SYNTHETIC GLYCOLIPIDS AS MODULATORS OF CARBOHYDRATEPROTEIN INTERACTIONS

Míriam Salvadó Molero

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Els sotasignants Prof. Sergio Castillón Miranda, Catedràtic d'Universitat, del Departament de Química Analítica i Química Orgànica de la Universitat Rovira i Virgili, i Dr. Omar Boutureira Martín:

Fem Constar que aquesta memòria, titulada '*Synthetic Glycolipids as Modulators of Carbohydrate-Protein Interactions*', que presenta Míriam Salvadó Molero, i que la fa optar al grau de Doctor en Química per la Universitat Rovira i Virgili, ha estat realitzada sota la nostra direcció i que compleix els requeriments per poder optar a la Menció Europea.

Tarragona, 4 de Març de 2016

Prof. Sergio Castillón Miranda

Dr. Omar Boutureira Martín

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Míriam Salvadó

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INTERACTIONS
Míriam Salvadó Molero

Que mai és tard per començar de nou,

per atrapar els teus somnis.

Que avui és sempre, encara

però tota la vida és ara (...)

No t'adormis-Txarango

UNIVERSITAT ROVIRA I VIRGILI
SYNTHETIC GLYCOLIPIDS AS MODULATORS OF CARBOHYDRATEPROTEIN
INTERACTIONS
Míriam Salvadó Molero

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

A

Ac	Acetyl
app	apparent
ax	axial

B

bs	broad signal

C

COSY	correlation spectroscopy
Cys	cysteine

D

d (in NMR)	doublet
Dha	dehydroalanine

E

eq	equatorial
equiv.	equivalents
ESI-TOF	electrospray ionization- time-of-flight resolution spectrometry

G

g	gram(s)

Gal	D-galactose
Glc	D-glucose

H

h	hour(s)
HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum coherence
Hz	Hertz

I

IR	infrared

J

J	coupling constant

L

L	litre(s)
LC-MS	liquid chromatography-mass spectrometry

M

m (in NMR)	multiplet
m/z	mass under charge
Man	mannose
min	minute(s)
m.p.	melting point
MRI	magnetic resonance imaging
MS	mass spectrometry

N

NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
Nu	nucleophile

P

PET	positron emission tomography
ppm	parts per million
PTC	phase-transfer catalyst

Q

q (in NMR)	quadruplet
quin (in NMR)	quintet

R

R_f	retention factor
RT	room temperature

S

s (in NMR)	singlet

T

t (in NMR)	triplet
T	temperature
TLC	thin layer chromatography

U

UV	ultra-violet

V

vs	versus

LIST OF PUBLICATIONS

I **Topological defects in hyperbranched glycopolymers enhance binding to lectins**

Salvadó, M.; Reina, J. J.; Rojo, J.; Castillón, S.; Boutureira, O. In preparation.

II **Synthesis of Fluorosugar Reagents for the Construction of Well-Defined Fluoroglycoproteins**

Salvadó, M.; Amgarte, B.; Castillón, S.; Bernardes, G. J. L.; Boutureira, O. *Org. Lett.* **2015**, *17*, 2836-2839.

III **Deciphering the Non-Equivalence of Serine and Threonine O-Glycosylation Points: Implications for Molecular Recognition of the Tn Antigen by an anti-MUC1 Antibody**

Martínez-Sáez, N.; Castro-López, J.; Valero-González, J.; Madariaga, D.; Compañón, I.; Somovilla, V. J.; Salvadó, M.; Asensio, J. L.; Jiménez-Barbero, J.; Avenoza, A.; Busto, J. H.; Bernardes, G. J. L.; Peregrina, J. M.; Hurtado-Guerrero, R.; Corzana, F. *Angew. Chem. Int. Ed.* **2015**, *54*, 9830-9834.

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SYNTHETIC GLYCOLIPIDS AS MODULATORS OF CARBOHYDRATEPROTEIN
INTERACTIONS
Míriam Salvadó Molero

SUMMARY

CHAPTER 1. INTRODUCTION

Carbohydrates play numerous roles in Nature. Many of them are present on the outer cellular surface and modulate cell-cell, cell-matrix and, cell-molecule interactions as well as acting as mediators in the interactions between different organisms.[1,2] Usually, carbohydrates are covalentlyattached to other biomolecules such as proteins or lipids forming the so-called glycoconjugates. The interaction between carbohydrates and proteins is unusually weak compared to others in Nature.[3] For this reason, the weakness of the monovalent binding is solved by the increase of the strength and the specificity of the interaction using multivalency.[4]

They are now being identified in many biological responses and frequently they mediate the first step of infection processes, for the specific recognition between host and bacteria. The study of these interactions could provide useful information about how ligand bind to receptors and how the information is transferred to the cell to finally be able to synthesize multivalent ligand in order to treat diseases.

Protein-carbohydrates interactions mediate the first step of many diseases and the study of these interactions has provided a powerful tool for the design of drugs with therapeutic properties.[5] Mainly, proteins interact with carbohydrates by apolar interactions and hydrogen bonding. Proteins that interact with carbohydrates could be classified as binding or processing proteins depending on their mode of action.

Importance of glycolipids and their interactions with proteins.

Glycolipids are a group of glycoconjugates in which the carbohydrate moiety is linked by a glycosidic bond to alkyl chain(s). Glycolipids together with glycoproteins and proteoglycan components form the glycocalyx, a

thick outer layer of the plasma membrane involved in all cell communication events.

Glycolipids are amphiphilic derivatives composed by a hydrophilic polar head, sugar segment, and a lipophilic alkyl chain, which can undergo self-assembly in aqueous media, forming a glycolipid bilayer, glycoliposomes, glycovesicles and glycomicelles, depending on the length and size of the segments.[6] In order to study carbohydrate-protein interactions these architectures were used as multivalent scaffolds. In most cases, the glycolipid monomer is constructed based on a sugar head group an anchor moiety, normally alkyl chain(s), and a spacer or linker between them. The lengths of these spacers or linkers are going to be crucial for the sugar presentation at the surface. Moreover the incorporation of poly(ethylene glycol) (PEG) spacers could favor the liposome stabilization, although it may lead to micelle formation.[7]

Synthetic approach to multivalent ligands

Access to multivalent ligands is required to further investigate the glycoside cluster effect. As monovalent ligands can only be effective in very high doses; to reduce the dose and increase the affinity binding, multivalent ligands should be studied. It is needed to take into account that synthetic multivalent glycoconjugates are not going to reproduce exactly the valency and topology of natural ligands, although they can be used as a model to understand the binding and in a future develop them into therapeutics. In order to construct an appropriate multivalent system, it is useful to look at the multivalent lectin structure, to best fit with the lectin topology, what it is the so-called a lectin-based design strategy. Moreover, the conjugation of a potent monovalent ligand with a multivalent scaffold will provide a good synthetic strategy for drug discovery.[8]

CHAPTER 3. GLYCOLIPIDS, CLUSTERS AND HYPERBRANCHED POLYMERS AS β-GLYCOSIDASE INHIBITORS

Glycosidases, enzymes that catalyse the hydrolysis of glycosidic bonds, are important enzymes present in almost all living organisms. Wolfenden *et al.* found that the hydrolysis of a glycosidic bond was enhanced 10^{17}-fold by glycosidases compared to the uncatalyzed reaction.[9] It has been also reported that multivalent systems are potent inhibitors of glycosidases, and that hydrophobic interactions enhance substrate-receptor binding. The research described in Chapter 3 aims to study and explore the chemical features of glycolipids and multivalent systems containing glycolipids as inhibitors.

Glycolipid inhibitors were synthesized in two different directions. Glycolipids with modifications at position C-2 (H, OH, NHAc, and F) were synthesized using different strategies. The glycosylation reaction to obtain 2-OH-β-glycolipids was studied using glycosyl donors with 2,6-diFBz and Ac groups at position C-2 and better results were obtained when the group 2,6-diFBz was present in the glycosyl donor. The synthesis of 2-F and 2-NHAc-glycolipids required microwave heating to achieve moderate yields.

These glycolipids, together with glycolipids with modification in the aglycone moiety (a short chain, an aliphatic chain, a polar chain, and a two aliphatic chains) and their multivalent structures were evaluated against α-glycosidase (baker's yeast), β-galactosidase (bovine liver), β-galactosidase (*E. coli*), α-galactosidase (green coffee) and β-glucosidase (almonds).

When synthetic glycolipids bearing different groups at C-2 were evaluated, it was found that the presence of fluorine is detrimental for inhibition as reflected in the strong increase of K_i values. Comparing glycolipids **5.5** (two aliphatic chains) and **5.25** (glycocluster presenting one glycolipid with two aliphatic chains), with and without azide group, it was found that in the presence of β-galactosidase (*E.coli*) **5.25** was hydrolyzed

while **5.5** was not, which indicates that **5.25** is a substrate for this enzyme. Higher relative inhibition potency per sugar was obtained for glycocluster presenting 4 glycolipids.

CHAPTER 4. HYPERBRANCHED GLYCOPOLYMERS AS MULTIVALENT INHIBITORS OF CARBOHYDRATE-LECTIN INTERACTIONS

The complexity of cellular glycocalyx has been shaped over millions of year of evolution by the action of cell's (*glyco*)machinery, an intricate pool of glycosyl hydrolases, transferases, and other carbohydrate-processing enzymes that together with a different set of post-translational modifications define the composition of the cellular membrane, and its components. Biological systems, and particularly glycoproteins, are heterogeneous in nature, yet the functions of redundant glycoforms, which represent structurally similar glycoproteins that differ in sugar structure, density, and/or glycosylation site, and decorate the outer surface of cells remains unknown. Heterogeneous glycoforms typically participate in a variety of surface recognition events. In one example, galectins are able to recruit and cross-link a heterogeneous collection of complex glycoconjugates that trigger important signalling events.[10]

In an attempt to mimic Nature and gain insight into the heterogeneous nature of natural multivalent sugar-protein interactions we sought to systematically evaluate the binding mechanism between a series of multivalent homogeneous (dendrimers) *vs.* heterogeneously (hyperbranched polymer-Boltorn H30) presented glycoconjugates (D-galactose) and a model plant toxin (RCA_{120}). We performed a thorough analysis *via* surface plasmon resonance (SPR) and dynamic light scattering (DLS) of the behaviour of hyperbranched glycopolymers[11] as a suitable family of multivalent glycomaterials with advanced properties not only in terms of accessibility

and processability but also as simplified defect-containing analogues with enhanced potency.

We found the nature of the core and the display of ligans enormously affects the binding potency and mechanism. For example, lower densities of receptors (lectins) as those found in natural systems favours the use of heterogeneous glycoconjugate presentations (*e.g.* those mimicking inherent defects in glycoproteins and/or glycoforms) that enhance the potency in a match scenario of interactions suggesting this might be one of the pivotal reasons for the evolution of glycans to such a complex, heterogeneous structures.

CHAPTER 5. GLYCODENDRIPROTEIN-BASED NANOCAPSULES AS MULTIVALENT INHIBITORS OF CARBOHYDRATE-LECTIN INTERACTIONS

Work by Davis, B. G. *et al.* demonstrated that glycodendrimers could mimic the carbohydrates presents in glycoproteins and glycodendrimers chemically attached to a protein in a specific place generating a new family of glycoconjugates called glycodendriproteins (protein platforms coated with glycan ligands).[12] Such glycodendriproteins, which were prepared by ligation of a chemically synthesized glycodendron and a protein-degrading enzyme (protease) reduced the binding ability of pathogens.[13] While this strategy is limited to display only a determined carbohydrate valency on a single protein, Ribero-Viana, R. *et al.* found that a high multivalent display of sugars could be achieved by a "nested polyvalency". [14] This novel strategy allows for the multivalent assembly of protein monomers carrying polyvalent glycodendrons. With this background, we aimed to found a promising novel strategy for the design of multivalent inhibitors of carbohydrate-binding proteins based on glycodendriprotein-based nanocapsules.

The binding affinities of a series of synthetic glycodendrons based on gallic and pyrogallic scaffolds, as monomers, towards a model lectin

(RCA_{120}) were evaluated using Bio-layer Interferometry (BLI). It was found that ligand presentation (shape/spatial arrangement/epitope density) is even more important.

Functionalization of BSA at Cys34 was studied in order to investigate in further experiments its aggregation to form the desired glycodendriprotein-based nanocapsules.

CHAPTER 6. SYNTHESIS OF FLUOROSUGAR REAGENTS FOR THE CONSTRUCTION OF WELL-DEFINED FLUOROGLYCOPROTEINS.

F-glycopeptides[15] and the more recently disclosed F-glycoproteins[16] are promising candidates for the preparation of synthetic carbohydrate vaccines[17] and hold great potential as a new generation of [^{18}F]-(glyco)radiopharmaceuticals[18,19] and tracers for the non-invasive imaging techniques positron emission tomography (^{18}F-PET)[20,21] and magnetic resonance/magnetic resonance imaging (^{19}F-NMR/MRI).[22] In addition to the development of more efficient methods for protein/peptide modification,[23] it is also pivotal to access pure F-sugar reagents and building blocks for such transformations.

With this background, in this chapter, we envisioned a "two-step" strategy for the preparation of 2-F-glycosyl iodides from ready available 1-*O*-Ac that may overcome such limitations enabling a more efficient preparation of this class of highly reactive glycosyl donors. We then sought to explore their reactivity for the preparation of fluorosugar reagents that are useful moieties to achieve site-selective chemical glycosylation of proteins.

We disclosed a general strategy to access a wide range of fluorosugars that are useful reagents for chemical-site selective protein glycosylation. The glycosyl iodide intermediates prepared here possess a seemly balance between stability and reactivity that facilitates their preparation, purification

and storage. Importantly, these intermediates also ensure product homogeneity by their exquisite β-control during stereoselective glycosylations with "soft" Nu (*via* S_N2-like reactions). The reported F-sugar reagents will find broad utility not only for building homogeneous ^{19}F- but also [^{18}F]-glycoprobes that are valuable tools in the fields of chemical biology and biomedical imaging.

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UNIVERSITAT ROVIRA I VIRGILI
SYNTHETIC GLYCOLIPIDS AS MODULATORS OF CARBOHYDRATEPROTEIN
INTERACTIONS
Míriam Salvadó Molero

CHAPTER 1

INTRODUCTION

UNIVERSITAT ROVIRA I VIRGILI
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1.1. GLYCOBIOLOGY

Carbohydrates play numerous roles in Nature. Many of them are present on the outer cellular surface and modulate cell-cell, cell-matrix and, cell-molecule interactions as well as acting as mediators in the interactions between different organisms (Figure 1.1).[1,2] Usually, carbohydrates are covalently bound to other biomolecules such as proteins or lipids forming the so-called glycoconjugates. The function of these carbohydrates and the enormous influence that can cause in biological response are of growing interest in the scientific community and led to the introduction of the concept of "sugar code".[3]

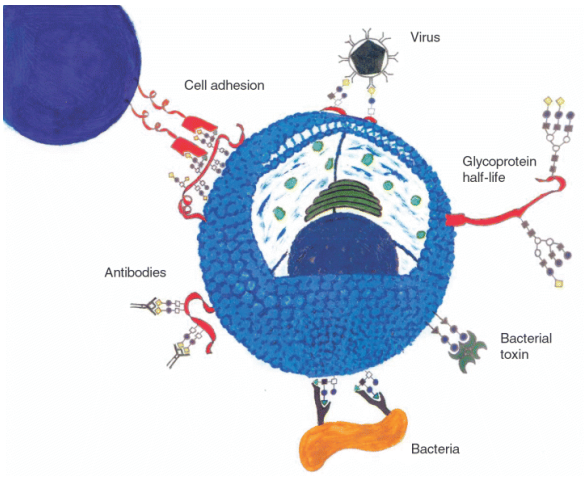


Figure 1.1. Schematic representation that illustrates the multiple interactions occurring in the cell surface (adapted from Holgersoon *et al.*).[4]

The interaction between carbohydrates and proteins is unusually weak compared to others in Nature.[5] For this reason, the weakness of the monovalent binding in Nature is solved by the increase of the strength and the specificity of the interaction using multivalency.[6] These interactions usually occur in the cell-surface where multivalent systems interact with

cellular receptors, which transfer the extracellular information to the internal machinery of the cell. They are now being identified in many biological responses and frequently they mediate the first step of the infection processes, for the specific recognition between host and bacteria. The study of these interactions could provide useful information about how ligand bind to receptors and how the information is transferred to the internal cell to finally be able to synthesize multivalent ligand in order to treat diseases.

Two distinguishable mechanisms could enhance the binding affinity: a statistical effect and a chelate effect (Figure 1.2). The statistical effect occurs when the multivalent compound give rise to a highly localized concentration of the ligand at the receptor binding site (Figure 1.2a), this mechanisms can also referred to as clustering effect. Whereas, chelate mechanisms are present when the multivalent ligand cross-links binding sites. This chelation effect could occur either by bridging an adjacent binding site (*i.e.* AB_5 toxins) with multivalent architectures (Figure 1.2b), or by bridging multivalent receptor within individual glycans, "miniclustering" or between more distant multivalent glycans, "maxiclustering" (Figure 1.2c), enhancing binding affinities.[7] Moreover, another binding mechanism that could be observed happens when the receptor possess a different binding subsite in addition to their primary site of interaction and it is called subsite binding mechanism (Figure 1.2d).[8,9]

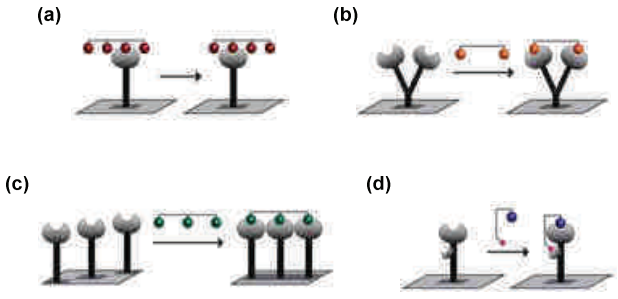


Figure 1.2. Cluster glycoside effect and its different mechanisms; (a) statistical effect, (b) chelation, (c) clustering and (d) subsite binding (adapted from Levine, P. M. *et al.*).[9]

Multivalent interactions offer a dramatically enhance of the binding on a molecular scale compared to weak monovalent binding.[10] Lee *et al.* found that the inhibitory power of a tetraantennary ligand was a million greater than that of a monoanternnary ligand while the total galactose concentration was only 4-fold higher, demonstrating the "cluster glycoside effect".[11]

1.2. PROTEIN-CARBOHYDRATE INTERACTIONS

Protein-carbohydrates interactions mediate the first step of many diseases and the study of these interactions has provided a powerful tool for the design of drugs with therapeutic properties. [12] Mainly, proteins interact with carbohydrates by apolar interactions and hydrogen bonding. Proteins that interact with carbohydrates could be classified as binding or processing proteins depending on their mode of action.

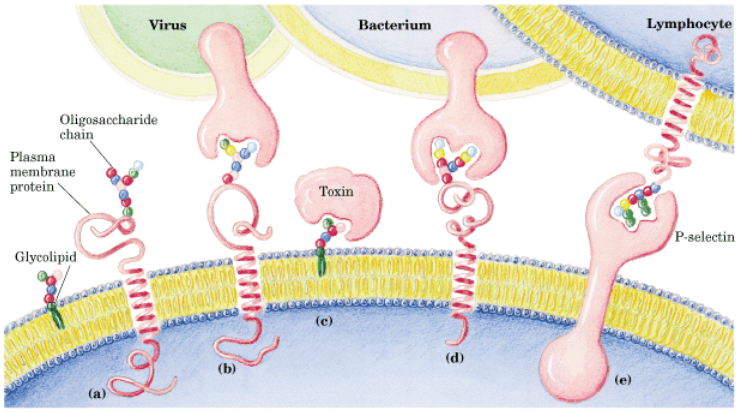


Figure 1.3. Carbohydrates recognition at cell surface (a) glycoprotein, (b) glycoprotein interaction with virus, (c) glycolipid interaction with toxin, (d) glycoprotein interaction with bacterium and (e) glycoprotein recognition mediating cell-cell interactions (adapted from Nelson, B. L. and Cox, M. M.).[13]

1.2.1. Carbohydrate-Binding proteins

Carbohydrate-specific enzymes, anticarbohydrate antibodies and lectins, are different examples of proteins that interact with carbohydrates in a non-covalent manner. In this thesis we are going to focus our efforts in lectins, perhaps the most widely studied molecules in Glycobiology, as well as glycosyl hydrolases (glycosidases).

Lectins can mediate cellular recognition due to its interaction with a specific carbohydrate presented as a glycoconjugate in a reversible and high specific way.[14] They are present in most organisms and have significant biological importance. Their binding with cell surface glycans initiates adhesion and infection.[15] Carbohydrate-binding proteins possess the so-called carbohydrate recognition domain (CRD), a specific binding site where the interaction occurs, and typically two or more CRD are present in each lectin.[16] Lectin CRDs (Figure 1.4a) recognize the non-reducing carbohydrate residues of glycans, presented as oligosaccharides or glycoconjugates. These interactions typically consist of hydrogen bonding, metal coordination, van der Waals, and hydrophobic interactions.[17]

Lectins and their corresponding ligands are usually presented in clustered arrays.[18] As multivalency has a binding benefit in protein-ligand interactions, multivalent ligands should be studied to block lectins for the treatment of certain diseases. In order to increase the inhibitor potency, it must be necessary the incorporation of multivalency in the design of lectin inhibitors.

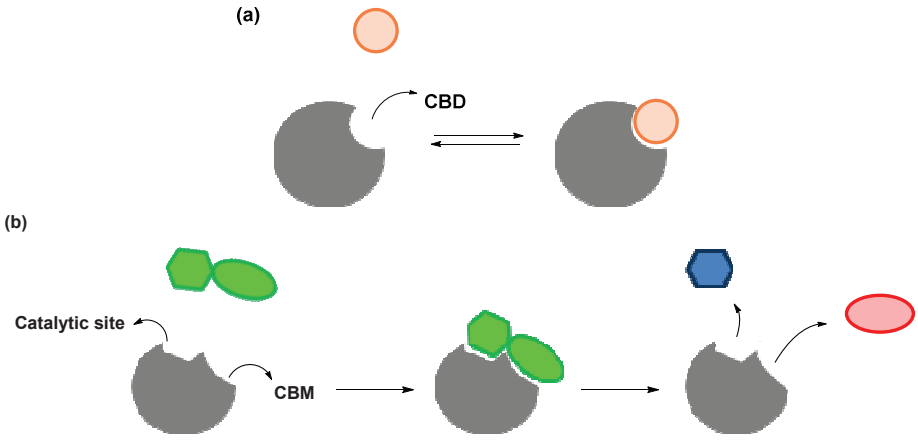


Figure 1.4. Carbohydrate interaction with (a) carbohydrate-binding proteins and its carbohydrate binding domain (CBD) and (b) carbohydrate-processing enzymes composed by a catalytic site and a carbohydrate-binding module (CBM).

1.2.2. Carbohydrate-Processing Enzymes

Among carbohydrate-processing proteins, glycoside hydrolases are enzymes that catalyse the cleavage of glycosidic bonds in glycoconjugates, disaccharides and oligosaccharides. They are involved in many biological processes such as degradation of polysaccharides to provide monosaccharides that are metabolized and absorbed by the organisms, lysosomal catabolism of glycoconjugates, glycoprotein processing and biosynthesis of oligosaccharides units of glycoproteins and glycolipids, which are involved in cell-cell interaction processes.[19,20] Their deregulation generally is translated into significant biological problems and pathogenic states. Thus, compounds that can interfere with their enzymatic activity are widely studied as potent drug candidates.[21]

A part from its catalytic site, some glycosidases also present noncatalytic carbohydrate-binding modules (CBMs) (Figure 1.4b). These CBMs enable the interaction with the carbohydrate portion and are typically located in the N- or C-terminal end of these proteins. The main function of these carbohydrate-binding modules is to recognize and bind specifically to

carbohydrates. The interaction of carbohydrates with these carbohydrate-binding modules are in symbiosis with the catalytic domain, their interaction has a significant biological influence in the catalytic activity of these enzymes; enhancing the hydrolysis of insoluble substrates, bringing the catalytic domain in close proximity to the substrate, cell protein anchoring, etc.[22]

Due to its participation in biological processes in all organisms and its affection in several diseases, glycoside hydrolase inhibition continues to be an interesting topic in order to find promising therapeutic routes.[23]

In general, the active site of glycosidases, which is normally unique, is not very accessible because it is buried within the protein, fact that prevents chelation, rebinding and all the typical multivalent binding phenomena. Thus, in contrast to most lectins, multivalent binding seams not to be appropriate for glycosidases. Thobhani *et al.* demonstrate for the first time the enhancement of catalytic activity of bacterial sialidases for multivalent ligands compared to their monovalent counterparts. This activity enhancement was attributed to the simultaneous interaction of both the catalytic and lectin domains.[24] Moreover, recent studies demonstrate an enhance inhibitor potency when inhibitors were present as multivalent ligands (Figure 1.5).[2526,27]

In 1999, Bols and co-workers investigated the possible effect of multivalency in glycosidase inhibitors. Surprisingly, a promising affinity enhance was obtained for the inhibition of sweet almond β-glucosidase when a tetravalent azafagomine was tested.[28] In 2009, the first evaluation of multivalent iminosugars as glycosidase inhibitors were performed using oligoethylene scaffold presenting 1-deoxynojirimycin (DNJ), a broad glycosidase inhibitor, as the peripheral ligand. In this sense, whereas an affinity decrease was observed for sweet almond β-glucosidase, a promising affinity enhance was obtained for Jack bean α-mannosidase.[29]

When a C_{60}-fullerene scaffold was used to symmetrically display twelve iminosugars against Jack bean α-mannosidase a 2147-fold inhibitor strength was found compared with the monomer-ligand and a 180-fold enhancement per sugar unit.[30] Based on the strong multivalent inhibitory effect produced by C_{60}-fullerene, β-cyclodextrin (βCD) derivatives, were studied as scaffolds offering a well-defined multivalent constructs of high valency. Iminosugars were conjugated to βCD, and it was found that multivalent effect increases with the valency and the linker length, reaching a nanomolar inhibition of Jack bean α-mannosidase.[31] Using these βCD, the 21-valent iminosugar was found to have the strongest affinity enhancement observed to date for multivalent glycosidase inhibitors (K_i = 19 nM, Jack bean α-mannosidase). Gouin *et al.* reported the synthesis of tetra- to octavalent click clusters based on different scaffolds such as porphyrin, calix[4]arene, glucose, galactose, trehalose and γ-cyclodextrin, with the same linker length (n=3) and ligand (DNJ).[32] In this sense, the conjugation of DNJ to porphyrin was found to be 160 times more potent than its conjugation to glucose. These strong multivalent effects gave interest of using rigid scaffolds to construct potent glycosidase inhibitors.[33]

Recently, the glycosidase inhibition by iminosugars displayed in biomimetic nanoparticles were studied by Ortiz-Mellet and co-workers.[34] The self-assembly of DNJ-based glycopeptide together with galactose-glycopeptide at different ratios gave a small library of micelles containing various iminosugars densities observing an iminosugar-content-dependent increase in the inhibitory potency.

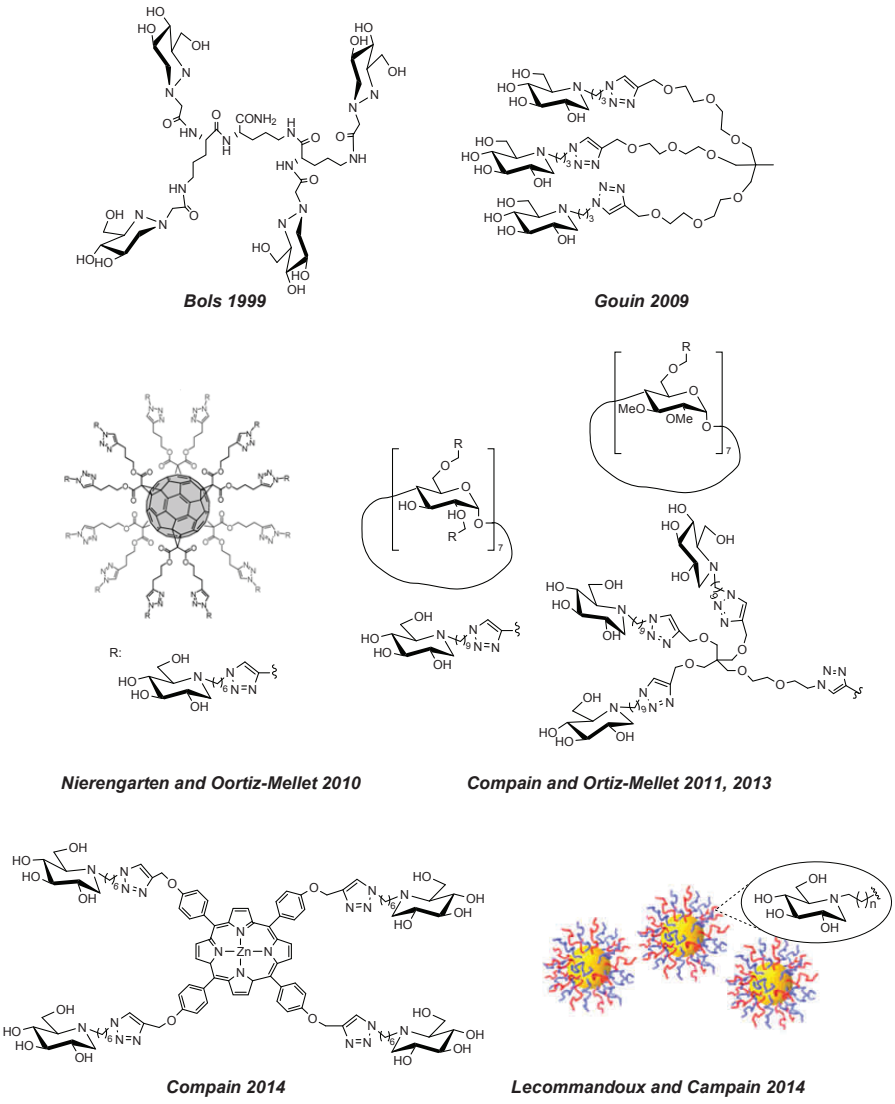


Figure 1.5. Multivalent glycosidase inhibitors.

