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Synthetic Galectin Inhibitors Selective O-galactosyl aldoximes, multivalent lactosides and galactose-mimicking mannosides

Tejler, Johan

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Synthetic Galectin Inhibitors
Selective *O*-galactosyl aldoximes, multivalent lactosides and galactose-mimicking mannosides

Abstract
This thesis describes the design, synthesis and evaluation of synthetic inhibitors for the carbohydrate binding proteins called galectins. Potent and selective inhibitors can be used as important research tools in the endeavor to chart the true role of galectins in biological events such as cancer and inflammation and possibly as lead for future galectin targeting drugs.
A panel of *O*-galactosyl aldoximes was synthesized and inhibitors with high affinity and selectivity for galectin-3 and -7 were identified. The best inhibitor for galectin-3, *O*-(β-D-galactopyranosyl)-indole-3-carbaldoxime, was combined with previously optimized galectin-3 targeting triazoles, in a fragment based approach, to give 3C-triazol-1-yl-*O*-galactopyranosyl aldoximes with high affinity and selectivity for galectin-3 (K_d down to 11 μM).
Furthermore, a glycoside clustering effect was observed for multivalent lactose containing inhibitors and galectin-1. The mechanisms behind this were investigated using mutated galectin-1, lacking the possibility to dimerize.
Finally, 1*H*-[1,2,3]-triazol-1-yl mannosides, with synthetic advantages over galactose based inhibitors, were synthesized and the 4-benzylaminocarbonyl-1*H*-[1,2,3]-triazol-1-yl β-D-mannopyranoside (K_d=540 μM, for galectin-9N) compared favorably with its galactoside counterpart.

Key words
Galectin, lectin, aldoxime, inhibitor, triazole, multivalence, cluster effect, fragment based, glycomimetic

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A doctoral thesis at a university in Sweden is produced as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (*in press, submitted or in manuscript*).

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So tell me quick and tell me true
or else I have no time for you!
Not how this study came to be
but what its news can do for me.
And if it is not meant for me
please tell me fast so I may flee!
From Alice in Wonderland by Lewis Carrolls

Mamma Alva Pappa Nellie och Snutte
Nellie 18/7-06 om familjen

Populärvetenskaplig sammanfattning

Galektin är en proteinfamilj med många funktioner i kroppen, varav en del är kopplade till sjukdomstillstånd, såsom inflammationer samt utvecklandet och fortskridandet av cancer.

Galektiners funktion styrs av en kolhydratbindande domän (CRD) som känner igen och binder till galaktos. Vissa galektiner kan hålla ihop olika celler genom att binda till galaktos på cellytor. Detta sker bland annat då cancerceller sprids och bildar nya tumörer vid metastasering. Det finns även galektiner med funktioner inne i cellen där det till exempel har visats att galektin-3 hindrar cytostatika från att döda cancerceller vilket motverkar cancerbehandling. Mekanismerna bakom dessa och andra galektinfunktioner är till stor del okända.

Syntetiska molekyler, inhibitorer, som hindrar inbindningen av galektin till naturligt förekommande ligander kan användas som kemiska verktyg för att undersöka vilka funktioner galektin har. Det är möjligt att en inhibitor som binder till galektin-3 blockerar proteinet så att cytostatikabehanlingen av cancerceller blir effektivare.

Vi har utvecklat en metod för att tillverka små modifierade galaktosmolekyler som binder galektin-3 upp till 20 gånger bättre än laktos (mjölksocker). Laktos är en naturlig galektinligand som innehåller galaktos. Några av dessa syntetiska inhibitorer är selektiva för galektin-3, vilket innebär att de binder galektin-3 men inte andra galektiner. Att bara påverka ett galektin i taget är viktigt för att undersöka just det proteinets funktioner. Detta tillsammans med inhibitorernas fördelaktiga kemiska struktur innebär ett steg mot läkemedel som blockerar galektin-3 vid cancer- och inflammationsrelaterade sjukdomar.

Den syntesmetod vi utvecklat för galektin-3 inhibitorer kan vara möjlig att använda även för att göra selektiva inhibitorer för andra galektin.

List of Papers

This thesis summarizes the following papers. Papers I and III are reprinted with kind permission from the publisher.

I. Synthesis of *O*-galactosyl aldoximes as potent LacNAc-mimetic galectin-3 inhibitors
Johan Tejler, Hakon Leffler, Ulf J. Nilsson. *Bioorg. Med. Chem. Lett.* **2005**, 15, 2343-2345.
Contribution: Experimental work and manuscript preparation were performed by Johan Tejler.

II. A fragment based approach towards triazole-substituted *O*-galactosyl aldoximes as selective and low micromolar small molecule galectin-3 inhibitors
Johan Tejler, Bader Salameh, Hakon Leffler, Ulf J. Nilsson. *In manuscript*.
Contribution: Major experimental work, except triazole formation performed by Bader Salameh, manuscript preparation shared between Johan Tejler and Ulf J. Nilsson.

III. Synthesis of multivalent lactose derivatives by 1,3-dipolar cycloadditions: selective galectin-1 inhibition
Johan Tejler, Erik Tullberg, Torbjörn Frejd, Hakon Leffler, Ulf J. Nilsson. *Carbohydr. Res.* **2006**, 341, 1353-1362.
Contribution: Experimental work and manuscript preparation were equally shared between Johan Tejler and Erik Tullberg.

IV. Interaction of a divalent lactose ligand with monomeric galectin-1. Mechanisms contributing to the glycoside clustering effect
Emma Salomonsson, Johan Tejler, Erik Tullberg, Anders Sundin, Areej Khabut, Torbjörn Frejd, Yura Lobsanov, James Rini, Ulf J. Nilsson, Hakon Leffler. *In manuscript*.
Contribution: Evaluation of results shared with Emma Salomonsson and Erik Tullberg, synthesis of supplied inhibitors equally shared with Erik Tullberg, manuscript preparation was shared between Johan Tejler, Emma Salomonsson, Erik Tullberg and Hakon Leffler.

V. Synthesis of galactose-mimicking 1*H*-(1,2,3-triazol-1-yl)-mannosides as selective galectin-3 and 9N inhibitors
Johan Tejler, Fredrik Skogman, Hakon Leffler, Ulf J. Nilsson. *In manuscript*.
Contribution: Experimental work shared with Fredrik Skogman, manuscript preparation was done by Johan Tejler.

Paper not included in this thesis

Synthesis of a 3'-naphthamido-LacNAc fluorescein conjugate with high selectivity and affinity for galectin-3
Karolina Aplander, Johan Tejler, Jörgen Toftered, Susanne Carlsson, Barbro Kahl-Knutson, Anders Sundin, Hakon Leffler, Ulf J. Nilsson. *Carbohydr. Res.* **2006**, 341, 1363-1369.
Contribution: Synthetic work shared with Karolina Aplander.

Abbreviations

aa	Amino Acid
Ac	Acetyl
AcOH	Acetic Acid
AlaX	Alanine number X
ArgX	Arginine number X
Bz	Benzoyl
CRD	Carbohydrate Recognition Domain
Cpd	Compound
D	Dimensional
DIPEA	*N,N*-Diisopropylethylamine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
dHumGal1	Homodimeric Human Galectin-1
dRatGal-1	Homodimeric Rat Galectin-1
ER	Endoplasmatic Reticulum
FP	Fluorescence Polarization
Gal	D-Galactopyranose
Glu	D-Glucopyranose
GluX	Glutamate number X
HisX	Histidine number X
HPLC	High Performance Liquid Chromatography
K_d	Dissociation constant
LeuX	Leucine number X
Man	D-Mannopyranose
Me	Methyl
MCP	Modified Citrus Pectin
mHumGal1	Monomeric Human Galectin-1
MS	Mass Spectroscopy
mRatGal-1	Monomeric Rat Galectin-1
N.D.	Not Determined
N.I.	Non Inhibitory
NMR	Nuclear Magnetic Resonance
Ph	Phenyl
RP	Reversed Phase
PBA	Phenyl-Bis-Alanine
PTA	Phenyl-Tris-Alanine
THF	Tetrahydrofuran
Thr	Threonine number X
TLC	Thin Layer Chromatography
TrpX	Tryptophan number X
TsCl	*p*-Toluenesulfonyl chloride
ValX	Valine number X

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1 Introduction to glycobiology and galectins

Carbohydrates, or sugars, are the most abundant organic substance on our planet. The structural complexity of carbohydrates gives them a unique role in nature. Large polymers of saccharides like cellulose and starch build up plants and trees and a polysaccharide called chitin forms the exoskeleton of insects and crabs. The nucleotides in DNA and RNA are attached to sugar-phosphate polymers and carbohydrates are included in the determining antigens in the ABO blood group system.[1] Most carbohydrates in cells are attached to proteins or lipids, these glycoproteins and glycolipids are a major component of the mammalian cell surface. Viruses often use these cell surface oligosaccharides as receptors when they infect cells. The influenca A virus known as H5N1 causes the bird flu by first attaching to cell surface glycoproteins or glycolipids comprising sialic acid.[2] The noroviruses that causes the winter vomiting disease bind to carbohydrates present in the blood group antigens.[2,3] Furthermore, the glycoproteins and glycolipids are essential in numerous of other biological events such as fertilization, cell growth, cell-cell adhesion (cell-cell binding), immune defense and inflammation.[4]

Lectins are proteins that bind to carbohydrates.[5,6] They serve to mediate biological recognition events in organisms ranging from plants to humans. Galectins are an intriguing family of lectins and this thesis summarizes our attempts to find synthetic molecules that inhibit galectins and thus can be used to deduce their biological roles.

1.1 The galectin history

In 1975, a 14 kDa protein was isolated from tissue extracts of electric eel.[7] The research group was looking for proteins that recognized cell surface carbohydrates involved in cell

adhesion. The eel extract was subjected to affinity chromatography on immobilized β-galactosides and bound protein was eluted with lactose. Lactose was used since it resembled known carbohydrate structures on the cell surface. The protein eluted, now known as galectin-1, was the first protein found in a still growing family of intra- and extra-cellular lectins now known as galectins.

Over the years it turned out that galectins are spread throughout nature and they have been found in various organisms such as viruses, sponges, nematodes, fungi, plants, amphibians, fish, worms and birds. About 15 galectins and 5 galectin like-proteins are found in mammals.[8,9] Because galectins are so widely spread throughout both plant and animal kingdom, it is reasonable to say that they are a very ancient family of proteins.

Mammalian galectins are numbered 1-15. So far no protein has been denoted galectin-11 although two galectin like proteins OvGal11 and GRIFIN (galectin-related-inter-fiber protein) have been proposed as candidates for this slot.[10,11] However, GRIFIN has not been shown to have carbohydrate-binding activity and thus is not a galectin. OvGal11 was later announced as galectin-15 after being shown to display affinity for lactose.[12]

Galectins are often described as a large protein family with highly conserved sequence elements.[13] However, with the completed genome sequences available, a more accurate description might be that it is a moderately sized family[8] and that galectins are not more conserved than other functional proteins.

For the simplicity of discussion the galectins referred to from now on and throughout this thesis will be mammalian galectins.

1.2 Galectin structure

The galectins were defined in 1994 as follows; “Membership in the galectin family requires fulfillment of two criteria: affinity for β-galactosides and significant sequence similarity in the carbohydrate-binding site...”.[14] The carbohydrate recognition domain, most often referred to as the CRD, is a beta-sandwich of about 135 amino acids. One side of the CRD contains the carbohydrate binding site that can hold about a linear tetrasaccharide and can schematically be divided into the four subsites A, B, C and D.[8] Subsite C is built from most of the conserved amino acids characteristic for galectins and binds selectively the β-galactoside with many interactions.

In 1993 Hirabayashi and Kasai divided galectins into three subfamilies as proto-, chimera- and tandem repeat type (Figure 1).[15] The prototype galectins have one CRD and galectins-1, -2, -5, -7, -10, -13, -14, and -15 belong to this subfamily. The prototype galectins-1, -2, -5, and -7 can be found as dimers or actually homodimers where two galectins are held together and therefore constitute two identical CRD:s.[16] In contrast, the tandem repeat type galectins, galectin-4, -6, -8, -9, and -12, have two non identical CRD:s joined by a peptide chain. Finally, galectin-3 is the only known chimera type galectin in vertebrates, it has one CRD as the prototype galectins, but also an extended peptide domain rich in proline and glycine. Galectin-3 can form multimers via interactions of these peptide domains.[17]

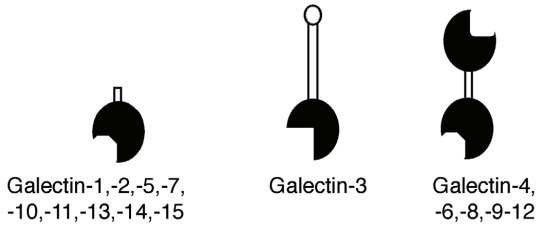


Figure 1. Galectin structures showing prototype, chimera type and tandem repeat type. The CRD are represented as black illustrations and the extended peptide chains as white lines.

1.3 Biological location and function

Galectins can be found in various tissues throughout the body (Table 1), where they display both intra- and extra-cellular activities. While some galectins like galectin-1, -3, and -8 are expressed in a wide range of tissues other galectins, for example galectin-10 is only expressed in the eosinophiles.

Galectins have been implicated to play roles in numerous biological events and many of these concerns cancer[18-23] and the regulation of immunity[24,25] and inflammation.[26] Animal experiments have supported several of these roles.[27-30] Galectins tend to be overexpressed in cancerous cells and different galectins display regulatory effects in different biological events. While some galectins, as galectin-3, display anti-apoptotic activities several other galectins, including galectin-1, -2, -7, and -9, tend to display pro-apoptotic effects.[31] It has been emphasized that some galectins induce apoptosis (cell death) extracellularly through cell surface glycoproteins and other regulate apoptosis intracellularly through interactions

with intracellular proteins.[22] Galectins play an important role in a range of other mechanisms related to pathological conditions, such as tumor transformation, cell growth regulation and cell adhesion.[32]

Table 1. Schematic representation of distribution and functional properties of galectins.[12,16,33,34]

Galectin	Distribution	Example of function
1	Most tissues: skeletal, muscle, heart, placenta, lymphatic tissues, etc.	Head and neck tumors, induces T-cell apoptosis, inhibits acute inflammation, suppresses chronic inflammation and autoimmunity
2	Gastro-intestinal tract, tumors	Myocardial infarction
3	Macrophages, epithelia, tumors	Breast, colon and ovarian tumors, anti-apoptotic and pro-inflammatory, modulates cell adhesion and migration
4	Gastro-intestinal tract	Liver, gastric and colon tumors
5	Blastocyst at implantation, reticulocytes	
6	Gastro-intestinal tract	
7	Epithelia	Breast, bladder and skin tumors
8	Liver, kidney, cardiac muscle, lung, brain, colon	Pancreas, liver, skin and colon tumors
9	Eosinophiles, monocytes, macrophages, gastro-intestinal tract, kidney	Hodgkins lymphomas, allergic response, melanoma tumors
10	Eosinophiles	
12	Adipocytes	Induces apoptosis
13	Placenta	
14	Eosinophiles	Allergic response
15	Endometrium	

1.3.1 Extra-cellular function

The mammalian cell surface is coated with glycoconjugates and some of these display galactose containing oligosaccharides that are suitable binding sites for galectins. These cell-surface glycoconjugates can be crosslinked via the bivalent or multivalent properties of galectins, and this crosslinking can trigger a cascade of transmembrane signaling events. The bivalent and multivalent properties of galectins enable aggregation of cells and binding of proteins to the cell surface.[22]

1.3.2 Intra-cellular function

Inside the cell, galectins interact with a number of intracellular proteins involved in essential cellular functions such as regulation of cell growth, apoptosis and cell cycle progression. However, the exact mechanisms and functions of galectins in these intriguing events are not known.[22]

1.3.3 Multivalency

The binding of glycoconjugates by lectins is often of low affinity and biological systems use multivalent interactions to circumvent these low affinities.[35] By using multivalent interactions large cross-linked structures can be formed where the ligand structure and type of galectin determines the structure of the formed aggregate. Cross-linking of cell-surface receptors is important in cell-signaling events[13,36] and the use of multivalent interactions seems to be important for the biological response in certain situations. As an example, the observation that galectin-3 inhibits the apoptotic effect of galectin-1 in cells can not be explained by different affinity requirements between galectin-1 or -3 and cell surface inhibitors. Indeed, the explanation might be that the formation of more stable aggregates between cell surface inhibitors and galectin-3 will competitively displace galectin-1 from the cell surface.

