835	0.8
27	NMD3A_HUMAN	Glutamate receptor ionotropic, NMDA 3A OS=Homo sapiens GN=GRIN3A PE=1 SV=2	29	126525	0.6
28	TICRR_HUMAN	Treslin OS=Homo sapiens GN=TICRR PE=1 SV=2	28	212721	0.8
29	PARL_HUMAN	Presenilins-associated rhomboid-like protein, mitochondrial OS=Homo sapiens GN=PARL PE=1 SV=2	28	42562	1.3
30	RBBP6_HUMAN	E3 ubiquitin-protein ligase RBBP6 OS=Homo sapiens GN=RBBP6 PE=1 SV=1	28	202354	0.3
31	CADH9_HUMAN	Cadherin-9 OS=Homo sapiens GN=CDH9 PE=2 SV=2	21	88976	1.1

Table B.3 List of proteins identified using 2'FL immobilized with DVS and lysate from Jurkat cells shotgun approach (continued)

Entry	Accesion number	Protein description	Score	Mass (Da)	Coverage (%)
32	ADA22_HUMAN	Disintegrin and metalloproteinase domain-containing protein 22 OS=Homo sapiens GN=ADAM22 PE=1 SV=1	18	102991	1.2
33	WNT7A_HUMAN	Protein Wnt-7a OS=Homo sapiens GN=WNT7A PE=1 SV=2	17	40405	4.3

The data shown in this table was collected using the shotgun method described in pages 123-126

Table B.4 List of proteins identified using LNT immobilized with DVS and lysate from Jurkat cells shotgun approach

Entry	accession number	Protein description	Score	Mass (Da)	Coverage (%)
1	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	1378	66170	32.5
2	K1C10_HUMAN	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	1011	59020	27.2
3	K1C9_HUMAN	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	754	62255	31.8
4	K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	642	65678	25.7
5	LEG9_HUMAN	Galectin-9 OS=Homo sapiens GN=LGALS9 PE=1 SV=2	565	39835	27.6
6	LEG3_HUMAN	Galectin-3 OS=Homo sapiens GN=LGALS3 PE=1 SV=5	493	26193	33.6
7	LEG9B_HUMAN	Galectin-9B OS=Homo sapiens GN=LGALS9B PE=2 SV=3	390	39977	15.2
8	K1C13_HUMAN	Keratin, type I cytoskeletal 13 OS=Homo sapiens GN=KRT13 PE=1 SV=4	367	49900	17.7
9	H4_HUMAN	Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2	274	11360	47.6
10	K1C14_HUMAN	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4	267	51872	14.4
11	K1C16_HUMAN	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4	227	51578	13.7
12	ALBU_HUMAN	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	222	71317	8.2
13	K1C15_HUMAN	Keratin, type I cytoskeletal 15 OS=Homo sapiens GN=KRT15 PE=1 SV=3	169	49409	14.5
14	SRSF2_HUMAN	Serine/arginine-rich splicing factor 2 OS=Homo sapiens GN=SRSF2 PE=1 SV=4	157	25461	11.3
15	K2C6B_HUMAN	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5	141	60315	14.9
16	H15_HUMAN	Histone H1.5 OS=Homo sapiens GN=HIST1H1B PE=1 SV=3	130	22566	18.1

Table B.4 List of proteins identified using LNT immobilized with DVS and lysate from Jurkat cells shotgun approach (continued)

Entry	Accesion number	Protein description	Score	Mass (Da)	Coverage (%)
17	H2B1C_HUMAN	Histone H2B type 1-C/E/F/G/I OS=Homo sapiens	118	13898	52.4
18	H2B1B_HUMAN	Histone H2B type 1-B OS=Homo sapiens GN=HIST1H2BB PE=1 SV=2	110	13942	52.4
19	K1C17_HUMAN	Keratin, type I cytoskeletal 17 OS=Homo sapiens GN=KRT17 PE=1 SV=2	105	48361	9.3
20	H2A1C_HUMAN	Histone H2A type 1-C OS=Homo sapiens GN=HIST1H2AC PE=1 SV=3	89	14097	32.3
21	TRAP1_HUMAN	Heat shock protein 75 kDa, mitochondrial OS=Homo sapiens GN=TRAP1 PE=1 SV=3	88	80345	2
22	HS90B_HUMAN	Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4	88	83554	3.2
23	K2C5_HUMAN	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3	81	62568	12.4
24	K2C4_HUMAN	Keratin, type II cytoskeletal 4 OS=Homo sapiens GN=KRT4 PE=1 SV=4	68	57649	9.4
25	ACTB_HUMAN	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1	66	42052	18.1
26	SRSF1_HUMAN	Serine/arginine-rich splicing factor 1 OS=Homo sapiens GN=SRSF1 PE=1 SV=2	65	27842	7.7
27	H12_HUMAN	Histone H1.2 OS=Homo sapiens GN=HIST1H1C PE=1 SV=2	62	21352	19.2
28	K2C8_HUMAN	Keratin, type II cytoskeletal 8 OS=Homo sapiens GN=KRT8 PE=1 SV=7	62	53671	8.1
29	LEG8_HUMAN	Galectin-8 OS=Homo sapiens GN=LGALS8 PE=1 SV=4	62	35957	9.1
30	K2C75_HUMAN	Keratin, type II cytoskeletal 75 OS=Homo sapiens GN=KRT75 PE=1 SV=2	59	59809	6.5
31	H31_HUMAN	Histone H3.1 OS=Homo sapiens GN=HIST1H3A PE=1 SV=2	53	15509	27.2
32	K2C3_HUMAN	Keratin, type II cytoskeletal 3 OS=Homo sapiens GN=KRT3 PE=1 SV=3	51	64549	5.6