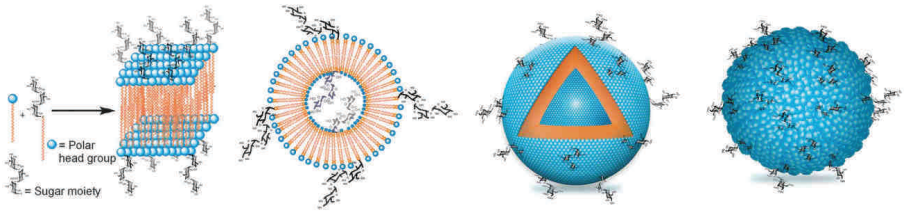
Due to these findings the lectin-like behaviour of glycosidases has gained importance. However, mechanistic studies to understand how these multivalent ligands enhance inhibition potency are required.[35]

1.3. GLYCOLIPID RECEPTORS AND SUGAR-PROTEIN INTERACTIONS

Glycolipids are a group of glycoconjugates in which the carbohydrate moiety is linked by a glycosidic bond to alkyl chain(s). Glycolipids together with glycoproteins and proteoglycan components form the glycocalix, a thick outer layer of the plasma membrane involved in all cell communication events.

As it has been mentioned previously, the interaction of pathogens to the host membrane is the first stage of an infectious process. For this reason, the field of glycolipid research has been addressed as a strategy for preventing different diseases promoted by microbial infections such as verotoxines,[36] cholera toxin,[37] HIV-CD4(–)[38] as well to other processes in which they are involved.

Glycolipids are amphiphilic derivatives composed by a hydrophilic polar head, sugar segment, and a lipophilic alkyl chain, which can undergo self-assembly in aqueous media, forming a glycolipid bilayer, glycoliposomes, glycovesicles and glycomicelles, depending on the length and size of the segments (Figure 1.6).[39] In order to study carbohydrate-protein interactions these architectures were used as multivalent scaffolds. In most cases, the glycolipid monomer is constructed based on a sugar head group an anchor moiety, normally alkyl chain(s), and a spacer or linker between them. The lengths of these spacers or linkers are going to be crucial for the sugar presentation at the surface. Moreover the incorporation of poly(ethylene glycol) (PEG) spacers could favor the liposome stabilization, although it may lead to micelle formation.[40]



(a) Bilayer **(b)** Glycoliposome **(c)** Glycovesicle **(d)** Glycomicelle

Figure 1.6. Bilayer assemblies formed in aqueous media by amphiphilic derivative (adapted from Jayaraman, N. *et al.*).[39]

The influence of sequentially increasing spacer length and flexibility in liposome-phagocyte interactions were studied by Engel *et al.* A number of glycolipids varying the spacer length between the sugar moiety and the lipid anchor were synthesized and incorporated into liposomes to study their interactions with human phagocytic cells. Liposomes that present glycolipids with short PEG spacers were recognized by ConA, whereas longer spacers were required in the interaction with phagocytic cells.[41] The importance of the incorporation of alkyl chains,[42] fullerenes[43] and other different moieties[44] as the anchor was also explored.

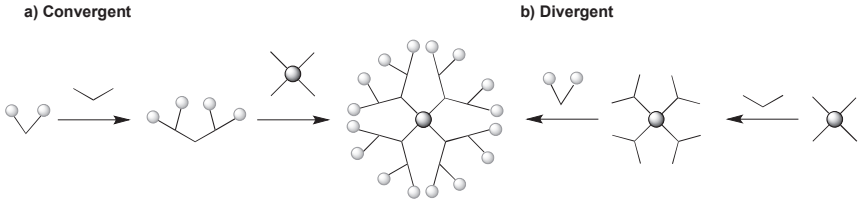
Murthy *et al.* studied the multivalent effect involving large assemblies and their multivalent interactions with ConA using surface plasmon resonance (SPR). Glycolipid monomers bearing one- or two-sugar moieties and varying the distance between the sugar moieties, constituted micelles. Favourable association rate constants were found for the two-sugars containing glycolipid-micelles demonstrating a beneficial effect of additional sugar unit at the binding site vicinity.[45]

1.4. SYNTHETIC APPROACH TO MULTIVALENT LIGANDS

The access to multivalent ligands is required to further investigate the glycoside cluster effect. As monovalent ligands can only be effective in very

high doses; to reduce the dose and increase the affinity binding, multivalent ligands should be studied. It is needed to take into account that synthetic multivalent glycoconjugates are not going to reproduce exactly the valency and topology of natural ligands, although they can be used as a model to understand the binding and in a future develop them into therapeutics. In order to construct an appropriate multivalent system, it is useful to look at the multivalent lectin structure, to best fit with the lectin topology, what it is the so-called a lectin-based design strategy.

Moreover, the conjugation of a potent monovalent ligand with a multivalent scaffold will provide a good synthetic strategy for drug discovery.[46] In order to create well-designed multivalent inhibitors for increasing potency and selectivity the differents mechanisms of multivalent ligand binding such as chelation, subsite binding, clustering and statistical rebinding need to be taken into account.[8] Multivalent systems can be synthesized in a step-wise and controlled manner with two mainly synthetic strategies: divergent and convergent synthesis (Scheme 1.1).[6,47,48]



Scheme 1.1. Divergent *vs.* convergent synthesis of multivalent systems.

There are many examples in the literature for both chemical and biochemical studies on the design and properties of multivalent systems displaying various carbohydrate-density and topological properties. In order to construct the best synthetic multivalent system it is really important to understand the complexity of natural multivalent ligands. For the reasons aforementioned, the synthesis of many unnatural glycoconjugates (neoglyconjugates) combining the carbohydrate head group with different

valences and spatial arrangement, such as polymers, gold nanoparticles, fullerenes dendrimers, aromatic scaffolds, lipids, proteins, have been extensively studied with respect to their interactions with (Figure 1.7).[49,50,51,52]

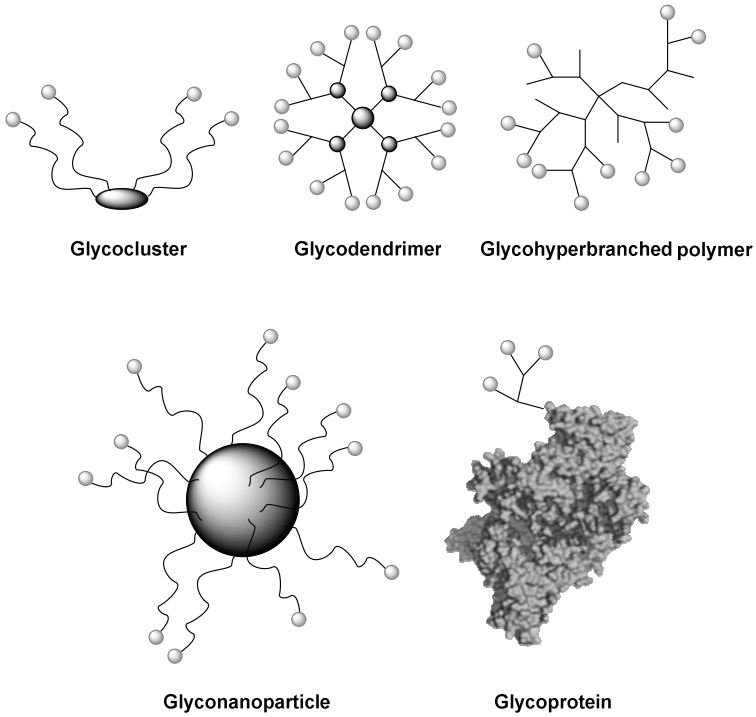


Figure 1.7. Schematic representation of multivalent systems with different central scaffolds.

Glycoclusters belong to a family of multivalent systems that display, in a synthetically controlled way, a limited number of binding epitopes. Glycoclusters have been studied for the inhibition of multivalent interactions in the first stage of infection, between pathogenic agents and host cells recognition.[53]

Hyperbranched polymers are polydisperse and include some linear units in their molecular structure,[54] feature that allow for mimicry the multivalent, heterogeneous displaying of natural receptors. Hyperbranched polymers can be easily synthesized *via* one-step reactions providing access to valuable products for both small-scale and large-scale industrial applications. Thus, it

seems certain that hyperbranched polymers will play a significant role in the future development of new biomedical devices for the treatment of human diseases.[55]

Dendrimers are branched synthetic polymers with high structural and chemical homogeneity. As hyperbranched polymers they also present high ligand density as they are able to display multiple copies of surface groups. First dendrimers structures were described by Newkomo and Tomalia in 1985.[56] Many different dendrimer structures has been used for different biological applications such as drugs, drug and gene delivery, carriers for magnetic resonance imaging, scaffolds for tissue repair, etc.[16] Dendrimers containing carbohydrates (glycodendrimers) could be classified in three different types depending on the carbohydrates present: carbohydrate-based, carbohydrate-centered and carbohydrate-coated structures.[57,58] Its globular symmetric distribution is effective for occupying multiple binding sites in a single receptor, although they main not be able to cluster with multiple proteins due to the large distance needed for this.

Carbohydrates are present in many proteins in Nature, and their correct glycosylation is crucial for the correct protein folding. If the protein folds incorrectly, the glycans will not be displayed correctly and this will let to an expulsion of the protein through the degradation pathway.[59] Moreover, glycans attached to proteins can have other functions; they can act as recognition motifs for carbohydrate-binding proteins (lectins), mediate cell-cell-interactions, provide the protein to which are attached protease protection, etc.[60]

Glycoprotein/glycopeptide synthesis was introduced in order to study the role that this structures plays in Nature.[61] The first example of multivalency introduced into a protein was done using glycolMTS method, leading into the concept of glycodendriproteins.[62] Many examples of the use of proteins as

central scaffolds in the synthesis of multivalent glycoconjugates are reported in the literature.[63]

In this sense, in order to develop modified proteins, chemical protein modification has emerged as an invaluable tool. Modifications of natural amino acids and non-natural amino acids and the reactivity of carboxylic acids, amides, amines, alcohols and thiols have been extensively studied.[64,65] As an inconvenient, when a protein is used to anchor ligands it is nearly impossible to control the orientation of the epitope although some strategies to control it, such as introduction of different functionalities to viral coat proteins,[66,67] have been studied.

1.5. SECONDARY INTERACTIONS

The affinity and specificity of multivalent binding events between ligands and proteins are known to be influenced by the carbohydrate residue, the template of the central scaffold upon the residues are displayed and the relative spatial orientation of the saccharide. Among these, the different protein domains confer optimal ligand disposition in order to enhance binding (Figure 1.8). Typical carbohydrate interactions take place in the primary and secondary binding site in lectins or in the catalytic site in glycosyl hydrolases. Moreover, as it has been mentioned before, glycosyl hydrolases also present a CBM, directly correlated with the enhance in binding when multivalent systems were tested. However, this enhance in binding affinity could also be observed due to non-specific interactions (hydrophobic patches).

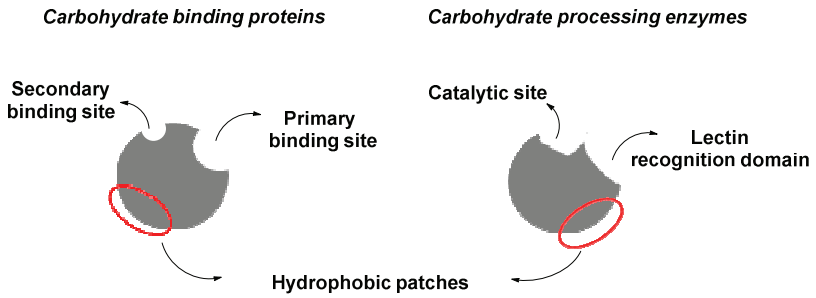


Figure 1.8. Specific and non-specific interactions.

Polizzotti *et al.* found that, when a hydrophobic chain was used as a linker, the inhibition of Cholera toxin (lectin that recognize GM1) was enhanced, suggesting that the increase in length of the linker resulted in an improvement in accessibility to the binding pocket. Moreover, apart from improved accessibility, hydrophobic interactions between the aliphatic regions of the linker with residues located in the binding pocket provide favorable interactions.[68]

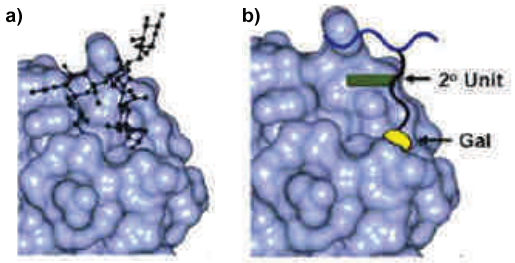


Figure 1.9. CTx crystal structure (a) binding with GM1 and (b) interaction with synthetic glycopolymers presenting a secondary binding motif (adapted from Jones, M. W. *et al.*).[69]

Recently, glycopolymers with a second binding motif were also studied in the inhibition of Cholera toxin. Although the carbohydrate residue was presented in an optimized linkage length in this case they focused in the study of these variable second binding groups. CTx crystal structure revealed a primary (where galactose residue is placed) and a secondary binding pocket

(Figure 1.9). Surprisingly, inhibition potency and selectivity towards other lectins increased due to specific hydrophobic interaction of the secondary motif.[69]

Dynamic combinatorial chemistry (DCC) has been applied in multiple studies as a screening method for the identification of potent multivalent recognition systems and to probe secondary interactions with biomolecules.[70]

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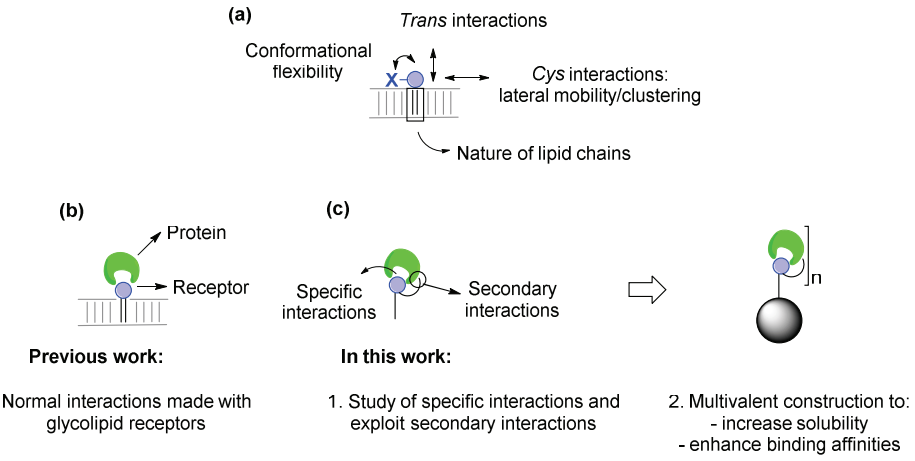
CHAPTER 2

OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
SYNTHETIC GLYCOLIPIDS AS MODULATORS OF CARBOHYDRATEPROTEIN
INTERACTIONS
Míriam Salvadó Molero

With this background and focusing on the importance that glycoconjugates, and in particular glycolipid receptors, have in the first stage of infections, the relevance of multivalency in the enhancement of binding affinities and the significance of carbohydrate presentation and secondary (specific and non-specific) interactions in protein binding studies, the main goal of this thesis is to synthesize a series of synthetic glycolipids (mono and multivalent) systems to evaluate them against carbohydrate-binding proteins and sugar processing enzymes.

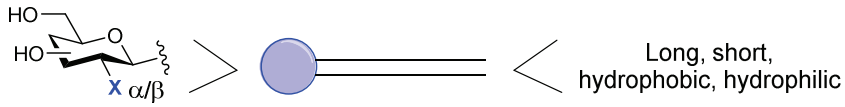


The research described in Chapter 3 aims to study and explore the chemical features of glycolipids as inhibitors. As it has been mentioned in the introduction, the catalytic site of glycosidases was found to be unique and not easily accessible. Moreover, it was also found that they have a lectin-like domain and some multivalent systems were found to be potent inhibitors. Those findings together with the relevant importance of hydrophobic interactions to enhance binding gave us to think about the possibility to construct glycolipid-based inhibitors and their corresponding multivalent presentation, which will modulate sugar presentation and therefore binding properties. In particular the specific objectives of this work are:

(i) To synthesize glycolipid-based inhibitors in two different directions, introducing modifications in the pyranose ring and in the

algycone moiety, and evaluate their inhibition potency against glycosidases. This will give us a general idea about how the C-2 position of the pyranose ring could affect the binding and if the length and polarity of the linker will improve the accessibility and/or the inhibition potency.

(ii) To synthesize multivalent systems based on glycolipid monomers to study the influence the carbohydrate density and the nature of the linker could have in glycosidases inhibition.



X: H, OH, F, NHAc

The research described in Chapter 4 aims to systematically evaluate the binding affinities between a series of multivalent homogeneous *vs.* heterogeneously presented glycoconjugates against a model plant toxin (RCA_{120}). In particular the specific objectives of this work are:

(i) To synthesize multivalent structures using different Boltorn central cores in order to obtain a homogeneous *vs.* a heterogeneous presentation of the carbohydrate residues. Boltorn (hyperbranched polymer and homogeneous glycodendrimer) was chosen as a central core to construct two different families of multivalent systems that could allow for the presentation of D-galactose residues in a polydisperse and a monodisperse manner.

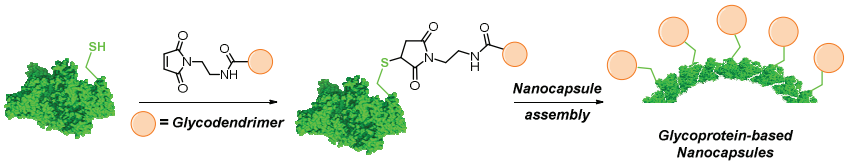
(ii) Evaluate the behaviour of the different synthetic multivalent systems using surface plasmon resonance (SPR) and dynamic light scattering (DLS) against a model plant toxin (RCA_{120}). Evaluation of the topology and carbohydrate density will be studied.

The research described in Chapter 5 aims to found a promising novel strategy for the design of multivalent inhibitors of carbohydrate-binding proteins based on glycodendriprotein-based nanocapsules. In particular, the specific objectives of this work are:

(i) To determine the binding affinities of a series of synthetic glycodendrons based on gallic and pyrogallic acid scaffolds towards a model lectin (RCA_{120}) using Bio-layer Interferometry (BLI).

(ii) To anchor the promising glycodendron to BSA using site-selective protein strategies to finally obtain a glycodendriprotein.

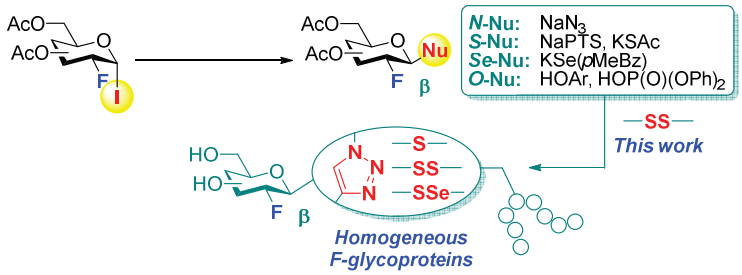
(iii) To construct glycodendriprotein-based nanocapsules using controlled sonication in order to obtain the desired BSA-nanoparticles size and evaluate and compare their binding affinities with the synthetic glycodendrons.



The research described in Chapter 6 aims to found a novel strategy for the preparation of Fluoroglycoproteins using F-sugars building blocks that were prepared with the idea of modifying the sugar in glycolipid ligands (see Chapter 3). In this context, the specific goals of this chapter are:

(i) To optimize the halogenation conditions for obtaining 2-F-carbohydrate halides and to study their reactivity employing phase-transfer catalyzed reactions (PTC) for the ultimate preparation of 1-β-"soft Nu"-2-deoxy-2-fluoroglycosides.

(ii) To explore the reactivity of some F-sugar derivatives with a model single-Cys protein (Annexin V) to provide a useful platform to disulfide-linked Fglycoproteins.



CHAPTER 3

GLYCOLIPIDS, CLUSTERS AND HYPERBRANCHED POLYMERS AS β-GLYCOSIDASE INHIBITORS

UNIVERSITAT ROVIRA I VIRGILI
SYNTHETIC GLYCOLIPIDS AS MODULATORS OF CARBOHYDRATEPROTEIN
INTERACTIONS
Míriam Salvadó Molero

3.1. INTRODUCTION

Glycosidases, enzymes that catalyse the hydrolysis of glycosidic bonds, are important enzymes present in almost all living organisms. Wolfenden *et al.* found that the hydrolysis of a glycosidic bond was enhanced 10^{17}-fold by glycosidases compared to the uncatalyzed reaction.[1] Glycosidases can be classified depending on their mode of action as endoglycosidases or exoglycosidases. Moreover, they can also be classified as inverting or retaining glycosidases, referring to the mechanism used for their catalytic activity (Figure 3.1).[2]

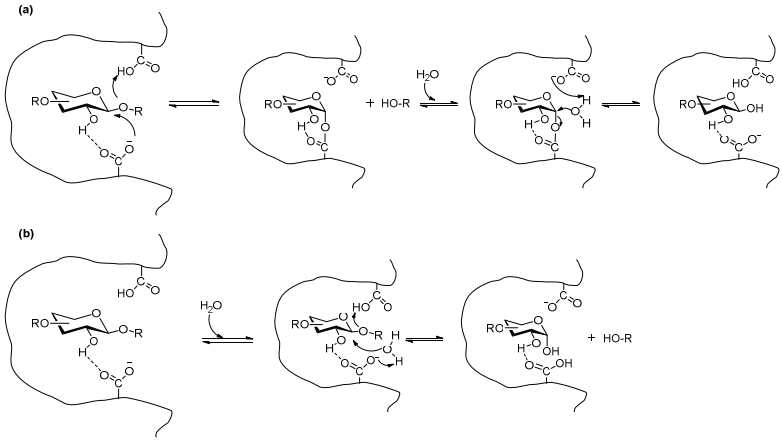


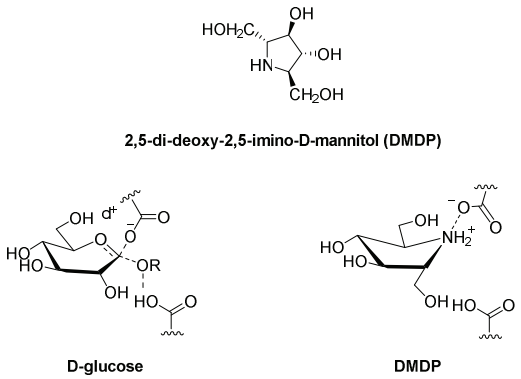
Figure 3.1. Catalytic mechanism of glycosidases; (a) inverting β-glycosidase and (b) retaining β-glycosidase.

One of the most active fields in Glycobiology is to look for compounds to modulate the activity of glycosidases. Thus, glycosidase inhibitors are used in order to understand the biological processes involving biosynthesis, metabolism and recognition of carbohydrates.

Glycosidase inhibitors could be classified, broadly, as noncovalent or covalent inhibitors, depending on their mode of action. As the name suggests, compounds that forms a covalent bond with the enzyme are considered

covalent inhibitors. Typically, an enzyme-based nucleophile attacks an electrophilic region of the inhibitor forming a covalent bond, which links each other in an irreversible way. This produces the enzyme inactivation because the access to the active site is blocked, or because a specific residue of the active site, that it is crucial for the catalytic function, is modified.[2] Whereas, noncovalent inhibitors binds to the enzyme in a reversible way and they have the greatest potential as therapeutics.[3] It was found that some saccharides that behave as noncovalent inhibitors have a trigonal anomeric carbon that adopt a half chair conformation that, together with hydrogen bonds, aid the stabilization of the oxocarbenium-ion transition state.[4,5]

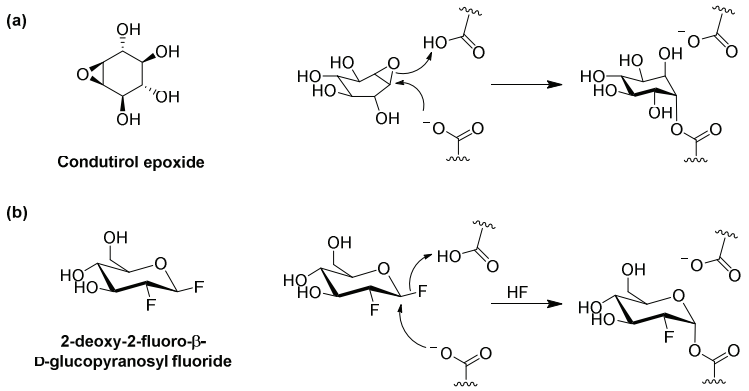
2,5-di-deoxy-2,5-imino-D-manitol (DMDP), is an example of reversible inhibitor of α-glucosidases I and II, such as almond β-glucosidase, bovine liver β-galactosidase, invertase and PFP (pyrophosphate-D-fructose-phosphate-1-phosphotransferase). Its flat 5-member ring structure with a unique *C2* symmetric axis mimics the transition state observed during substrate hydrolysis. Moreover, the high affinity found against glucosidase could be due to the spatial arrangement of the hydroxyl groups compared to the natural substrate, glucose (Scheme 3.1).[6,7]



Scheme 3.1. Transition state of the hydrolysis of a D-glucoside by α-glucosidase and its interaction with the reversible inhibitor DMDP.

As a model of irreversible inhibitor, Condutirol B epoxide, a synthetic compound, was found to bind to β-glucosidase in an irreversible way (Scheme 3.2a). Its trans-diaxal orientation seams to play a key role in the inhibition, since it shows a potent activity against β-glucosidase.[3,8]

Activated fluorinated glycosides are other examples of covalent inhibitors. The substitution of the OH group at position C-2 by fluor, leads to a destabilization of the oxocarbenium ion-like transition state due to the loss of hydrogen-bonding interactions, by far the most important interactions at position 2, as well as its electron-withdrawing nature destabilizing both glycosylation and deglycosylation transition states. Moreover, the addition of an activated leaving group (*e.g.* fluorine) at the anomeric centre, selectively favours de glycosylation reaction leading to an accumulation of highly stable covalent glycosyl-enzyme intermediate,[2] in retaining β-glycosidases (Scheme 3.2b).



Scheme 3.2. Proposed inhibition mechanism of the active site of β-glycosidases for (a) Condutirol epoxide and (b) 2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride.

Glycosidases, and indeed their catalytic activity, are indispensable for all organisms and are involved in many biological processes and diseases. Lactose, a disaccharide found in milk, is hydrolysed by a β-gycosidase into monosaccharides (galactose and glucose) that would be absorbed into the

bloodstream. Lactase deficiency causes lactose intolerance. Gaucher's disease is also caused by the mutation of the glucocerebrosidase gene, another β-glycosidase. Problems in glucose absorption can cause high blood glucose levels that can derive in a chronic metabolic disorder as diabetes. A-Glycosidases are the enzymes responsible of the hydrolytic cleavage of oligosaccharides; inhibition of this enzyme could retard the absorption of glucose. Moreover, glycosidase inhibitors have also been studied in the treatment of cancer, cystic fibrosis and virus infections.[9]

In 1940, Pauling postulated that to construct the best inhibitor this had to mimic the structure of the transition state.[10,11] Many examples of glycosidase inhibitors mimicking the transition state have been found and this fact has been corroborated by transition state energy calculations.[12] Different classes of carbohydrate-based transition state mimics were postulated by Gloster, T. M. and Vocadlo, D. J.[13] Compounds that have a basic nitrogen in a five-, six- or seven-membered rings are proposed to mimic the charge distribution of the oxocarbenium ion-like transition state (Figure 3.2a). Compounds that incorporate a trigonal centre at the anomeric centre and/or in the endocyclic oxygen, emulate the planar geometry of the transition state (Figure 3.2b). More complex compounds (Figure 3.2c) mimic both shape and charge incorporating planarity and positive charge. Finally, appending different groups onto inhibitors mimicking shape and/or charge can capture binding energy (Figure 3.2d).