The dimeric galectins, whether they are homodimers like galectin-1 or tandem repeat type, can form two types of cross-linked aggregates (Figure 2). Combination of dimeric galectins with divalent inhibitors gives a chain called type-1 aggregate. Dimeric galectins combined with multivalent inhibitors can form 2 and 3D aggregates known as type-2a aggregates. The last scenario arises when pentamers (or other multimers) of chimera type galectin-3 is combined with multivalent inhibitors in type-2b aggregates.[17]

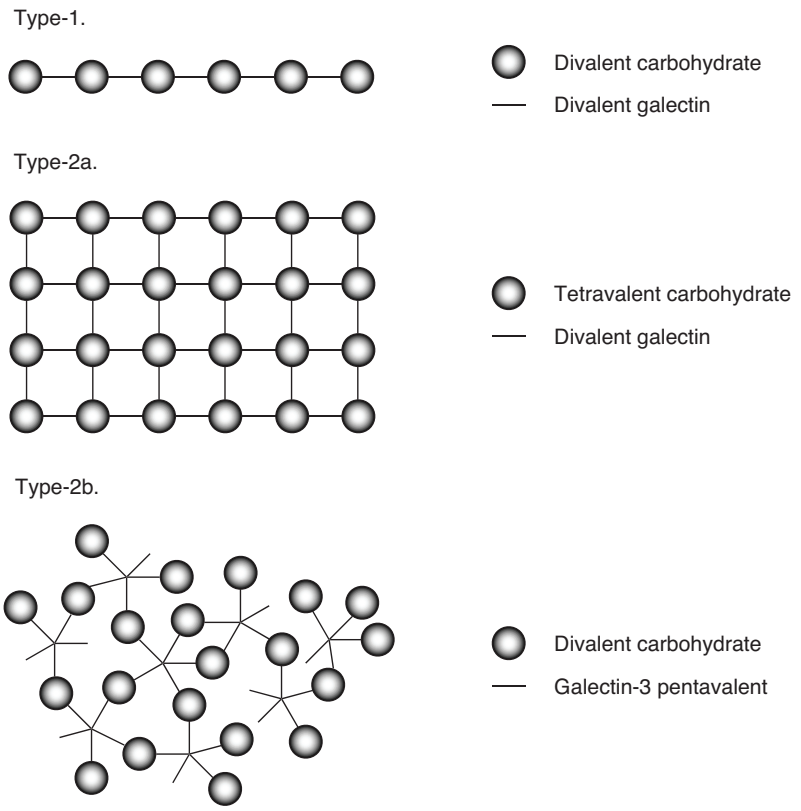


Figure 2. Multivalent inhibitors may form type 1 or type 2a and b aggregates with dimeric or multimeric galectins.

1.4 Galectins and non-classical secretion

Protein secretion in mammalian cells generally occurs via the classical secretory pathway via the endoplasmatic reticulum (ER) and Golgi apparatus. Proteins are synthesized in the ribosomes, and then directed to the ER, the ER transports the proteins to the Golgi apparatus where the proteins are modified and prepared for secretion. This secretion requires proteins with a signal sequence typically built from 13-30 hydrophobic amino acids allowing passage across the membrane of the ER.

Galectins have both intra-and -extra cellular functions. They are synthesized on cytosolic ribosomes intracellularly, however, they lack the signal sequence required for the described classical secretion into the extacellular matrix.[37,38] Furthermore, the absence of galectins in the ER and Golgi apparatus and the fact that galectins were still secreted from

cells where the classical secretory pathway had been deactivated led to the proposal of non-classical secreation.[39-44]

Different distinct mechanisms have been proposed for non-classical secretion. One of these called ectocytosis or membrane blebbing has been proposed for the secretion of galectin-1 and -3. Herein, the galectins are accumulating below the plasma membrane, and then included into the plasma membrane into vesicles called exovesicles ("blebs"). The exovesicles are then released into the extracellular space where they release their load of galectins.[44,45]

Other proposed mechanisms for secretion are by embedding the galectins in exosomes (small vesicles), the exosomes are transported to and fused with the plasma membrane and the protein content is released into the extracellular space.[46] The third mechanism suggests transportation via membrane transporters.[47,48]

The Golgi apparatus transports many glycoproteins and the unusual secretory pathways of galectins might prevent premature interactions of oligosaccharides on nascent glycoproteins.

1.5 Galectin ligands

As previously stated, galectins interact with cell surface oligosaccharides or glycoproteins intra- and extracellularly. The highly conserved subsite C of galectins, binds galactose that in natural saccharides typically glycosylates another pyranoside that occupy subsite D, the second most conserved feature (see appendix A for some basic carbohydrate chemistry). This pyranoside is typically (1→4)-linked Glc (**1**) (and becomes lactose) or GlcNAc[49] (**2**) (and becomes LacNAc-II, referred to as LacNAc) or (1→3)-linked GlcNAc (**3**) (and becomes LacNAc-I) or GalNAc (**4**) (and becomes T-antigen) (Figure 3). Further extension at C3 of galactose, reaching into subsite B and then farther into subsite A, varies the specificity of the galectins.[8]

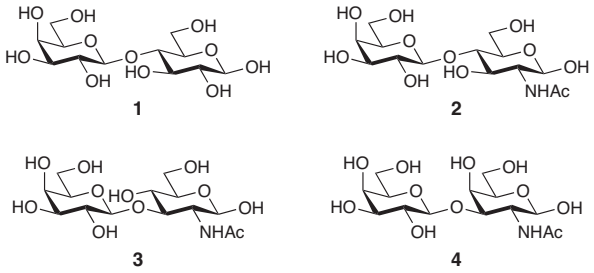


Figure 3. Structures of natural disaccharides that interact with galectins.

The X-ray structures of a range of galectins have been solved.[50] In some cases, inhibitors are included in the crystals which provides valuable information on how inhibitors bind to galectin.[51,52] Easier access to computers with enough power for modeling of inhibitor protein complexes is today a valuable research tool in the search for new high affinity galectin inhibitors, as well as for the understanding of why and how certain inhibitors bind to galectins. However, the complexity of the molecular world and chemical synthesis tend to complicate things as will be described for some of the projects presented in this thesis.

2 Synthetic galectin inhibitors

Galectins have emerged as promising molecular targets for cancer therapy and galectin inhibitors have the potential to be used for therapeutic purposes in cancer treatment, as well as in inflammation and immunity disorders. A range of molecules have been synthesized and evaluated as inhibitors of galectins. Indeed, potent galectin inhibitors can be used as basic research tools in order to further analyze the functional role of galectins in biological systems. In the long run, it is predicted that these inhibitors can serve as lead compounds for future pharmaceuticals used in cancer treatment leading to delays in tumor progression and improvements in overall survival.

2.1 Synthetic inhibitors

Ever since the pioneering study in 1996 where Glinsky *et al.*[53] reduced metastasis in a mouse model with glycoamines, numerous molecules with different properties have been synthesized and evaluated as inhibitors of galectins.

2.1.1 Small synthetic galectin inhibitors

The use of small synthetic molecules as galectin inhibitors have certain advantages as compared with natural saccharides. In order to serve as lead compounds, the inhibitors have to possess high affinity towards galectins. Selectivity is important in order to avoid unwanted side effects, as some galectins can have other functions. A smaller size, combined with lower polarity and less hydrogen bonding of a synthetic molecule as compared to natural saccharides, is advantageous, *i.e.* Lipinski's rule of five.[54]

Our group have previously presented 3'-derivatised N-Acetyllactosamines (LacNAc) (*e.g.* **5**) with K_d as low as 0.32 μM for galectin-3 (Figure 4).[52,55] The C2-symmetric thiodigalactoside **6** had a K_d of 33 nM for galectin-3[56] and a monosaccharide, phenyl thio-β-D-galactopyranoside **7**, had a K_d of 140 μM for galectin-7.[57] Monosaccharides with a

triazole substituent at C3 of galactose (*e.g.* **8**) have been presented as galectin-3 inhibitors with K_d values as low as 107 μM.[58] Recently, Roy *et al.*[59] presented monosaccharide based triazole- and isoxazole-derivatives (*e.g.* **9**) with the advantage of a shorter synthesis as compared to triazoles at C3. The same group recently presented a QSAR model for galectin-3[60] that might prove useful in the future design of galectin-3 inhibitors. In 2006, Hindsgaul *et al.* presented 2 and 2'- LacNAc-I derivatives (*e.g.* **10**)[61] as galectin-3 inhibitors with K_d down to 11 μM. Earlier in 2006, Kiss *et al.* showed that a concomitant addition of a lactosylated steroid (**11**)[62] and cis-platin increased the therapeutic benefits of each component alone in a mouse lymphoma model. Synthetic lactulose amines (*e.g.* **12**)[32] have been shown to inhibit tumor-cell apoptosis and galectin-mediated cell aggregation. Some of these lactulose amines were divalent structures and thus also belong in the next section on multivalent compounds.

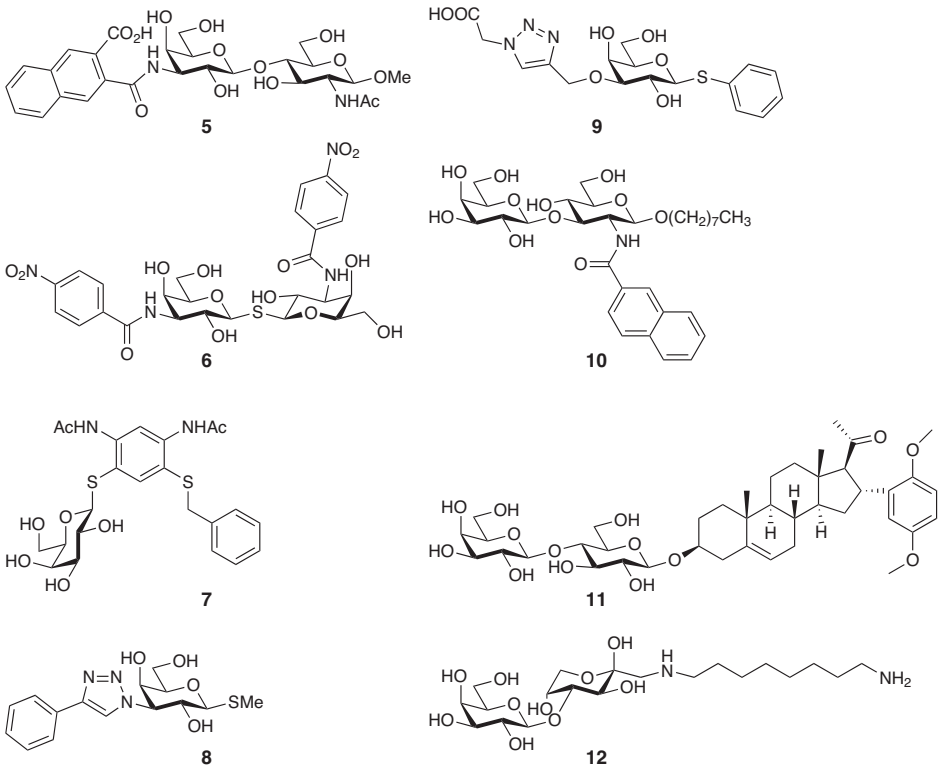


Figure 4. A selection of previously reported synthetic monovalent galectin inhibitors.

2.1.2 Multivalent galectin inhibitors

Multivalency has been described previously (chapter 1.3.3) and the affinity enhancement, accomplished when multivalent inhibitors interact with galectins, has been utilized in the design of numerous synthetic inhibitors. Multivalent galectin inhibitors possess a valuable resource as research tools. However, compared to small galectin inhibitors, some disadvantages, when it comes to the development of drugs are their size and hydrogen bonding properties. Large starbust dendrimers with up to 128 lactose units have been reported with affinity enhancements of up to 130 times per lactose unit, as compared to free lactose, for galectin-1.[63] Tetravalent wedgelike dendrimers (**13**) have been reported to inhibit galectin-1 more than 6000 times better than free lactose (Figure 5).[64]

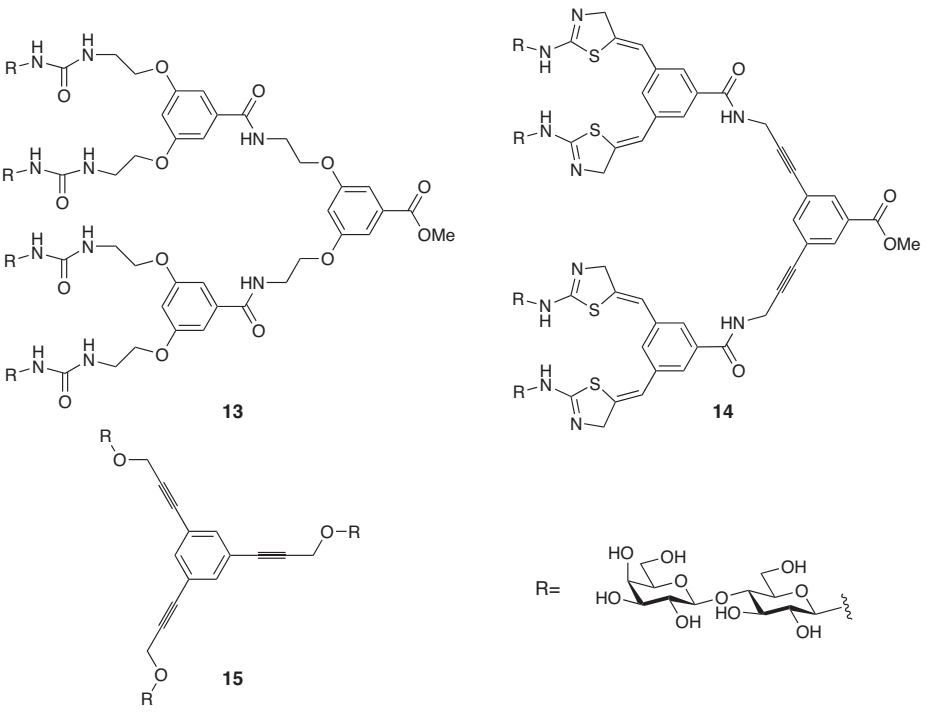


Figure 5. Selection of previously reported synthetic multivalent galectin inhibitors.

Furthermore, Pieters *et al.*[65] have presented rigid multivalent lactose molecules (**14**) together with a valuable comparison of the variations in the observed multivalent effects seen with different assays used for evaluation of inhibitors. This clearly showed that results from different assays should be compared with caution. Their solid phase inhibition assay

showed an affinity enhancement of up to 1071 times for a tetravalent inhibitor as compared with free lactose, whereas, a fluorescence binding assay showed an enhancement of 11 times. Roy *et al.*[66] showed that the trimer **15** had a relative potency of 7.6 times per lactose unit as compared with free lactose for galectin-3. The investigations by Pieters and Roy reveal that rigid structures are preferred by galectin-3 while more flexible structures suits galectin-1 better. A more thorough discussion about multivalency and the interpretation of the glycoside clustering effect will follow in section 7.

2.1.3 Galectin fragments

Truncated galectin-3, galectin-3C, has been shown to significantly suppress tumor growth and to inhibit metastasis in a mouse model of human breast cancer.[27] Galectin-3C lacks the N-terminal peptide tail, used in the formation of type-2b aggregates, and it is possible that galectin-3C disrupts the cross-linked aggregates of native galectin-3 and thereby prevents protein and cell interactions.

2.1.4 Modified citrus pectin, MCP

Pectin is a branched polysaccharide fiber, rich in galactoside residues, that is present in all plant cell walls. The native form has no affinity for galectins, however hydrolysis of citrus pectin, using moderate heating at alkaline conditions followed by acidification, yields shorter water-soluble fibers called modified citrus pectin (MCP). When MCP was orally administered to mice, tumor growth was reduced as well as metastasis. It was shown that MCP inhibits the binding of galectin-3 and galectin-3 expressing cells to vein endothelium.[67] Another MCP called GCS-100 has been shown to induce apoptosis in cells resistant to conventional therapeutic agents.[68] Effects on cell adhesion and angiogenesis have been observed as well (Cell adhesion plays a key role in metastasis, and angiogenesis is the process where cancer cells recruit a blood supply.). However, so far no link to galectin-3 has been established.

2.2 Fluorescence polarization (FP)

We have discussed the importance of finding high affinity inhibitors (dissociation constants in low μM to nM range) for galectins but how do we measure their affinity? There are a number of methods with different advantages and disadvantages, and our method of choice was fluorescence polarization.[69,70] Fluorescence is the emission of light derived from

the excess energy emitted when an electron returns from its excited singlet state back to its ground state. In FP measurements, a fluorescent probe (in this case a fluorescein-tagged saccharide) is excited with plane-polarized light and the degree of polarization remaining in the emitted light is measured. The decrease in polarization depends on, among other things, how much the fluorescent probe moves during the excited state (in average 4 ns for fluorescein). Large molecules move slower as compared to small molecules, therefore, if the fluorescent probe is bound by a large protein the remaining polarization will be larger as compared to a free probe.[71]

In direct binding studies, the carbohydrate of interest is tagged with a fluorophore and the relation of bound and free carbohydrate to a protein is measured directly. This has been done for all fluorescent probes later used for competitive FP (Figure 6A).[70,72-75]

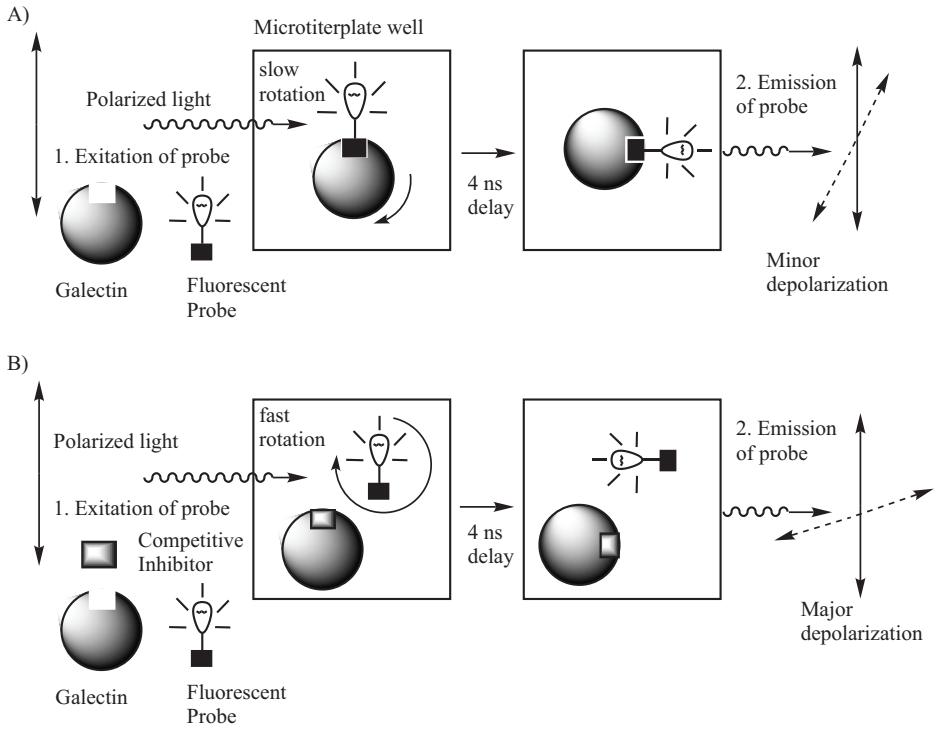


Figure 6. Fluorescence polarization assay. A) Direct fluorescence polarization measured without a competitive inhibitor. B) Competitive fluorescence polarization measured with a competitive inhibitor. (Reprinted with kind permission from Erik Tullberg).