Table B.4 List of proteins identified using LNT immobilized with DVS and lysate from Jurkat cells shotgun approach (continued)

Entry	Accesion number	Protein description	Score	Mass (Da)	Coverage (%)
33	PEX1_HUMAN	Peroxisome biogenesis factor 1 OS=Homo sapiens GN=PEX1 PE=1 SV=1	51	143804	0.7
34	TRY3_HUMAN	Trypsin-3 OS=Homo sapiens GN=PRSS3 PE=1 SV=2	45	33306	4.3
35	KRT36_HUMAN	Keratin, type I cuticular Ha6 OS=Homo sapiens GN=KRT36 PE=1 SV=1	44	53354	4.7
36	K1H2_HUMAN	Keratin, type I cuticular Ha2 OS=Homo sapiens GN=KRT32 PE=1 SV=3	44	51793	3.6
37	CCD87_HUMAN	Coiled-coil domain-containing protein 87 OS=Homo sapiens GN=CCDC87 PE=2 SV=2	42	96741	0.8
38	YBOX1_HUMAN	Nuclease-sensitive element-binding protein 1 OS=Homo sapiens GN=YBX1 PE=1 SV=3	41	35903	5.9
39	TBA1A_HUMAN	Tubulin alpha-1A chain OS=Homo sapiens GN=TUBA1A PE=1 SV=1	39	50788	5.1
40	ASAP2_HUMAN	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 2 OS=Homo sapiens GN=ASAP2 PE=1 SV=3	34	112835	0.8
41	ZEP2_HUMAN	Transcription factor HIVEP2 OS=Homo sapiens GN=HIVEP2 PE=1 SV=2	33	271108	0.2
42	S7A6O_HUMAN	Probable RNA polymerase II nuclear localization protein SLC7A6OS OS=Homo sapiens GN=SLC7A6OS PE=1 SV=2	33	35235	2.3
43	WIPF3_HUMAN	WAS/WASL-interacting protein family member 3 OS=Homo sapiens GN=WIPF3 PE=2 SV=4	33	49655	1.7
44	MBL2_HUMAN	Mannose-binding protein C OS=Homo sapiens GN=MBL2 PE=1 SV=2	33	26526	3.6
45	S14L2_HUMAN	SEC14-like protein 2 OS=Homo sapiens GN=SEC14L2 PE=1 SV=1	31	46629	4.7
46	ARI5A_HUMAN	AT-rich interactive domain-containing protein 5A OS=Homo sapiens GN=ARID5A PE=1 SV=2	30	64718	1.9

Table B.4 List of proteins identified using LNT immobilized with DVS and lysate from Jurkat cells shotgun approach (continued)

Entry	Accesion number	Protein description	Score	Mass (Da)	Coverage (%)
47	NMD3A_HUMAN	Glutamate receptor ionotropic, NMDA 3A OS=Homo sapiens GN=GRIN3A PE=1 SV=2	30	126525	0.6
48	ANXA6_HUMAN	Annexin A6 OS=Homo sapiens GN=ANXA6 PE=1 SV=3	29	76168	2.8
49	XYLB_HUMAN	Xylulose kinase OS=Homo sapiens GN=XYLB PE=1 SV=3	28	59086	2.4
50	LUC7L_HUMAN	Putative RNA-binding protein Luc7-like 1 OS=Homo sapiens GN=LUC7L PE=1 SV=1	28	44100	3

The data shown in this table was collected using the shotgun method described in pages 128-130