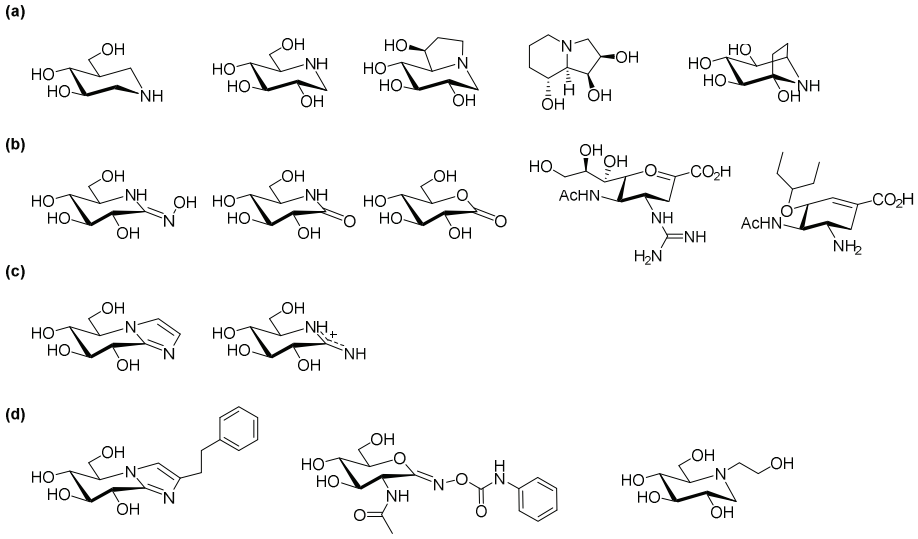


Figure 3.2. Carbohydrate-based transition state mimics as glycosidase inhibitors.[13]

Mutations in glycosidase can cause lysosomal storage disorders (LSDs). Twenty years ago a pharmacological chaperone (PC) therapy was launched.[14] Pharmacological chaperones are small molecules able to bind to the mutant glycosidase in order to induce their proper folding, restore trafficking and increase their enzymatic activity and substrate processing in the lysosome. Ligands as PCs designed as a conjugation of a sugar with a lipophilic moiety that mimic both the transition state towards glycosidic cleavage and ceramide aglycone of the natural substrate (glycosylceramides) are extensively studied.[15] The stabilization of hydrophobic non-glycone interactions leads to the development of strong glycosidase inhibitors (Figure 3.3).

Brusmshtein B. *et al.* studied the binding of *N*-alkyl iminosugars (Figure 3.3a) towards GCase (β-glucocerebroside) finding that alkyl chains participate in favourable hydrophobic contacts with amino acid residues favouring the binding and the corresponding correct folding and trafficking to the lysosome.[16] Based on this scaffold Ye and co-workers developed a new *N*-alkylated iminosugar lactam (Figure 3.3c).[17] When moving the alkyl chain

from the N-atom to the adjacent C-atom in 1-azasugars, promising GCase inhibitors were found (Figure 3.3b,g).[18,19] Glycosidase inhibitors containing two nonyl chain were also studied by Butters and Llebaria (Figure 3.3f).[20]

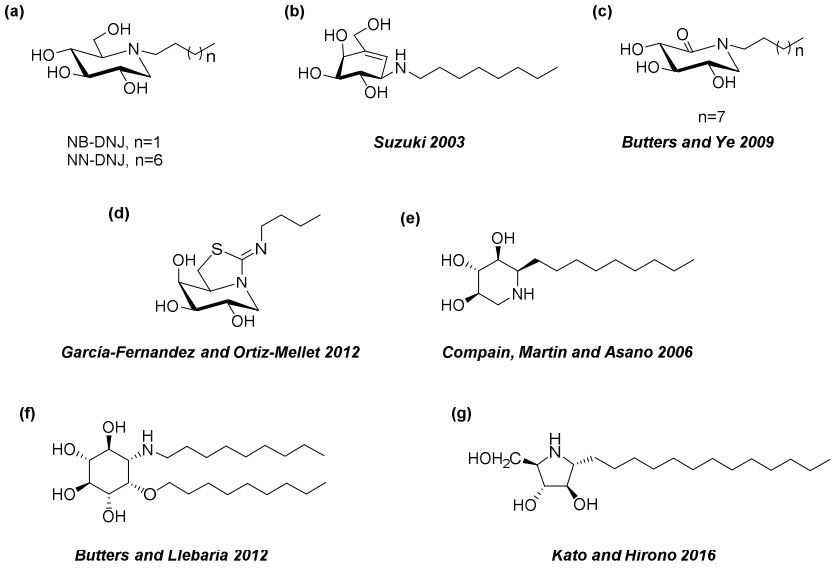


Figure 3.3. Examples of glycosidase inhibitors with alkylated chain(s).

The design of glycosidase inhibitors have been focused on monovalent structures that mimic the natural substrate or the transition state, due to the unique, buried and not very accessible active site of glycosidases.[13,21] Thus, in principal, the construction of multivalent structures as glycosidase inhibitors would negatively affect the enzyme-inhibitor complex formation due to sterics. Even though, when three copies of 1-deoxynojirimycin (DNJ) onto a dendritic core were presented against glycosidases, in some cases an enhanced inhibition potency was found.[22] This opened a new line to study multivalent ligands as glycosidase inhibitors and the lectin behaviour of glycosidase due to their carbohydrate-binding modules (CBMs) (see Chapter 1, 1.3.2. Carbohydrate-processing enzymes). For example, it was found that when a monomer (glycosidase substrate) was presented to the same enzyme clustered into nanodiamond particles not only remains stable towards

hydrolysis, but they are also able to competitively and reversibly inhibit this enzyme.[23]

3.2. OUTLOOK AND OBJECTIVES

With this background, in this chapter, we were focus in obtaining different glycosidase inhibitors. For that reason, the inhibition potency of glycolipids, clusters and hyperbranched polymers was evaluated. In this context, the goals of this chapter are:

(i) To synthesize, and evaluate against glycosidases a series of glycolipids presenting modifications at the C-2 position of the pyranose ring (OH, H, F, and NHAc). The OH group at position 2 of the carbohydrate plays a key role in the enzymatic active site due to hydrogen bond interactions.[24] The evaluation of this family of glycosidase inhibitors will allow us to study the effect of such groups in the inhibition properties.

(ii) To explore whether the nature of the aglycone plays any role in the inhibition process. Glycolipids modified in the aglycone moiety (hydrophilic, hydrophobic, and chain length) will be tested as glycosidase inhibitors.

(iii) To evaluate the effect of presenting glycolipids as multivalent systems towards glycosidases. The glycolipid presentation using either a cluster or a hyperbranched polymer will be compared.

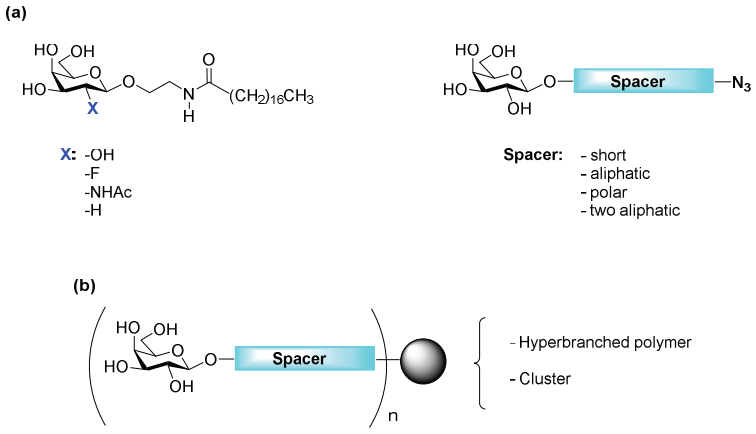


Figure 3.4. Synthetic inhibitors: (a) glycolipid monomers either presenting modifications in the pyranose ring or the aglycone moiety and (b) glycolipid-multivalent systems using either hyperbranched polymers or clusters as central scaffolds.

3.3. RESULTS AND DISCUSSION

One of our main goals was to synthesize glycolipids with different substituents at the C-2 position of the pyranose ring in order to evaluate the role of this position and how particular groups, OH, F, NHAc and H, influence the specific recognition of the desired glycolipids against different glycosidases. These groups were chosen because they may interfere in H-bonding and may be involved in polar hydrophobic contacts. Moreover, subtle conformation changes to due to the presence of such electronegative groups could favour their inhibition potency. Due to its specific properties, fluorine influence the biological function of the molecules to which is incorporated, see Chapter 6.

3.3.1. Glycolipid synthesis

The choice of the most appropriate protecting groups is crucial in order to obtain the desired glycoside with the correct configuration at the anomeric position, and in particular acyl groups are selected as participating groups in order to obtain β-glycosides. Moreover these protecting groups should be

easy to remove since harsh deprotection conditions may cause β-elimination[25] and epimerization of α-stereocenters.[26] Acetyl group has been extensively studied although there are some limitations associated to this group such as migration and orthoester formation.[27,28] Benzoate group was found to be more effective but, in contrast, their removal could lead to side reactions. In a study about the glycosylation of ceramides starting from the per-acetyl-glycosyl iodides and stannylated ceramides we found that the orthoester was exclusively formed when Bu_4NI was used as a promoter. That implied a further acid treatment necessary to get the β-glycosides. In order to circumvent this limitation and aiming to get directly the glycosides, we decided to use fluorobenzoyl groups as protecting groups at position C-2. These groups can avoid orthoester formation during glycosylation reactions due to its electron-withdrawing nature that destabilize the dioxolenium ion intermediate. Specifically, the 2,6-difluorobenzoyl group combines the advantages of the benzoyl group in formation of glycosidic bonds with the ease removal.

In an effort to improve the direct glycosylation of lipids applying the experience in our group with stannyl ethers,[29] we decided to investigate the one step glycosylation reaction using glycosyl donors carrying a 2,6-di-F-benzoyl[30] group at C-2, a highly disarmed glycosyl donor, to access direct β-glycosylation without orthoester formation.

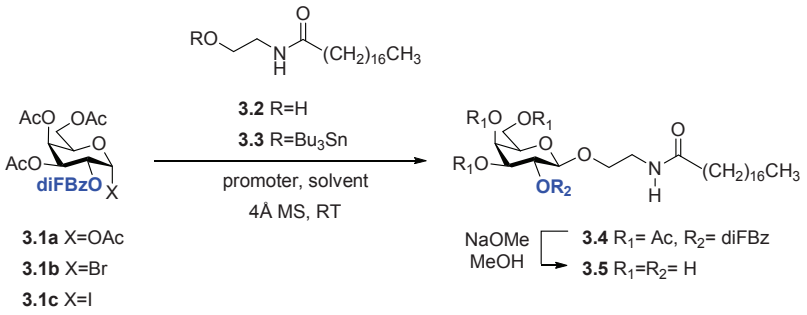
Thus, stannyl lipid **3.3** was treated with glycosyl iodide **3.1c** in refluxing toluene and in the presence of Bu_4NI, under the conditions previously optimized in our group.[29,31,32,33] However, glycosylation of stannyl lipid **3.3** did not provide the glycosylated product. Similar results were found when AgOTf was used as a promoter (Table 3.1, entries 1 and 2). These results are consistent with the fact that glycosyl iodide **3.1c** is hydrolyzed before reacting with the stannyl derivative.

We then tested a classical glycosylation procedure starting from 1-*O*-acetylgalactosyl donor **3.1a** and lipid **3.2** employing Brönsted acids as a promoter. Thus, the glycosyl donor **3.1a** was treated with acceptor **3.2** in the presence of TfOH to render a poor yield of glycosylated product (Table 3.1, entry 3). Starting from bromo glycoside **3.1b** and using AfOTf/$Sn(OTf)_2$ as a promoter, however, the glycosylated product **3.4** was obtained in a moderate 58% yield (Table 3.1, entry 4).

We then explored the reaction of **3.1a** and **3.1b** as glycosyl donors with stannyl ether **3.3** under different reaction conditions (entries 5–14). Thus, the reaction of **3.1a** with **3.3** in the presence of 3 equivalents of $BF_3.OEt_2$ provided only a product resulting from the hydrolysis of the donor (Table 3.1, entry 5). When 6 equivalents were used after 3 h at room temperature only hydrolysed glycosyl donor was isolated (4:1 α/β ratio).

However, when 3 equivalents of TfOH were added to the reaction mixture the desired β-glycoside **3.4** was obtained in a 60% yield (Table 3.1, entry 6). The use of smaller amount of acid decreased the yield. The replacement of CH_2Cl_2 by toluene as solvent produced a drastic decrease in yield (Table 3.1, entry 5). An excess of TfOH was detrimental since partial deprotection of galactose **3.1a** was observed. In order to simplify the purification step, 1 equivalent of amide **3.2** was used and the glycosylated product **3.4** was directly deprotected to afford **5** in a good 85% overall yield for the two steps (Table 3.1, entry 8).

Table 3.1. Glycosylation of lipids **3.2** and **3.3** with 2-di-F-Bz glycosyl donors **3.1a**, **3.1b** and **3.1c**.[a]

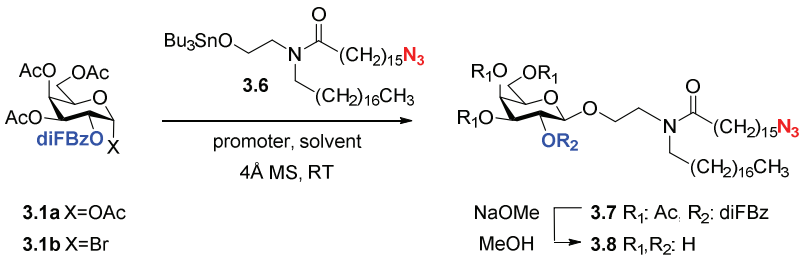


Entry	Donor (equiv)	Acceptor (equiv)[b]	Promoter (equiv)	Solvent	*t* [h]	Product [%][c]
1[d]	**3.1c** (1)	**3.3** (1.2)	TBAI (0.3)	PhMe	15	[e]
2	**3.1c** (1)	**3.3** (1.2)	AgOTf (3)	CH_2Cl_2	24	[e]
3	**3.1a** (1.2)	**3.2** (1)	TfOH (3)	CH_2Cl_2	3	**3.5** (37)[f]
4	**3.1b** (1.2)	**3.2** (1)	[g]	CH_2Cl_2	24	**3.5** (58)[f]
5	**3.1a** (1)	**3.3** (1.1)	$BF_3 \cdot Et_2O$ (3)	CH_2Cl_2	4	[e]
6	**3.1a** (1.2)	**3.3** (1)	TfOH (3)	CH_2Cl_2	0.5	**3.4** (60) (>99)
7	**3.1a** (1.2)	**3.3** (1)	TfOH (3)	PhMe	1	**3.4** (20)
8	**3.1a** (1.2)	**3.3** (1)	TfOH (3)	CH_2Cl_2	2	**3.5** (85)[f]
9[h]	**3.1b** (1)	**3.3** (1.2)	TBAI (0.3)	PhMe	24	[e]
10	**3.1b** (1.2)	**3.3** (1)	AgOTf (3)	CH_2Cl_2	24	**3.4** (30)[i]
11	**3.1b** (1)	**3.3** (1.2)	AgOTf (3)	CH_2Cl_2	24	**3.4** (35)[i]
12	**3.1b** (1)	**3.3** (1.2)	$Hg(OTf)_2$ (3)	CH_2Cl_2	24	**3.4** (30)[i]
13	**3.1b** (1.2)	**3.3** (1)	[g]	CH_2Cl_2	24	**3.4** (53)
14	**3.1b** (1.2)	**3.2** (1)	[g]	CH_2Cl_2	24	**3.5** (90)[f]

[a]*General conditions*: Promoter was added to a mixture of donor, tributylstannyl acceptor, and 4Å molecular sieves in the corresponding solvent at room temperature unless otherwise indicated. [b]Tributylstannyl acceptor **3.3** was prepared *in situ* prior to glycosylation reaction; see Experimental section for full details. [c]Isolated yield. In parenthesis yield calculated by ^{19}F NMR analysis of the crude reaction mixture. [d]T=reflux. [e]54–89% of the corresponding 3,4,6-tri-*O*-acetyl-2-*O*-(2,6-difluorobenzoyl)-D-galactose was also obtained. [f]Yield over two steps. [g]AgOTf/$Sn(OTf)_2$ (3:3). [h]T=80 ºC [i]Starting material recovered. PhMe=toluene. TBAI=tetrabutylammonium iodide. NR=No reaction.

Reaction of glycosyl bromide **3.1b** with **3.3** was initially tested in the presence of TBAI as a promoter, but a mixture of hydrolysed product and lipid **3.3** was obtained (Table 3.1, entry 9). However, the use of triflate salts such as AgOTf or $Hg(OTf)_2$, afforded glycoside **3.4** in a low yield due to the competitive formation of orthoester and hydrolyzed products (Table 3.1, entries 10–12). These results indicate that, unexpectedly, orthoester remains intact in a slightly acidic solution after long reaction times. However, a mixture of AgOTf and $Sn(OTf)_2$ as Lewis acids favours the orthoester rearrangement.[34] Thus, when this promoter mixture was applied, quantitative formation of glycosylated product **3.4** was obtained. However, after purification **3.4** was recovered only in a moderate 53% yield. In contrast, when the crude mixture was deprotected to afford **3.5**, 90% yield over two steps was obtained (Table 3.1, entries 13 and 14).

Table 3.2. Glycosylation of lipid **3.6** with 2-di-F-Bz glycosyl donors **3.1a, 3.1b**.[a]



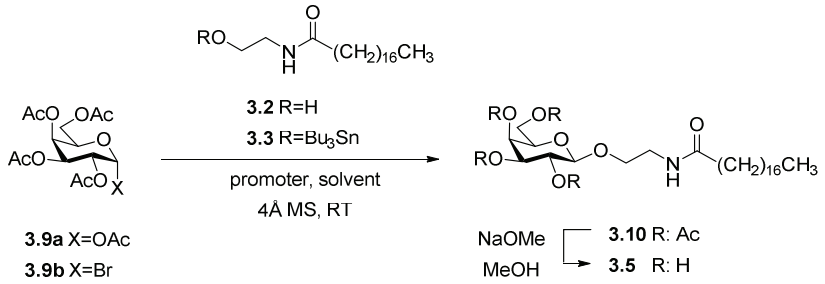
Entry	Donor (equiv)	Acceptor (equiv)[b]	Promoter (equiv)	Solvent	*t* [h]	Product [%][c]
1	**3.1a** (1.2)	**3.6** (1)	TfOH (3)	CH_2Cl_2	2	**3.8** (40)[d]
2	**3.1b** (1.2)	**3.6** (1)	AgOTf (3) $Sn(OTf)_2$ (3)	CH_2Cl_2	40	**3.8** (8)[d]
3[e]	**3.1b** (1.2)	**3.6** (1)	AgOTf (3) $Sn(OTf)_2$ (3)	CH_2Cl_2	24	**3.8** (63)[d]

[a]*General conditions*: Promoter was added to a mixture of donor, acceptor, and 4Å molecular sieves in the corresponding solvent at room temperature unless otherwise indicated. [b]Tributylstannyl acceptor **3.6** was prepared *in situ* prior to glycosylation reaction; see Experimental Section for full details. [c]Isolated yield. [d]Yield over two steps. [e]T=40 ºC.

Then, we decided to use these conditions for the glycosylation of more complex lipid **3.6**. When, TfOH was used as a promoter, the yield of glycosylation followed by hydrolysis was moderate (Table 3.2, entry 1) compared to the glycosylation of stannyl lipid **3.3** (85% yield). When AgOTf/$Sn(OTf)_2$ was used as a promoter in glycosylation at room temperature of the more complex lipid **3.6**, yield was also lower (Table 3.2, entry 3), which indicates the reduced reactivity of these lipids and the necessity to warm up the reaction for improving the yield.

In parallel and in order to compare the effectiveness of using a 2,6-difluorobenzoate group at position C-2, the glycosylation reaction was studied using fully acetylated glycosyl donors (Table 3.3).

Table 3.3. Glycosylation reaction of **3.2** and **3.3** with acetylated glycosyl donors **3.9a** and **3.9b**.[a]



Entry	Donor (equiv)	Acceptor (equiv)[b]	Promoter (equiv)	Solvent	*t* [h]	Product [%][c]
1	**3.9a** (1.2)	**3.3** (1)	TfOH (3)	CH_2Cl_2	2	**3.5** (18)[d]
2	**3.9a** (1.2)	**3.2** (1)	TfOH (3)	CH_2Cl_2	3	**3.5** (15)[d]
3	**3.9b** (1.2)	**3.3** (1)	AgOTf (3) $Sn(OTf)_2$ (3)	CH_2Cl_2	24	**3.5** (42)[d]
4	**3.9b** (1.2)	**3.2** (1)	AgOTf (3) $Sn(OTf)_2$ (3)	CH_2Cl_2	24	**3.5** (34)
5	**3.9a** (1.2)	**3.2** (1)	$BF_3.OEt_2$ (6)[e]	CH_2Cl_2	7	**3.10** (72)

[a]*General conditions*: Promoter was added to a mixture of donor, acceptor, and 4Å molecular sieves in the corresponding solvent at room temperature unless otherwise indicated. [b]Tributylstannyl acceptor **3.3** was prepared *in situ* prior to glycosylation reaction. [c]Isolated yield. [d]Yield over two steps. [e]Promoter was added at 0 ºC.

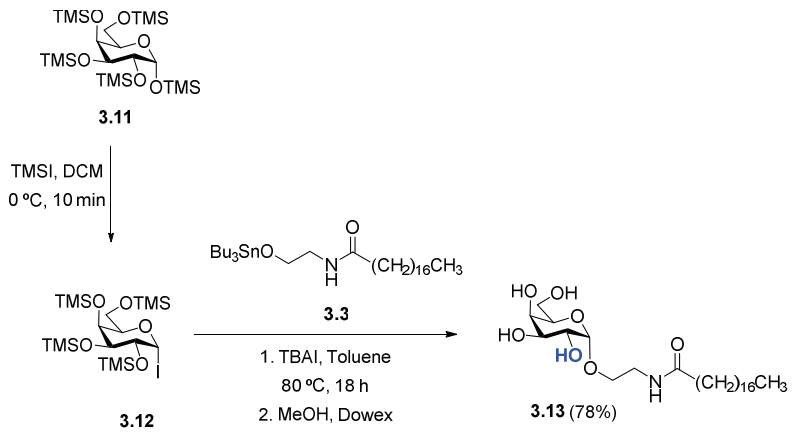
Initially, the reaction was tested with fully acetylated galactose **3.9a** and lipid **3.3**, in the presence of 3 equiv. of TfOH as a promoter, under similar reaction conditions (Table 3.3, entry 1). A mixture of hydrolyzed, glycosylated product, and orthoester were detected in the ^{1}H NMR spectrum of the reaction crude. After deprotection and purification a low 18% yield was obtained. When **3.2** was used as glycosyl acceptor using the same reaction conditions a poor 15% yield was also obtained (Table 3.3, entry 2).

Acetyl-galactosyl bromide **3.9b** was treated with **3.3** under optimized reaction conditions using AgOTf/$Sn(OTf)_2$ as a promoter system and the yield after protecting groups removal was moderate 42–34% (Table 3.3, entries 3 and 4), in contrast with the good results obtained when using 2,6-di-F-Bz (90% yield) (Table 3.1, entry 14). The yield was lower than that of the reaction with acceptor **3.2** under similar conditions (entry 4).

The glycosylation of **3.2** proved better using donor **3.9a**, 6 equivalents of $BF_3.OEt_2$ as a promoter and following the methodology previously described in our group.[33b] Under these conditions, a 72% yield was obtained from fully acetylated galactose **3.9a** and **3.2** as glycosyl acceptor.

From these results it can be concluded that, disarmed glycosyl donors provide better yield in the glycosylation reaction of stannyl-lipids, and could be considered an improvement to access β-glycolipids.

Next, we tackled the synthesis of the corresponding α-anomer, and for this purpose the conditions reported by our group, starting from persilylated donors, were used.[31] Thus, TMS-Gal **3.11** was treated with TMSI in DCM at 10 ºC for 10 min to form the corresponding TMS-Gal iodide **3.12** which was added to the stannyl ether in the presence of TBAI as a promoter to afford, after deprotection, glycoside **3.13** in a 78% yield over three steps (Scheme 3.3).



Scheme 3.3. Synthesis of compound **3.13** using TMS-galactose as glycosyl donor.

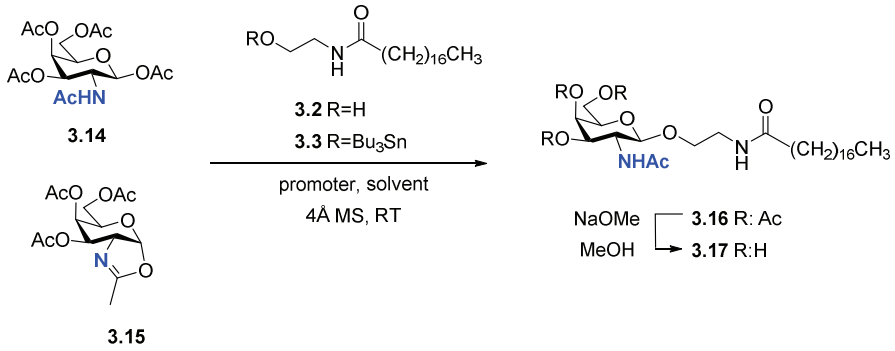
In order to access the 2-NHAc glycolipid, traditionally, a temporary protecting group, such as *N*-Troc, *N*-Phth, azide, etc. is placed at position C-2 and it is then synthetically converted into the biologically relevant 2-NHAc group. To obtain specifically β-selectivity, oxazolines have been also explored as glycosyl donors, as they can minimise the number of synthetic steps because they can directly yield the acetyl moiety. Moreover, Christensen *et al.* studied the direct glycosylation of *N*-acetyl donors using rare earth metal triflates.[36]

Microwave heating was found to be a powerful tool to increase reaction rates in carbohydrate chemistry. Many glycosylation reactions have been studied using microwave heating instead of conventional heating.[35,36]

To obtain the desired 2-NHAc-glycolipid we used conditions described by Christensen *et al*. Thus, when donor **3.14** was treated with **3.2** in the presence of $Sc(OTf)_3$ (15 mol%) under microwave irradiation the reaction did not evolve after 1 h. When the same conditions were applied to oxazoline **3.15**, a moderate overall yield (23%) was obtained after deprotection (Table 3.4, entries 1 and 2). The reaction of the corresponding stannyl lipid **3.3** as acceptor and oxazoline **3.15** as a donor in the presence of TfOH under

microwave irradiation, provided, after deprotection, the desired product **3.17** in very low yield (5%), probably due to tedious reaction manipulation and purification (Table 3.4, entry 4). However, starting from **3.2** as glycosyl acceptor and **3.14** as glycosyl donor, **3.16** was obtained in 42% yield. Using the same reagents with $BF_3.OEt_2$ as a promoter and without heating, only oxazoline **3.15** was formed after 48 h (Table 3.4, entry 6).

Table 3.4. Synthesis of 2-deoxy-2-NHAc-galactolipid.[a]



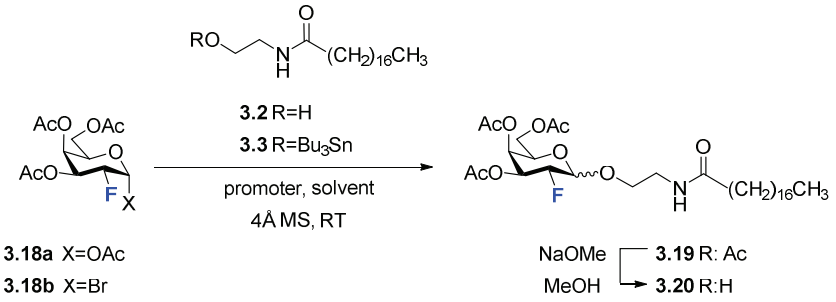
Entry	Donor (equiv)	Acceptor (equiv)[b]	Promoter (equiv)	Solvent	*t* [h]	Product [%][c]
1[f]	**3.14** (1.2)	**3.2** (1)	$Sc(OTf)_3$ (0.15)	CH_2Cl_2	1	NR[d]
2[f]	**3.15** (1.2)	**3.2** (1)	$Sc(OTf)_3$ (0.15)	CH_2Cl_2	1	**3.17** (23)[e]
3	**3.15** (1.2)	**3.3** (1)	TfOH (3)	CH_2Cl_2	1	NR
4[f]	**3.15** (1.2)	**3.3** (1)	TfOH (3)	CH_2Cl_2	1	**3.17**[g]
5	**3.14** (1.2)	**3.2** (1)	$BF_3.OEt_2$ (6)	CH_2Cl_2	48	**3.15**
6[f]	**3.14** (1.2)	**3.2** (1)	TfOH	CH_2Cl_2	2	**3.16** (42)

[a]*General Conditions*: Promoter was added to a mixture of donor, acceptor, and 4Å molecular sieves in the corresponding solvent at room temperature unless otherwise indicated. [b]Tributylstannyl acceptor **3.3** was prepared *in situ* prior to glycosylation reaction; see Experimental Section for full details. [c]Isolated yield. [d]Starting material recovered. [e]Yield over two steps. [f]T=80 ºC using microwave irradiation. [g]Low yield. NR=No reaction.