In competitive fluorescence polarization, a mixture of a fluorescent probe and a proposed inhibitor are allowed to competitively bind to the galectin (Figure 6B). The ratio between bound and free fluorescent probe towards the protein is measured, and from these results, the dissociation constant (K_d) of the inhibitor is calculated.[57,70]

3 Objectives

Potent and selective inhibitors are important in the endeavor of the true role of galectins and numerous molecular structures have been synthesized and evaluated as galectin inhibitors.

The objective of this thesis was to design and synthesize galectin inhibitors with improved properties such as high affinity and selectivity towards various galectins.

A second objective was to study the glycoside clustering effect between galectins and multivalent inhibitors, including monomeric galectin mutants lacking the possibility to form aggregates.

4 *O*-galactosyl aldoximes as galectin inhibitors

4.1 Strategy for the synthesis of O-galactosyl aldoximes as galectin inhibitors

Natural saccharides have been proposed as inhibitors of galectins but their use are typically hampered by problematic synthesis, sensitivity towards enzymatic hydrolysis and high polarity. The selectivity that galectins display for β-galactose occupying subsite C, comes from many interactions between the protein and the saccharide (Figure 7). This makes β-galactose an excellent scaffold for the synthesis of new inhibitors. On the other hand, the saccharide that normally occupies subsite D binds with few interactions to galectins. Thus, it is reasonable to replace this saccharide with simpler and less polar structures in order to circumvent the disadvantages of natural saccharides.

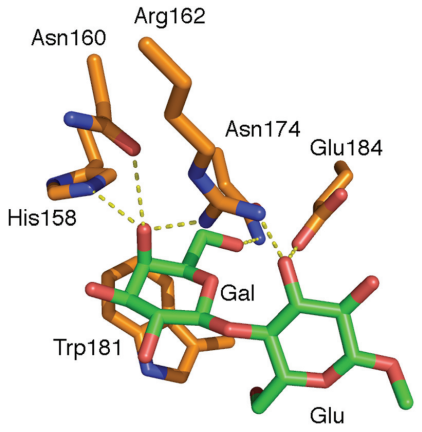
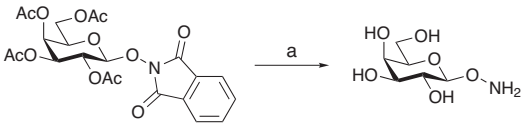


Figure 7. Methyl lactoside in modeled complex with Galectin-3. The hydrophobic surface of galactose stack on the Trp181 and hydrogen bonds are visualized with yellow beads.

In our quest for galectin inhibitors, we first decided to synthesize a panel of *O*-galactosyl aldoximes. The anomeric oxime ethers are expected to possess improved stability against enzymatic hydrolysis, as compared to natural saccharides, they are fairly stable at physiological pH and they can be prepared in water.[76,77] The synthesis is robust with comparably simple purifications and easy access to starting material.

4.2 Synthesis of starting material

The synthesis of *O*-β-D-galactopyranosyl hydroxylamine[76-78] (**17**) is a well known procedure starting with galactosylation of *N*-hydroxyphthalimide. In the final step, the phthalimide derivative **16** is deprotected and the hydroxylamine moiety is unmasked using hydrazine hydrate (Scheme 1). However, many hydrazine derivatives are carcinogenic and their use are therefore regulated. Together with the tedious purification of the final product and the consumption of large quantities of solvents an improvement of this step was desirable. Indeed, treating **16** with methylamine[79] in methanol greatly simplified the workup from a tedious flash chromatography to a very simple short column flash chromatography.



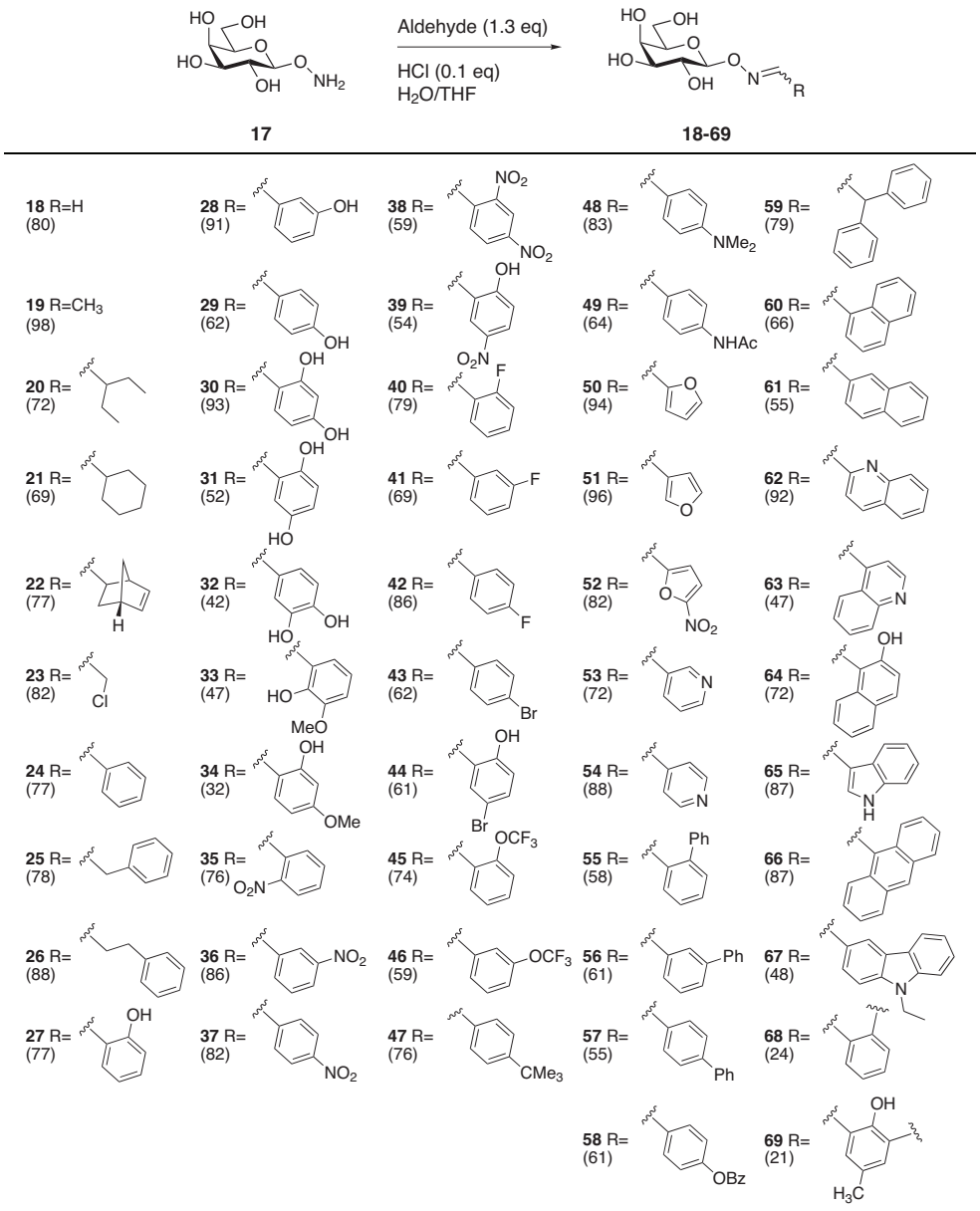
16 **17**

Scheme 1. (a) $MeNH_2$ in MeOH, yield 78%.

4.3 Synthesis of O-galactosyl aldoximes

A panel of 52 aldoximes was synthesized[80] in good to moderate yields by condensation of **17** with a diverse collection of aldehydes under acidic conditions in a mixture of water/THF as solvent (Scheme 2).

In order to validate whether the strategy of replacing the saccharide occupying subsite D with oxime ethers would be feasible for galectins, the aldehydes were chosen in order to be diverse in respect to size, polarity, and geometry.



Scheme 2. Synthesis of aldoximes **18**-**69**. (Yield) in %.

One drawback of the aldoxime synthesis is the formation of *E*- and *Z*-isomers although the *E*-isomer is predominantly formed. However, a few oxime ethers (**20**, **21**, **22**, **23**, **36**, **51**, **52**, and **65**) were obtained as mixtures (*E*/*Z* 10/1 to 3/1). Assignment of *E*/*Z* configurations was based on the chemical shift of the oxime proton in ^{1}H NMR.[81-83] The isomers are

expected to interact with galectins in separate ways, which results in different affinities. Therefore, the isomers of one selected compound, the indole-3-carboxaldoxime **65**, were separated using C-18 RP-HPLC.

4.4 Evaluation of aldoximes as galectin inhibitors

Dissociation constants for the proposed inhibitors (**18**-**69**) were determined by competitive fluorescence polarization[57,70] and compared to the known reference compounds D-galactose, lactose, and *N*-acetyllactosamine as methyl β-glycosides.[84] Evaluation against five galectins, -1, -3, -7, and the N-terminal domain of galectin-8 and -9 allowed for the selectivity of the aldoximes to be studied as well as their affinity for these galectins. The aim was to find less complex structures with the same affinity for galectins as shown by the carbohydrates normally occupying subsite D (*i.e.* glucose in lactose or gluNAc in LacNAc).

The following section describes in more detail the affinity of the aldoximes for each galectin. This will be followed by a "comparison between galectins" where both similarities and differences of the aldoxime binding to the galectins will be compared. A complete list of K_d values can be found in appendix B.

4.4.1 Galectin-1

The affinity of the aldoximes for galectin-1 was generally low and methyl lactoside had about 5 times higher affinity for galectin-1 as compared to the best oxime ethers.

Since phenyl oxime ether **24** displayed no or low affinity enhancement as compared to methyl galactoside (Figure 8 and Table 2), it is reasonable to assume that the affinity enhancement in many substituted aromatic analogues comes from favorable interactions between the substituents on the phenyl ring and subsite D. Thus, the affinity enhancement in **28** (K_d=1000 μM) is probably due to hydrogen bonding between the hydroxy group in *meta* position and subsite D. A nitro group in *meta* position (**36**, K_d=1400 μM) gives almost as good affinity as seen for **28**. Neither a hydroxy group in *ortho* nor *para* position (**27** and **29**) gives any affinity enhancement as compared to phenyl analogue **24**. Therefore the higher affinity of 4-bromo-2-hydroxy phenyl **44** (K_d=1200 μM) might be explained by favorable interactions from the bromo substituent that possibly redirects the *ortho* hydroxyl into a more favorable posture.

The chloromethyl analogue **23** (K_d=1100 μM) belongs to the best inhibitors for galectin-1. However, this analogue turned out to decompose and is therefore not suitable as a galectin inhibitor.

To conclude, the affinity was low for galectin-1 although the best oxime ethers showed about 10 times higher affinity for galectin-1 as compared to MeGal.

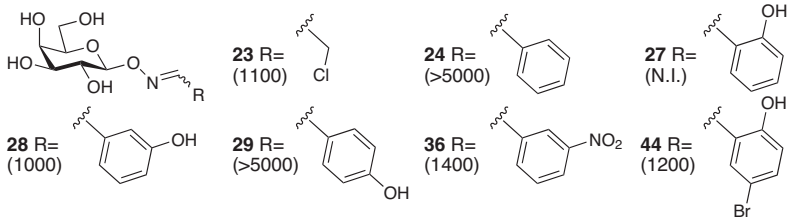


Figure 8. Structures discussed for galectin-1 binding (K_d in μM).

4.4.2 Galectin-3

The phenyl substituted **24** showed no affinity enhancement for galectin-3 as compared to methyl galactoside (Figure 9). However, a hydroxy group in *ortho* position (**27**, K_d=1800 μM) appeared more important than a hydroxy group in *meta* **28** (K_d=2800 μM) or *para* **29** (K_d=4500 μM) position. The dihydroxylated compounds **30**, **31** and **32** displayed further affinity enhancements (K_d=840 μM, 780 μM and 550 μM, respectively). A nitro group in *meta* position (**36**, K_d=530 μM) was 4 times better as compared to *meta* hydroxylated phenyl **28**. An additive effect from hydroxy and nitro groups was observed, comparing *ortho* hydroxy **27** and *meta* nitro phenyl **36** with the second best galectin-3 inhibitor **39** (K_d=360 μM). A bromo substituent in *meta* or *para* position conferred low K_d values (**43**, **44**, K_d=610 μM and 650 μM, respectively). Three of the best inhibitors contained bicyclic aromatic moieties and it is clear that the carbohydrate should be attached on the position next to the second aromatic ring, that is carbon 1 on naphthalene, compare **60** (K_d=370 μM) with **61** (K_d=2300 μM), or carbon 4 on quinoline, compare **63** (K_d=620 μM) with **62** (K_d=1800 μM). Another bicyclic analogue the indole **65** (K_d=330 μM), turned out to be the best inhibitor for galectin-3 with 13 times affinity enhancement as compared to methyl galactoside and almost as good as methyl lactoside (K_d=220 μM).

The purified [*E*]- and [*Z*]-indole-3-carbaldoximes **65** displayed different affinity for galectin-3, with the *E*-isomer (K_d=180 μM) being better than the *Z*-isomer (K_d=550 μM).

Indeed, the [*E*]-indole-3-carboaldoxime provided an improved affinity for galectin-3 similar to methyl lactoside, and 24 times affinity enhancement as compared to methyl galactoside.

From the observation that a hydroxy group in *ortho* position on a phenyl ring (**27**) conferred low K_d values, as did the naphthyl analogue **60**, we set out to synthesize 2-hydroxy naphthyl analogue **64**. However, the affinity was about the same as for compound **60** (480 μM and 370 μM, respectively).

To conclude, the oxime ethers were suitable as galectin-3 inhibitors and many inhibitors showed high affinity for galectin-3. Aldoximes with bicyclic ring systems showed high affinity, as did many disubstituted phenyl oxime ethers.

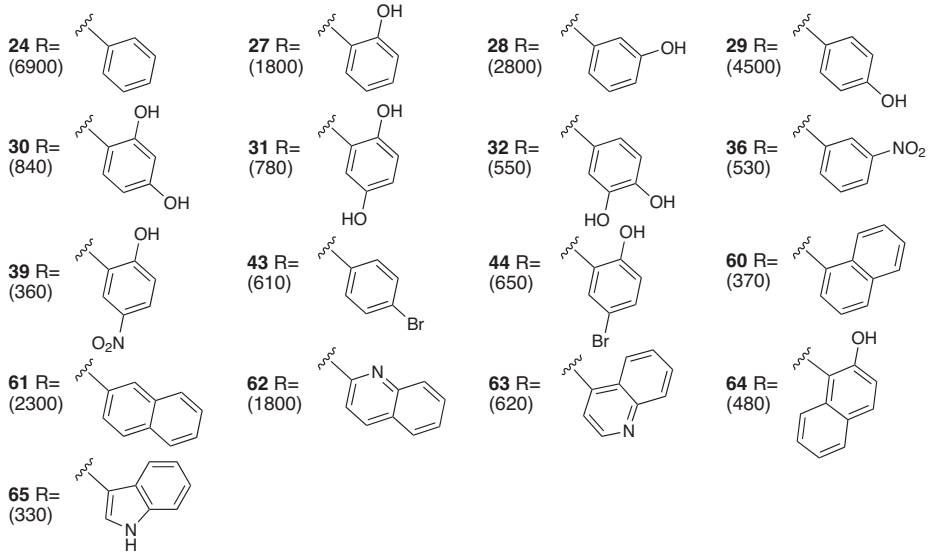


Figure 9. Structures discussed for galectin-3 binding (K_d in μM).

4.4.3 Galectin-7

Tricyclic oxime ether analogues, anthracene **66** (K_d=340 μM) and carbazole **67** (K_d=780 μM) conferred low K_d values for galectin-7 (Figure 10). Compound **66** had 14 times higher affinity as compared to methyl galactoside and compared favorable with LacNAc. Hydroxy substituted naphthyl **64** (K_d=840 μM) showed high affinity in contrast to naphthyl aldoxime **60** (K_d>2000 μM). The third best inhibitor for galectin-7 was 2,5-dihydroxylated **31** (K_d=510 μM). *Para* substituted phenyl analogues like bromo **43** and benzoyloxy **58** (K_d=1300 μM and 1000 μM, respectively) conferred low K_d values and

para-acetamido phenyl **49** (K_d=390 μM) was the second best galectin-7 inhibitor with 14 times higher affinity than methyl lactoside.