The next objective of our work was the synthesis of the 2-F-glycolipid. With this purpose, acetyl-2-F-galactose was synthesized from acetyl-galactal using Selectfluor® as the fluorinating agent.[37] The reaction of 2-deoxy-2-

fluoroderivative **3.18a** with acceptor **3.2** at room temperature and TfOH as a promoter did not evolved after 12 h (Table 3.5, entry 1). Microwave heating did not improved the results and after 1 h of reaction no evolution was observed when TfOH was used as activator, whereas when $Sc(OTf)_3$ was tested a complex mixture of products was found. The product of hydrolysis of **3.18a** was detected by ^{1}H NMR but not glycoside **3.19** (Table 3.5, entries 2 and 3). The reaction of **3.18a** with **3.2** in toluene at 110 ºC under microwave irradiation, in the presence of MgO as an acid scavenger and TMSOTf as a promoter, led to decomposition of the glycosyl donor (Table 3.5, entry 4). Thus, the reaction was studied using a better leaving group at

Table 3.5. Synthesis of 2-deoxy-2-fluoro-galactolipid.[a]



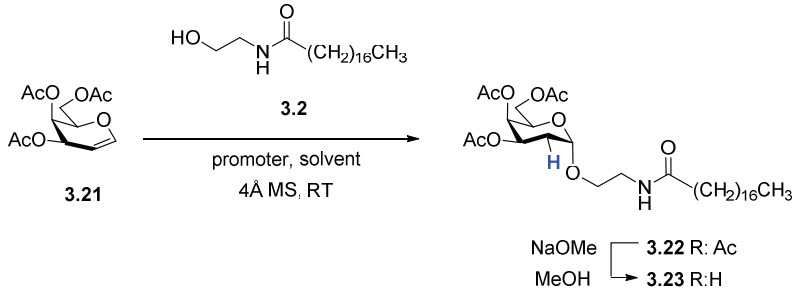
Entry	Donor (equiv)	Acceptor (equiv)[b]	Promoter (equiv)	Solvent	*t* [h]	Product [%][c]
1	**3.18a** (1.2)	**3.2** (1)	TfOH (3)	CH_2Cl_2	12	NR[d]
2[e]	**3.18a** (1.2)	**3.2** (1)	TfOH (3)	CH_2Cl_2	1	NR[d]
3[e]	**3.18a** (1.2)	**3.2** (1)	$Sc(OTf)_3$ (0.15)	CH_2Cl_2	0.5	[f]
4[e]	**3.18a** (1.2)	**3.2** (1)	TMSOTf/MgO	Toluene	1	Decomp.
5	**3.18a** (1.2)	**3.3** (1)	TfOH (3)	CH_2Cl_2	1.5	Decomp.
6[g]	**3.18b** (1.2)	**3.3** (1)	TfOH (3)	Toluene	2	**3.19** (35)[h]

[a]*General conditions*: Promoter was added to a mixture of donor, acceptor, and 4Å molecular sieves in the corresponding solvent at room temperature unless otherwise indicated. [b]Tributylstannyl acceptor **3.3** was prepared in situ prior to glycosylation reaction. [c]Isolated yield. [d]Starting material recovered. [e]T=80 ºC using microwave irradiation. [f]Complex mixture. NR=No reaction. [g]T=110 ºC using microwave irradiation. [h]Yield over two steps.

the anomeric position. Using **3.18b** as glycosyl donor with stannyl lipid and TfOH as a promoter in toluene under microwave irradiation, **3.19** was obtained in 35% yield (Table 3.5, entry 6).

In order to access the desired 2-deoxy-glycolipid, direct glycosylation reaction from *O*-acetyl-galactal **3.21** was studied using HBr as a promoter.[38]

Table 3.6. Synthesis of 2-deoxy-galactolipid **3.23**.[a]



Entry	Donor (equiv)	Acceptor (equiv)	Promoter	Solvent	*t* [h]	Product [%][b]
1	**3.21** (1.2)	**3.2** (1)	HBr (48% Aq)	CH_2Cl_2 /CH_3CN	3	[c]
2[d]	**3.21** (1.2)	**3.2** (1)	HBr (48% Aq)	CH_2Cl_2 /CH_3CN	12	[e]
3	**3.21** (1.2)	**3.2** (1)	HBr (33% AcOH)	CH_3CN	3	**3.22** (14)
4	**3.21** (1.2)	**3.2** (1)	HBr (33% AcOH)	CH_3CN	12	**3.22** (30)

[a]*General conditions*: Promoter was added to a mixture of donor, acceptor, and 4Å molecular sieves in the corresponding solvent at room temperature unless otherwise indicated. [b]Isolated yield. [c]3,4,6-tri-*O*-acetyl-2-deoxy-D-galactopyranose. [d]T= –78 ºC. [e]3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl bromide.

We found that, when **21** was treated with **2** and aqueous HBr (48% w/w), 3,4,6-tri-*O*-acetyl-2-deoxy-D-galactopyranose was obtained as result of the addition of water to double bond (Table 3.6, entry 1). When the reaction was carried out at –78 ºC (Table 3.6, entry 2) no glycosylation product was

observed and instead bromide intermediate was formed. When a solution of HBr in acetic acid was used the desired 2-deoxy-glycolipid **22** was formed in 14% yield after 3 h, achieving 30% yield when the reaction was left over night (Table 3.6, entries 3 and 4). As it was expected, complete α-selectivity was obtained, as it is known, glycals give preferentially α-2-deoxy-glycosides.

3.3.2. Multivalent galactose glycoclusters and hyperbranched glycopolymers

The synthesis of glycolipids with different spacers, glycolipids anchored to clusters and hyperbranched glycopolymers to obtain multivalent ligands was carried out by Dr. Cobo and it was presented in his thesis.[39]

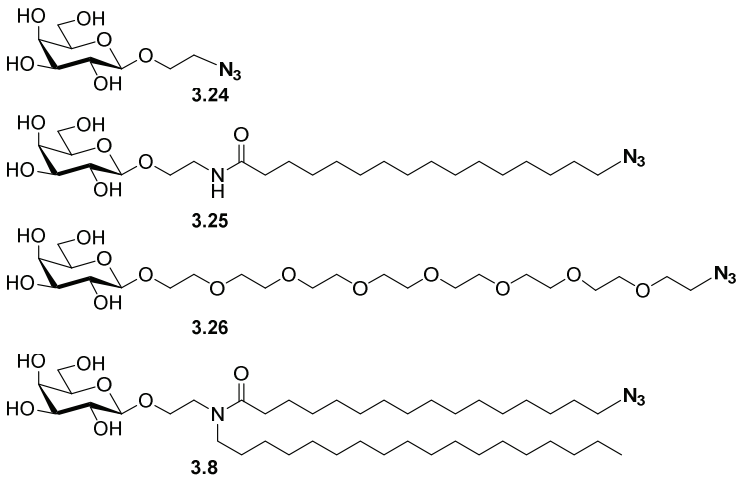


Figure 3.5. β-GalCer analogs with different spacers and terminal azide group.

Glycoclusters mimicking glycolipids with galactose as sugar and different spacers, which play the role of the lipidic moiety were selected for evaluation in order to study the difference in glycosidase inhibition. Glycolipids varying the aglycone moiety with a short chain (2-azidoethanol), a long hydrophobic chain, or two long hydrophobic chains and a polar (PEG)

chain bearing an azide group at the ω carbon (**3.8**, **3.24**–**3.26**) were synthesized (Figure 3.5).

Briefly, to obtain the glycoclusters, synthetic glycolipids with a terminal azide group were anchored using [2+3]-cycloaddition catalysed by copper (click chemistry) to both clusters and hyperbranched polymers. A family of propargylic cores for the synthesis of glycolipid-clusters were used as central scaffolds (Figure 3.6). Glycolipid **3.8** with a two aliphatic chain spacer was chosen as monomer to construct glycoclusters **3.30**–**3.32** (Scheme 3.4).

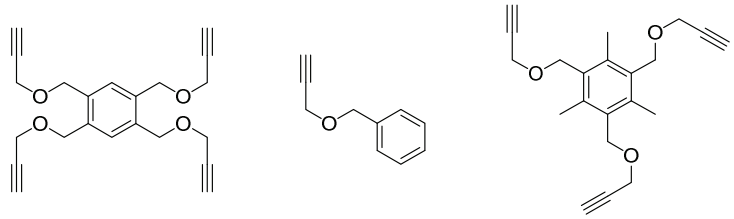
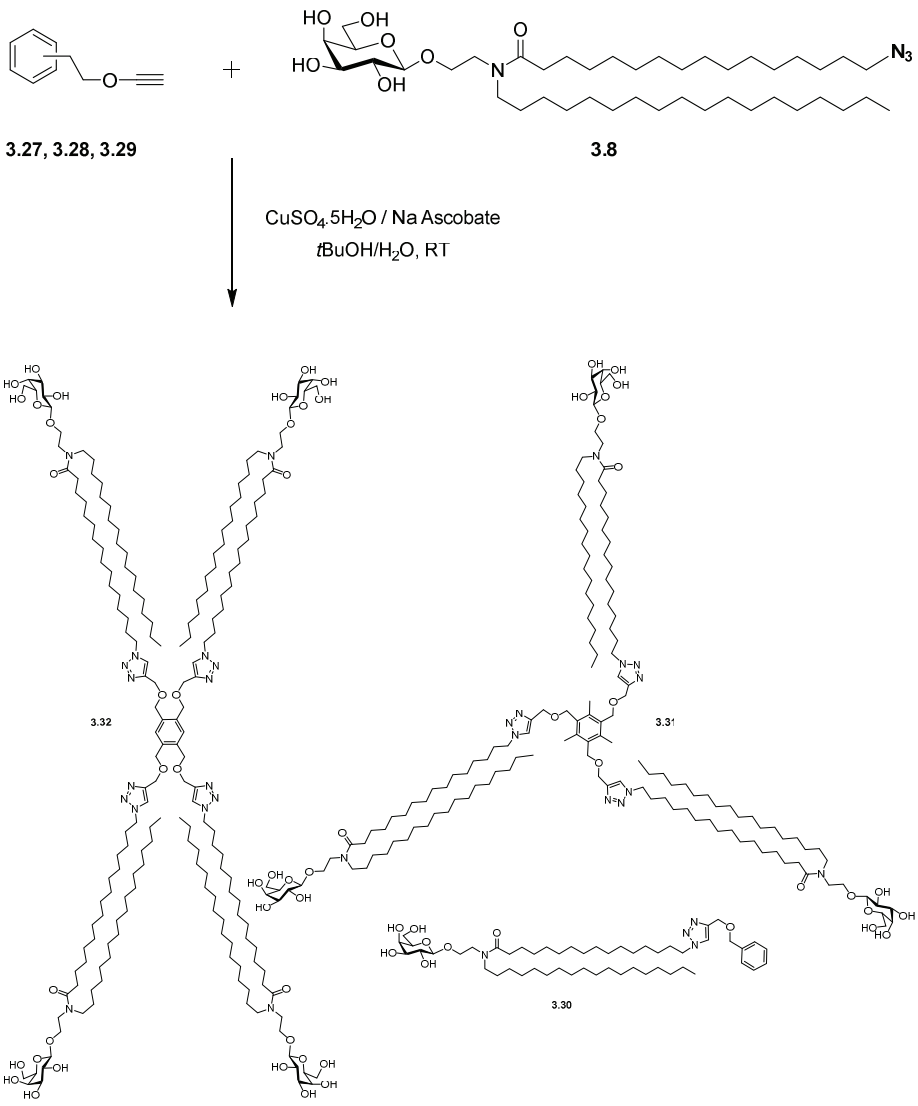


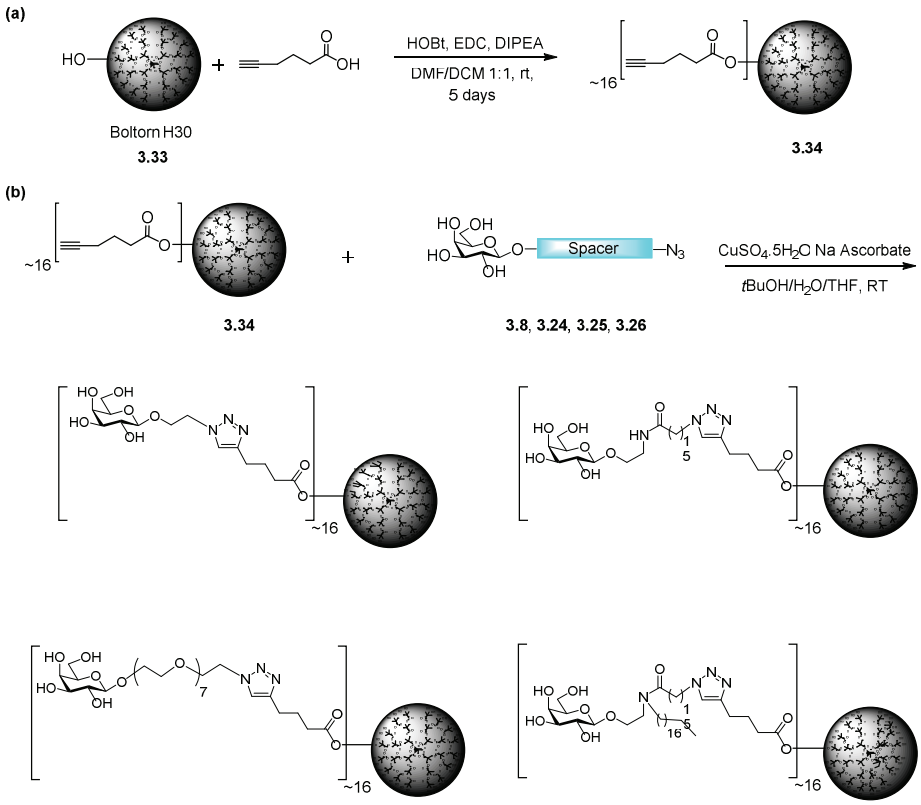
Figure 3.6. Propargylic-based central scaffolds to construct glycoclusters.



Scheme 3.4. Synthesis of glycolipids clusters employing CuAAC reaction.

The azide group of the glycolipids with different spacers, allowed to anchor these glycolipids analogues into the central scaffolds using [2+3]-cycloaddition catalysed by copper (I) (click chemistry), to construct the desired multivalent systems. Commercial Boltorn H30 (**3.29**) was functionalized to obtain the library of glyco-hyperbranched polymers. Alkynyl moieties were introduced into the polymer by partial esterification of

hydroxylic groups with 5-hexynoic acid (Scheme 3.5a). The average number of hydroxyl modified groups was determined using a procedure described by Fernández-Francos and co-workers.[40] Hyperbranched polymers were then prepared using the methodology previously developed in our group.[41] Thus modified Boltorn H30 was reacted with β-GalCer analogs **3.8**, **3.28**, **3.29** and **3.30** using click chemistry to obtain hyperbranched polymers **3.33**, **3.34**, **3.35** and **3.36** (Scheme 3.5b).



Scheme 3.5. Hyperbranched polymers as central scaffold for multivalent glycolipid presentation; (a) Boltorn H30 modification, (b) Synthesis of hyperbranched glycopolymers using CuAAC reaction.

Finally, glycoclusters and hyperbranched glycopolymers based on glycolipids were evaluated against comercial glycosidases, the results obtained are presented below.

3.3.3. Evaluation of glycolipids, glycoclusters and hyperbranched glycopolymers

Table 3.7. Hydrolysis resistance against β-galactosidase (*E.coli*)

Analyte	Galactose units	Concentration	DMSO/H_2O	Hydrolysis resistance
3.5	1	3 mM	10:90	Yes
3.8	1	3 mM	10:90	Yes
3.13	1	3 mM	10:90	Yes
3.17	1	3 mM	10:90	Yes
3.23	1	3 mM	10:90	Yes
3.20α	1	3 mM	10:90	Yes
3.20β	1	3 mM	10:90	Yes
3.30	1	3 mM	20:80	Yes
3.31	3	1 mM	30:70	Yes
3.32	4	750 μM	30:70	Yes
3.24	1	3 mM	20:80	**NO**
3.25	1	3 mM	10:90	**NO**
3.26	1	3 mM	20:80	**NO**
3.35	16	187.5 μM	20:80	Yes
3.36	16	187.5 μM	20:80	**NO**
3.37	16	187.5 μM	20:80	**NO**
3.38	17	176.5 μM	20:80	Yes

The different glycolipids were initially evaluated against hydrolysis using β-galactosidase from *E. coli* to test their ability to act as substrates. Glycolipids, glycoclusters and hyperbranched glycopolymers were incubated

with β-galactosidase in Phosphate buffer for 30 minutes at 37 ºC prior to analysis. GC-FID analysis revealed that for all substrates tested only **3.24**, **3.25**, **3.26**, **3.36** and **3.37** (Table 3.7) are substrates for β-galactosidase (Table 3.7).

Next, glycolipids, and multivalent systems were evaluated against commercial glycosidases including β-glucosidase (almonds), α-glucosidase (baker's yeast), β-galactosidase (bovine liver and *E.coli*) and α-galactosidase (green coffee). Results are presented in the following sections.

3.3.3.1. Evaluation of glycolipids against glycosidases

Glycosides **3.5**, **3.13** and **3.20β** act as inhibitors against α-glucosidase (baker's yeast) and β-galactosidase (bovine liver) (Table 3.8). β-Glycoside **3.5** provided K_i values significantly lower for β-selective enzyme than for α-enzymes. It was also found that for α-glucosidase inhibition, compounds with α-configuration (**3.13**, **3.23**, **3.20α**) give better inhibitions than those with β-configuration (**3.5**, **3.17**, **3.20β**). Moreover, the specific group at position C-2 plays and important role in binding for all of them. Thus, fluorine at position C-2 plays also an important role in potency in comparison to an OH group (OH>>F), 93 *vs*. 525μM K_i value, meaning that probably in the case of β-galactosidase, the pyranose ring is the part of the molecule that gave this inhibition response. Whereas in the case of α-glucosidase, as no significant evidence could be extracted for all compounds tested, the inhibition may involve a non-specific interaction between the aglycone moiety and the enzyme.

Surprisingly, inhibition against α-glycosidases is only detectable for compound **3.17**, probably due to the conformational changes and/or positive H-bonds by the presence of a NHAc group at position C-2.

Table 3.8. K_i Values (μM) against glycosidases for glycolipids monomers.

Enzymes	3.5	3.13	3.17	3.20α	3.20β	3.23
α-glucosidase (baker's yeast)	443	196	455	304	507	204
β-galactosidase (bovine liver)	93 (IC_{50} 188)	154 (IC_{50} 338)	n.i.	n.i.	525	n.i.
β-galactosidase (*E. coli*)	n.i.	n.i.	n.i.	n.i.	n.i	n.i.
α-galactosidase (green coffee)	n.i.	n.i.	592	n.i.	n.i.	n.i.

When β-glycolipid monomers with modifications in the aglycone part were evaluated it was found that **3.24**, **3.25** and **3.26** were substrates for β-galactosidase from *E.coli* (Table 3.7). Surprisingly, while glycolipid **3.25** was hydrolysed, a similar glycolipid without the azide group (**3.5**) was not, although both of them are β-galactosidase inhibitors. A similar behaviour was found in the literature using glycosyl transferases,[42] when an azido glycoside incubated with B16 cells it was processed faster than the alkyl glycoside, and also it was degraded less efficiently.

For β-galactosidase (bovine liver) an inhibition trend was found corresponding to a combination between accessibility and hydrophobicity. The best inhibition potency was achieved when using a long and hydrophobic spacer (Table 3.9). Thus, for compound **3.25** with a long aliphatic spacer K_i = 35 μM, while for compound **3.26** bearing a long polar spacer K_i value was 14.7-fold higher (less potent). Moreover, a 10.7-fold increase in K_i value was found for compound **3.8** having two aliphatic non-polar chains (Table 3.9). No evidence that the amide group was favouring the interaction between the enzyme and the glycolipids due to H-bond interactions was found.

The only azido glycolipid that showed inhibition against α-galactosidase was **3.26** that present a polar chain spacer.

Table 3.9. K_i Values (μM) against glycosidases for azido glycolipids.

Enzymes	3.8	3.24	3.25	3.26
α-glucosidase (baker's yeast)	n.i.	n.i.	n.i.	n.i.
β-galactosidase (bovine liver)	377	373	35	516
β-galactosidase (E. coli)	n.i.	n.i.	n.i.	n.i.
α-galactosidase (green coffee)	n.i.	n.i.	n.i.	485

3.3.3.2. Evaluation of multivalent compounds

Recently, it was found that multivalent iminosugar-based glycoclusters increase inhibitor activity against its monomeric compound.[23,43] In an attempt to increase the inhibition potency of our glycolipids multivalent model glycolipids were explored.

When glycoclusters were tested against commercial glycosidase (Table 3.10) no inhibition was found for glycoclusters **3.30** and **3.31**, which present 1 and 3 galactose residues, respectively. In contrast, for glycocluster **3.32** a K_i = 15 μM was obtained when tested against mammalian β-galactosidase. Moreover, this compound also presented inhibition against α-glucosidase (K_i = 263 μM).

The simple addition of an aromatic ring to a distal position in glycolipid **3.8** to form **3.30**, changed its inhibition potency (Table 3.9 and Table 3.10). While **3.8** gave inhibition against β-galactosidase (bovine liver) no inhibition was detected for **3.30**.

Table 3.10. K_i Values (μM) against glycosidases for glycoclusters.

Enzymes	3.30	3.3	3.32
α-glucosidase (baker's yeast)	n.i.	n.i.	263
β-galactosidase (bovine liver)	n.i.	n.i.	15 (IC_{50} 31)
β-galactosidase (E. coli)	n.i.	n.i.	n.i.
α-galactosidase (green coffee)	n.i.	n.i.	n.i.

In the inhibition study of hyperbranched polymers against glycosidases, a control experiment with Boltorn **3.33** was performed to discard any positive effect due to the central core. When glycopolymers **3.36**–**3.38** were evaluated against β-galactosidase (*E. coli*) it was found **3.36** and **3.37** were substrates but no inhibitors, whereas **3.35** and **3.38** were inhibitors but not substrates. This could be explained due to non-specific interactions between hyperbranched polymers and the enzyme. A better potency was obtained for compounds with short aliphatic spacers than the one with two hydrophobic chains (Table 3.11).

For the α-glucosidase from baker's yeast just compound **3.35** and **3.36** gave inhibition (Table 3.11). The same trend as for α-glucosidase was found for α-galactosidase (green coffee). Hyperbranched polymer **3.35** with short spacer gave better inhibition than hyperbranched polymers **3.36** and **3.38** with hydrophobic chains (Table 3.11). This could indicate that access to the catalytic site is key; glycopolymers with a short spacer may fit well whereas those with long aliphatic spacer may have detrimental hydrophobic interaction that would preclude the correct disposition within the narrow active site.

Table 3.11. K_i Values (μM) against glycosidases for hyperbranched glycopolymers.

Enzymes	3.33	3.35	3.36	3.37	3.38
α-glucosidase (baker's yeast)	n.i.	1.2	12	n.i.	n.i.
β-galactosidase (bovine liver)	n.i.	123	23 (IC_{50} 33)	n.i.	42
β-galactosidase (E. coli)	n.i.	67	n.i.	n.i.	19
α-galactosidase (green coffee)	n.i.	110	597	n.i.	605

These results reflected a variable multivalent effect. While inhibition of compound **3.8** against β-galactosidase from bovine liver has K_i = 377 μM, the glycocluster **3.32**, presenting 4 galactose units, or the hyperbranched polymer **3.38**, that present 16 galactose units, present inhibition constants of 25- and 9-fold enhanced respectively. This means that a small multivalent effect is obtained when the multivalent system was not too crowded. If the corresponding monomers are compared with the hyperbranched structures, higher inhibition was found for the multivalent systems in all cases exhibiting a 1.5-fold for long aliphatic chain, 3-fold for short chain and 9-fold for the two aliphatic chains. However, the relative sugar potency is lower for hyperbranched polymers, and higher for the glycocluster **3.32**.

Moreover, some of the compounds studied (all of them presenting galactose) were found to be inhibitors of α-glucosidase, due to the flexibility in accommodating galactose. Indeed, one of the best inhibition constant was found when compound **3.35** was tested against α-glucosidase.

3.4. CONCLUSIONS

In summary, glycosylation reactions to obtain a series of glycolipids with different substituents at C-2 position of pyranose ring were studied. To study the importance of multivalent carbohydrate presentation, two different platforms, clusters and hyperbranched polymers, were employed as central scaffolds. Glycolipids with variations at C-2, glycolipids with different aglycone moieties, glycoclusters and hyperbranched glycopolymers were tested as glycosidase inhibitors against commercial glycosidases. Thus, the following conclusions were extracted:

(i) The use of disarmed glycosyl donors, with a 2,6-diFBz group, enable a direct glycosylation of stannyl lipids and inactivated lipids providing access to β-glycolipids. Microwave irradiation was found to be crucial in the glycosylation reaction with 2-NHAc and 2-F glycosyl donors. 2-F-glycolipids were obtained when a good leaving group (-Br) was introduced at the anomeric position. Furthermore, in order to obtain glycosidase inhibitors based on glycolipids with modifications in the aglycone moiety, galactose was glycosylated with different chains that allow us to study the influence of length and polarity.

(ii) When glycolipids bearing different groups at C-2 were tested, compound **3.5** (β, 2-OH) presented the best K_i value against β-galactosidase (bovine liver) although its inhibition potency was enhanced when an azide group was introduced at the end of the aliphatic chain (**3.25**).

(iii) β-Galactosidase (bovine liver) inhibition was found for all glycolipids probably due to the aglycone part. Compound **3.25** with a long aliphatic spacer presented a positive combination between accessibility and hydrophobicity.

(iv) When glycoclusters **3.30**, **3.31** and **3.32** were tested against glycosidases, inhibition was found for compound **3.32** (clustering 4 glycolipids). In this sense, the best inhibition potency per sugar unit was obtained.

(v) When hyperbranched glycopolymers (**3.33**, **3.35**, **3.36**, **3.37** and **3.38**) were tested as inhibitors, the best K_i value (1.2 μM) was obtained when compound **3.33** presenting galactose with a short aliphatic spacer was tested towards α-glucosidase. This result suggested that **3.33** could be interacting with the CBMs and not with the catalytic site. The same trend was observed for compound **3.36** (K_i = 12 μM).

(vi) Moreover, compound **3.36** were found to inhibit β-galactosidase (bovine liver). As it was a substrate for β-galactosidase (E. coli) no inhibition was found against this enzyme.

In order to understand how ligands affect the catalytic activity of those enzymes, further studies are required. Saturation transfer different, STD-NMR, experiments and molecular dynamics (MD) simulations could be performed to understand the interactions of the hydrophobic tails with the enzyme. Moreover, dynamic light scattering (DLS) is also needed to discard if the inhibition is due to ligand aggregates.

3.5. EXPERIMENTAL PART

General Remarks

Proton (^{1}H NMR), carbon (^{13}C NMR), fluor (^{19}F NMR), and phosphorus (^{31}P NMR) nuclear magnetic resonance spectra were recorded on a Varian Mercury spectrometer (400 MHz for ^{1}H), (100.6 MHz for ^{13}C), (376.5 MHz for ^{19}F), and (162 MHz for ^{31}P). Spectra were fully assigned using COSY, HSQC, HMBC and NOESY. All chemical shifts are quoted on the δ scale in

ppm using either Me_4Si (1H NMR: $CDCl_3$ = 0.00) or the residual solvent as internal standard (^{1}H NMR: $CDCl_3$ = 7.26 and 13C NMR: $CDCl_3$ = 77.00) and $CFCl_3$ and 85% H_3PO_4 (^{19}F NMR: $CDCl_3$ = 0.00 and ^{31}P NMR: $CDCl_3$ = 0.00) as external standards, respectively. Coupling constants (*J*) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet and app = apparent. Infrared (IR) spectra were recorded on a Jasco FT/IR-600 Plus ATR Specac Golden Gate spectrophotometer. Absorption maxima (ν_{max}) are reported in wavenumbers (cm^{-1}). Elemental analyses (C, H, N, and S) were performed with a Carlo Erba EA 1108 Analyzer. Optical rotations were measured on a Perkin–Elmer 241 polarimeter with a path length of 1.0 dm and are reported with implied units of 10^{-1} $degcm^2g^{-1}$. Concentrations (c) are given in g/100 mL. High resolution mass spectra (HRMS) were recorded on a Agilent 1100 Series LC/MSD mass spectrometer with electrospray ionization (ESI) by the Servei de Recursos Científics (URV). Nominal and exact m/z values are reported in Daltons. Thin layer chromatography (TLC) was carried out using commercial aluminium backed sheets coated with 60F254 silica gel. Visualization of the silica plates was achieved using a UV lamp (λ_{max} = 254 nm) and/or 6% H_2SO_4 in EtOH and/or 2% $PdCl_2$ and 15% H_2SO_4 in water. Flash column chromatography was carried out using silica gel 60 A CC (230–400 mesh). Radial chromatography was performed on 1, 2, or 4 mm plates of Kieselgel 60 PF254 silica gel, depending on the amount of product. Mobile phases are reported in relative composition (*e.g.* 1:1 EtOAc/*n*-hexane v/v). HPLC grade dichloromethane, tetrahydrofuran (THF) and dimethylformamide (DMF) were dried using a solvent purification system (Pure SOLV system-400). All other solvents were used as supplied (Analytical or HPLC grade), without prior purification. Distilled water was used for chemical reactions and Milli–QR purified water for protein manipulations. All reagents were used as received from commercial suppliers. All reactions using anhydrous conditions were performed using flame-dried apparatus under an atmosphere

of argon. Brine refers to a saturated solution of sodium chloride. Anhydrous magnesium sulphate ($MgSO_4$) was used as drying agent after reaction work-up, as indicated.

General glycosylation procedure for 1-*O*-acetylglycosides/glycosyl halides with stannyl lipids

The following protocol was followed prior to the glycosylation reaction. The glycosyl aceptor and the glycosyl donor were azeotroped out with dry toluene (3 x 5 mL) each one in independently dried flasks and placed under vacum for 1 h.

To a stirred mixture of amidoalcohol (0.11 mmol), and 4Å MS (36 mg) in dry CH_2Cl_2 (440 μL) under argon atmosphere at room temperature allyltributyltin (0.14 mmol) and TfOH (0.03 mmol) were added. After stirring for 2 h, glycosyl donor (0.14 mmol) and more TfOH (0.30 mmol) were added to the mixture. The crude was then washed with sat. aq. $NaHCO_3$. The aqueous layer was extracted with EtOAc and the combined organic layers were washed with brine. The organic extract was dried with $MgSO_4$ and concentrated *in vacuo* to yield a crude oil which was purified by column chromatography or submitted to subsequente hydrolysis.