To conclude, galectin-7 preferred inhibitors with large ring systems as well as *para* substituted phenyl oxime ethers. Hydroxylated phenyl aldoximes were not preferred except for the 2,5-dihydroxylated **31**.

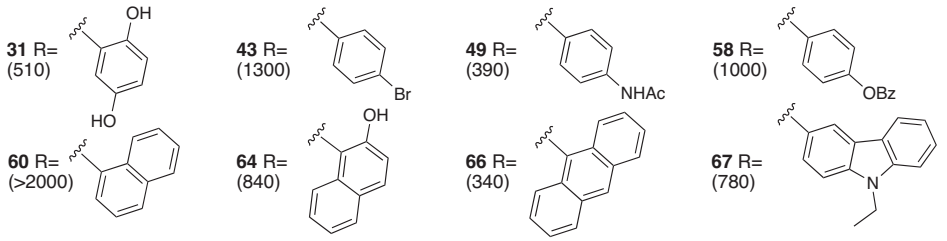


Figure 10. Structures discussed for galectin-7 binding (K_d in μM).

4.4.4 Galectin-8N

Only three oxime ethers displayed good affinity for galectin-8N (Figure 11). The anthracene oxime ether **66** (K_d=780 μM) had the same K_d as LacNAc and was 7 times better than MeGal. The second best inhibitor, hydroxy naphthyl **64** (K_d=370 μM) carried a hydroxy group *ortho* to the aldoxime group as did the best inhibitor, 2,5-dihydroxy phenyl aldoxime **31** (K_d=160 μM). Furthermore, **31** showed 33 times affinity enhancement as compared to MeGal and 4 times as compared to LacNAc.

To conclude, the affinity for galectin-8N was low for all but three oxime ethers, which in contrast, showed rather high affinity for galectin-8N.

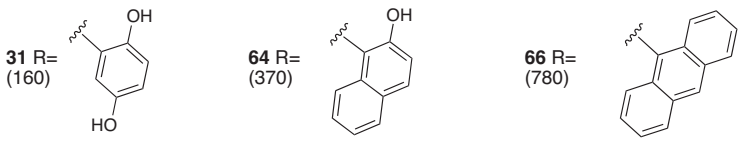


Figure 11. Structures discussed for galectin-8N binding (K_d in μM).

4.4.5 Galectin-9N

Even the best aldoximes showed low affinity for galectin-9N (Figure 12). The anthracene analogue **66** had a K_d of 950 μM and although being the best analogue, it was

only 3.5 times better than MeGal and worse than both LacNAc and MeLac. The 2,5-dihydroxylated **31** and benzoyloxy phenyl **58** had about the same affinity for galectin-9N as did **66**.

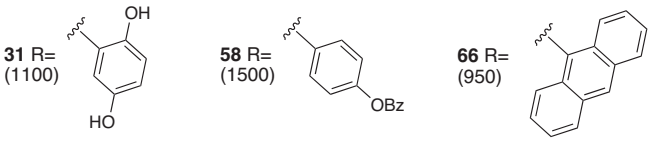


Figure 12. Structures discussed for galectin-9N binding (K_d in μM).

4.4.6 Comparison between galectins

Except for galectin-1, the other galectins evaluated bind methyl galactoside with a K_d between 4 and 5 mM (10 mM for galectin-1) (Table 2). However, while galectin-1 and -3 show higher affinity for methyl LacNac, galectin-7, -8N, and -9N prefer methyl lactoside.[57,70,84] Evaluation of the oxime ether showed, not surprisingly, that the structure of the aldehyde component greatly influenced the affinity for the different galectins.

Comparing the 3 best inhibitors for each galectin, *i.e.* lowest K_d, revealed selectivity towards galectin-1, -3, -7, and -8N. In contrast, the best inhibitors for galectin-9N showed higher affinity for some of the other galectins (Table 2).

The aminoxy moiety had probably no or only low influence on the K_d values of the aldoximes since neither aminoxy compound **17** nor the smallest oxime ether **18** showed any improved binding to the galectins, as compared to methyl galactoside, with the exception of **17**, which bound to galectin-7 with a K_d of 2200 μM.

The aldoximes showed the same low affinity for galectin-1 as for galectin-9N. However, *ortho* trifluorometoxy **45** showed some selectivity for galectin-1.

Bicyclic systems are advantageous for galectin-3 inhibition. Three of the four aldoximes with highest affinity for galectin-3 are bicyclic aldoximes (**60**, **64**, and **65**). Two of them, the indole-3-carboxaldehyde **65** (K_d=330 μM) and naphthyl **60** (K_d=370 μM), showed high selectivity for galectin-3. The hydroxylated naphthyl **64** also showed high affinity for galectin-7 and -8N, which is difficult to explain since neither the *ortho* hydroxylated phenyl **27** nor the naphthyl **60** showed any affinity for these galectins.

Table 2. Dissociation constants (μM) for the three best inhibitors of each galectin (bold). N.I.= no inhibition measurable at an inhibitor concentration of 5 mM for galectin-1 and -3, and 2 mM for galectin-7, -8N, and -9N as determined by fluorescence polarization[a].

Cpd	Galectin-1	Galectin-3	Galectin-7	Galectin-8N	Galectin-9N
MeGal	≈10000	4400	4800	5300	3400
MeLac	190	220	91	52	23
LacNac	70	67	490	700	500
28	**1000**	2800	1900	N.I	N.I
44	**1200**	650	>2000	>2000	N.I
45	**910**	6300	N.I	N.I	>2000
39	8400	**360**	>2000	>2000	>2000
60	>5000	**370**	>2000	N.I	N.I
65	>5000	**330**	>2000	>2000	N.I
31	2300	780	**510**	**160**	**1100**
49	1200	>5000	**390**	N.I	N.I
66	N.I[b]	4600	**340**	**780**	**950**
58	N.I	820	1000	>2000	**1500**
64	—[c]	480	840	**370**	>5000

[a]At 4°C except for galectin-3 and -8N, which were evaluated at room temperature.

[b] non inhibitory.

[c] not determined.

The relatively high flexibility of the *O*-galactosyl aldoximes makes it difficult to evaluate structure-affinity relations using computer models of the oxime ethers in complex with galectins. Nevertheless, energy minimization of 2-naphthyl aldoxime **60** in complex with galectin-1 and -3 visualizes the possibility of large variations in the binding mode of the aldoxime moiety to the different galectins (Figure 13). The narrow subsite D in galectin-1 may accommodate the naphthyl group in the vicinity of unfavorable interactions from Glu71 while, in the wider subsite of galectin-3, the naphthyl is in a different posture, which suggests better complementarity with the protein surface. The Arg162 and Glu165 in galectin-3 give no or less unfavorable interactions with the naphthyl substituent, because Glu165 is neutralized by ion-pairing with Arg186.

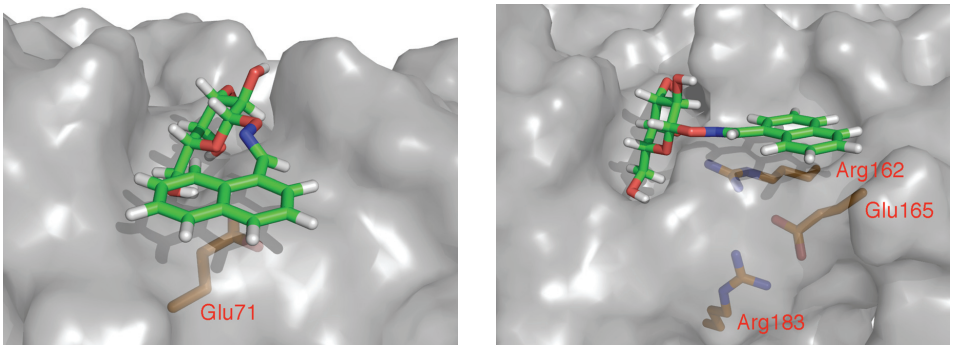


Figure 13. Naphthyl aldoxime **60** in modeled complexes with (a) galectin-1 and (b) galectin-3. The narrow subsite-D in galectin-1 places the naphthyl moiety in an unfavorable posture over Glu71. Whereas the wider subsite in galectin-3 places the naphthyl near Arg162 and Glu165 that is neutralized by ion-pairing with Arg183.

The anthracene aldoxime **66** (K_d=340 µM) showed high affinity but moderate selectivity for galectin-7, because this aldoxime bound both galectin-8N and -9N. However, the *para* substituted acetamidophenyl **49** (K_d=390 µM) had 3 times higher affinity for galectin-7, as compared to galectin-1, and displayed no or low affinity for the other galectins.

The best inhibitor for galectin-8N, a 2,5-dihydroxylated aldoxime (**31**, K_d=160 µM), had 3 times higher affinity for galectin-8N than for galectin-3 and -7.

None of the aliphatic aldoximes displayed any affinity towards the galectins.

4.5 Conclusions

The glucose moiety of lactose and LacNAc has been replaced with less polar structures. The aldoximes have lower polarity and a proposed increased stability towards enzymatic degradation as compared to natural saccharides. In combination with the robust synthesis and improved selectivity for various galectins this characteristics pave the way for further advancements towards novel galectin inhibitors with possibly improved pharmacological properties.

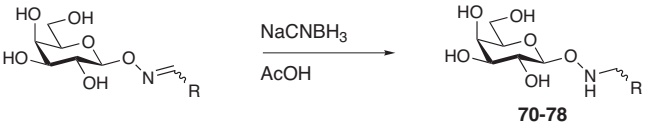
5 Hydroxylamines as galectin inhibitors

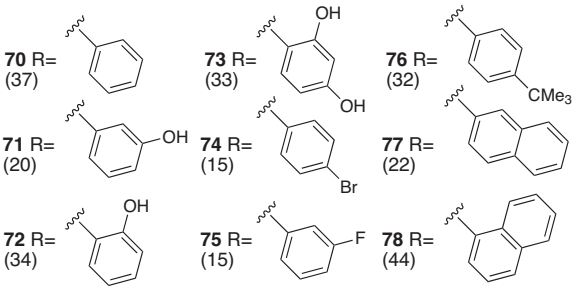
Some of the oxime ethers were as good inhibitors for galectins as natural disaccharides. Nevertheless, an attempt to further increase the affinity and selectivity of the aldoximes were undertaken by altering the chemical structures and properties of the oxime ethers.

Reduction of the oxime ethers would result in hydroxylamines that provide a different functionality for binding to galectins, as well as a new possibility for diversification. The hydroxylamines are more flexible as compared to the aldoximes, thus they may more easily find beneficial interactions with subsite D. However, any improved interaction may be counterbalanced by the increased flexibility of the aminoxy functionality as compared to the oximes.

5.1 Reduction and acylation

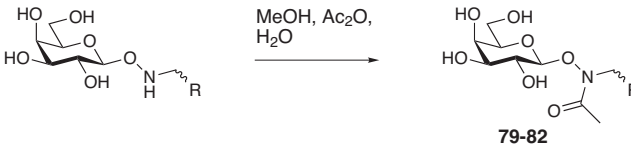
Reduction of the oxime ether bond turned out to be more challenging than expected and various conditions were tested. Reduction using pyridine-borane complex[85] initially looked promising, but turned out to result in significant formation of byproducts as well as cleavage of the N-O bond. Reduction with $NaCNBH_3$ in various acidic media are known in literature and the use of glacial acetic acid and $NaCNBH_3$[86,87] gave the products, although large excess and repeated additions of $NaCNBH_3$ where needed, and the yields varied due to starting material recoveries. (Scheme 3).

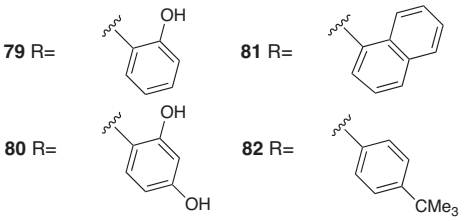




Scheme 3. Synthesis of hydroxylamines **70**-**78**. (Yield) in %.

Four of the hydroxylamines were quantitatively acylated in a mixture of methanol, water and acetic anhydride (scheme 4).





Scheme 4. Synthesis of acetylated hydroxylamines **79**-**82** in quantitative yields.

5.2 Evaluation of hydroxylamine derivatives as galectin inhibitors

In most cases the hydroxylamines lost their affinity for galectin-1, -3, and -7 as compared to their parent oxime ethers (Table 3). One exception was the naphthyl analogue **77**, which has higher affinity for galectin-1, -3, and -7 (K_d=1900 µM, 820 µM, and 490 µM, respectively) as compared to the parent aldoxime **61** (K_d=N.I., 2300 µM, and >2 µM

respectively). While the affinity for galectin-1 and -3 is still moderate, **77** has the same affinity for galectin-7 as that of LacNAc.

No conclusions about the affinity for galectin-8N and -9N can be drawn since neither the hydroxylamines nor their parent aldoximes showed any affinity for these galectins.

Table 3. Dissociation constants (μM) for hydroxylamines **70-78** as determined by fluorescence polarization[a].

Cpd	Galectin-1	Galectin-3	Galectin-7	Galectin-8N	Galectin-9N
70	>5000	4100	1200	>2000	>2000
71	N.I[b]	4600	>2000	>2000	>2000
72	N.I	4600	>2000	>2000	N.I
73	N.I	3700	2400	>2000	>2000
74	N.I	2200	>2000	>2000	>2000
75	N.I	4100	2100	>2000	N.I
76	N.I	4600	>2000	N.I	>2000
77	1900	820	490	—[c]	—
78	N.I	3200	2500	>2000	>2000

[a] At 4°C except for galectin-3 and -8N, which were evaluated at room temperature.

[b] non inhibitory.

[c] not determined.

All hydroxylamines lost their affinity for galectins after acylation with one exception (Table 4). The naphthyl analogue **81** (K_d=1700 μM) had similar affinity for galectin-7 as compared to the parent hydroxylamine **78** (K_d= 2500 μM). Interestingly, naphthyl aldoxime **60** was one of the best inhibitors for galectin-3 (K_d=370 μM) but lost most of its activity after reduction (**78**, K_d=3200 μM) and showed no affinity for galectin-3 after acylation (**81**).

Table 4. Dissociation constants (μM) for acylated hydroxylamines **79-82** as determined by fluorescence polarization[a].

Cpd	Galectin-1	Galectin-3	Galectin-7	Galectin-8N	Galectin-9N
79	N.I[b]	>5000	>2000	>N.I	>2000
80	>5000	>5000	3200	>2000	>2000
81	>5000	>5000	1700	1800	3200
82	N.I	N.I	>2000	N.I	N.I

[a] At 4°C except for galectin-3 and -8N, which were evaluated at room temperature.

[b] non inhibitory.

To conclude, since no affinity enhancement was seen for the reduced oxime ethers towards the galectins, except in one case, this project was soon abandoned due to the modest efficiency of the reduction, as well as for the disheartening galectin affinities. Instead we turned our attention towards other ways of increasing the efficacy of the oxime ethers.

6 A fragment based approach

The successful replacement of the saccharide occupying subsite D in galectin-3 with oxime ethers represents an advancement towards small galectin inhibitors with improved affinity.[80] However, the affinity still remained low and an improvement was requested.

A fragment based approach, in which previous optimization of the structure occupying subsite B in galectin-3 is combined with the presented targeting of subsite D, was hypothesized to lead to monosaccharide based inhibitors with high selectivity and affinity for galectin-3.

6.1 Galactosyl aldoximes with subsite B-binding substituents

Previous targeting of subsite B in galectin-3 with aromatic amides at C3' of LacNAc,[52,55] as well as 1,2,3-triazoles at C3 of galactose,[58] resulted in inhibitors with high affinity for galectin-3. Thus, combining the subsite D-binding indole-3-carbaldoxime **65**, with the efficient subsite B-binding 3,5-dimethoxy benzamide analogue **83** in a fragment based approach, would possibly result in a monosaccharide based inhibitor with high affinity as well as selectivity for galectin-3 (Figure 14). Furthermore, combining **65** with methyl amide triazole **85** and phenyl triazole **86** could also yield inhibitors with increased selectivity as well as affinity for galectin-3 (Figure 15). The resulting monosaccharides **84**, **88** and **89** are less polar and probably more stable towards enzymatic hydrolysis as compared to **83** and **87**.

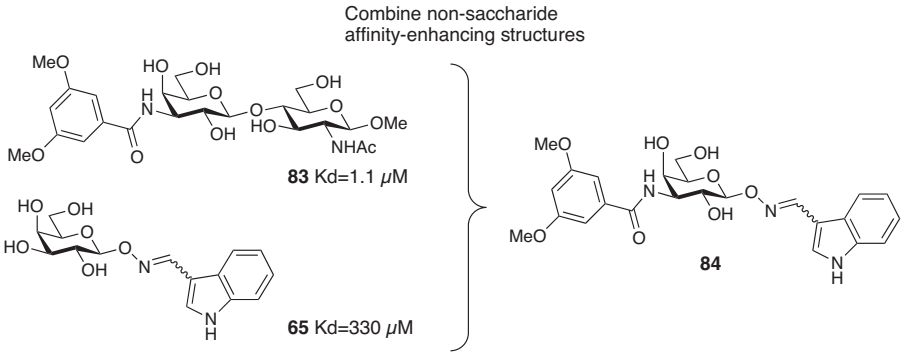


Figure 14. Combination of subsite B-binding aromatic amides with a subsite D-binding indole-3-carbaldoxime for efficient and selective galectin-3 inhibition.

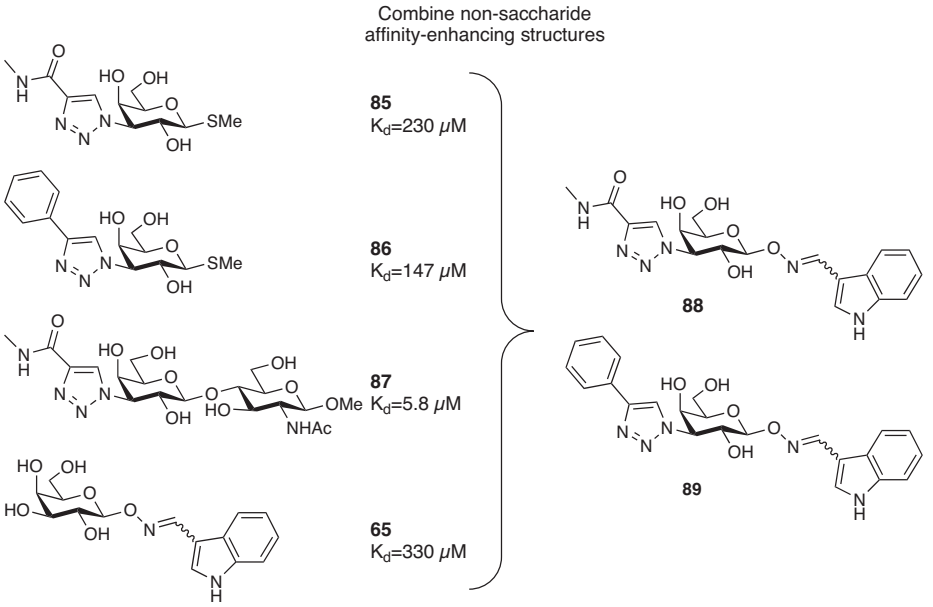
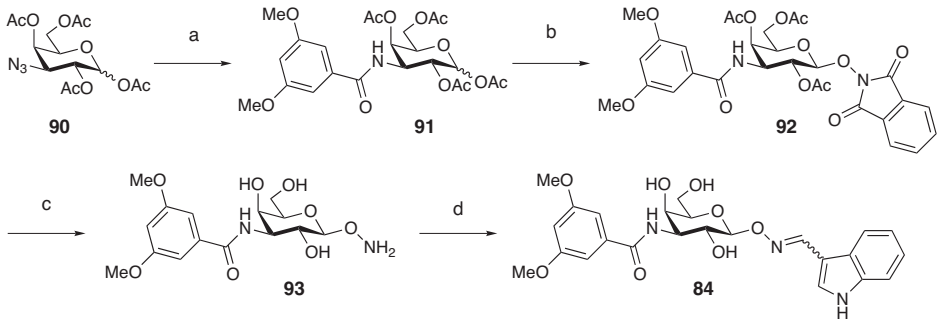


Figure 15. Combination of subsite B-binding triazoles with a subsite D-binding indole-3-carbaldoxime for efficient and selective galectin-3 inhibition.

6.2 Synthesis of a galactosyl aldoxime with an aromatic subsite B-binding amide at C3

The 3,5-dimethoxy amide **91** was prepared by catalytic hydrogenation in ethanol/HCl over Pd/C of the azido group in the known structure **90**,[88] followed by acylation with 3,5-dimethoxybenzoyl chloride (Scheme 5). The reaction time of the hydrogenation was limited to 70 minutes and it is important to work fast and to avoid heating in order to limit acetyl migration from neighboring *O*-acetate protecting groups.[55]

Bromination of **91** followed by glycosylation with *N*-hydroxyphthalimide in a two phase system gave **92**. Deprotection using hydrazine hydrate gave the aminoxy compound **93**, which was condensed with indole-3-carboxaldehyde under acidic conditions to form **84** in 74% yield. The product was obtained as a E/Z 5/1 mixture and an attempt to separate the isomers using C-18 RP-HPLC was done without success.

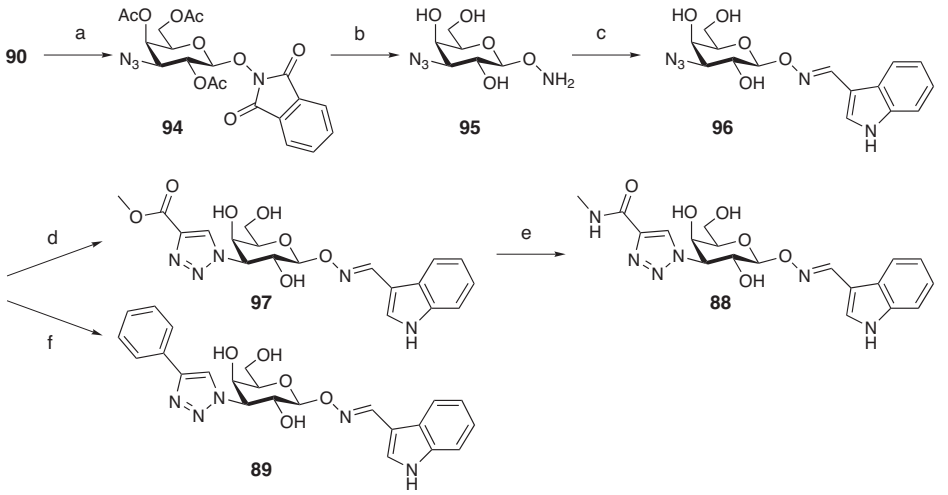


Scheme 5. Synthesis of a galactosyl oxime with a subsite B-binding amide at C3. a) i. H_2, Pd/C, HCl, EtOH. ii. 3,5-dimethoxybenzoyl chloride, pyridine, CH_2Cl_2. 91%; b) i. HBr, CH_2Cl_2. ii. *N*-hydroxyphthalimide, tetrabutylammonium hydrogensulfate, CH_2Cl_2, Na_2CO_3, 55%; c) Hydrazine hydrate, MeOH. 72%; d) Indole-3-carboxaldehyde, H_2O/THF, HCl. 74%, E/Z 5:1.

6.3 Synthesis of galactosyl aldoximes with subsite B-binding 1,2,3-triazoles at C3

Compound **94** was obtained in 33% yield from glycosylation of **90**[88] using boron trifluoride diethyl etherate and *N*-hydroxyphthalimide (Scheme 6). The reaction gave low yields and the α-anomer of the starting material was recovered, which explains the low

yield. Deprotection with methylamine in methanol gave hydroxylamine **95** that was reacted with indole-3-carboxaldehyde in an acidified H_2O/THF mixture to form **96**. Two Cu(I)-catalyzed 1,3-dipolar cycloaddition reactions were performed.[89,90] The first by reacting **96** with methyl propiolate gave **97**, which after treatment with methylamine resulted in **88**. The second triazole was prepared by cycloaddition between phenyl acetylene and **96** to furnish **89**.



Scheme 6. Synthesis of galactosyl oximes with subsite B-binding 1,2,3-triazoles at C3. a) *N*-hydroxyphthalimide, boron trifluoride diethyl etherate, CH_2Cl_2, 33%; b) 2.3M $MeNH_2$ in MeOH, 88%; c) Indole-3-carboxaldehyde, HCl (0.1 eq), H_2O/THF, 67%; d) i. Cu(I), methyl propiolate, propanol; e) 40% $MeNH_2$ in H_2O, 65%; f) Cu(I), phenyl acetylene, propanol, 45%.

6.4 Evaluation of galactosyl aldoximes with subsite B binding substituents at C3

The use of aromatic amides at C3 indeed supported our hypothesis, indicating that a subsite B-binding amide combined with a subsite D-binding oxime ether, could lead to efficient monosaccharide based inhibitors of galectin-3. The affinity of **84** (K_d=46 μM) for galectin-3 was 7 times higher as compared to the oxime ether analogue **65** (Table 2) although much lower as compared to 3,5-dimethoxybenzylamido **83** (K_d=1.1 μM). This came as no surprise since LacNAc showed about 5 times higher affinity for galectin-3 as

compared to the oxime ether **65** indicating that GlcNAc has higher affinity for subsite D than the oxime ether group. Nevertheless, **84** compared favorable with LacNAc (K_d=67 μM) and further analysis showed a significant selectivity of about 8 times for galectin-3 over galectin-7, -8N and -9N (Table 5).

Table 5. Dissociation constants (μM) for galactosyl aldoximes with an amide or 1,2,3-triazole substituent at C3, as determined by fluorescence polarization[a].

Cpd	Galectin-3	Galectin-7	Galectin-8N	Galectin-9N
84	46	390	410	370
88	11	—[b]	870	920
89	17	>600	>3000	>3000

[a] At 4°C except for galectin-3 and -8N, which were evaluated at room temperature.

[b] not determined.

The C3 1,2,3-triazoles **88** (K_d=11 μM) and **89** (K_d=17μM) were significantly better as galectin-3 inhibitors as compared to **84** (Table 5). An additive effect between the substituents targeting subsite B and D is clearly seen by comparing previously published results for the parent 3-deoxy-3-(1*H*-1,2,3-triazole-1-yl)-1-thio-galactosides[58] **85** and **86** (K_d=230 μM and 147 μM, respectively) and oxime ether **65**[80] (K_d=330 μM). Furthermore, the affinity of **88** and **89** for galectin-3 was 6 respectively 4 times higher as compared to the methyl glycoside of LacNAc (K_d=67 μM) and 20 respectively 13 times higher than the methyl glycoside of lactose (K_d=220 μM). The triazole moiety of **88** gave a 30-fold affinity enhancement as compared to **65**, which surpasses the 12-fold enhancement seen for a LacNac derivative with the same triazole group (**87**) as compared to LacNAc. Indeed, **88** and **89** display higher selectivity for galectin-3 over galectin-7, -8N, and -9N.

Energy minimization of **89** in complex with galectin-3 reveals a low energy conformation where the phenyl triazole at galactose C3 and the indole-3-carboxaldehyde at the anomeric center pinch Arg144 (Figure 16).

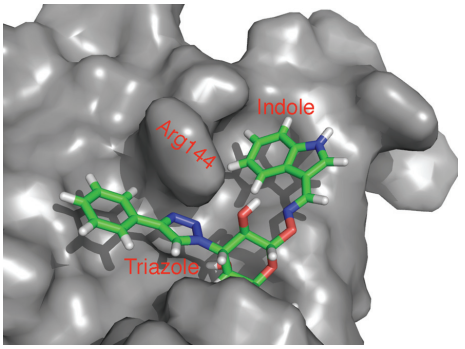


Figure 16. Energy minimization of **89** in modeled complex with galectin-3. Arg144 is pinched between the phenyl triazole at C3 of galactose and indole-3-carboxaldoxime at C1.

An observation from this modeling was that the cleft harboring the indole moiety of **89** could be suitable for hosting other bicyclic aromatic structures as well. This was experimentally observed for the high affinity aldoximes **60**, **63** and **64** (Table 2), where the carbohydrate was attached to position 1 on the bicyclic ring. In contrast, the aromatic bicycles with the carbohydrate attached to position 2 would probably not project into the cleft and this might be the explanation for the poor affinities seen for aldoxime **60** and **61**, however, these observations remain to be modeled.

6.5 Conclusions

Combining the subsite D-binding oxime ethers with subsite B-binding triazoles gave selective monosaccharide based inhibitors with low micromolar affinity for galectin-3. This may be an important step towards novel research tools for investigations of the proposed roles of galectin-3 in cancer and metastasis.

Utilizing the developed fragment based approach in which the oxime ethers displaying selectivity as well as high affinity for various galectins, is combined with different structures targeting subsite B of different galectins, may lead to inhibitors with high affinity and selectivity towards other galectins.

7 Multivalent lactose derivatives and the glycoside clustering effect

Events controlled by carbohydrate interactions *in vivo* often require significantly higher affinity as compared to the low affinity often seen for protein-carbohydrate interactions. The multiple binding utilized in nature to circumvent this disadvantage can be exploited in the design of high affinity multivalent inhibitors for galectins.

7.1 The glycoside clustering effect

The compensation of low affinity by multiple binding sometimes results in an increased affinity beyond what would be expected from the increased number of interactions.[91,92] This observation is termed the glycoside clustering effect and several mechanisms may operate in this enhancement of relative potency seen for some multivalent inhibitors. An aggregative effect may arise when multivalent inhibitors bind multivalent receptors to form aggregates.[35] The different types of aggregates have previously been described. In contrast, a chelate effect is observed when a multivalent inhibitor binds to multiple binding sites within a protein. Finally, the increased local concentration of binding epitopes around a single binding site is referred to as a statistical effect. That means, when the binding epitope leaves the binding site, there is a new binding epitope in the vicinity. This results in a slower off-rate of the inhibitor as a whole,[93] by a faster on-rate of the individual binding epitope. However, for future discussion I will also refer to a fourth effect called "non-specific effect" by *Kiessling et al.*.[93] In "non-specific effect" the aglycon and/or another binding epitope of a multivalent inhibitor interacts with the surface of the protein and not, as in the chelate effect, with a previously defined binding site.

Several research groups have exploited the idea of using multivalent inhibitors as galectin inhibitors[94] and multivalent enhancement has been seen for galectin-1,[63,65,95] galectin-3,[63,65,66,93,95] galectin-4 (N-terminal),[96] and galectin-5.[65]

7.2 Interpreting the glycoside clustering effect

Although the glycoside clustering effect is well-known and numerous reports of multivalent inhibitors have been published there are still few hard facts in this research area. Many suggestions have been made both for the interpretation of the glycoside clustering effect as well as which of the above mentioned mechanisms, contributing the most in each specific case. The glycoside clustering effect is generally reported as a relative potency of the inhibitor as compared to a reference compound. The relative potency is calculated by dividing the K_d value of the inhibitor with the K_d value of the reference compound. If the inhibitor is multivalent, this value is further divided with the number of binding epitopes in order to give the valency corrected glycoside clustering effect. The literature on cluster effects between galectins and multivalent inhibitors invariably reports the cluster effect relative to lactose. This may sometimes be misleading as effects from direct interactions between the galectins and the aglycon of the inhibitor are not taken into account.[65,97] Thus, large cluster effects, as compared to lactose, may not be significant when accounting for the interaction of the aglycon with the protein.

7.3 Multivalent lactose derivatives

In order to gain an affinity profit from the glycoside clustering effect between multivalent inhibitors and galectins, we set out to synthesize a panel of monovalent and multivalent inhibitors.

By utilizing modified mono- and polyfunctional unnatural amino acids based on phenyl-bis-alanine (PBA) and phenyl-tris-alanine (PTA), from Frejd *et al.,*[98-100] we had access to scaffolds that could rather easily be modified, and that contained two orthogonal functionalities (amine and ester) that could enable derivatisation with two different molecular entities (for example a galectin inhibitor and a fluorescent tag). Since peptide coupling of the PBA or PTA and an unprotected carbohydrate can be difficult, we turned our attention to other ways of attaching carbohydrate residues onto the PBA or PTA.

The copper(I) catalyzed 1,3-dipolar cycloaddition[90,101-103] between terminal alkynes and azides has been used for the synthesis of intricate carbohydrate structures. The ready availability of 2-azidoethyl lactoside,[104] together with the robust features and compatibility with many functional groups, makes the 1,3-dipolar cycloaddition ideal for our purposes.

Suitable alkyne precursors from the above mentioned PBA and PTA were synthesized from two acetylenic precursors: propiolic acid and propargyl chloroformate (Figure 17).[97] The panel was designed so that a limited number of scaffolds would provide as much information as possible for the optimization and studying of the glycoside clustering effect.