General glycosylation procedure for 1-*O*-acetylglycosides/glycosyl halides with lipids

The following protocol was followed prior to the glycosylation reaction. The glycosyl aceptor and the glycosyl donor were azeotroped out with dry toluene (3 x 5 mL) each one in independently dried flasks and placed under vacum for 1 h.

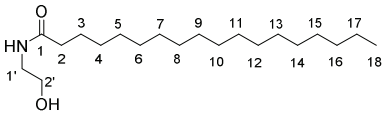
To a mixture of lipid (0.11 mmol), glycosyl donor (0.14 mmol) and 4Å MS in dry solvente, promoter was added under argon atmosphere at room temperature. The crude was then washed with sat. aq. $NaHCO_3$. The aqueous

layer was extracted with EtOAc and the combined organic layers were washed with brine. The organic extract was dried with $MgSO_4$ and concentrated *in vacuo* to yield a crude oil which was purified by column chromatography or submitted to subsequente hydrolysis.

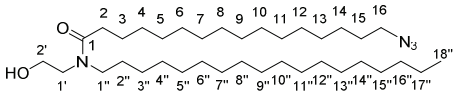
General deacetylation procedure

The hydrolysis of the glycosylation product was carried out by addition of a 7% solution of NaOMe in MeOH/CH_2Cl_2 (3:1 or 1:0, 5 mL/mmol) at room temperature. The reaction was quenched by adding DOWEX-50WX2 until pH 7 was reached. The solvent was removed under *vacuo*, and the resulting reside was purified by column chromatography.

Synthesis of *N*-(2-hydroxyethyl)stearamide (3.2): A solution of stearoyl



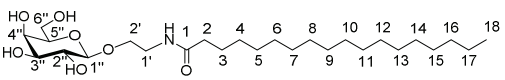
chloride (50 g, 0.165 mol) in 250 mL of dry CH_2Cl_2 was cooled to 0°C. To this solution 2-amino ethanol (100.78 g, 1.65 mol) was added in a slow drop-wise manner over a period of 30 min, resulting in the precipitation of a white solid. The reaction was stirred for 4 h at room temperature and then the mixture was filtered. The solid was washed with *n*-hexane and diethyl ether and dried *in vacuo*. The resulting crystalline material was recrystallized from CH_2Cl_2 to give **3.2** (51.26 g, 95%) as a white solid. R_f (*n*-hexane/EtOAc/MeOH 60:30:10): 0.25; m.p.: 53-55 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ in ppm 5.94 (br s, 1H, OH), 3.72 (t, $J_{2',1'}$ = 5.2 Hz, 2H, H-2'), 3.42 (td, $J_{1',2'}$ = 5.2, 1.0 Hz, 2H, H-1'), 2.18 (t, $J_{2,3}$ = 7.2 Hz, 2H, H-2), 1.62 (quint, $J_{3,2}$ = 7.2 Hz, $J_{3,4}$ = 6.5 Hz, 2H, H-3), 1.28 (m, 28H, CH_2), 0.87 (t, $J_{18,17}$ = 6.8 Hz, 3H, H-18); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 172.6 (C=O, C-1), 61.0 (C-2'), 41.6 (C-1'), 36.5 (C-2), 31.8 (CH_2), 29.5 (CH_2), 28.6 (CH_2), 25.6 (CH_2), 22.7 (CH_2), 14.1(C-18); FT-IR (neat) ν in cm^{-1}: 3370, 3291, 3086, 2954, 2916, 2847, 1636, 1551, 1470, 1383, 1275, 1055, 720; Anal. Calcd. for $C_{20}H_{41}NO_2$: C, 73.34; H, 12.62; N, 4.28; found: C, 73.30; H, 12.69; N, 4.20.

Synthesis of *N*-(2-hydroxyethyl)-*N*-octadecylstearamide: A 250 mL,  three-necked, roundbottomed flask with a mechanical stirrer was fitted with a reflux condenser with drying tube, and a stoppered pressure-equalizing dropping funnel. The system was flushed with nitrogen or argon, and the flask was charged with 75 mL of dry THF and $LiAlH_4$ (1.45 g, 38.22 mmol). A mixture of 75 mL of THF and *N*-(2-hydroxyethyl)stearamide **3.2** (10 g, 30.58 mmol) was added, with stirring, at a rate sufficient to reach and maintain refluxing. After the addition was completed, the reaction mixture was kept boiling for 18 h. The flask was immersed in an ice bath, and 30 mL of water, 15 mL of 10% aqueous potassium hydroxide, and again 30 mL of water were added cautiously with very vigorous stirring. The reaction mixture was stirred for an additional 1h, filtered with suction, and the solid was washed with several 100-mL portions of ethyl acetate. The two layers were separated, and the aqueous phase was extracted with ethyl acetate (3 x 100 mL). The combined organic layers were dried over anhydrous sodium sulphate and concentrated *in vacuo* to get crude residue of 2-(ocatadecylamino) ethanol (6.7 g) as a white solid.

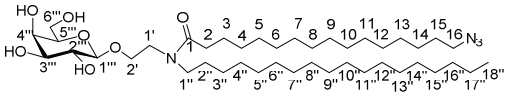
A solution of 2-(octadecylamino) ethanol (1 g, 3.194 mmol), HOBt (586 mg, 3.832 mmol), EDC (734 mg, 3.832 mmol) and DIPEA (494 mg, 3.832 mmol) in 30 mL of dry CH_2Cl_2 was cooled to 0 °C. Stearic acid (908 mg, 3.194 mmol) in 20 mL of dry CH_2Cl_2 was added drop wise over 6 h at 0 °C and then the reaction was stirred under argon for 18 h at room temperature. The mixture was diluted with ethyl acetate (75 mL) and washed successively with HCl (10 % aqueous, 2 x 30 mL), $NaHCO_3$ (7 % aqueous, 2 x 30 mL), K_2CO_3 (7% aqueous, 2 x 30 mL) and brine (3 x 30 mL). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using *n*-hexane/EtOAc/MeOH (85:10:5) as the eluent to give 1.67 g of pure *N*-(2-hydroxyethyl)-*N*-octadecylstearamide (80 % over two steps) as a waxy solid. R_f (*n*-

hexane/EtOAc/MeOH 60:30:10): 0.70; m.p. 50-52 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ in ppm 3.75 (t, $J_{2',1'}$ = 5.2 Hz, 2H, H-2'), 3.52 (t, $J_{1',2'}$ = 5.2 Hz, 2H, H-1'), 3.26 (t, $J_{1'',2''}$ = 7.6 Hz, 2H, H-1''), 2.32 (t, $J_{16,15}$ = 7.6 Hz, 2H, H-16), 1.62-1.54 (m, 4H, CH_2), 1.25 (m, 58H, CH_2), 0.87 (t, 6H, $J_{18'',17''}$ = 6.5 Hz, H-18''); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 173.0 (C=O, C-1), 58.9 (C-2'), 50.2 (CH_2N_3), 50.2 (C-1''), 47.3 (C-1'), 34.3 (C-2), 31.8 (CH_2), 30.4 (CH_2), 29.6 (CH_2), 29.3 (CH_2), 28.9 (CH_2), 28.6 (CH_2), 27.7 (CH_2), 22.7 (CH_2), 14.1 (C-18''); FT-IR (neat) ν in cm^{-1}: 3397, 2915, 2847, 2090, 1609, 1469, 1424, 1363, 1312, 1211, 1075, 718; Anal. Calcd. for $C_{38}H_{77}NO_2$: C, 78.69; H, 13.38; N, 2.41. Found: C, 78.73; H, 12.98, N, 2.45.

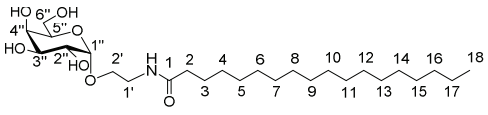
1-(β-D-Galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol (3.5): Starting



from *N*-(2-hydroxyethyl)stearamide **3.2** (36 mg, 0.110 mmol) and using a mixture of AgOTf (3 equiv.) and $Sc(OTf)_2$ (3 equiv.) glycolipid **3.5** was obtained as a syrup according to glycosylation procedure from *halo*-glycosides and following deacetylation procedure (46 mg, 90%). R_f (CH_2Cl_2/MeOH 9:1): 0.16; $[\alpha]_D^{25}$: +6.24º (0.125, CH_2Cl_2/MeOH 2:1); ^{1}H NMR ($CDCl_3$/CD_3OD, 1:2, 400 MHz) δ in ppm: 4.21 (d, $J_{1'',2''}$ = 7.6 Hz, 1H, H1''), 3.96 (m, 1H, H-2a'), 3.84 (d, $J_{4'',3''}$ = 2.0 Hz, 1H, H-4''), 3.76-3.73 (m, 2H, H6a'', H6b''), 3.61 (m, 1H, H-2b'), 3.56-3.48 (m, 4H, H-2'', H-3'', H-5'', H-1a'), 3.35 (m, 1H, H-1b'), 2.17 (t, $J_{2,3}$ = 6.8 Hz, 2H, H-2), 1.57 (t, $J_{3,2}$ = 6.8 Hz, 2H, H-3), 1.40-1.25 (m, 28H, CH_2), 0.86 (t, $J_{18,17}$ = 6.8 Hz, 3H, H-18); ^{13}C NMR ($CDCl_3$/CD_3OD, 1:2, 100.6 MHz) δ in ppm: 176.1 (C=O, C-1), 104.5 (C-1''), 76.1 (C-5''), 74.3 (C-3''), 72.2 (C-2''), 69.9 (C-4''), 69.5 (C-2'), 62.5 (C-6''), 40.3 (C-1'), 37.0 (C-2), 32.8-26.8 (14 CH_2), 23.5 (C-17), 14.5 (C-18); FT-IR (neat) ν in cm^{-1}: 3486, 2918, 2848, 1603, 1466, 1259, 1232, 1171, 1034, 630; HRMS (TOF ES$^+$) for $(M + Na)^+$ $C_{26}H_{51}NNaO_7^+$ (*m*/*z*): calc. 512.3557; found 512.3551.

1-(β-D-Galactopyranosyl)-*N*-(16-azidohexadecanoyl)-*N*-octadecanyl-2-amino-ethanol (3.8): 
Sarting from *N*-(2-hydroxyethyl-)-*N*-actadecylstearamide (65 mg, 0.11 mmol) the glycoside **3.8** was obtained as a syrup according to the general procedure (33 mg, 0.043 mmol, 40% according to the glycosylation procedure from *O*-acetylglycosides or 52 mg, 0.069 mmol, 63% according to the glycosylation procdeure from *halo*-glycosides). R_f (9:1 EtOAc/MeOH): 0.22; $[\alpha]_D^{20}$: –5.8 (0.65, Pyridine); ^{1}H NMR (1:2 $CDCl_3$/CD_3OD, 400 MHz) δ in ppm: 4.20 (t, $J_{1''',2'''}$ =7.6 Hz, 1H, H-1'''), 3.96 (m, 1H, H-2a'), 3.84 (dd, $J_{4''',3'''}$ = 2.0 Hz, $J_{4''',5'''}$ = 1.8 Hz, 1H, H-4'''), 3.78-3.61 (m, 4H, H-6a''', H-6b''', H-2b', H-1a'), 3.56-3.33 (m, 6H, H-2''', H-5''', H-3''', H-1b', H-1''), 3.25 (t, $J_{16,15}$ = 6.8 Hz, 2H, H-16), 2.42 (t, $J_{2,3}$ = 8.0 Hz, 2H, H-2), 2.34 (dt, $J_{2,3}$ = 8.0 Hz, 2H, H-2)[1], 1.61-1.50 (m, 8H, H-3, CH_2, H-2''), 1.40-1.25 (m, 46H, 23 CH_2), 0.86 (t, $J_{18'',17''}$ = 6.8 Hz, 3H, H-18''); ^{13}C NMR (1:2 $CDCl_3$/CD_3OD, 100.3 MHz) δ in ppm: 175.7 (C=O, C-1), 105.2 (C-1'''), 76.7 (C-2'''); 75.0 (C-4'''), 72.4 (C-5'''), 70.2 (C-3'''), 68.5 (C-2'''), 62.5 (C-6'''), 52.4 (C-16), 50.2 (C-1''), 47.0 (C-1'), 34.2 (C-2''), 33.9 (C-2), 33.1-30.0, 28.5, 28.0, 27.9, 27.8, 26.8, 26.2, 23.8 (CH_2), 14.5 (C-18); FT-IR (neat) ν in cm^{-1}: 3330, 2917, 2848, 2812, 2776, 2682, 2099, 1614, 1466, 1350, 766; HRMS (TOF ES$^+$) for $(M+Na)^+$ $C_{42}H_{82}N_4NaO_7^+$ (*m/z*): calc: 777.6081; found: 777.6901.

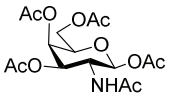
1-(α-D-Galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol (3.13): [31] To a solution of 1,2,3,4,6-penta-*O*-trimethylsilyl-D-galactopyranose **3.11** (151.6 mg, 0.28 mmol) in CH_2Cl_2 (2 mL), TMSI (51 μL, 0.375 mmol) was added at 0 ºC, and the



[1] Signal associated to H-2 is doubled at room temperature due to the tertiary amide.imnio alcoholate equilibrium. The signal coalesces once temperature is increased over 50 ºC.

reaction mixture was stirred under argon pressure for 20 min. The reaction was stopped adding anhydrous toluene. The solvent was removed and the crude of the reaction was diluted in toluene (1.2 mL).Starting from **3.2** (92 mg, 0.28 mmol) the general procedure for glycosylation for stannyl lipids was followed using toluene as solvent. After 18 h at 80 ºC, the solvent was evaporated. MeOH (2.8 mL) and Dowex-H^+ (1.9 g) were added to the crude mixture and stirred at room temperature for 4 h. The ion-exchange resin was removed by filtration and the crude was purified by flash column chromatography (EtOAc/MeOH/H_2O 10:2:1) to afford **13** (78%) as a white solid. R_f (CH_2Cl_2/MeOH/H_2O 7:2:1): 0.35; $[\alpha]_D^{25}$: +47.7 (0.13, CH_2Cl_2/MeOH 2:1); ^{1}H NMR ($CDCl_3$/CD3OD, 2:1, 400 MHz) δ in ppm: 4.82 (d, $J_{1'',2''}$ = 3.7 Hz, 1H, H-1''), 3.86 (m, 1H, H-2a'), 3.74 (m, 6H, H-4'', H-6'', H-2'', H-3'', H-2b'), 3.56 (m, 1H, H-5''), 3.44 (m, 1H, H-1a'), 3.19 (m, 1H, H-1b'), 2.14 (t, $J_{2,3}$ = 7.6 Hz, 2H, H-2), 1.55 (t, $J_{3,2}$ = 7.6 Hz, 2H, H-3), 1.40-1.25 (m, 28H, CH_2), 0.86 (t, $J_{18,17}$ = 6.8 Hz, 3H, H-18); FT-IR (neat) ν in cm^{-1}: 3294, 2914, 2848, 1646, 1558, 1471, 1058, 1038, 620; HRMS (TOF ES^+) for $(M + Na)^+$ $C_{26}H_{51}NNaO_7^+$ (*m*/*z*): calc. 512.3557; found 512.3547.

1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-*N*-acetamido-D-β-galactopyranose



(3.14):[44] To D-galactosamine hydrochloride salt (1.0 g, 4.64 mmol), pyridine (10 mL) was added and the suspension was cooled to 0 ºC, and acetic anhydride (12 mL) was added dropwise. After 48 hours stirring at room temperature, the reaction solution was cooled to 0 ºC and poured into distilled water (140 mL). After stirring vigorously at 4 ºC, thus obtained white precipitate was separated by filtration. The obtained white solid was dried under reduced pressure to obtain compound **3.14** (1.5 g, 86%). R_f (EtOAc): 0.35; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.70 (d, $J_{1,2}$ = 9.0 Hz, 1H, H-1), 5.38 (d, $J_{4,5}$ = 1.0 Hz, $J_{4,3}$ = 3.3 Hz, 1H, H-4), 5.35 (d, $J_{NH,2}$ = 9.5 Hz, 1H, NH), 5.08 (dd, $J_{3,4}$ = 3.2 Hz, $J_{3,2}$ = 11.1 Hz, 1H, H-3), 4.45 (dt, $J_{2,1}$ = 9.0 Hz, $J_{2,3}$ = 11.1 Hz, 1H, H-2), 4.14 (m, 2H, H-6), 4.02 (dt, $J_{5,4}$ = 1.0

Hz, $J_{5,6a} = J_{5,6b} = 6.6$ Hz, 1H, H-5), 2.17, 2.13, 2.05, 2.02, 1.94 (s, 15H, $5CH_3$, Ac). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.9, 170.6, 170.4, 170.3, 169.8 (5C, C=O); 93.2 (C-1), 72.0 (C-5), 70.5 (C-3), 66.5 (C-4), 61.4 (C-6), 49.9 (C-2), 23.5, 21.1, 20.9, 20.88 ($5CH_3$, Ac). HRMS (TOF ES^+) for $(M + Na)^+$ $C_{16}H_{23}NNaO_{10}^+$ (*m*/*z*): calc. 412.1214, found 412.1286; spectroscopic data was identical to that previously reported.[45]

1,2-Dihydrooxazole-3,4,6-tri-*O*-acetyl-α-D-galactopyranoside (3.15): 2-deoxy-2-*N*-acetylamino-D-galactose (1.62 g, 7.33 mmol) was diluted in pyridine (25 mL) and then acetic anhydride was added (6 mL). The reaction was stirred for 30 minutes under argon pressure. Once the reaction was completed, the flask was cooled by an ice/water bath and some water drops were added. The mixture was washed with 10% HCl solution, sat. aq. $NaHCO_3$ and brine and extracted with CH_2Cl_2. The organic layers were dried over $MgSO_4$ and concentrated. The crude was then diluted in dichlorometane (12.3 mL) and TMSOTf (0.3 mL, 1.66 mmol) was added under argon pressure. After 16 h runder reflux the solvent was removed under reduced pressure to afford the desired compound **3.15** (2.0 g, 83%) used in the next step without further purification. R_f (EtOAc/CH_2Cl_2 2:1): 0.35; ^1H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.89 (d, *J* = 7.4 Hz, 1H), 5.17 (t, *J* = 3.1 Hz, 1H), 4.84 (m, 1H), 4.14-3.98 (m, 3H), 3.52 (m, 1H), 2.03, 2.01, 2.00 (s, 12H, $4CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.3, 169.3, 168.9, 166.4, 99.1, 70.0, 68.0, 67.2, 64.6, 63.0, 20.7, 20.6, 20.5, 13.7; spectroscopic data was identical to that previously reported.[46]

1-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-acetamido-β-D-galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol (3.16): Sarting from 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-*N*-acetylamino-β-D-galactopyranose **3.14** (154 mg, 0.369 mmol), *N*-(2-hydroxyethyl)stearamide **3.2** (108 mg, 0.330 mmol),

TfOH (26 µl, 0.118 mmol), under microwave irradiation (150 W, 80 ºC) and after purification (EtOAc/*n*-hexane 10:2) **3.16** (110 mg, 42%) was afforded following the general glycosylation procedure. R_f (EtOAc): 0.29; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 6.20 (t, $J_{NHAc,2''}$ = 5.4 Hz, 1H, NHAc), 6.04 (d, $J_{NH,1''}$ = 8.8 Hz, 1H, NH), 5.33 (d, $J_{4'',3''}$ = 3.4 Hz, 1H, H-4''), 5.10 (dd, $J_{3'',2''}$ = 11.6 Hz, $J_{3'',4''}$ = 3.4 Hz, 1H, H-3''), 4.58 (d, $J_{1'',2''}$ = 8.6 Hz, 1H, H-1''), 4.10 (m, 3H, H-2'', H-6''), 3.90 (td, $J_{5'',4''}$ = 1.0 Hz, $J_{5'',6''}$ = 6.4 Hz, 1H, H-5''), 3.85 (m, 1H, H-2a'), 3.66 (m, 1H, H-2b'), 3.55 (m, 1H, H-1a'), 3.31 (m, 1H, H-1b'), 2.16 (m, 2H, H-2a, H-2b), 2.14 (s, 3H, CH_3, NHAc), 2.03, 1.99, 1.95 (s, 9H, 3CH_3, Ac), 1.60 (t, $J_{3,2}$ = 6.4 Hz, 2H, H-3), 1.23 (m, 28H, CH_2), 0.86 (t, $J_{18,17}$ = 6.4 Hz, 3H, H-18). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 173.7 (C=O, C-1), 170.9, 170.8, 170.6, 170.4 (4C=O, Ac, NHAc), 101.6 (C-1''), 70.9 (C-5''), 70.4 (C-3''), 68.6 (C-2'), 66.7 (C-4''), 61.7 (C-6''), 51.1 (C-2''), 39.0 (C-1'), 36.8 (C-2), 32.0-29.5 (CH_2), 25.9 (C-3), 23.6, 22.8, 20.8 (4CH_3, Ac, NHAc), 14.2 (C-18); FT-IR (neat) ν in cm^{-1}: 3306, 2916, 2849, 1744, 1661, 1646, 1548, 1370, 1224, 1076, 1047. HRMS (TOF ES$^+$) for $(M + Na)^+$ $C_{34}H_{60}N_2NaO_{10}^+$ (*m*/*z*): calc. 679.4140; found 679.4139.

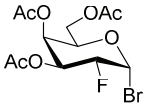
1-(2-Deoxy-2-acetamido-β-D-galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol (3.17): Sarting from **3.16** (17 mg, 0.025 mmol) and following the general deacetylation procedure, **3.17** was obtained (13.5 mg, 98 %). R_f (CH_2Cl_2/MeOH 9:1): 0.16; $[\alpha]_D^{20}$: –1.21 (0.14, CH_2Cl_2/MeOH 2:1); ^{1}H NMR ($CDCl_3$/CD_3OD 2:1, 400 MHz) δ in ppm: 4.31 (d, $J_{1'',2''}$ = 8. 3 Hz, 1H, H-1''), 3.85 (m, 2H, H-2'', H-4''), 3.75 (m, 3H, H-6'', H-2a'), 3.62 (m, 1H, H-2b'), 3.53 (dd, $J_{3'',2''}$ = 10.6 Hz, $J_{3'',4''}$ = 3.2 Hz, 1H, H3''), 3.45 (appt, $J_{1'',2''}$ = 5.98 Hz, 1H, H-5''), 3.34 (m, 2H, H-1'), 2.15 (t, $J_{2,3}$ = 7.3 Hz, 2H, H-2), 1.98 (s, 3H, CH_3, NHAc), 1.56 (t, $J_{3,2}$ = 7.3 Hz, 2H, H-3), 1.27-1.20 (m, 28H, CH_2), 0.83 (t, $J_{18,17}$ = 6.8 Hz, 3H, H-18); ^{1}H NMR ($CDCl_3$/CD_3OD 2:1, 100.6 MHz) δ in ppm: 175.4, 173.8 (2C=O, C-1, NHAc), 101.8 (C-1''), 75.2 (C-5''), 72.5 (C-3''), 68.7 (C-2'), 68.5 (C-4''), 61.7 (C-6''), 53.4 (C-2''),

39.8 (C-1'), 36.6 (C-2), 32.2 (C-3), 30.0-23.0 (CH_2), 22.9 (CH_3, NHAc), 14.2 (C-18); FT-IR (neat) ν in cm^{-1}: 3308, 2916, 2849, 2359, 1631, 1558, 1418, 1257, 1034, 630; HRMS (TOF ES^+) for $(M + Na)^+$ $C_{26}H_{54}N_2NaO_7^+$ (m/z): calc. 553.3829; found 553.3849.

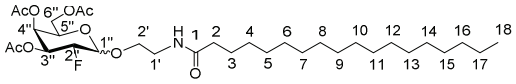
1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-fluoro-D-galactose (3.18a):[37] 3,4,6-Tri-*O*-acetyl-D-galactal (3.9 g, 14.384 mmol) was dissolved in CH_3NO_2/H_2O (5:1) at room temperature. Selectfluor® (6.4g, 17.26 mmol) was added and the mixture was stirred for 4 hours. After the completion of the reaction, the mixture was refluxed at 120 ºC during an hour. After five hours the reaction was completed and the mixture was washed with 10% HCl, saturated aqueous $NaHCO_3$ and brine. The organic layer was then dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. To acetylate the anomeric position acetic anhydride (2 ml) was added and the mixture was dissolved in dry pyridine (14 ml). After one hour the reaction was complete and water was added to stop it, with the flask previously cooled in an ice/water bath. The reaction was treated with the previous work up. The product was then purified by column chromatography using 1:1 EtOAc/*n*-hexane as the eluent to afford the product **3.18a** (2.21 g, 67%) in a 1:1 α/β ratio. R_f (*n*-hexane/EtOAc 3:1): 0.32; Alpha anomer; ^1H NMR ($CDCl_3$, 400 MHz) δ in ppm: 6.32 (d, $J_{1,F}$ = 4.06 Hz, 1H, H-1), 5.38 (td, $J_{4,F}$ = 3.6 Hz, $J_{4,5}$ = 1.6 Hz, 1H, H-4), 5.26 (td, $J_{3,2}$ = 10.4 Hz, $J_{3,4}$ = 3.72 Hz, 1H, H3), 4.85-4.69 (ddd, $J_{2,F}$ = 49.0 Hz, $J_{3,2}$ = 10.4 Hz, $J_{2,1}$ = 3.7 Hz, 1H, H-2), 4,21 (td, $J_{5,6}$ = 6.61 Hz, $J_{4,5}$ = 1.6 Hz, 1H, H-5), 4-3.90 (m, H-6 α/β), 1.96 (m, 18H, $6CH_3$, Ac α/β); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 88.7 (d, $J_{1,F}$ = 25.0 Hz, C-1), 84.0 (d, $J_{2,F}$ = 190.0 Hz, C-2), 68.3 (C-5), 68.0 (d, $J_{3,F}$ = 19.5 Hz, C-3), 67.7 (d, $J_{4,F}$ = 8.6 Hz, C-4), 60.8 (C-6), 20.6-20.2 ($6CH_3$, Ac α/β). ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: -209.17 (ddd, $J_{2,F}$ = 50.0 Hz, $J_{1,F}$ = 11.3 Hz, $J_{3,F}$ = 3.5 Hz). Beta anomer: ^1H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.71 (dd, $J_{1,2}$ = 7.8 Hz, $J_{1,F}$ = 4.2 Hz, 1H, H-1), 5.32 (t, $J_{4,5}$ = 3.0 Hz, 1H, H-4), 5.11 (td, $J_{3,2}$ = 9.1 Hz $J_{3,4}$ =3.5 Hz, 1H, H-3), 4.59-4.40 (ddd, $J_{2,F}$ = 51.6

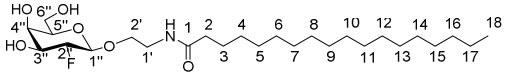
Hz, $J_{3,2}$ = 9.1 Hz, $J_{1,2}$= 7.8 Hz, 1H, H-2), 4.04-3.90 (3H, H-5, H-6 α/β), 1.96 (m, 18H, $6CH_3$, Ac α/β); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 91.2 (d, $J_{1,F}$ = 25.0 Hz, C-1), 86.7 (d, $J_{2,F}$ = 190.0 Hz, C-2), 71.4 (C-5), 67.4 (d, $J_{3,F}$ = 19.0 Hz, C-3), 67.4 (d, $J_{4,F}$ = 8.6 Hz, C-4), 60.7 (C-6), 20.6-20.2 ($6CH_3$, Ac α/β); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: -208.29 ppm (ddd, $J_{2,F}$ = 50.0 Hz, $J_{1,F}$ = 13.5 Hz, $J_{3,F}$ = 2.6 Hz); spectroscopic data was identical to that previously reported.[47]

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl bromide



(3.18b): 33% HBr in AcOH (2.1 mL) was added to a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-galactopyranose (1:1 α/β) **15** (105 mg, 0.304 mmol) in dry CH_2Cl_2 (2.0 mL) at 0 ºC. The reaction mixture was allowed to stir at room temperature for 8 h. The crude was then diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford **3.18b** (82 mg, 72%) as a yellowish oil. Used in the next step without further purification. R_f (EtOAc/*n*-hexane 1:1): 0.56; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 6.60 (d, $J_{1,2}$ = 4.2 Hz, 1H, H-1), 5.51 (td, $J_{4,3}$ = 3.4 Hz, $J_{4,5}$ = 1.2 Hz, 1H, H-4), 5.44 (td, $J_{3,2}$ = 10.1 Hz, $J_{4,3}$ = $J_{3,F}$ = 3.4 Hz, 1H, H-3), 4.74 (ddd, $J_{2,F}$ = 50.5 Hz, $J_{3,2}$ = 10.1 Hz, $J_{1,2}$ = 4.2 Hz, 1H, H-2), 4.49 (t, $J_{5,6}$ = 6.5 Hz, 1H, H-5), 4.12 (m, 2H, H-6), 2.12, 2.04, 2.03 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.4, 169.8 (3C=O, Ac), 86.9 $J_{2,F}$ = 25.6 Hz, C-1), 84.3 (*d,* $J_{1,F}$ = 194.6 Hz, 1H, C-2), 71.3 (C-5), 69.0 (d, $J_{3,F}$ = 18.0 Hz, C-3), 67.5 (d, $J_{4,F}$ = 7.5 Hz, C-4), 60.7 (C-6), 20.7, 20.6 (s, $3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: -195.0 (ddd, $J_{2,F}$ = 50.5 Hz, $J_{1,F}$ = 10.3 Hz, $J_{3,F}$ = 3.4 Hz, F-2); spectroscopic data was identical to that previously reported.[48]

1-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol (3.19): *N*-(2-hydroxyethyl)stearamide **3.3**,  obtained from **3.2** (58.9 mg, 0.18 mmol) and using the general procedure to obtain the stannyl lipid, in toluene (3.6 mL), 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl bromide **3.18b** (67 mg, 0.18 mmol) and following the general glycosylation procedure for stannyl lipid **3** (38.9 mg, 35%, α/β ratio: 1:1) was obtained after purification by flash column chromatography (EtOAc/*n*-hexane 1:1). Alpha anomer: R_f (EtOAc/*n*-hexane 1:1): 0.15; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.89 (t, J = 5.2 Hz, 1H, NH), 5.47 (td, $J_{4'',3''}$ = 3.4 Hz, $J_{4'',5''}$ = 1.3 Hz, 1H, H-4''), 5.41 (td, $J_{4'',3''}$ = 3. 4Hz, $J_{3'',2''}$ = 10.3 Hz, 1H, H-3''), 5.10 (d, $J_{1'',2''}$ = 3.9 Hz, 1H, H-1''), 4.77 (ddd, $J_{2'',F}$ = 50.0 Hz, $J_{2'',3''}$ = 10.3 Hz, $J_{1'',2''}$ = 3.9 Hz, 1H, H-2''), 4.23 (m, 1H, H-5''), 4.08 (d, $J_{6a'',6b''}$ = 6.6 Hz, 2H, H-6''), 3.85 (m, 1H, H-2a'), 3.61 (m, 2H, H-1a', H-2b'), 3.42 (m, 1H, H-1b'), 2.19 (m, 2H, H-2), 2.14, 2.05 (s, 9H, 3CH_3, Ac), 1.60 (m, 2H, H-3), 1.24 (s, 28H, CH_2), 0.88 (t, $J_{18,17}$ = 7.1 Hz, 3H, H-18); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: -208.05 (ddd, $J_{2'',F}$ = 50.0 Hz, $J_{1'',F}$ = 11.0 Hz, $J_{3'',F}$ = 3.9 Hz, F-2''). Beta anomer R_f (EtOAc/*n*-hexane 1:1): 0.1; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.88 (t, J = 5.5 Hz, 1H, NH), 5.42 (m, 1H, H-4''), 5.08 (dd, $J_{3'',2''}$ = 9.8 Hz, $J_{3'',4}$ = 3.6 Hz, 1H, H-3''), 4.56 (m, 1H, H-1''), 4.50 (ddd, $J_{2'',F}$ = 54.0 Hz, $J_{3'',2''}$ = 9.8 Hz, $J_{2'',1''}$ = 7.6 Hz, 1H, H-2''), 4.12 (m , 2H, H-5'', H-6a''), 3.95 (m, 2H, H-6b'', H-2a'), 3.74 (m, 1H, H-2b'), 3.60 (m, 1H, H-1b'), 3.44 (m, 1H, H-1b'), 2.18 (m, 2H, H-2), 2.15, 2.15, 2.05 (s, 9H, 3CH_3, Ac), 1.63 (m, 2H, H-3), 1.24 (s, 28H, CH_2), 0.92 (t, $J_{18,17}$ = 7.10 Hz, 3H, H-18); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: -206.69 (m, F-2'').

1-(2-Deoxy-2-fluoro-β-D-galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol (3.20β): Sarting from  **3.19β** (42 mg, 0.067 mmol) and following the deacetylation procedure, **3.20β** (40.6 mg, 98%) was obtained as

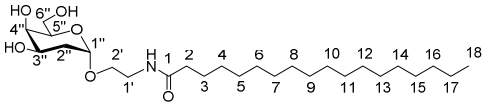
a white solid. R_f (CH_2Cl_2/MeOH/H_2O 7:2:1): 0.57; $[\alpha]_D^{20}$: –5.6 (*0.1*, CH_2Cl_2/MeOH 2:1); ^{1}H NMR ($CDCl_3$/CD_3OD 1:2, 400 MHz) δ in ppm: 4.43 (d, $J_{1'',2''}$ = 7.5 Hz, $J_{1'',F}$ = 4.2 Hz, 1H, H-1''), 4.34 (m, 1H, H-2''), 3.87 (m, 2H, H-4'', H-2-a'), 3.70 (m, 3H, H-2b', H-6''), 3.63 (m, 1H, H-3''), 3.50 (t, $J_{5'',4''} = J_{5'',6''}$ = 5.9Hz, 1H, H-5''), 3.40 (t, J = 5.0 Hz, 2H, H-1'), 2.14 (t, $J_{2,3}$ = 7.3 Hz, 2H, H-2), 1.56 (t, $J_{2,3}$ = 7.3 Hz, 2H, H-3), 1.27-1.20 (m, 28H, CH_2), 0.82 (t, $J_{17,18}$ = 6.8 Hz, 3H, H-18). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 174.6 (C=O, C-1), 100.6 (d, $J_{1'',F}$ = 23.4 Hz, C-1''), 91.4 (d, $J_{2'',F}$ = 181.6 Hz, C-2''), 74.7 (C-5''), 71.6 (d, $J_{3'',F}$ = 17.6 Hz, C-3''), 69.1 (d, $J_{4'',F}$ = 8.9 Hz, C-4''), 68.3 (C-2'), 60.6 (C-6''), 39.1 (C-1'), 35.9 (C-2), 31.5-22.2 (CH_2), 13.6 (C-18); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: -207,41 (dtd, $J_{2'',F}$ = 51.0 Hz, $J_{3'',F}$ = 14.4 Hz, $J_{1'',F}$ = 2.6 Hz, F-2); FT-IR (neat) ν in cm^{-1}: 3293, 2917, 2848, 1645, 1555, 1468, 1067, 1039, 719; HRMS (TOF ES$^+$) for $(M + Na)^+$ $C_{26}H_{50}NNaO_6^+$ (*m*/*z*): calc.514.3514; found 514.3519.

1-(2-deoxy-2-fluoro-α-D-galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol (3.20α): Sarting from **3.19α** (15.1 mg, 0.025 mmol) and following the deacetylation procedure, **3.20α** (12.2 mg, 90%) was obtained as a white solid. R_f (CH_2Cl_2/MeOH/H_2O 7:2:1): 0.57; $[\alpha]_D^{20}$: +42.5 (0.48, CH_2Cl_2/MeOH 2:1); ^{1}H NMR ($CDCl_3$/CD_3OD 1:2, 400 MHz) δ in ppm: 7.28 (t, J = 5.2 Hz, 1H, NH), 4.99 (d, $J_{1'',2''}$ = 4.0 Hz, 1H, H-1''), 4.60 (ddd, $J_{2'',F}$ = 50.0 Hz, $J_{2'',3''}$ = 9.4 Hz, $J_{1'',2''}$ = 4.0 Hz, 1H, H-2''), 3.97 (m, 2H, H-4'', H-3''), 3.81 (t, $J_{2a'',2b''}$ = 5.6 Hz, 1H, H-2a'), 3.72 (m, 3H, H-2b', H-6''), 3.56 (m, 1H, H-5''), 3.46 (m, 1H, H-1a'), 3.34 (m, 1H, H-1b'), 2.14 (t, $J_{2,3}$ = 7.5 Hz, 2H, H-2), 1.55 (t, $J_{2,3}$ = 7.5 Hz, 2H, H-3), 1.25-1.18 (m, 28H, CH_2), 0.82 (t, $J_{17,18}$ = 6.8 Hz, 3H, H-18). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 174.6 (C=O, C-1), 96.5 (d, $J_{1'',F}$ = 20.9 Hz, C-1''), 88.7 (d, $J_{2'',F}$ =185.6 Hz, C-2''), 70.4 (C-5''), 70.0 (d, $J_{4'',F}$ = 8.3 Hz, C-4''), 67.8 (d, $J_{3'',F}$ = 17.6 Hz, C-3''), 67.0 (C-2'), 61.1 (C-6''), 38.8 (C-1'), 36.0 (C-2), 31.5-22.3 (CH_2), 13.6 (C-18); ^{19}F NMR

($CDCl_3$, 376.5 MHz) δ in ppm: -209.6 (ddd, $J_{2'',F}$ = 50.0 Hz, $J_{3'',F}$ = 11.7 Hz, $J_{1'',F}$ = 3.5 Hz, F-2); FT-IR (neat) ν in cm^{-1}: 3290, 2917, 2848, 1645, 1556, 1468, 1097, 1037, 664; HRMS (TOF ES^+) for $(M + Na)^+$ $C_{26}H_{50}NNaO_6^+$ (*m*/*z*): calc. 514.3514; found 514.3517.

1-(3,4,6-Tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol (3.22): The general glycosylation procedure for lipids was used. To a stirred solution of 3,4,6-tri-O-acetyl-D-galactal **3.21** (100 mg, 0.30 mmol), and 4Å molecular sieves in acetonitrile (1.14 ml) under argon atmosphere at room temperature, a 5% solution of HBr (33%) in Acetic acid (57 µl) and *N*-(2-hydroxyethyl)stearamide **3.2** (82.6 mg, 0.25 mmol) were added. The crude was purified by column chromotography (1:3 to 1:1 EtOAc/*n*-hexane) to yield **3.22** (46 mg, 26%) as a white solid. R_f (EtOAc/*n*-hexane 1:1): 0.28; ^1H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.82 (t, *J* = 6.2 Hz, 1H, NH), 5.29 (d, $J_{4'',3''}$ = 2.7 Hz, 1H, H-4''), 5.25 (m, 1H, H-3''), 5.01 (d, $J_{1'',2a''}$ = 3.1 Hz, 1H, H-1''), 4.10 (m, 3H, H-6a'', H-6b'', H-5''), 3.70 (m, 1H, H-2a'), 3.52 (m, 2H, H-2b', H-1a'), 3.40 (m, 1H, H-1b'), 2.19 (m, 2H, H-2a, H-2b), 2.13, 2.05, 1.99 (s, 9H, $3CH_3$, Ac), 2.07 (m, 1H, H-2a''), 1.88 (m, 1H, H-2b''), 1.63 (m, 2H, H-3), 1.24 (s, 28H, CH_2), 0.87 (t, $J_{18,17}$ = 6.7 Hz, 3H, H-18). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 173.4 (C=O, C-1), 170.7, 170.5, 170.3 (3C=O, Ac), 97.9 (C-1''), 67.3 (C-2'), 67.0 (C-5''), 66.6 (C-4''), 66.2 (C-3''), 62.6 (C-6''), 39.2 (C-1'), 37.0 (C-2), 32.1 (C-2''), 30.3-29.5 (CH_2), 25.9 (C-3), 21.1-20.9 ($3CH_3$, Ac), 14.3 (C-18). FT-IR (neat) *v* in cm^{-1}: 3301.05, 2916.32, 2849.31, 1754.74, 1644.02, 1371.14, 1238.56, 1039.93, 773.797. HRMS (TOF ES^+) for $(M + Na)^+$ $C_{32}H_{57}NNaO_9^+$ (*m*/*z*): calc. 622.3926; found 622.3914.

1-(α-D-galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol (3.23):



Following the general deacetylation procedure, to a solution of 1-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-*N*-octadecanoyl-2-amino-ethanol **3.22** (12 mg, 0.02 mmol) in Methanol (200 µl), NaOMe (10 µl, 1M) was added. The reaction was stirred for 30 minutes and neutralized with Dowex 50-WX2. The solvent was removed under *vacuo*, and the resulting residue purified by flash column chromatography (EtOAc/MeOH/H_2O 10:1:0.5) to obtain **3.23** (9.5 mg, 98%) as a white solid. R_f (EtOAc/MeOH/H_2O 7:2:1): 0.56; $[\alpha]_D^{20}$: +44.2 (*0.1*, CH_2Cl_2/MeOH 2:1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 4.91 (d, $J_{1'',2a''}$ = 3.5 Hz, 1H, H-1''), 3.95 (ddd, $J_{3'',2a''}$ = 11.8 Hz, $J_{3'',2a''}$ = 5.3 Hz, $J_{3'',4''}$ = 2.9 Hz, 1H, H-3''), 3.76 (m, 1H, H-4''), 3.74 (m, 1H, H-5''), 3.70 (dd, $J_{6a'',6b''}$ = 13.8 Hz, $J_{6a'',5''}$ = 6.4 Hz, 1H, H-6a''), 3.61 (td, $J_{6b'',6a''}$ = 13.8 Hz, $J_{6b'',5''}$ = 5.6 Hz, 1H, H-6b''), 3.57 (m, 2H, H-2'), 3.36 (m, 2H, H1'), 2.16 (t, $J_{2a,2b}$ = 7.6 Hz, 2H, H-2), 1.90 (ddd, $J_{2a'',2b''}$ = 12.8 Hz, $J_{2a'',3''}$ = 11.8 Hz, $J_{2a'',1''}$ = 3.5 Hz, 1H, H-2a''), 1.80 (dd, $J_{2b'',2a''}$ = 12.8 Hz, $J_{2b'',3''}$ = 5.3 Hz, 1H, H-2b''), 1.58 (t, $J_{3,2}$ = 6.8 Hz, 2H, H-3), 1.24 (s, 28H, CH_2), 0.85 (t, $J_{18,17}$ = 6.8 Hz, 3H, H-18). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 174.8 (C=O, C-1), 97.6 (C-1''), 70.5 (C-5''), 68.1 (C-4''), 65.7 (C-2'), 64.9 (C-3''), 61.9 (C-6''), 38.8 (C-1'), 35.8 (C-2), 31.5 (C-2''), 32.0-28.8 (CH_2), 25.5 (C-3), 22.2 (C-17), 13.3 (C-18); FT-IR (neat) ν in cm^{-1}: 3521.89, 3413.87, 3288.52, 2918.25, 2848.35, 1709.59, 1419.35, 1359.09, 1220.72, 700.51. HRMS (TOF ES^+) for $(M + Na)^+$ $C_{26}H_{51}NNaO_6^+$ (*m*/*z*): calc. 496.3609; found 496.3610.

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SYNTHETIC GLYCOLIPIDS AS MODULATORS OF CARBOHYDRATEPROTEIN
INTERACTIONS
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CHAPTER 4

HYPERBRANCHED GLYCOPOLYMERS AS MULTIVALENT INHIBITORS OF CARBOHYDRATE-LECTIN INTERACTIONS

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4.1. INTRODUCTION

The complexity of cellular glycocalyx has been shaped over millions of year of evolution by the action of cell's (*glyco*)machinery, an intricate pool of glycosyl hydrolases, transferases, and other carbohydrate-processing enzymes that together with a different set of post-translational modifications define the composition of the cellular membrane, and its components. Biological systems, and particularly glycoproteins, are heterogeneous in nature, yet the functions of redundant glycoforms, which represent structurally similar glycoproteins that differ in sugar structure, density, and/or glycosylation site, and decorate the outer surface of cells remains unknown. Heterogeneous glycoforms typically participate in a variety of surface recognition events. In one example, galectins are able to recruit and cross-link a heterogeneous collection of complex glycoconjugates that trigger important signalling events.[1] In another example, partially deglycosylated protein structures have been related to disease states *via* reduction of the high-density shell surface in mucins that reveals truncated, short epitopes (*e.g.* Tn antigen). The presence of such composition and spatial organization defects enables preferential binding modes with particular lectins and reveals higher-order/supramolecular recognition patterns.[2] Thus, glycosylated proteins resulting from aberrant glycosylation patterns and/or with glycosylation defects render otherwise hidden structures surface exposed and sufficiently accessible to bind entities such as enzymes, antibodies, lectins or pathogens with particularly high avidities.

Chemical glycobiologists have traditionally focused their efforts on the development of homogeneous multivalent structures to precisely determine carbohydrate-protein binding events.[3] However, recent findings demonstrate that steric clash usually diminishes potency of homogeneous, yet sterically congested high-generation glycoconjugates and has driven the attention to

alternative, heterogeneous multivalent structures not only at the glycan level[4] but also related to the central core architecture.[5] Examples by Cloninger and co-workers highlight the presence of topological defects that enhance binding towards their targeted receptors.[6]

In an attempt to mimic Nature and gain insight into the heterogeneous nature of natural multivalent sugar-protein interactions we sought to systematically evaluate the binding mechanism between a series of multivalent homogeneous *vs.* heterogeneously presented glycoconjugates and a model plant toxin (RCA_{120}). We performed a thorough analysis *via* surface plasmon resonance (SPR) and dynamic light scattering (DLS) of the behaviour of hyperbranched glycopolymers[7] as a suitable family of multivalent glycomaterials with advanced properties not only in terms of accessibility and processability but also as simplified defect-containing analogues with enhanced potency.

We found the nature of the core and the display of ligans enormously affects the binding potency and mechanism. For example, lower densities of receptors (lectins) as those found in natural systems favours the use of heterogeneous glycoconjugate presentations (*e.g.* those mimicking inherent defects in glycoproteins and/or glycoforms) that enhance the potency in a match scenario of interactions suggesting this might be one of the pivotal reasons for the evolution of glycans to such a complex, heterogeneous structures.

We believe the data presented herein will be relevant to develop cheap, ready available multivalent carbohydrate therapeutics designed and utilized as anti-infective agents against common human diseases.

4.2. OUTLOOK AND OBJECTIVES

With this background, Boltorn (hyperbranched polymer and homogeneous glycodendrimer) was chosen as a central core to construct two different families of multivalent systems that could allow for the presentation of D-galactose residues in a polydisperse and a monodisperse manner. In particular the specific objectives of this work are:

(i) To synthesize multivalent structures using different Boltorn central cores in order to obtain a homogeneous *vs.* a heterogeneous presentation of the carbohydrate residues.

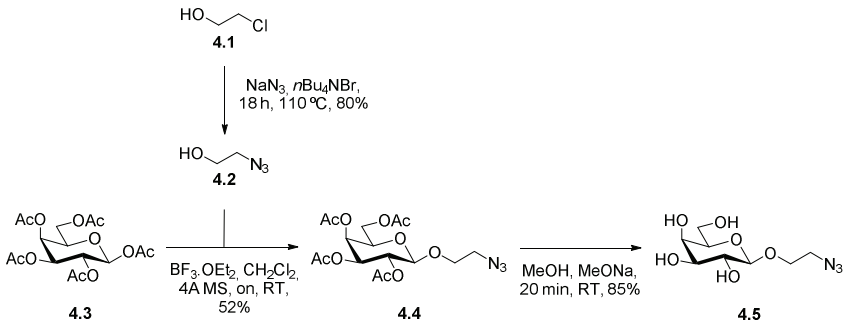
(ii) Evaluate the behaviour of the different synthetic multivalent systems using surface plasmon resonance (SPR) and dynamic light scattering (DLS) against a model plant toxin (RCA_{120}). Evaluation of the topology and carbohydrate density will be studied.

4.3. RESULTS AND DISCUSSION

As it is well known, high valency does not always correlate with high biological activity and the scaffold for carbohydrate presentation is indeed important. In this study we want to compare the efficient binding of two families of multivalent systems with different carbohydrate presentation towards the binding of a model plant toxin (RCA_{120}). Binding interactions of these compounds with a D-Galactose-binding protein have been analysed at the molecular level using surface plasmon resonance (SPR) and dynamic light scattering (DLS).

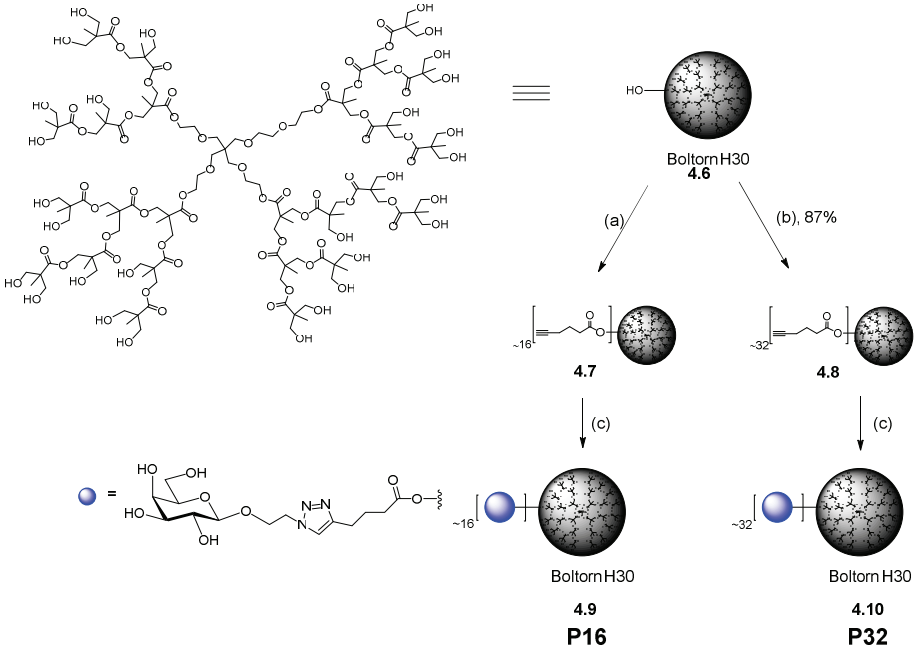
4.3.1. Multivalent systems synthesis

Multivalent systems were synthesized based on two different Boltorn central scaffolds (hyperbranched Boltorn H30 – **P**, and monodisperse Boltorn dendrimer – **M**) with two different D-galactose loadings (16 and 32 D-Galactose residues). Monomer **4.6** was synthesized bearing an azide group to be employed in the subsequent CuAAC ligation. Thus, 2-chloroethanol **4.1** was treated with sodium azide to obtain the desired 2-azidoethanol **4.2** in 80% yield. Penta-acetyl galactose **4.3** was treated with **4.2** and $BF_3.OEt_2$ as a promoter and glycoside **4.4** was obtained in 52% yield. After deprotection under Zemplén conditions (1M NaOMe in MeOH) the desired monomer **4.5** was obtained in good 85% yield (Scheme 4.1).



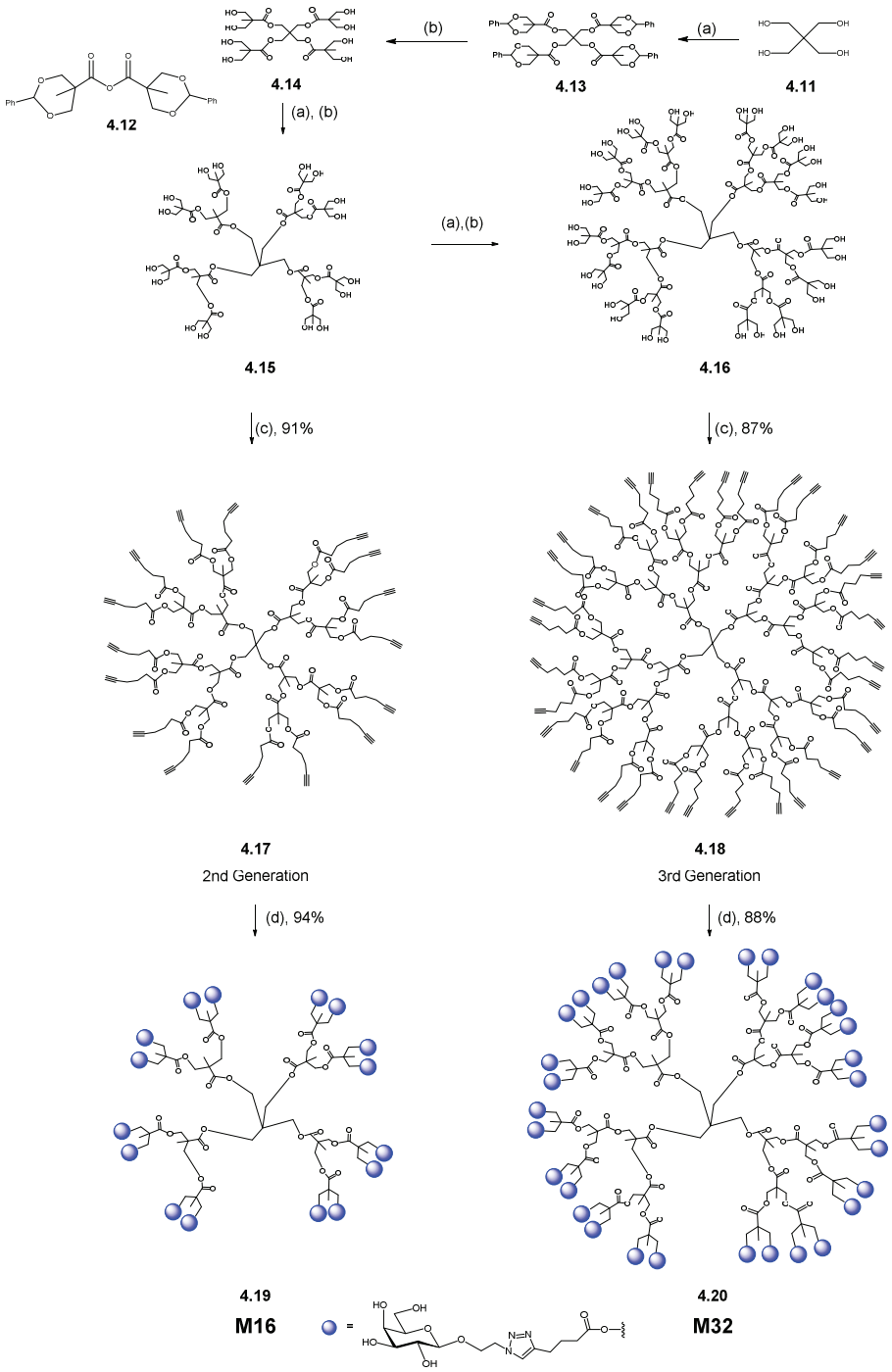
Scheme 4.1. 1-*O*-(2-Azidoethyl)-β-D-galactopyranoside **4.5** synthesis.

To obtain the desired heterogeneous multivalent presentation, Boltorn-H30 was used as the central scaffold. In this sense, Boltorn-H30 was treated with 5-hexynoic acid to obtain **4.7** and **4.8** with approximately 16 and 32 modified hydroxyl groups respectively. These alkyne groups reacted with monomer **4.5** using CuSO4 and sodium ascorbate *via* click chemistry. Compounds **4.9** (**P16**) and **4.10** (**P32**) were obtained after purification by dialysis and lyophilisation (Scheme 4.2).



Scheme 4.2. Synthesis of heterogeneous multivalent systems P16 (4.9) and P32 (4.10). *Conditions*: (a) 5-Hexynoic acid, DCM/DMF (1:1), HOBt, EDC, DIPEA, 5 days, RT, 71%. (b) 5-Hexynoic acid, DCM, DPTS, DCC, 12 h, 40 ºC. (c) **4.5**, TFH/H_2O (1:1) $CuSO_4.5H_2O$, AscNa, **P16** (72 h, RT, 94%), **P32** (2 h, 60 ºC µwave, 94%).

The synthesis of homogeneous multivalent systems was performed by Dr. J. J. Reina and Prof. J. Rojo (IIQ–CSIC, Sevilla). Central cores of 2nd (**4.15**) and 3rd (**4.16**) generations were synthesized from phentaerythriol using the methodology described by Prof. J. Rojo.[8] The subsequent functionalization with 5-hexynoic acid and monomer **4.5** was carried out in an similar manner as that for the functionalization of heterogeneous compounds **4.9** and **4.10** (Scheme 4.3). Thus, the synthesis of homogeneous multivalent systems requires more synthetic steps.



Scheme 4.3. Synthesis of homogeneous multivalent systems **M16** (**4.19**) and **M32** (**4.20**). *Conditions:* (a) Anhydride **4.12**, DMAP, Pyr/DCM, RT, 97%, (b) H_2, Pd(C), MeOH/DCM, RT, 95%. (c) 5-Hexynoic acid, DCM, DPTS, DCC, 12 h, 40 ºC. (d) **4.5**, TFH/H_2O (1:1) $CuSO_4.5H_2O$, sodium ascorbate, 60 ºC µwave, 2h.

4.3.2. Dynamic light scattering (DLS)

We first investigated the aggregation properties and binding specificity of RCA_{120} towards ligands **P** and **M** in solution by DLS. *Ricinus communis agglutinin* (RCA) is a heterodimeric D-galactose specific lectin with four subunits with a BAAB composition (Figure 4.1). Whereas in A-chain resides the cytotoxic activity, the B-chain contains two D-galactose binding sites separated by *ca.* 100 Å and composed by two globular domains.[9]

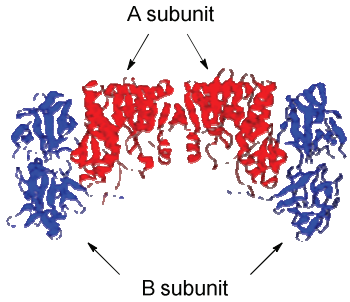


Figure 4.1. RCA_{120} crystal structure (1RZO).[10]

Both lectin and ligands (**P16**, **P32**, **M16** and **M32**) were checked prior to analysis to determine the size and confirm the absence of aggregates (Figure 4.2).

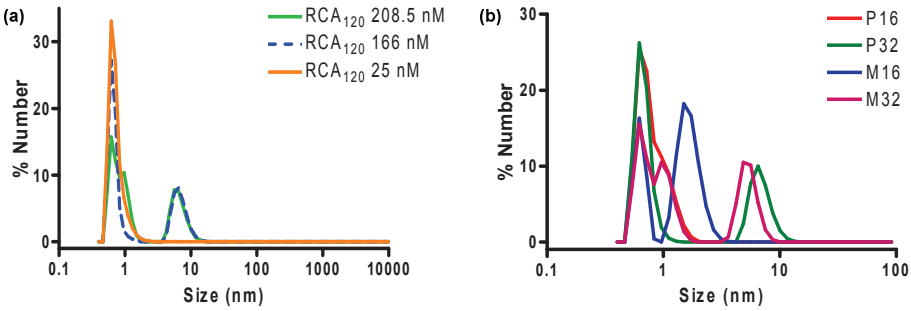


Figure 4.2. DLS measurements for (a) RCA_{120} (25–208.5 nM) and (b) **P16**, **P32**, **M16** and **M32** (400 nM).