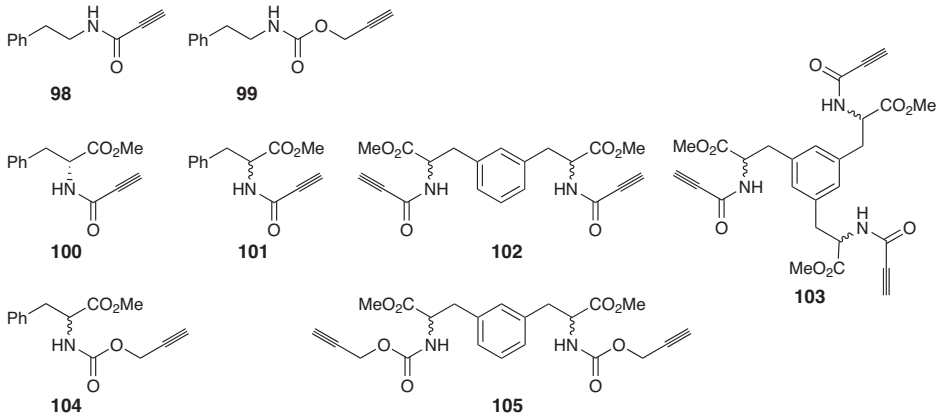
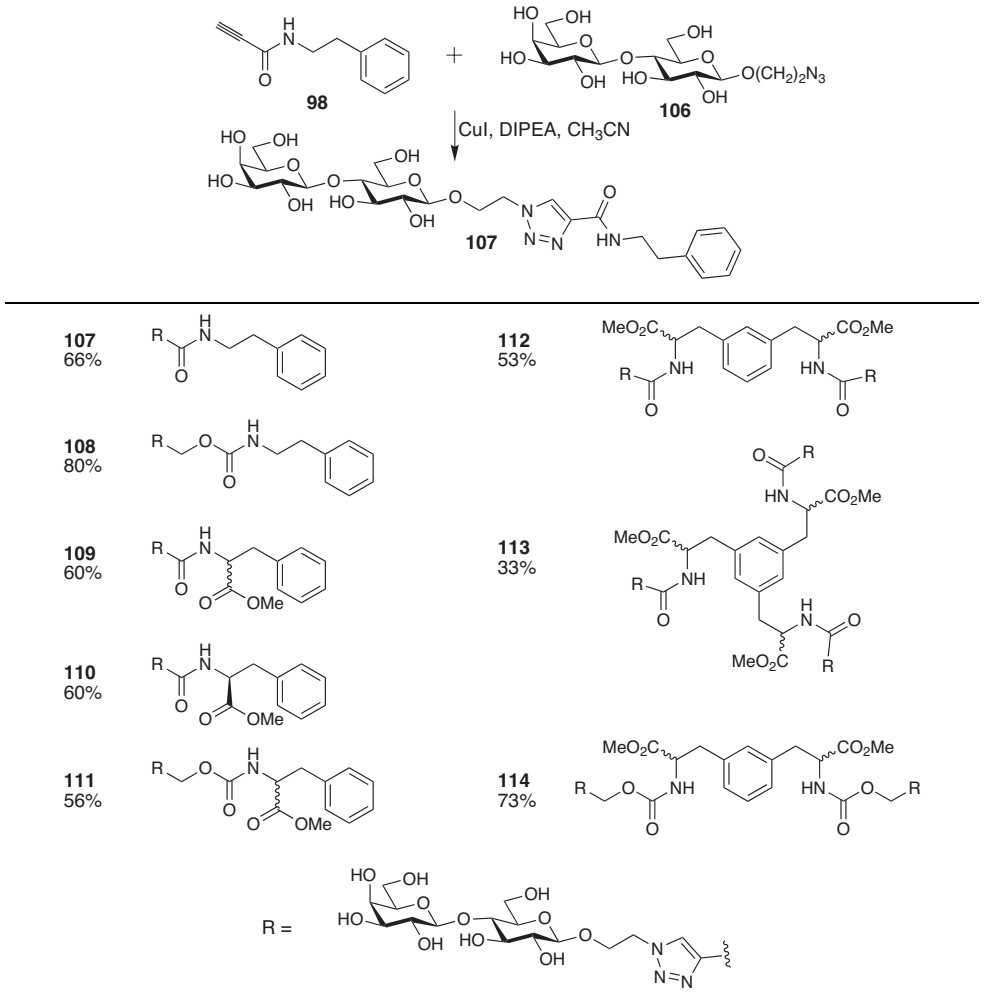


Figure 17. Acetylenic precursors **98**-**105**.

Information about a possible glycoside clustering effect was expected to be obtained by comparing monovalent **101** and **104** with di- and trivalent **102**, **103** and **105**, respectively. The influence of a longer carbamate linker in **99**, **104**, and **105** was compared with the shorter amide linker in **98**, **100**, **101**, **102**, and **103**. The racemic properties of these scaffolds constitute a possible disadvantage. Thus racemic **101** was compared with enantiomerically enriched **100** and finally the effect of the ester functionality was investigated by comparing **98** with **100** and **101**, and **99** with **104**.

The alkynes **98**-**105** were reacted with 2-azidoethyl lactoside according to literature procedures[101] (Scheme 7). The use of Cu(I) as catalyst not only lowers the activation energy of the reaction, it also ensures the regioselectivity of the reaction so that the 1,4-heterocyclic derivative is favored.[89,90,103,105]



Scheme 7. Synthesis of 1,4-substituted 1,2,3-triazoles **107-114** via 1,3-dipolar cycloaddition reactions exemplified with **107**.

7.4 Evaluation of lactose derivatives

The inhibitors were evaluated as inhibitors of galectin-1, -3, and -7, the C-terminal domain of galectin-4 as well as the N-terminal domain of galectin-4, -8, and 9 using fluorescence polarization as previously discussed.[57,70]

It was apparent that the aglycon fragment had a profound effect on the affinity of the inhibitors as all monovalent inhibitors showed higher affinity towards galectin-1, -3, and -

4N, as compared to methyl β-lactoside (Table 6). Galectin-1 showed a consistent preference for the aglycons without the ester moiety (**107** and **108**), as well as for the carbamate linkers (**108** and **111**), a preference not generally observed for the other galectins. In particular, ester free carbamate **108** (K_d=24 μM) showed a pronounced affinity for galectin-1 with a relative potency of 8 as compared to MeLac (K_d=190 μM).

The preference for the diastereomerically enriched **110** over **109** was most profound for galectin-4N and -4C.

Galectin-4N showed a significantly higher affinity for trivalent **113** as compared to the monovalent inhibitor **107**. The valency corrected glycoside clustering effect relative to the monomer **109** was 7 for trivalent **113** and 2.1 for divalent **112** (25 and 5 respectively, as compared to MeLac) (Table 7).

Table 6. Dissociation constants (μM) for **107**-**114** and relative potencies compared to MeLac as determined in a fluorescence polarization assay[a].

Cpd	Valency	Galectin-1		Galectin-3		Galectin-4N		Galectin-4C	
		K_d	Rel	K_d	Rel	K_d	Rel	K_d	Rel
MeLac	1	190	1	220	1	540	1	1200	1
107	1	80	2.4	66	3.3	230	2.3	2200	0.5
108	1	24	7.9	66	3.3	220	2.5	1600	0.8
109	1	120	1.6	75	2.9	460	1.2	2300	0.5
110	1	80	2.4	70	3.1	200	2.7	1100	1.1
112	2	8.3	23	30	7.3	110	4.9	710	1.7
113	3	7.4	26	17	13	22	25	360	3.3
111	1	49	3.9	86	2.6	220	2.5	1700	0.7
114	2	3.2	59	27	8.1	110	4.9	570	2.1

		Galectin-4		Galectin-7		Galectin-8N		Galectin-9N	
		K_d	Rel	K_d	Rel	K_d	Rel	K_d	Rel
MeLac	1	1400	1	91	1	52	1	23	1
107	1	1300	1.1	380	0.2	63	0.8	51	0.5
108	1	1100	1.3	100	0.9	61	0.9	48	0.5
109	1	1700	0.8	210	0.4	94	0.6	97	0.2
110	1	1400	1	270	0.3	62	0.8	85	0.3
112	2	750	1.9	42	2.2	29	1.8	27	0.9
113	3	360	3.9	32	2.8	19	2.7	18	1.3
111	1	1300	1.1	240	0.4	58	0.9	110	0.2
114	2	630	2.2	96	0.9	35	1.5	31	0.7

[a]At 4°C except for galectin-3 and -8N, which were evaluated at room temperature.

Table 7. Valency corrected glycoside clustering effects for **112**, **113** and **114** calculated relative to MeLac and to the corresponding monomers, Mon = **109** and **111**

Cpd	Mon	Valency	Galectin-1 Cluster effect		Galectin-3 Cluster effect		Galectin-4N Cluster effect		Galectin-4C Cluster effect	
			MeLac	Mon	MeLac	Mon	MeLac	Mon	MeLac	Mon
112	**109**	2	11	7.2	3.7	1.3	2.5	2.1	0.8	1.6
113	**109**	3	8.6	5.4	4.3	1.5	8.2	7.0	1.1	2.1
114	**111**	2	30	7.7	4.1	1.6	2.5	1.0	1.1	1.5

			Galectin-4 Cluster effect		Galectin-7 Cluster effect		Galectin-8N Cluster effect		Galectin-9N Cluster effect	
			MeLac	Mon	MeLac	Mon	MeLac	Mon	MeLac	Mon
112	**109**	2	0.9	1.1	1.1	2.5	0.9	1.6	0.4	1.8
113	**109**	3	1.3	1.6	0.9	2.2	0.9	1.6	0.4	1.8
114	**111**	2	1.1	1.0	0.5	1.3	0.7	0.8	0.4	1.8

Galectin-1 showed a marked preference for the divalent inhibitors in contrast to galectin-4N. **112** and **114** had a valency corrected cluster effect of 7.2 respectively 7.7 as compared to their monovalent analogues **109** and **111** (11 and 30 as compared to MeLac). Trivalent **113** showed a glycoside clustering effect of 5.4 relative to the monovalent inhibitor **109** (8.6 as compared to MeLac).

We have previously argued that the glycoside clustering effect can, at least partly, be related to interactions of the aglycon with the protein. This is particularly clear with galectin-3, in which a valency corrected glycoside clustering effect of 4.3, as compared to MeLac, was seen for trivalent **113** but compared to monovalent **109** the effect is diminished to a valency corrected clustering effect of 1.5. The corresponding value for divalent **112** is 1.3.

7.5 Conclusions

The copper(I)-catalyzed 1,3-dipolar cycloaddition proved to be a convenient way of attaching unprotected lactose residues to PBA and PTA derivatives. The limited number of inhibitors gave us valuable insights about the features of the scaffolds, information that can be utilized in future optimization of inhibitors with high affinity for galectins.

We suggested that the observed glycoside clustering effect came from the formation of aggregates. Since the length of the linkers is too short to span over the two binding sites of homodimeric galectin-1, a contribution from the chelate effect is unlikely. Furthermore, the

lower glycoside clustering effect seen for the trivalent inhibitor, as compared to the divalent inhibitor, contradicts a major contribution from the statistical effect.

The use of different assays for affinity measurements makes it difficult to compare results in the literature, although the cluster effect remains, the size of it varies.[65,106]

7.6 Investigation of the glycoside clustering effect with mutated galectin-1

The previous project was left with a feeling of unsatisfaction because the mechanism behind the observed glycoside clustering effect for galectin-1 remained unclear. In an attempt to further investigate it, a model system, based on "different" galectin-1 molecules, was created.

The previously used galectin-1 came from rat and formed homodimers (dRatGal-1) at the concentration used in the fluorescence polarization assay. The formation of homodimers is essential for an aggregative effect, allowing for the formation of chains or lattice aggregates. Cummings *et al.*[107] have presented mutated human galectin-1 (mHumGal-1) that do not form homodimers at the concentration used in FP. They used site directed mutagenesis to replace cysteine-3 with a serine and valine-6 with aspartic acid. The exchanged amino acids are close to the N-terminus and this is where the surface of the two galectins fuse when a homodimer is formed.[6,50] This method could be used to prepare mutated rat galectin-1 that stays monomeric (mRatGal-1) at a concentration necessary for FP.

If the observed glycoside clustering effect in dRatGal-1 disappears in mRatGal-1 a contribution of an aggregative effect may be concluded.

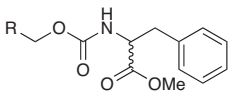
The discrepancies between galectins from different species and the access to dHumGal-1 and mHumGal-1 led us to examine if the glycoside clustering effect, seen in dRatGal-1,[97] was also prevailing in dHumGal-1.

7.7 Evaluation of inhibitors

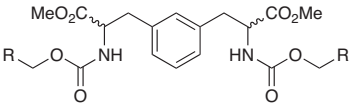
Two inhibitors (**111** and **114**) were evaluated as inhibitors of dHumgal-1, mHumGal-1, dRatGal-1 and mRatGal-1 with competitive fluorescence polarization. However, a different fluorescent probe was used for these new measurements, with better shelf stability than the previously used. This probe had lower affinity for the galectin which means that a higher concentration of the galectin had to be used. In theory this should not affect the observed dissociation constants, though some variations between the K_d values were seen. However, the same trends in glycoside clustering effect for dRatGal-1 remained.

7.7.1 Human galectin-1

To our surprise, no glycoside clustering effect was observed when comparing divalent **114** with monovalent **111** (Figure 18) for dHumGal-1 (Table 8 and 9). Interestingly, the affinities of **114** and **111** were the same as for both dHumGal-1 and mHumGal-1, while dHumGal-1 had about three times higher affinity for methyl β-lactoside as compared to dRatGal-1 (K_d=62 μM and 180 μM respectively).



111



114

Figure 18. Mono- and divalent inhibitors **111** and **114**.

7.7.2 Rat galectin-1

The valency corrected glycoside clustering effect seen for **114** compared to **111** (5.8) in dRatGal-1 was also observed for mRatGal-1 (8.0) (Table 9). Thus, the disruption of dimerisation did not affect the glycoside clustering effect, which suggests that an aggregative effect can be excluded.

Table 8. Dissociation constants (μM) for **111** and **114** and relative potencies compared to MeLac as determined in a fluorescence polarization assay at 4°C.

Cpd	Valency	dRatGal-1		mRatGal-1		dHumGal-1		mHumGal-1	
		K_d	Rel	K_d	Rel	K_d	Rel	K_d	Rel
MeLac	1	180	1	290	1	62	1	64	1
111	1	140	1.3	350	0.8	59	1.1	49	1.3
114	2	12	15	22	13	23	2.7	18	3.6

Table 9. Valency corrected glycoside clustering effects of divalent compound **114** calculated relative to MeLac and to the corresponding monomer **111**.

Cpd	Valency	dRatGal-1 Cluster effect		mRatGal-1 Cluster effect		dHumGal-1 Cluster effect		mHumGal-1 Cluster effect	
		MeLac	111	MeLac	111	MeLac	111	MeLac	111
114	2	7.5	5.8	6.5	8.0	1.4	1.3	1.8	1.4

7.8 Discussion

The unexpected absence of glycoside clustering effect in dHumGal-1 made us conclude that the clustering effect seen for RatGal-1 was not due to a statistical effect. Furthermore, the small differences, seen in crystal structures of galectin-1 from the two species, can not explain the absence of a statistical effect in one species and the presence of it in the other (Figure 19).

From galectin-1 models, it is apparent that the length of our dimeric inhibitor is sufficient for binding to two galectins in an aggregative fashion but not to span between the two binding grooves of dGal-1 in a chelate effect. However, the remaining clustering effect seen for dimeric **114** contradicts an aggregative effect.

Since neither the statistical-, aggregative- nor chelate effect explains the glycoside clustering effect, seen for carbamate **114**, this might be explained by a "non-specific effect" where one lactose binds to subsite C and D, and the aglycon and/or the second lactose interacts with the surface of the protein and not with a second defined carbohydrate binding site, *i.e.* an alternative chelate effect. Because of the large number of degrees of freedom in **114** an exhaustive conformational search has not yet been made. However, a sampling of low energy conformers of divalent **114** and ratGal suggests that while one lactose (Lac1) is bound in subsite C and D the other lactose (Lac2) is part of the time projected out from the surface and into the solution (light blue in Figure 19). However, part of the time the inhibitor is wrapped around the protein surface (green in Figure 19) and while lactose1 remains in subsite C-D lactose2 is placed close to threonine75 (red) with a possibility to

form hydrogen bonds. Interestingly, this interaction may not be formed in humGal1 where Thr75 is replaced with alanine. This is also true for the next aminoacid (aa76), which is valine in humGal1 and alanine in ratGal1. These findings support the contribution of an alternative chelate effect for the observed glycoside clustering effect.

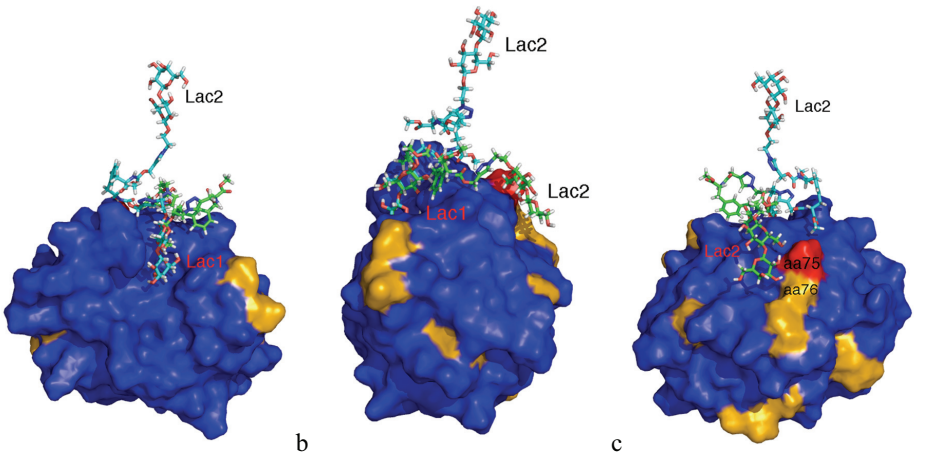


Figure 19. Fused RatGal-1 and HumGal-1 in complex with two low energy conformations of **114** derived from conformational search of **114** on RatGal-1. The conserved parts of the two galectins are colored blue and the differences are yellow and red. (a) The "carbohydrate binding side" with lactose1 bound to subsite C and D, (b) Side view, (c) The "back side" where lactose2 is either projected out into solution or wrapped around the protein near aa75, which is Thr in ratGal1 and Ala in humGal1.

We have previously argued that comparing multivalent inhibitors with a corresponding monomeric inhibitor, instead of with *e.g.* lactose, gives a more adequate picture of the glycoside clustering effect. Indeed, we have shown that the aglycon binds to the surface of the protein. I believe that this is often the case when a statistical- or chelate effect is involved, however, when aggregates are formed, the aglycon will not interact with the protein to the same degree as it spans from one protein over to another. Hence, comparing multivalent inhibitors with monomeric inhibitors when aggregates are formed, may not be straightforward, although not less so than comparing with lactose.

To conclude, the use of mutated monomeric galectin-1 has provided a valuable insight into the binding mode of one set of mono- and divalent inhibitors, where the observed glycoside clustering effect might be explained by an alternative chelate effect.

8 Galactose-mimicking mannosides as galectin inhibitors

Optimized galectin inhibitors have been shown in the preceding chapters and different structures, occupying subsite B and D, have been investigated throughout literature in order to find suitable structures for binding to galectins, while the β-galactoside occupying subsite C has remained mainly untouched. However, mannose has some structural features in common with galactose making mannose an interesting galactose-mimetic. Within this context, it is worthwhile to mention that galectin-10 shows only weak β-galactoside binding activity and a few reports have instead suggested affinity for mannosides.[108,109]

8.1 Molecular modeling of mannose derivatives in subsite C

Mannose and galactose have some structural features in common. The stereochemical relationship of the axial O4 and equatorial O3 in galactose resembles that of the axial O2 and equatorial O1 in mannose (Figure 20).[110] Easier access to position C1 in mannose, as compared to C3 in galactose, makes it interesting and feasible to exploit the synthesis of β-mannosides as galectin inhibitors.

The affinity enhancement seen for β-D-galactopyranosides substituted at C3 with triazole-[58] or amide-substituents,[52,55,56] encouraged us to utilize these functionalities for the derivatisation of the anomeric position of β-mannoside.

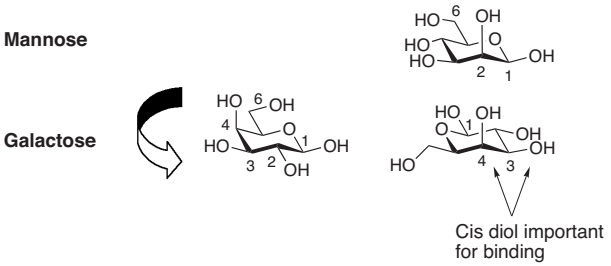


Figure 20. Structural features in common for β-mannopyranoside and β-galactopyranoside. The O1 and O2 in mannose resembles O3 and O4 in galactose.

The observed hydrogen-bond interactions between HO4 of galactose and Arg162 and His158, respectively in a galectin-3-LacNAc complex crystal structure resemble the hydrogen-bond interaction from HO2 of amido and triazolyl β-mannosides suggested in computer modeling of galectin-3 (Figure 21). Furthermore, the anomeric amide or triazolyl substituents gave favorable interactions with Arg144 in the same manner as has been observed for the corresponding galactose derivatives (Figure 21).[52] Modeling of the proposed triazole derivatives showed that an extended binding groove in galectin-3 could harbor a propylamide triazole (Figure 22) whereas the corresponding methyl and ethyl analogues were too small and a butyl analogue was too large.

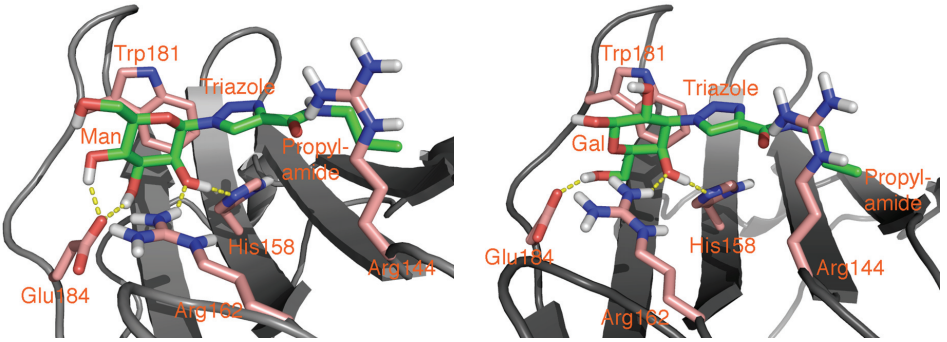


Figure 21. a) Mannose and b) galactose propylamide triazoles in modeled complexes with galectin-3. Mannose HO2 and galactose HO4 are hydrogen bonded (yellow beads) with Arg162 and His158, mannose HO3 and HO4 and galactose HO6 are hydrogen bonded to Glu184. The hydrophobic surface on both saccharides stack onto Trp181 and the triazole residues interact with Arg144.

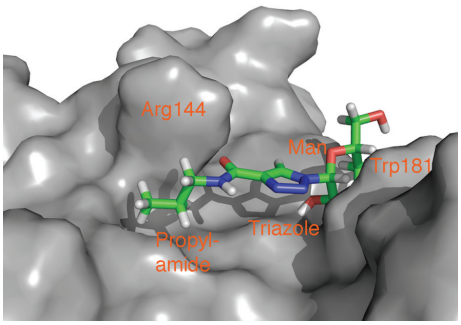
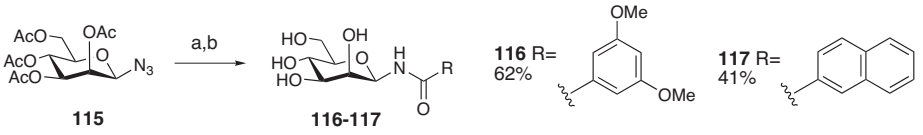


Figure 22. Propylamide trizole in modeled complex with galectin-3.

The modeling results supported our hypothesis that mannose indeed may mimic galactose, which encouraged us to synthesize amido and 1*H*-(1,2,3)-triazol-1-yl β-mannosides as galectin inhibitors. The amido β-mannosides were evaluated as galectin-1 and -3 inhibitors and the 1*H*-(1,2,3)-triazol-1-yl β-mannosides as galectin-1, -3, -7, -8N, and -9N inhibitors.

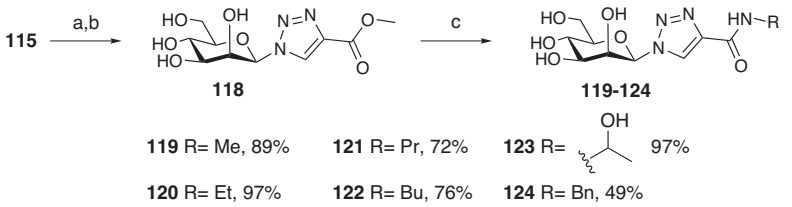
8.2 Synthesis of mannose based inhibitors

Catalytic hydrogenation of the azide **115**[111] gave the intermediate amine. Subsequent acylation, followed by deprotection, gave the corresponding aromatic amides **116** and **117** (Scheme 8).



Scheme 8. Synthesis of amide analogues **116** and **117**: a) i. Pd/C, H_2, EtOH (**116**) or THF (**117**), 200 psi, 75 minutes. ii. Acid chloride, CH_2Cl_2 (**116**) or THF (**117**), pyridine, over night; b) MeOH, NaOMe.

The panel of triazole amides (**119-124**) was synthesized via copper(I) catalyzed 1,3-dipolar cycloaddition[90,101-103] of **115** with methyl propiolate, followed by treatment of the methyl ester **118** with different amines (Scheme 9).[101]



Scheme 9. Synthesis of triazole analogues **119-124**; a) CuI, toluene, DIPEA, methyl propiolate, over night (67%); b) MeOH, NaOMe; c) RNH_2 in H_2O or MeOH.

8.3 Evaluation of mannose based inhibitors

Disappointingly, the two amides **116** and **117** showed almost no affinity for galectin-1 and -3. Fortunately, the triazoles **119-124** displayed no or low affinity for galectin-1, -7, and -8N, however, on the other hand good affinity for galectin-3 and -9N (Table 10).

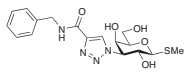
Interestingly, our prediction that the propyl analogue **121** would bind galectin-3 the best was confirmed with our K_d measurements, thus supporting the hypothesized binding mode in subsite C of galectin-3. The 2-hydroxyl propylamide analogue **123** had about the same K_d as propyl **121**, 1.5 mM for **123** compared to 1.4 mM for **121**.

For galectin-9N, the best inhibitor was benzyl analogue **124** with a K_d of 540 μM, similar to the affinity of LacNAc.[84] Furthermore, **124** compared favorably with the corresponding galactose derivative **125**.[58]

To conclude, we have shown that galactose mimicking mannosides can act as selective galectin-3 and -9N inhibitors as long as they are properly designed.

Table 10. Dissociation constants (μM) as determined in a fluorescence polarization assay[a].

Cpd	Galectin-1	Galectin-3	Galectin-7	Galectin-8N	Galectin-9N
118	N.I.[b]	>5000	6600	N.I.	6100
119	7300	≈8000	7000	N.I.	6500
120	6300	3800±600[d]	4200	N.I.	2700±140
121	8400	1400±210	3200	N.I.	1900±410
122	N.I.	2100±210	4600	N.I.	1800±190
123	5100	1500±330	4600	N.I.	1400±390
124	N.I.	1900±450	4500	N.I.	540±250
125	—[c]	110	2400	>5000	670



[a]At 4°C except for galectin-3 and -8N, which were evaluated at room temperature.

[b]Non inhibitory

[c] Not measured

[d] Average and standard deviation from four measurements.

8.4 Further outlook for the use of mannose as galactose mimetica

Based on the stereochemical relationship between galactose and mannose, we exploited the simple derivatisation of the anomeric position in mannose, as compared to C3 in galactose. In this model, the hydroxyl group of the anomeric position of galactose resembles C6 in mannose and computer modeling shows a binding groove in galectin-3, suitable for derivatisation of mannose C6. The binding groove is large enough to host various structures and a naphthylmethyl sulfide, various benzamides and a naphthamide looked most promising. Favorable interactions from arg162 with the sulfide or amide linkers might be suggested. Various triazoles could also be hosted in the binding groove although their rigid structures looked hampering (Figure 23).

Henceforth, we set out to derivatise the propyl amide derivative **121** at C6 in order to investigate a possibility to further enhance the affinity for galectin-3 and -9N.

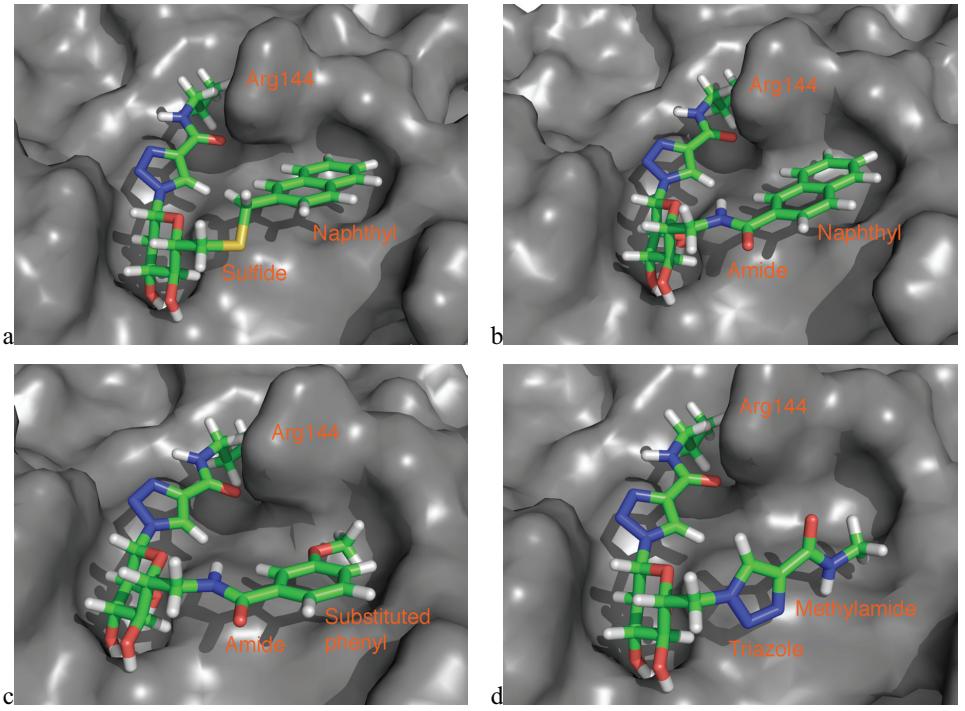
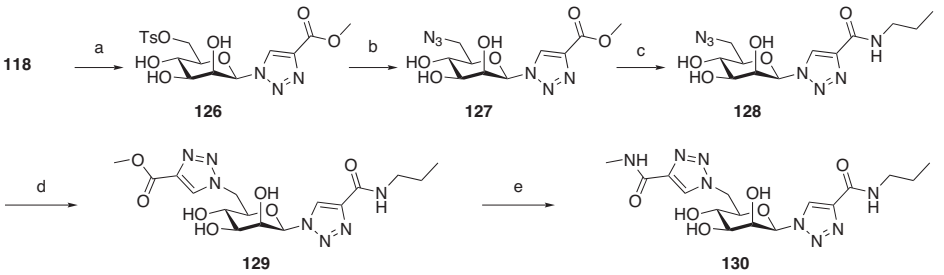


Figure 23. 1,6-Disubstituted mannose in modeled complexes with galectin-3. (a) naphthylmethyl sulfide at C6, (b) naphthyl amide at C6, (c) *m*-methoxy benzylamide at C6, (d) methyl amide triazole at C6.

8.5 Synthesis of a C6 triazole

Insertion of an azide at C6 of **118** was straightforward following literature procedures. Compound **126** was formed by reacting **118** with *p*-toluenesulfonyl chloride at 0°C (Scheme 10).[112] After four hours byproducts became visible at TLC, and the reaction was quenched by adding MeOH. Reacting **126** with NaN_3 in DMF at 100°C[113] gave the azide **127**. At this stage it was time to convert the ester triazole in **127** at the anomeric position to the amide triazole **128** by adding propylamine. Preparing the C6 azide, before converting the anomeric ester triazole to different amides, gives less synthetic steps when different anomeric triazole amide analogues are to be synthesized. The C6 triazole was formed by Copper(I) catalyzed 1,3-dipolar cycloaddition[90,101-103] of **128** with methyl propiolate, followed by treatment of the methyl ester **129** with methylamine to form the bis-triazole **130**.



Scheme 10. Synthesis of **130**. a) TsCl, CH_2Cl_2, pyridine, 0°C, 76%; b) DMF, NaN_3, 100°C, 88%; c) Propylamine, MeOH, 40°C, 82%; d) CuI, acetonitrile, DIPEA, methyl propiolate, over night, 28%; e) MeOH, 40% $MeNH_2$ in MeOH.

8.6 Evaluation of the mannose bis-triazole as a galectin-3 and 9N inhibitor

To our disappointment, the bis-triazole **130** showed no affinity for galectin-3. Encouraging, some affinity was observed for **130** (K_d=5.4 mM) towards galectin-9N although diminished as compared to **121**. These results can only be seen as preliminary depending on problems to dissolve the inhibitor before fluorescence polarization, which may be solved by adding small amounts of DMSO. Indeed, the results show that it is worthwhile to synthesize a few more bis-triazoles for more accurate K_d measurements with a properly dissolved inhibitor. And most important, to synthesize a few C6 amides and sulfides since computer modeling suggests better complementarity with galectin-3 for C6 amides and sulfides, as compared to C6 triazole derivatives.

9 Summary and prospects

Inhibitors with high affinity and selectivity towards galectins hold the potential to be used as important research tools in experiments designed to elucidate the role of galectins in various pathological conditions. By proper design, synthetic inhibitors can serve as lead for future development of galectin targeting drugs.

Natural saccharides, like LacNAc, have been proposed as inhibitors of galectins. However, the drawbacks of tedious synthesis for many natural saccharides, their sensitivity to enzymatic hydrolysis and high polarity make them unsuitable as drugs. Thus, we investigated the possibility to replace the glucose moiety of LacNAc with simpler and less polar structures. Some of these *O*-galactosyl aldoximes have the advantage of being less polar and more selective for various galectins as compared to natural saccharides. Another possible advantage of the aldoximes is their expected increased stability towards enzymatic hydrolysis. Although the aldoximes might constitute an advancement towards better galectin inhibitors, their affinity had to be further improved.

Therefore, we developed a fragment based approach where suitable structures from the panel of aldoximes targeting subsite D were combined with amide and triazole moieties optimized for targeting subsite B in galectin-3. This proved to be successful as one of the C-3 triazoles (**89**) showed high affinity as well as high selectivity for galectin-3. Together with the moderate polarity and small size (K_d 17 μM, MW 449, and clogP 2.3), inhibitor **89** represents a significant progress towards potentially important research tools and as lead for future galectin-3 targeting pharmaceuticals. The described method may be utilized for the synthesis of inhibitors with selectivity for various galectins.

Many carbohydrate-mediated interactions in biological systems depend on multivalent binding. Thus, various scaffolds were investigated for the prospect of using multivalent structures as high affinity inhibitors for galectins. We claimed that the aglycon can not be seen as an innocent bystander and its role varies depending on the mechanism behind the observed glycoside clustering effect. A prerequisite for aggregate formation is the presence

of at least two binding sites. Thus, using mutated monomeric galectin-1 led us to conclude that the inhibitor with the longer carbamate linker (**114**) did not form aggregates. Instead, the glycoside clustering effect observed was suggested to come from a chelate-like effect.