A solution of RCA_{120} (166 nM) in HBS-EP buffer was treated with increasing ligand concentrations (100–400 nM). At high concentrations polydisperse ligands, **P16** and **P32**, were able to form aggregates while no aggregate formation was observed for monodisperse ligands, **M16** and **M32** (Figure 4.3) supporting the fact under same concentration range and generation, polydisperse ligands proved more effective to cross-link RCA_{120}. For **P32** the largest aggregates were observed at 200 nM (1:1.2 ratio), confirming that the ligand was acting as the nucleating agent for RCA_{120} aggregation.[11] While using the same concentrations no aggregates were found for monodisperse ligands although presenting a similar amount of carbohydrates in the surface. These results demonstrate the importance of carbohydrate presentation against receptor binding.

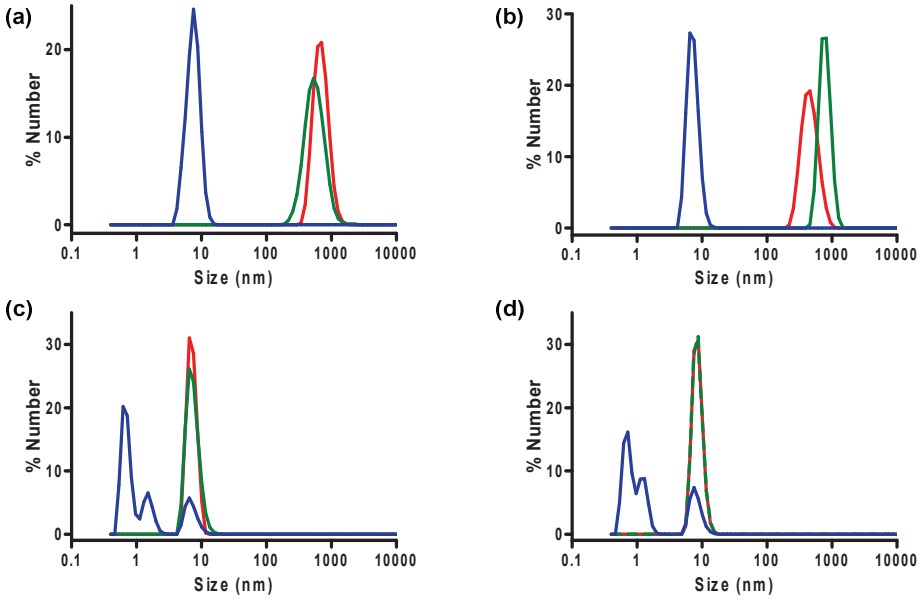


Figure 4.3. DLS measurements RCA_{120} **(**166 nM**)** and (a) **P16**, (b) **P32**, (c) **M16** and (d) **M32**. — RCA_{120} + **P16/32** or **M16/32** (400 nM) — RCA_{120} + **P16/32** or **M16/32** (200 nM) — RCA_{120} + **P16/32** or **M16/32** (100 nM)**.**

A series of control experiments were also performed to confirm the binding specificity of RCA_{120} towards D-galactose residues. A solution of **P16** with RCA_{120} in HBS-EP buffer (2.4:1 ratio) was chosen for the study. The solution was titrated with D-galactose (from 1 mM to 250 µM) and the particle size was determined. For concentrations of D-galactose ranging from 500 µM to 1 mM reversion of aggregate formation was observed. While titration of the same solution with the same amount of D-glucose as well as the equivalent volume of buffer (HBS-EP) did not influence aggregate formation, indicating that our ligands were able to bind selectively to RCA_{120} due to the presence of non-reducing D-galactose residues (Figure 4.4).

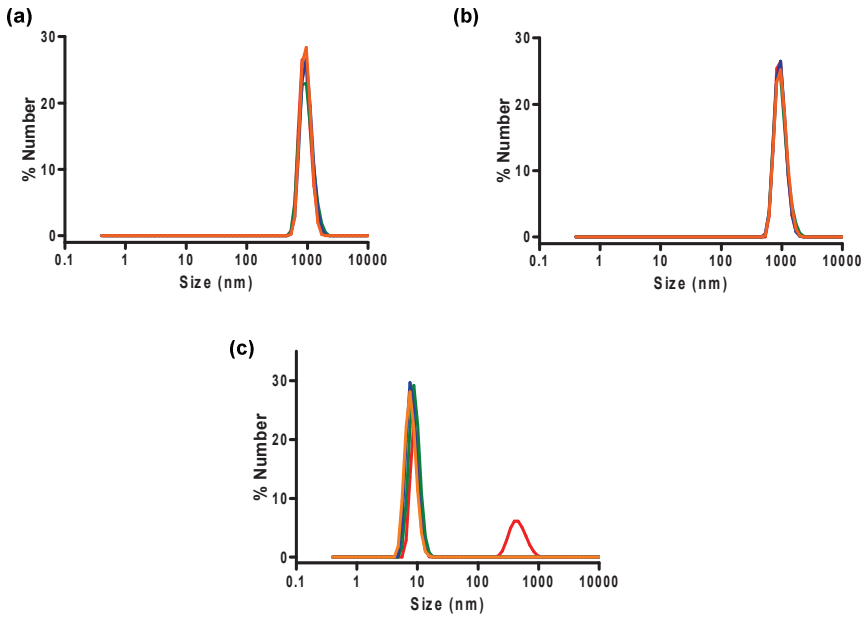


Figure 4.4. DLS measurements of RCA_{120} (166 nM) + **P16** (400 nM) solution titrations with (a) D-Glucose, (b) HBS-EP and (c) D-Galactose. — RCA_{120} + **P16** + D-Glc, HBS-EP or D-Gal (250 µM) — RCA_{120} + **P16** + D-Glc, HBS-EP or D-Gal (500 µM) — RCA_{120} + **P16** + D-Glc, HBS-EP or D-Gal (750 µM) — RCA_{120} + **P16** + D-Glc, HBS-EP or D-Gal (1 mM**)**.

4.3.3. Surface plasmon resonance (SPR)

With these preliminary results in hand the sought to study how the monodisperse and polydisperse D-galactose presentation significantly influences the binding kinetics and binding mechanism.

To obtain kinetic data of multivalent interactions, SPR direct binding experiments were performed. This technique enables a real-time evaluation of multivalent interactions occurring at the cell surface that could mimic the binding events that occur *in vivo* (in particular for glycolipid-lectin binding). Here, we report a thorough SPR-based kinetic analysis of the interaction between multivalent analytes and their surface receptor.

RCA_{120} was covalently attached to a polycarboxylated CM5 sensor chip to generate three different lectin surfaces at various levels of functionalization (RCA_{120}-HD, RCA_{120}-MD and RCA_{120}-LD where HD, MD and LD = high, medium and low densities, respectively). Binding data were collected at 5 μL/min flow rate using different analyte concentrations. Prior to analysis, binding tests using RCA_{120}-HD at different flow rates were performed to discard mass transport effects that could influence the shape of sensograms.[12]

Table 4.1. Binding data from direct binding analysis of P/M(n)-Gal ligands with RCA_{120}-MD/LD: k_{on} (x10^4 M^{-1} s^{-1}), k_{off} (x10^4 s^{-1}) and K_D (nM); n.d. = not detectable.

	RCA_{120}-LD			**RCA_{120}-MD**			
Analyte	k_{on}	k_{off}	K_D	k_{on} *1*	k_{on} *2*	k_{off}	K_D
P16	1.44	17.9	125	10.6	1.93	6.35	11.7
M16	n.d.	n.d.	n.d.	217	24.9	56.2	3.23
P32	13.8	45	32.6	57.5	8.0	8.73	2.71
M32	n.d.	n.d.	n.d.	66.7	9.21	18.6	5.93

As expected, complex binding profiles were reflected in the sensograms with RCA_{120}-MD according to the multivalent nature of the analytes tested. Our attempt to fit the results obtained to conventional binding models failed, yet following the procedure described by Fernandez-Megia *et al.*, a detailed kinetic analysis of the sensograms at early association and late dissociation times was performed.[13]

During the dissociation, a two-phase process was observed. Initially a fast dissociation was shown followed by an extremely slow dissociation phase suggesting the presence of both weak and strong binding events. This fast dissociation is more pronounced in monodisperse than in polydisperse analytes. More analyte reminds attached to the surface of the lectin when polydisperse structures are tested. In both cases, attempts to fit the dissociation phase to conventional binding models failed. While the mayor degree of heterogeneity was found at early dissociation times (t = 180–250 s), late dissociation times ($t \geq 250$ s) showed good fitting to 1:1 Langmuir binding model. For polydisperse analytes, k_{off} values were found to be lower than for monodisperse. Significantly different values in k_{off} resulted when comparing multivalent structures presenting the same number of D-galactose units. **M16** and **M32** showed 8.9- and 2.1-fold slower dissociation rates than **P16** and **P32**, respectively. When comparing dispersity, in the case of polydisperse analytes a 1.4-fold slower dissociation was observed when the number of D-galactoses was increased. In contrast, **M16** showed a 3.0-fold slower dissociation than the corresponding 3rd generation monodisperse structure (**M32**). The same trend was observed for polydisperse analyte **P16** when the early dissociation phase was evaluated as the percentage of SPR signal decay (t = 180–250 s). For **P16** the percentage observed was 1.9- and 1.1-fold lower than for **P32** and **M16** (Table 4.2).

Table 4.2. Percentage of signal decay.

Analyte	% signal decay	
	RCA_{120}-HD	**RCA_{120}-MD**
P16	14	19
M16	60	37
P32	25	22
M32	41	41

Analyte (5 μM) at early dissociations times (R_t=250/R_t=180)x100, where R_t=180 is the SPR response at the end of analyte injection at RCA_{120}-HD (9000 RU) and RCA_{120}-MD (6400 RU).

The slow dissociation of polydisperse Boltorn structures (**P16** and **P32**) and their low percentage of signal decay from RCA_{120}-MD surface represented an enhanced binding activity compared to monodisperse structures (**M16** and **M32**). The significant low value of off rates could be explained due to the fact that a nearby analyte could quickly replace a bound analyte in close proximity. This specific mechanism is called "statistical-rebinding"[14] and is favoured when D-galactose is presented to the receptor in a polydisperse manner.

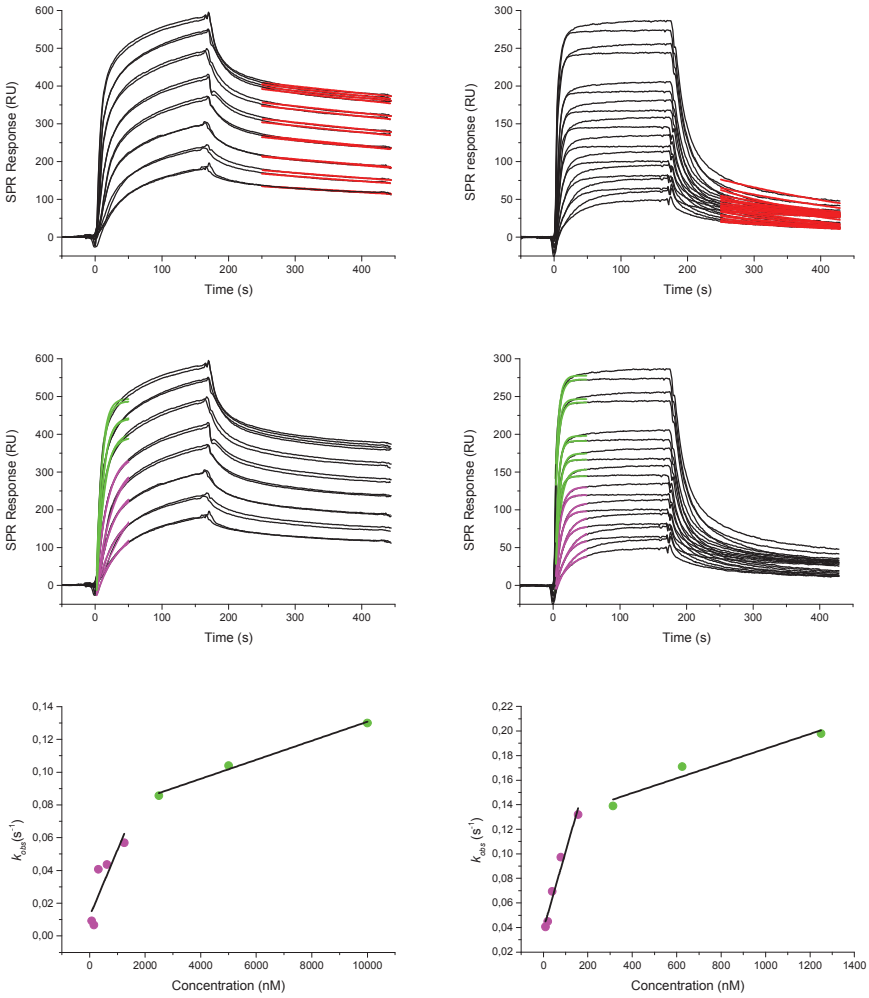


Figure 4.5. SPR of the interaction of **P16** (78 nM–10 μM) in the left hand side and **M16** (9.7 nM–10 μM) in the right hand side; separate kinetic analysis.

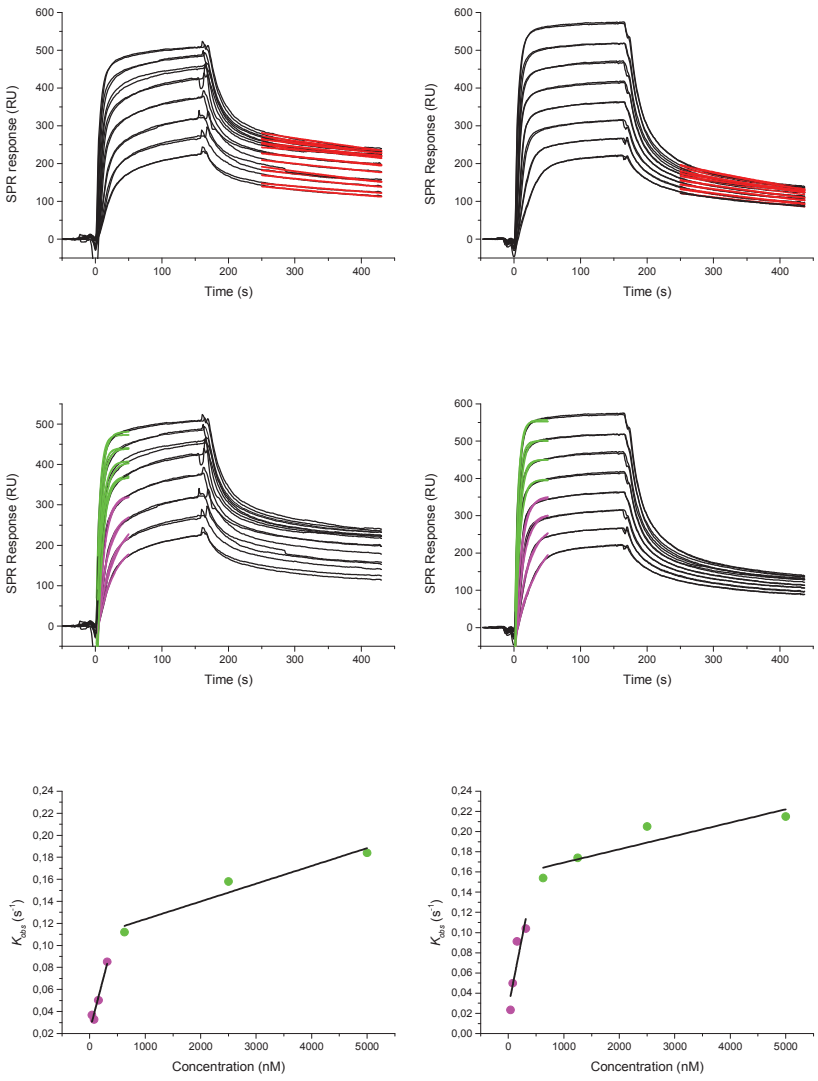


Figure 4.6. SPR of the interaction of **P32** in the left hand side and **M32** in the right hand side (39 nM–5 μM); separate kinetic analysis.

After an exhaustive evaluation of the dissociation phase of the sensogram, a kinetic evaluation of the association was also performed. The linear increase observed during the association phase, also indicates a strong binding. The early association phase ($t \leq 50$ s) was fitted to a 1:1 Langmuir binding model and k_{obs} *vs.* concentration was plotted (Figure 4.5). Deviations

from the linear behaviour were found for all analytes evaluated and two different slopes were observed (Figure 4.7).

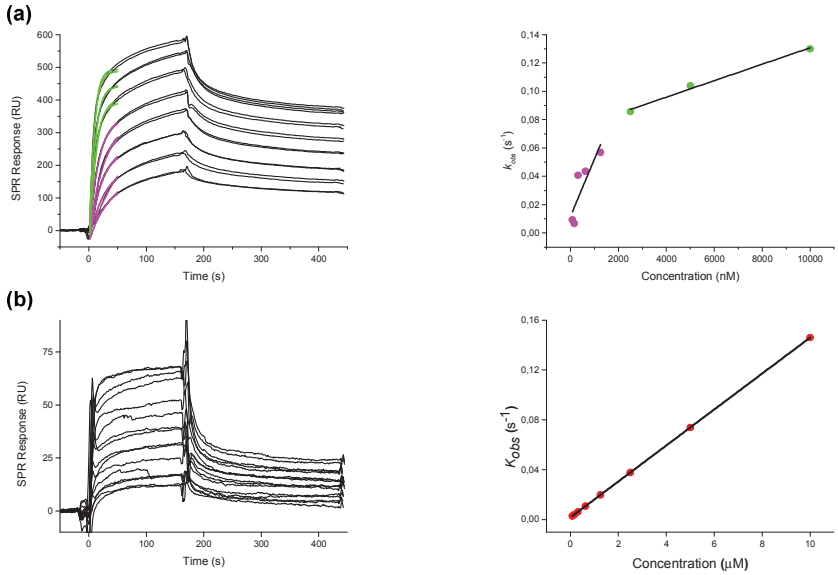


Figure 4.7. SPR data and detailed kinetic analysis of the association phase for (a) **P16**-RCA_{120}-MD and (b) **P16**-RCA_{120}-LD.

Low concentrations present a fast association (largest slope, high order complexes) while high concentrations present a slow association resulting in complex kinetic interactions due to the multivalent nature of the analytes.

The 2-fold increase of D-galactose residues in polydisperse analytes (from **P16** to **P32**) resulted in a 5-fold increase in $k_{on}1$. This trend was not observed in monodisperse analytes, when the glycodendrimer surface is coated with 32 D-galactose-units $k_{on}1$ value for **M32** (66.7×10^4 M^{-1} s^{-1}) decreased in 3.2-fold compared to **M16** (217×10^4 M^{-1} s^{-1}), which at low concentrations yielded a $k_{on}1$ value 20.4-fold higher than **P16** (10.6×10^4 M^{-1} s^{-1}) and a $k_{on}2$ (24.9×10^4 M^{-1} s^{-1}) 12.9-fold higher at high concentrations. For **M32**, $k_{on}1$ and $k_{on}2$ were only 1.2-fold higher compared to **P32**. The shape of sensograms was indicative of the high increase of binding for monodisperse compared to polydisperse analytes (at early association a more pronounced

increase is observed in **M16** and **M32** sensograms than in **P16** and **P32**). In hyperbranched polymers (**P**) when the number of carbohydrates presented in the surface increases their effective multivalency is also amplified. This effect could favour rebinding processes and the fact that one analyte could simultaneously bind to two proteins might explain why the affinity constant increase when the number of carbohydrates presented in the surface is higher. However in the case of homogeneous glycodendrimers it is known that structures with higher generations (high valency) not always give better affinities.[15] The drop in affinity has been usually attributed to steric hindrance between carbohydrate units.

In previous studies the binding constant of D-galactose towards RCA_{120} has been determined by ITC with a K_D of $4.5x10^{-4}$ M.[16] **P16**, **M16**, **P32** and **M32** yielded a K_D in the nanomolar range, 4 orders of magnitude lower than its monomeric counterpart. These results suggest that our multivalent ligands are able to simultaneously bind to two RCA_{120} lectins in the MD-surface.[17,18]

The detailed kinetic evaluation of RCA_{120}-MD gave important information about the real-time binding of this type of analytes, showing a heterogeneous binding with distinguishable weak and strong affinities caused by the clustering and rebinding phenomena.

SPR direct binding analysis was also performed in RCA_{120}-LD (1300 RU) for our four analytes, yet conversely to MD-surface no binding was detected for monodisperse ligands (Figure 4.8). The analyte does not remain bounded on the chip surface after injection, meaning that no binding was observed and therefore the increase in RUs during association is due to a change in the refractive index caused by the change in concentration. Exhaustive analysis of the polydisperse analytes were also performed at different concentrations. The same sensogram profile was observed as that for RCA_{120}-MD. According to the dissociation and association phase, the

same trend is observed as in RCA_{120}-MD, a fast dissociation followed by a slow dissociation and a linear increase in association.

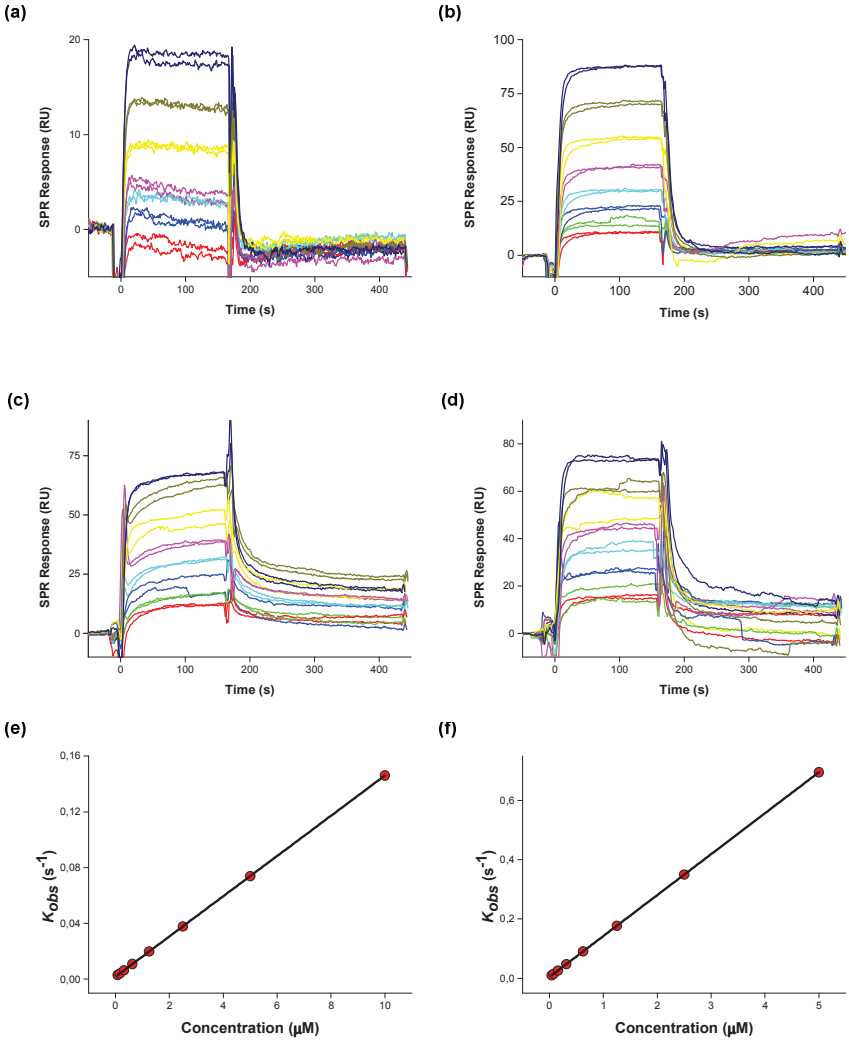


Figure 4.8. SPR data for the interaction of (a) **M16**, (b) **M32**, (c) **P16** and (d) **P32**, with RCA_{120}-LD; linear analysis of (e) **P16** and (f) **P32**.

The decrease in lectin density showed a less complex binding profile and the sensograms could be fitted well to a 1:1 Langmuir binding model. In contrast to what happened with RCA_{120}-MD, RCA_{120}-LD surface showed a single slope that equals to k_{on} when plotting k_{obs} *vs*. concentration for **P16** and

P32 indicating no binding heterogeneity (Figure 4.8). The binding efficiency between generations is maintained but the decrease in lectin density impedes the analyte to simultaneously bind two RCA_{120} lectins. The high affinity binding modes could only be explained by rebinding and clustering with the lectin secondary binding site.

Thus, the study of the binding affinities with different lectin density gave an idea of the importance of carbohydrate presentation front its mode of action.

Accordingly to the results obtained, this real-time-dependent analysis suggests that the different binding mechanisms described depend on the local concentration of analyte and its proximity to the immobilized lectin and not only to the analyte multivalency and the lectin surface density.

4.4. CONCLUSIONS

In summary, to the best of our knowledge this study is the first example that evaluates the interaction of synthetic multivalent systems that present the same amount of carbohydrates in a monodisperse and polydisperse manner with a model plant toxin (RCA_{120}). Modifications of the central core of multivalent systems give relevant binding differences both in DLS and SPR experiments. Thus, the conclusions that can be extracted are:

(i) Homogeneous systems (**M16** and **M32**) required more synthetic effort than heterogeneous systems (**P16** and **P32**) due to the nature of the central core. Whereas for the synthesis of heterogeneous systems, a commercial hyperbranched polymer (Boltorn-H30) was employed as a central scaffold, homogeneous systems required the synthesis of the central core.

(ii) A divergent behaviour in aggregate formation between monodisperse and polydisperse structures and specific D-

galactose binding against RCA_{120} was observed in solution by DLS. The only aggregates observed were formed with heterogeneous ligands (**P16** and **P32**).

(iii) A detailed kinetic binding analysis at different lectin densities gave evidence about the chelate and rebinding mechanisms that contribute to the complex profile interactions.

(iv) When testing the binding in RCA_{120}-LD, whereas binding was detected when heterogeneous ligands (**P16** and **P32**) were analysed, no binding was detected for homogeneous ligands (**M16** and **M32**).

The information extracted from these promising multivalent systems will be beneficial for further studies in order to design the most appropriate multivalent compounds that match the characteristics of the study system.

4.5. EXPERIMENTAL PART

General Remarks

Proton (^{1}H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance spectra were recorded on a Varian Mercury spectrometer (400 MHz for ^{1}H and 100.6 MHz for ^{13}C). Spectra were fully assigned using COSY, HSQC, HMBC, and NOESY. All chemical shifts are quoted on the δ scale in ppm using either Me_3Si (^{1}H NMR: $CDCl_3$ = 0.00) or the residual solvent as internal standard (^{1}H NMR: $CDCl_3$ = 7.26, D_2O = 4.79, CD_3OD = 3.31 and ^{13}C NMR: $CDCl_3$ = 77.00, CD_3OD = 49.00). Coupling constants (J) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, and app = apparent. Infrared (IR) spectra were recorded on a Jasco FT/IR-600 Plus ATR Specac Golden Gate spectrophotometer. Absorption maxima (ν_{max}) are reported in wavenumbers (cm^{-1}). High resolution mass spectra (HRMS) were recorded on an Agilent 1100 Series LC/MSD mass spectrometer with electrospray

ionization (ESI) by the Servei de Recursos Científics (URV). Nominal and exact *m/z* values are reported in Daltons. Gel permeation chromatography (GPC) for determination of polymer molecular weights and their distributions was performed in a Waters equipment provided with RI and UV detectors using 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, 99% Apollo Scientific Lim.) containing sodium trifluoroacetate (98%, Sigma-Aldrich Co.; 6.8 g/L) as a mobile phase. A total of 10 μL of 0.1% (w/v) sample solution was injected and chromatographed with a flow of 0.5 mL/min. HR5E Waters linear Styragel columns (7.8 × 300 mm, pore size 103–104 Å) packed with cross-linked polystyrene and protected with a precolumn (VanGuard, 1.8 μm, 2.1 × 5 mm) were used. Molecular weight averages and distributions were evaluated against poly(methyl methacrylate) standards. Thin layer chromatography (TLC) was carried out using commercial aluminium backed sheets coated with 60F254 silica gel. Visualization of the silica plates was achieved using a UV lamp (λ_{max} = 254 nm) and/or 6% H_2SO_4 in EtOH and/or 2% $PdCl_2$ and 15% H_2SO_4 in water. Flash column chromatography was carried out using silica gel 60 A CC (230–400 mesh). Mobile phases are reported in relative composition (*e.g.* 1:1 EtOAc/hexane v/v). HPLC grade dichloromethane, tetrahydrofuran (THF), and dimethylformamide (DMF) were dried using a solvent purification system (Pure SOLV system-400). All other solvents were used as supplied (Analytical or HPLC grade), without prior purification. All reagents were used as received from commercial suppliers. All reactions using anhydrous conditions were performed using flame-dried apparatus under an atmosphere of argon. Boltorn H30 was supplied by Perstorp Company. RCA_{120} was purchased from Sigma Aldrich. Methyl-β-D-galactopyranoside (Me-Gal) was purchased from Carbosynth. CM5 sensor chip and HBS-EP buffer were purchased from GE Healthcare. All binding studies were performed at 25 ºC in a Biacore 3000 spectrometer optical biosensor. DLS measurements were performed using a Zetasizer Nano ZS from Malvern.