The next step would be to change the binding entity of the multimeric inhibitors from simple lactose to some C3 derivatised lactoses with increased affinity for galectin-1.

The possibility to use galactose-mimicking mannosides as galectin inhibitors were investigated and while a propylamide triazole **121** (K_d=1.4 mM) showed the highest affinity for galectin-3 a benzylamide triazole **124** compared favorable with its galactoside counterpart **125** for binding to galectin-9N (K_d=540 μM and 670 μM, respectively). The easier access to mannose C1 as compared to galacose C3 is advantageous. Computer modeling suggests a possibility to further increase the affinity for galectin-3 by derivatisation of mannose C6.

Finally, my time with these fascinating projects has come to an end. Questions have been answered, new have been raised. The ultimate experiment remains where these inhibitors will be evaluated *in vivo*.

10 Appendix

A. Basic carbohydrate nomenclature

Carbohydrates exist in various sizes and shapes. The family name is *-ose* and the most common carbohydrates are based on a five- or six-carbon chain and are called pentoses or hexoses. The sugars we encounter in this thesis are aldohexoses, which means that they are built up of six carbons and have an aldehyde functional group, whereas ketoses have a ketone group (Figure 24). Many open-chain carbohydrates are in equilibrium with their cyclic forms, where five-membered furanoses and six-membered pyranoses are most easily formed. By combining the furanose form of fructose with the pyranose form of glucose a disaccharide called sucrose, commonly known as table sugar, is formed.

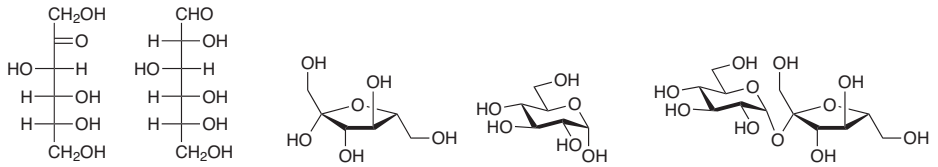


Figure 24. Chemical structures of open chain hexoses, cyclic monosaccharides and a disaccharide.

Since the aldohexoses have 4 stereogenic centers there can be 16 (2^4) possible stereoisomers, these are divided into eight D,L-pairs of enantiomers (mirror images, like a pair of gloves) where the most abundant in nature are D sugars (Figure 25).

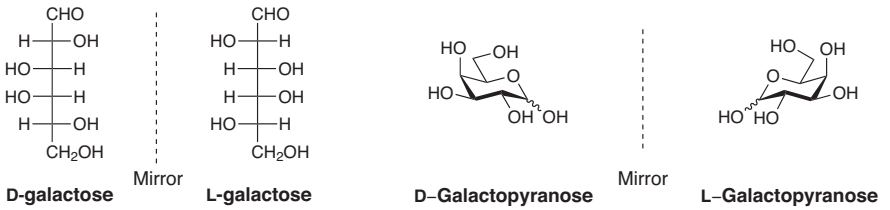


Figure 25. One set of D,L enantiomers in open- and cyclic-forms.

The carbons are numbered starting from 1 at the anomeric center, which is the carbon in the hemiacetal (Figure 26). The anomeric center can be α or β depending on if the OH at C1 is trans to CH_2OH at C5 (α-anomer) or if it is cis (β-anomer). The three monosaccharides that will be discussed in this thesis are galactose, glucose and mannose and their stereochemical relationship can be seen in Figure 26.

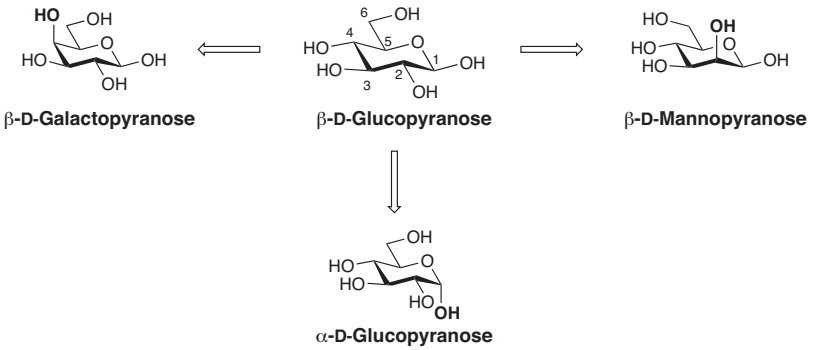


Figure 26. Numbering of carbons in monosaccharides and the differences in stereochemistry of galactose, mannose and glucose, as well as the differences of α- and β-anomers. The differences are displayed in **bold** as compared to β-D-glucopyranoside.

B. Dissociation constants for oxime ethers

Dissociation constants (μM) of galactosyl aldoximes as determined in a fluorescence polarization assay[a].

Cpd	Galectin-1	Galectin-3	Galectin-7	Galectin-8N	Galectin-9N
17	>5000	5500	2200	N.I.	N.I.
18	>5000	>5000	N.I.	N.I.	N.I.
19	>5000	>5000	N.I.	N.I.	N.I.
20	7800	>5000	>2000	N.I.	N.I.
21	>5000	>5000	>2000	N.I.	N.I.
22	>5000	>5000	>2000	N.I.	>2000
23	1100	>5000	N.I.	N.I.	N.I.
24	>5000	6900	>2000	N.I.	N.I.
25	>5000	>5000	N.I.	>2000	>2000
26	>5000	>5000	>2000	N.I.	N.I.
27	N.I.[b]	1800	>2000	>2000	N.I.
28	1000	2800	1900	N.I.	N.I.
29	>5000	4500	>2000	N.I.	>2000
30	>5000	840	>2000	N.I.	>2000
31	2300	780	510	160	1100
32	2200	550	—[c]	>2000	N.I.
33	N.I.	1600	N.I.	>2000	N.I.
34	N.1.	2000	>2000	N.I.	N.I.
35	N.I.	>5000	>2000	N.I.	N.I.
36	1400	530	>2000	>2000	>2000
37	N.I.	2500	2200	N.I.	N.I.
38	>5000	3200	>2000	>2000	>2000
39	8400	360	>2000	>2000	>2000
40	>5000	7300	>2000	N.I.	>2000
41	6700	2500	1600	>2000	>2000
42	>5000	4700	>2000	N.I.	>2000
43	7100	610	1300	N.I.	N.I.
44	1200	650	>2000	>2000	N.I.
45	910	6300	N.I.	N.I.	>2000
46	N.I.	3900	>2000	N.I.	>2000
47	>5000	>5000	>2000	N.I.	N.I.
48	7800	>5000	>2000	>2000	N.I.
49	1200	>5000	390	N.I.	N.I.
50	>5000	>5000	>2000	>2000	>2000
51	>5000	>5000	>2000	N.I.	N.I.
52	N.I.	2200	>2000	>2000	2200
53	N.I.	4400	N.I.	N.I.	N.I.
54	>5000	6000	N.I.	N.I.	N.I.
55	N.I.	5200	N.I.	N.I.	N.I.
56	N.I.	2900	N.I.	4300	N.I.
57	1800	>5000	1800	>2000	>2000
58	N.I.	820	1000	>2000	1500
59	>5000	>5000	N.I.	N.I.	N.I.
60	>5000	370	>2000	N.I.	N.I.
61	N.I.	2300	>2000	>2000	N.I.
62	N.I.	1800	N.I.	N.I.	N.I.

63	N.I.	620	2000	N.I.	>2000
64	N.M.	480	840	370	>2000
65	>5000	330	>2000	>2000	N.I.
66	N.I.	4600	340	780	950
67	>5000	4700	780	N.I.	N.I.
68	9200	5300	—	—	—
69	>5000	4600	—	—	—

[a] At 4°C except for galectin-3 and -8N, which were evaluated at room temperature.

[b] non inhibitory.

[c] not determined.

11 Acknowledgements

Mina fem år på avdelningen för bioorganisk kemi, sedemera organisk kemi, är snart slut och det är många nya och gamla kollegor som bidragit till att göra min tid både rolig och lärorik och jag vill rikta ett speciellt tack till:

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12 Experimentals

General methods. All commercial chemicals were used without further purification. Thin layer chromatography (TLC) was carried out on $60F_{254}$ silica (Merck) and visualization was made by UV light followed by heating with aqueous sulphuric acid. Column chromatography (CC) was performed on silica (Amicon 35-70 μm, 60 Å). NMR experiments were recorded with Bruker DRX 400 MHz spectrometers at ambient temperature. ^{1}H-NMR assignments were derived from COSY experiments. Chemical shifts are given in ppm relative to TMS, using the solvent residual peaks of MeOH at 3.31.[114] The optical rotations were measured with a Perkin-Elmer 341 polarimeter. HRMS (ESI) were recorded with a Micromass Q-TOF micro spectrometer.

General computational methods

Molecular modeling was performed with the MMFFs force field in water implemented in MacroModel (X. MacroModel; 9.1 ed.; Schrödinger, LLC: New York, NY, 2005). Models of the proposed inhibitors were constructed from X-ray crystal structures of galectin-1 or galectin-3 with bound lactose. The inhibitors were placed with the galactose or mannose in subsite C (and glucose in site D for lactose) and with the substituents in various postures that looked favorable for interactions. Energy minimization was performed with a Polak-Ribier Conjugate Gradient (PRCG) until the complex converged.

1,6-Dideoxy-1-[4-methoxycarbonyl-1*H*-(1,2,3)-triazol-1-yl]-6-*O*-[*p*-toluenesulfonyl]-β-D-mannopyranose 126

118 (80 mg, 0.28 mmol) was dissolved in dichloromethane (5 mL) and pyridine (5 mL) and cooled to 0 ^{O}C. *p*-Toluenesulfonyl chloride (260 mg, 1.37 mmol) was added and the reaction mixture was stirred for 4 hours, followed by addition of methanol (2 mL). Concentration and co-concentrated with toluene under reduced pressure followed by purification with flash chromatography (12:1, CH_2Cl_2/MeOH) gave **126** (94 mg, 76%): ^{1}H

NMR (400 MHz, MeOD): δ 8.43 (s, 1H, H-triazole), 7.76 (br d, 2H, *J* 6.6 Hz, Ar-H), 7.35 (br d, 2H, *J* 8.6 Hz, Ar-H), 6.04 (d, 1H, *J* 1.2 Hz, H-1), 4.36 (dd, 1H, *J* 10.6 Hz, *J* 1.5 Hz, H-6), 4.28 (dd, 1H, *J* 11.2 Hz, *J* 5.9 Hz, H-6), 4.05 (dd, 1H, *J* 1.3 Hz, H-2), 3.96 (s, 3H, CH_3), 3.73-3.66 (m, 3H), 2.40 (s, 3H, CH_3); ESI MS *m/z* calcd. for $[C_{17}H_{21}N_3O_9S+H]^+$: 444.1077. Found 444.1063.

6-Azido-1,6-dideoxy-1-[4-methoxycarbonyl-1*H*-(1,2,3)-triazol-1-yl]-β-D-mannopyranose 127

To **126** (160 mg, 0.36 mmol) dissolved in DMF (15 mL) was added sodium azide (118 mg, 1.82 mmol). The reaction mixture was heated to 100^{O}C under a nitrogen atmosphere for 2 hours, followed by concentration under reduced pressure. Purification with flash chromatography (12:1, CH_2Cl_2/MeOH) gave **127** (100 mg, 88%): $[\alpha]_D^{20}$ +53 (*c* 0.5, MeOH); ^{1}H NMR (400 MHz, MeOD): δ 8.59 (s, 1H, H-triazole), 6.19 (d, 1H, *J* 1.2 Hz, H-1), 4.13 (dd, 1H, *J* 2.8 Hz, *J* 1.3 Hz, H-2), 3.92 (s, 3H, CH_3), 3.82-3.70 (m, 3H), 3.65 (dd, 1H, *J* 13.5 Hz, *J* 2.3 Hz, H-6), 3.51 (dd, 1H, *J* 13.5 Hz, *J* 5.7 Hz, H-6); ESI MS *m/z* calcd. for $[C_{10}H_{15}N_7O_5+H]^+$: 315.1053. Found 315.1059.

6-Azido-1,6-dideoxy-1-[4-propylaminocarbonyl-1*H*-(1,2,3)-triazol-1-yl]-β-D-mannopyranose 128

To **127** (156 mg, 0.50 mmol) dissolved in methanol (15 mL) was added propylamine (1 mL). The reaction mixture was stirred at 40^{O}C for 22h, followed by concentration under reduced pressure. Flash chromatography (12:1, CH_2Cl_2/MeOH) gave **128** (139 mg, 82%): $[\alpha]_D^{20}$ $+61^{O}$ (*c* 0.5, MeOH); ^{1}H NMR (400 MHz, MeOD): 8.53 (s, 1H, H-triazole), 6.15 (d, 1H, *J* 1.2 Hz, H-1), 4.61 (s, 1H, H-amide), 4.11 (dd, 1H, *J* 2.9 Hz, *J* 1.3 Hz, H-2), 3.80-3.73 (m, 2H), 3.71 (ddd, 1H, *J* 9.3 Hz, *J* 6.0 Hz, *J* 2.3 Hz, H-5), 3.64 (dd, 1H, *J* 13.4 Hz, *J* 2.3 Hz, H-6), 3.51 (dd, 1H, *J* 13.4 Hz, *J* 6.0 Hz, H-6), 3.35 (t, 2H, *J* 7.3 Hz, CH_2), 1.63 (dt, 2H, *J* 7.3 Hz, CH_2), 0.97 (t, 3H, *J* 7.4 Hz, CH_3); ESI MS *m/z* calcd for $[C_{12}H_{20}N_7O_5+H]^+$: 342.1526. Found 342,1528.

1,6-Dideoxy-6-[4-methoxycarbonyl-1*H*-(1,2,3)-triazol-1-yl]-1-[4-propylaminocarbonyl-1*H*-(1,2,3)-triazol-1-yl]-β-D-mannopyranose 129

To **128** (130 mg, 0.38 mmol) dissolved in acetonitrile (20 mL) was added methyl propiolate (37 μL) and CuI (73 mg, 0.38 mmol). The reaction mixture was stirred for 10

minutes before addition of DIPEA (72 μL) followed by stirring over night. An additional 10 μL methylpropiolate was added, followed by stirring an additional 3 hours. After concentration under reduced pressure the reaction was purified by flash chromatography (12:1, CH_2Cl_2/MeOH) to give **129** (45 mg, 28%): $[\alpha]_D^{20}$ +56^O (*c* 0.5, MeOH); ^{1}H NMR (400 MHz, MeOD): 8.52 (s, 1H, H-triazole), 8.48 (s, 1H, H-triazole), 6.11 (d, 1H, *J* 1.1 Hz, H-1), 4.99 (dd, 1H, *J* 14.5 Hz, *J* 2.4 Hz, H-6'), 4.76 (dd, 1H, *J* 14.5 Hz, *J* 7.6 Hz, H-6), 4.59 (s, 1H, H-amide), 4.09 (dd, 1H, *J* 2.9 Hz, *J* 1.1 Hz, H-2), 3.97 (ddd, 1H, *J* 9.7 Hz, *J* 7.6 Hz, *J* 2.4 Hz, H-5), 3.88 (s, 3H, CH_3), 3.78 (dd, 1H, *J* 9.4 Hz, *J* 3.0 Hz, H-3), 3.64 (t, 1H, *J* 9.6 Hz, H-4), 3.35 (br t, 2H, *J* 7.1 Hz, CH_2), 1.64 (dt, 2H, *J* 7.3 Hz, CH_2), 0.97 (t, 3H, *J* 7.4 Hz, CH_3); ESI MS *m/z* calcd for $[C_{16}H_{24}N_7O_7+H]^+$: 426.1737. Found 426.1740.

1,6-Dideoxy-6-[4-methylaminocarbonyl-1*H*-(1,2,3)-triazol-1-yl]-1-[4-propylaminocarbonyl-1*H*-(1,2,3)-triazol-1-yl]-β-D-mannopyranose 130

To **129** (19 mg, 44 μmol) dissolved in MeOH (5 mL) was added 40% $MeNH_2$ in H_2O (1 mL) and the reaction mixture was stirred for 12 hours, followed by concentration under reduced pressure. Flash chromatography (10:1, CH_2Cl_2/MeOH) gave **130** (15 mg, 82%): ^{1}H NMR (400 MHz, MeOD): 8.55 (s, 1H, H-triazole), 8.30 (s, 1H, H-triazole), 6.11 (d, 1H, *J* 1.1 Hz, H-1), 4.98 (dd, 1H, *J* 14.5 Hz, *J* 2.3 Hz, H-6'), 4.86 (dd, 1H, *J* 14.6 Hz, *J* 7.6 Hz, H-6), 4.59 (br s, 2H, H-amide), 4.08 (dd, 1H, *J* 3.0 Hz, *J* 1.1 Hz, H-2), 3.94 (ddd, 1H, *J* 9.7 Hz, *J* 7.6 Hz, *J* 2.3 Hz, H-5), 3.78 (dd, 1H, *J* 9.4 Hz, *J* 3.1 Hz, H-3), 3.64 (t, 1H, *J* 9.6 Hz, H-4), 3.35 (br t, 2H, *J* 7.1 Hz, CH_2), 2.90 (s, 3H, N-CH_3), 1.64 (dt, 2H, *J* 7.3 Hz, CH_2), 0.97 (t, 3H, *J* 7.4 Hz, CH_3).

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