4.5.1. Surface plasmon resonance (SPR)

Preparation of RCA_{120}-coated sensor surface

Immobilization to generate three different lectin surfaces, namely RCA_{120}-HD, RCA_{120}-MD and RCA_{120}-LD was performed on a polycarboxylated dextran covalently attached to a gold surface (CM5 sensor chips) *via* amino-coupling using HBS-EP (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% P20) as running buffer at 5 µl/min flow rate. The caboxymethylated dextran layer in CM5 chip was activated by flowing a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide and 0.4 M *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide over the chip for 7 min at 25 ºC at 5 µL/min flow rate. RCA_{120} was immobilised on channels 2, 3 and 4 *via* injection of a solution (50 µg/ml in 10 mM acetate buffer pH 5) for 2, 5 and 3 min respectively resulting in functionalization of the surface at 1300, 9000 and 6400 response units (RUs). All channels were subsequently modified by flowing a solution of ethanolamine hydrochloride (1 M, pH 8.5) for 7 min at 5 µL/min to produce a blank on channel 1 and remove remaining reactive groups on channels 2–4.

Binding assays

P16, **M16** (78 nM–10 µM) and **P32**, **M32** (39 nM–5 µM) solutions were prepared in the same HBS-EP buffer. Sensograms for each multivalent system and concentration were recorded at 5 µL/ min, in all channels, with a 180 s injection (on period) followed by a 240 s of running buffer alone (off period). After each binding cycle, the sensor chip surface was regenerated with 2.5 mM methyl-β-D-galactopyranoside solution[19] prepared in the same running buffer. Two consecutive injections of 7 µL and 5 µL of Me-Gal solution were followed by an increase of the flow rate, 10 µL/ min, allowing 10 min for a completed regeneration. Duplicate injections of each concentration were performed. Data analysis were performed using

Biaevaluation 4.1 software, double referencing was used to remove all the artifacts.[20]

Mass transport effects assays

To test mass transport effects the more pronounced experimental conditions were selected, the analyte at the highest concentration and the highest RCA_{120} surface density (9000 RU). **P32** and **M32** using three different concentrations (5 μM, 2.5 μM and 1.25 μM) were injected in duplicate for 90 s of association and 240 s of dissociation at 100 μL/min flow rate followed by a 1 min injection of 2.5 mM Me-Gal regeneration solution and 10 min for a complete regeneration. The same sample concentrations were injected in duplicate for 180 s of association and 240 s of dissociation at 50 μL/min flow rate followed by the same regeneration step.

Detailed kinetic analysis method of the interaction between Gal(n)-M/P and RCA_{120}-MD surface

Separate kinetic analysis was employed.

First step: **analysis of the late dissociation phase.** Firstly, late dissociation phase ($t \geq 250$ s) was fitted to a 1:1 Langmuir binding model to obtain k_d values.

Second step: **non-linear analysis of the early association phase**. The k_d values obtained from the first step were employed when early association phase ($t \leq 50$ s) was fitted.

Third step: **linear analysis of the early association phase**. K_{obs} *vs.* concentration was plotted to evaluate the association phase using a linear model.

4.5.2. Dynamic light scattering (DLS)

To ensure the absence of aggregates, both lectin and ligands were checked previously under the same conditions. The size was measured using a Zetasizer Nano ZS (Malvern Instrument), each measurement was performed at 12 scans and repeated 3 times with 1 s delay at 25 ºC. BRAND Polystyrene (PS) Cuvettes were used. Various amounts of ligand (400, 200 and 100 nM) were added to a RCA_{120} solution (166 nM) and stirred for 1 h at room temperature. The results are given as distribution by number *vs.* size.

2-Azidoethanol (4.2):[21] A mixture of 2-chloroethanol **4.1** (10 mL, 149.17 mmol), NaN_3 (19.4 g, 298 mmol), and *n*-Bu_4NBr (961 mg, 2.98 mmol) was stirred vigorously for 18 h at 110 ºC. After cooling the mixture, the crude was purified by a short column chromatography (silica gel, Et_2O) to afford **4.2** as a colorless liquid (10.12 g, 80%). R_f (Et_2O): 0.77; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 3.75 (t, *J* = 5.2 Hz, 2H, $C\underline{H}_2OH$), 3.40 (t, *J* = 5.2 Hz, 2H, CH_2N_3), 2.94 (t, *J* = 5.2 Hz, 1H, OH); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 61.2 (CH_2OH), 53.2 (CH_2N_3); FT–IR (ATR) ν in cm^{-1}: 3356, 2934, 2877, 2092, 1441, 1347, 1286, 1061, 879, 629; HRMS (TOF ES^+) for $(M+H)^+$ $C_2H_6N_3O^+$ (*m/z*): calc. 88.0505; found 88.0530.

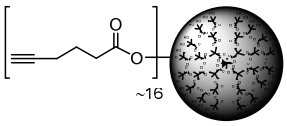
1-*O*-(2-Azidoethyl)-(2,3,4,6-tetra-*O*-acetyl)-β-D-galactopyranoside (4.4): To a mixture of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose **4.3** (1.0 g, 2.56 mmol), 2-azidoethanol **4.2** (668 mg, 7.68 mmol) and 4Å MS in dry CH_2Cl_2 (3.6 mL), $BF_3.OEt_2$ (1.89 mL, 15.36 mmol) was added at 0 ºC. The reaction mixture was warmed up to room temperature and stirred overnight. The crude was then diluted with CH_2Cl_2 and extracted with $NaHCO_3$ and brine. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (4:1 *n*-hexane/EtOAc) to afford **4.4** (555 mg, 52%). R_f (1:1 EtOAc/*n*-hexane): 0.35; $[\alpha]_D^{20}$: –12.0 (1.7, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.37

(dd, $J_{4',3'}$ = 3.5 Hz, $J_{4',5'}$ = 1.2 Hz, 1H, H-4'), 5.21 (dd, $J_{2',3'}$ = 10.4 Hz, $J_{2',1'}$ = 7.9 Hz, 1H, H-2'), 5.00 (dd, $J_{2',3'}$ = 10.4 Hz, $J_{4',3'}$ = 3.5 Hz, 1H, H-3'), 4.54 (d, $J_{2',1'}$ = 7.9 Hz, 1H, H-1'), 4.21–4.06 (m, 2H, H-6a', H-6b'), 4.02 (ddd, $J_{1a,1b}$ = 10.7 Hz, $J_{1a,2b}$ = 4.7 Hz, $J_{1a,2a}$ = 3.4 Hz, 1H, H-1a), 3.91 (td, $J_{5',6a'}$ = 7.2 = $J_{5',6b'}$ = 6.7 Hz, 1H, H-5'), 3.67 (ddd, $J_{1b,1a}$ = 10.6 Hz, $J_{1b,2a}$ = 8.5 Hz, $J_{1b,2b}$ = 3.3 Hz, 1H, H-1b), 3.48 (ddd, $J_{2a,2b}$ = 13.3 Hz, $J_{1b,2a}$ = 8.5 Hz, $J_{1a,2a}$ = 3.4 Hz, 1H, H-2a), 3.28 (ddd, $J_{2a,2b}$ = 13.3 Hz, $J_{1a,2b}$ = 4.7 Hz, $J_{1b,2b}$ = 3.3 Hz, 1H, H-2b), 2.15, 2.06, 2.04, 1.98 (s, 12H, 4CH_3, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.3, 170.1, 170.0, 169.4 (4C=O, Ac), 101.0 (C-1'), 70.8 (C-3'), 70.7 (C-5'), 68.4 (C-2'), 68.3 (C-1), 66.9 (C-4'), 61.1 (C-6'), 50.4 (C-2), 20.7, 20.6, 20.5, 20.4 (4CH_3, Ac); FT–IR (ATR) ν in cm^{-1}: 2915, 1741, 1469, 1372, 1224, 1135, 1087, 1057, 630; HRMS (TOF ES$^+$) for $(M+Na)^+$ $C_{16}H_{23}N_3NaO_{10}^+$ (*m/z*): calc. 440.1276; found 440.1267; spectroscopic data was identical to that previous reported.[22]

1-*O*-(2-Azidoethyl)-β-D-galactopyranoside (4.5): glycoside **4.4** (105.7 mg, 0.25 mmol) was azeotropically dried with anhydrous toluene and kept under high vacuum for 2 h. After that, it was dissolved in anhydrous MeOH (2.5 mL) and a freshly prepared 1 M solution of NaOMe in anhydrous methanol was added (1.5 mL). The mixture was stirred under argon pressure at room temperature for 20 min. The reaction was quenched with DOWEX 50WX2 until pH 7 was reached. After removing the solvent, the crude was purified by column chromatography (silica gel, 1:9 MeOH/DCM) to afford **4.5** (52.9 mg, 85%) as a syrup. R_f (7:2:1 EtOAc/MeOH/H_2O): 0.41; $[\alpha]_D^{20}$: +43.4 (0.38, 1:1 DCM/MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ in ppm: 4.27 (d, $J_{1b,1a}$ = 7.4 Hz, 1H, H-1'), 4.02 (dt, $J_{1b,1a}$ = 10.6 Hz, $J_{1b,1a}$ = $J_{1b,1a}$ = 5.1 Hz, 1H, H-1a), 3.84 (d, $J_{1b,1a}$ = 3.3 Hz, $J_{1b,1a}$ = 1.1 Hz, 1H, H-4'), 3.58–3.43 (m, 3H, H-1b, H-6a, H-6b), 3.56–3.46 (m, 5H, H-2', H-3', H-5', H-2a, H-2b); ^{13}C NMR (CD_3OD, 100.6 MHz) δ in ppm: 10.5 (C-1'), 76.7 (C-2'), 74.9, 72.4 (C-3', C-5'), 70.2 (C-4'), 69.1 (C-1), 62.4 (C-6'), 52.0 (C-2); FT–IR (ATR) ν in cm^{-1}: 3385, 2941, 1731, 1731,

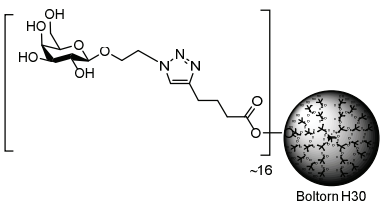
1468, 1378, 1232, 1131, 1064, 998; HRMS (TOF ES^+) for $(M+Na)^+$ $C_8H_{15}N_3NaO_6^+$ (*m/z*): calc. 272.0853; found 272.0847; spectroscopic data was identical to that previous reported.[22]

Synthesis of 2nd generation hyperbranched polymer (4.7):[23] To a solution



of 5-hexynoic acid (3347 μL, 29.42 mmol) and dialysed Boltorn H30 **4.6** (1558 mg, 0.432 mmol) in anhydrous 1:1 DCM/DMF (150 mL) was added HOBt (3975 mg, 29.42 mmol), EDC (5639 mg, 29.42 mmol), and DIPEA (4904 μL, 29.42 mmol). The reaction mixture was stirred at room temperature for 5 days and then concentrated under reduced pressure. The reaction was purified by dialysis against DMF (benzoylated tube flat width 32 mm (1.27'') for 3 days changing the solvent every 8 h to afford **4.7** (1572 mg, 70 %) as a syrup. The number of 5-hexynoate groups attached to the Boltorn's H30 surface corresponded to an average valency of 16, which represents a functionalization of 51% of total surface OH groups and was estimated by integration of signals in the ^{1}H NMR according to the methodology by Fernández-Francos and co-workers. ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 4.23-4.14 (m, 75H, H-1'), 3.67-3.37 (m, 32H, $\underline{CH_2}OH$), 2.43 (t, J = 6.8Hz, 32H, H-2), 2.21 (td, J = 7.2Hz, J = 2.8 Hz, 32H, H-4), 1.97 (s, 16H, H-6), 1.79 (appt, J = 7.2 Hz, 32H, H-3), 1.23–1.19 (m, 55 H, H-3'); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 173.1, 172.6, 172.4, 172.2, 171.9, 83.2 (C-5), 69.7 (C-6), 66.0-64.0 (C-1'), 48.7 (C-2'), 46.6–46.5 (C-2'), 32.7–32.6 (C-2), 23.6-23.5 (C-3), 17.9-17.5 (C-3', C-4); FT–IR (ATR) ν in cm^{-1}: 3529, 3285, 2942, 1730, 1468, 1380, 1237, 1130, 1053, 1011, 631; GPC: M_n = 2478 g/mol, M_w = 6801 g/mol, PDI = 2.78.

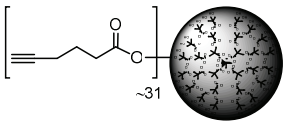
Synthesis of 2nd generation glycohyperbranched polymer P16 (4.9): To a



solution of dendritic polymer **4.7** (47 mg, 0.008 mmol) in 1:1 *t*-BuOH/H_2O (2.2 mL) was added a solution of glycoside **4.5**

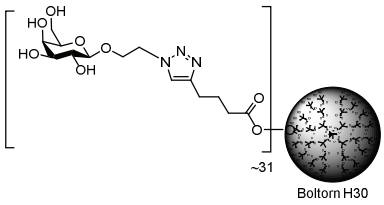
(11.75 mg, 0.22 mmol) in 1:1 *t*-BuOH/H_2O (2.2 mL) and a freshly prepared solution of $CuSO_4 \cdot 5H_2O$ (15 mg, 0.060 mmol) and sodium ascorbate (12.8 mg, 0.046 mmol) in 1:1 *t*-BuOH/H_2O (0.22 mL). The reaction mixture was stirred at room temperature for 72 h and then purified by dialysis (benzoylated tube flat width 32 mm (1.27'') from 1:1 *t*-BuOH/H_2O to 10 mM aqueous EDTA and finally to H_2O for 3 days changing the solvent every 8 h. After that, the sample was gently stirred with QuadraSil MP (220 mg) for 1 h. Then, the mixture was filtered off and the solution was freeze dried to obtain **4.9** (72.5 mg) as a white lyophilised. ^{1}H NMR (D_2O, 400 MHz) δ in ppm: 8.90–8.60 (bs, 16H, H-6), 4.50 (bs, 32H, H-8), 4.34–4.15 (bs, 16H, H-1''), 4.22–4.05 (bs, 109H, H-1', H-7, CH_2OH), 3.88–3.81 (bs, 16H, H-4''), 3.75 (bs, 16H, H-6''a), 3.65–3.52 (bs, 32H, H-2'', H-6''b), 3.60–3.34 (bs, 32H, H-3'', H-5''), 2.70 (bs, 32H, H-4), 2.35–2.26 (bs, 32H, H-2), 2.05–1.80 (bs, 32H, H-3), 1.44 (bs, 55H, H-3'); ^{13}C NMR (D_2O, 100.6 MHz) δ in ppm: 174.6–173.2 (CO, C-1), 102.9 (C-1''), 75.0 (C-2''), 72.5, 70.4 (C-5''or C-3''), 68.4 (C-4''), 67.6 (C-7), 65.5–63.9 (C-1', CH_2OH), 60.8 (C-6''), 50.8 (C-8), 48.5–46.4 (C-2'), 32.9 (C-2), 23.4 (C-3, C-4), 17.0 (C-3'); FT–IR (ATR) ν in cm^{-1}: 3385, 2941, 1734, 1608, 1390 1237, 1132, 1058; GPC: M_n = 7302 g/mol, M_w = 15963 g/mol, PDI = 2.19.

Synthesis of 3rd generation hyperbranched dendritic polymer (4.8): To a mixture of **4.6** (200 mg, 0.059 mmol), 5-hexynoic acid (322 μL, 2.84 mmol) and DPTS (278 mg, 0.944 mmol) in DCM (6 mL), DCC (585 mg, 2.84 mmol) was added and the reaction mixture was stirred overnight at 40 ºC under N_2. Then, the reaction mixture was filtered through a short path of Celite and the solvent was evaporated. The mixture was purified by size exclusion chromatography (Sephadex LH-20, 1:1 DCM/MeOH) to afford **4.8** (330 mg, 87%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 4.29–4.03 (m, 110H, H1', $OCH_{2\ Pentaerithritol}$), 3.65–332 (m, 20H, CH_2OH), 2.38 (t, 62H, *J*=7.5 Hz, H2), 2.17 (td, 64H,



J=7.1 Hz, 2.8 Hz, H4), 1.94 (s, 32H, H6), 1.75 (p, 64H, J=7.3 Hz, H3), 1.17 (bs, 84H, H3'); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 172.3 (C=O), 172.2 (C=O), 172.0 (C=O), 171.6 (C=O), 83.1 (C5), 69.4 (C6), 65.6, 65.5, 65.1, 64.9 (C1'), 46.6 (C2'), 46.5 (C2'),46.3 (C2'), 32.7, 32.5, 32.4 (C2), 23.4 (C3), 17.7, 17.7, 17.6 (C4, C3').

Synthesis of 3rd generation dendrimer P32 (4.10): To a solution of **4.8**



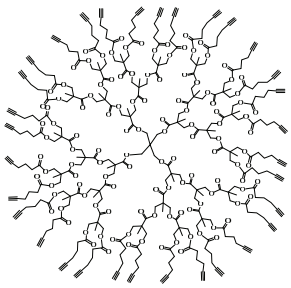
(12.4 mg, 1.93x10^{-3} mmol) and glycoside **4.5** (30.7 mg, 1.23x10^{-1} mmol) in 1:1 THF/H_2O (1 mL) was added a solution of $CuSO_4 \cdot 5H_2O$ (15.4 mg, 6.18x10^{-2} mmol) and TBTA (65 mg, 1.23x10^{-1} mmol) in 1:1 THF/H_2O (1 mL). Finally, sodium ascorbate (49 mg, 2.47x10^{-1} mmol) was added and the reaction mixture was microwave irradiated at 60 ºC for 2 h. After that, the sample was gently stirred with QuadraSil MP and purified by size exclusion chromatography (Sephadex G-25, 9:1 H_2O/MeOH) to afford **4.10** (24.7 mg, 89%) as a white lyophilized. ^{1}H NMR (D_2O, 400 MHz) δ in ppm: 7.76 (s, 32H, H6), 4.50 (bs, 64H, H8), 4.26 (d, 32H, J=7.7 Hz, H1''), 4.26-4.02 (m, 152H, H7, H1', $OCH_{2\ Pentaerithritol}$), 4.01–3.88 (m, 32H, H7b), 3.81 (d, 32H, J=3.4 Hz, H4''), 3.72–3.59 (m, 74H, H6'', CH_2OH), 3.59–3.48 (m, 74H, H3'', H5'', CH_2OH), 3.42 (t, 32H, J=8.8 Hz, H2''), 2.56 (bs, 64H, H2), 2.30 (bs, 64H, H4), 1.80 (bs, 64H, H3), 1.14 (s, 48H, H3'); ^{13}C NMR (D_2O, 100.6 MHz) δ in ppm: 174.3 (C=O), 172.9 (C=O), 147.2 (C5), 123.8 (C6), 103.1 (C1''), 75.1 (C5''), 72.7 (C3''), 70.6 (C2''), 68.5 (C4''), 68.0 (C7), 65.4 (C1', CH_2OH), 60.8 (C6''), 50.2 (C8), 46.5 (C2'), 33.0 (C4), 24.0 (C3, C2), 17.1 (C3'); GPC: M_n = 4913 g/mol, M_w = 7561 g/mol, PDI = 1.54.

Synthesis of 2nd generation dendrimer (4.17): To a mixture of **4.15** (100 mg, 0.065 mmol), 5-hexynoic acid (173 mL, 1.57 mmol) and DPTS (154 mg, 0.523 mmol) in DCM (4 mL), DCC (323 mg, 1.57 mmol) was added and the reaction mixture was stirred overnight at 40 ºC under N_2. Then, the reaction mixture was filtered through a short path of Celite and the solvent was evaporated. The mixture was purified by size exclusion chromatography (Sephadex LH-20, 1:1 DCM/MeOH) to afford **4.17** (180 mg, 91%) as a colourless oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 4.36–4.17 (m, 56H, H1'-G1, H1'-G2, $OCH_{2\ pentaerythritol}$), 2.47 (t, 32H, J=7.4 Hz, H2), 2.26 (td, 32H, J=7.4 Hz, 2.7 Hz, H4), 2.01 (t, 16H, J=2.6 Hz, H6), 1.83 (p, 32H, J=7.2 Hz, H3), 1.31 (s, 12H, H3'-G1), 1.26 (s, 24H, H3'-G2); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 172.4 (C=O), 172.0 (C=O), 171.6 (C=O), 83.2 (C5), 69.7 (C6), 65.0 (C1'), 64.9 (C1'), 46.8 (C2'-G1), 46.4 (C2'-G2), 32.6 (C2), 23.5 (C3), 17.8 (C3'-G1), 17.7 (C4), 17.5 (C3'-G2); LRMS (TOF ES$^+$) for $(M+Na)^+$ $C_{161}H_{204}NaO_{56}^+$ (*m/z*): calc. 3056.3; found 3057.7 $(M+Na)^+$, 1537.5 $(M+2Na)^{2+}$.

Synthesis of 2nd generation dendrimer M16 (4.19): To a solution of **4.17** (10 mg, 3.3×10^{-3} mmol) and glycoside **4.5** (20 mg, 7.9×10^{-2} mmol) in 1:1 THF/H_2O (1 mL) was added a solution of $CuSO_4\cdot5H_2O$ (6.6 mg, 2.6×10^{-2} mmol) and TBTA (28 mg, 5.3×10^{-2} mmol) in 1:1 THF/H_2O (1 mL). Finally, sodium ascorbate (21 mg, 1.06×10^{-1} mmol) was added and the reaction mixture was microwave irradiated at 60 ºC for 2 h. After that, the sample was gently stirred with QuadraSil MP and purified by size exclusion chromatography (Sephadex G-25, 9:1 H_2O/MeOH) to afford **4.19** (21.7 mg, 94%) as an white lyophilized. ^{1}H NMR (D_2O, 400 MHz) δ in ppm: 7.56 (s,

16H, H6), 4.50 (bs, 32H, H8), 4.26 (d, 16H, J=7.8 Hz, H1''), 4.26–4.05 (m, 72H, H7a, H1'-G1, H1'-G2, $OCH_{2\ pentaerythritol}$), 4.00–3.93 (m, 16H, H7b), 3.82 (d, 16H, H4''), 3.70–3.61 (m, 32H, H6''), 3.59–3.50 (m, 32H, H3'', H5''), 3.42 (t, 16H, J=8.8 Hz, H2''), 2.56 (t, 32H, J=7.4 Hz, H2), 2.29 (bs, 32H, H4), 1.79 (t, 32H, J=7.2 Hz, H3), 1.20 (s, 12H, H3'-G1), 1.14 (s, 24H, H3'-G2); ^{13}C NMR (D_2O, 100.6 MHz) δ in ppm: 174.6 (C=O), 173.3 (C=O), 172.5 (C=O), 147.1 (C5), 123.7 (C6), 103.0 (C1''), 75.1 (C5''), 72.7 (C3''), 70.6 (C2''), 68.5 (C4''), 68.0 (C7), 65.6 (C1'-G1, C1'-G2), 60.9 (C6''), 50.2 (C8), 46.8 (C2'-G1), 46.4 (C2'-G2), 38.7 (C2), 33.0 (C4), 24.0 (C3), 17.2 (C3'-G1), 16.9 (C3'-G2); LRMS (TOF ES^+) for $(M+H)^+$ $C_{288}H_{443}N_{48}O_{152}^+$ (*m/z*): calc. 7005.8; found 2363.7 $(M+2Na+K)^{3+}$, 2280.5 $(M–Gal+2Na+K)^{3+}$, 1778.4 $(M+3Na+K)^{4+}$, 2280.5 $(M–Gal+3Na+K)^4$.

Synthesis of 3rd generation dendrimer (4.18): To a mixture of **4.16** (200



mg, 0.059 mmol), 5-hexynoic acid (322 μL, 2.84 mmol) and DPTS (278 mg, 0.944 mmol) in DCM (6 mL), DCC (585 mg, 2.84 mmol) was added and the reaction mixture was stirred overnight at 40 ºC under N_2. Then, the reaction mixture was filtered through a short path of Celite and the solvent was evaporated. The mixture was purified by size exclusion chromatography (Sephadex LH-20, 1:1 DCM/MeOH) to afford **4.18** (330 mg, 87%) as a colorless oil. ^1H NMR ($CDCl_3$, 400 MHz) δ in ppm: 4.40–4.19 (m, 120H, H1'-G1, H1'-G2, H1'-G3, $OCH_{2\ Pentaerithritol}$), 2.48 (t, 64H, J=7.4 Hz, H2), 2.27 (td, 64H, J=7.1 Hz, 2.7 Hz, H4), 2.03 (t, 32H, J=2.7 Hz, H6), 1.84 (p, 64H, J=7.2 Hz, H3), 1.35 (s, 12H, H3'-G1), 1.29 (s, 24H, H3'-G2), 1.26 (s, 48H, H3'-G3); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 172.4 (C=O), 172.0 (C=O), 171.6 (C=O), 171.5 (C=O), 83.2 (C5), 69.5 (C6), 65.0 (C1'-G1, C1'-G2, C1'-G3), 46.7 (C2'-G1), 46.6 (C2'-G2),46.4 (C2'-G3), 32.6 (C2), 23.5 (C3), 17.8 (C3'-G3), 17.7 (C4), 17.6 (C3'-G2), 17.5 (C3'-G1); LRMS (TOF ES^+) for $(M+H)^+$ $C_{337}H_{429}O_{120}$ (*m/z*): calc. 6395.7; found 3222.2 $(M+2Na)^{2+}$.

Synthesis of 3rd generation dendrimer M32 (4.20): To a solution of **4.18** (12.4 mg, $1.93x10^{-3}$ mmol) and glycoside **4.5** (30.7 mg, $1.23x10^{-1}$ mmol) in 1:1 THF/H_2O (1 mL) was added a solution of $CuSO_4 \cdot 5H_2O$ (15.4 mg, $6.18x10^{-2}$ mmol) and TBTA (65 mg, $1.23x10^{-1}$ mmol) in 1:1 THF/H_2O (1 mL). Finally, sodium ascorbate (49 mg, $2.47x10^{-1}$ mmol) was added and the reaction mixture was microwave irradiated at 60 ºC for 2 h. After that, the sample was gently stirred with QuadraSil MP and purified by size exclusion chromatography (Sephadex G-25, 9:1 H_2O/MeOH) to afford **4.20** (24.5 mg, 88%) as an white lyophilized. ^{1}H NMR (D_2O, 400 MHz) δ in ppm: 7.74 (s, 32H, H6), 4.48 (bs, 64H, H8), 4.26 (d, 32H, J=7.8 Hz, H1''), 4.26–4.02 (m, 152H, H7a, H1'-G1, H1'-G2, H1'-G3, $OCH_{2\ pentaerythritol}$), 4.01–3.88 (m, 32H, H7b), 3.81 (d, 32H, J=3.4 Hz, H4''), 3.70–3.59 (m, 64H, H6''), 3.59–3.48 (m, 64H, H3'', H5''), 3.42 (t, 32H, J=8.8 Hz, H2''), 2.53 (bs, 64H, H2), 2.27 (bs, 32H, H4), 1.77 (bs, 64H, H3), 1.28 (s, 12H, H3'-G1), 1.19 (s, 24H, H3'-G2), 1.14 (s, 48H, H3'-G3); ^{13}C NMR (D_2O, 100.6 MHz) δ in ppm: 174.3 (C=O), 173.0 (C=O), 172.2 (C=O), 172.1 (C=O), 146.9 (C5), 123.7 (C6), 103.1 (C1''), 75.1 (C5''), 72.7 (C3''), 70.6 (C2''), 68.5 (C4''), 68.0 (C7), 65.4 (C1'-G3, C1'-G2, C1'-G1), 60.9 (C6''), 50.2 (C8), 46.8 (C2'-G1), 46.6 (C2'-G2), 46.4 (C2'-G3), 33.0 (C4), 24.1, 24.0 (C3,C2), 17.3, 17.1 (C3'-G1, C3'-G2, C3'-G3); MALDI–TOF MS for M^+ $C_{593}H_{908}N_{96}O_{312}^+$ (*m/z*): calc. 14365.8; found 3222.2 $(M+2Na)^{2+}$.

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CHAPTER 5

GLYCODENDRIPROTEIN-BASED NANOCAPSULES AS MULTIVALENT INHIBITORS OF CARBOHYDRATE-LECTIN INTERACTIONS

UNIVERSITY OF CAMBRIDGE

This work has been carried out in the laboratory of Dr. Gonçalo J. L. Bernardes at the University of Cambridge.

UNIVERSITAT ROVIRA I VIRGILI
SYNTHETIC GLYCOLIPIDS AS MODULATORS OF CARBOHYDRATEPROTEIN
INTERACTIONS
Míriam Salvadó Molero

5.1. INTRODUCTION TO GLYCODENDRIPROTEINS

In order to mimic Nature, synthetic multivalent systems are widely studied. As explained in Chapter 1, different central scaffolds have been studied to allow carbohydrate heat group presentation with different valences and spatial arrangement. Regarding the different families of compounds that have been used to develop multivalent glycoconjugates, glycodendrimers deserve special attention.

The word dendrimer is used to refer to a family of branched compounds with radial symmetry and monodisperse structures.[1] The first dendritic structure was reported by Fritz Vögtle[2] in 1978, and this field was developed quickly in the 1980s by Donald Tomalia.[3] They are monodisperse macromolecules containing symmetrically branched units built around either a polymer core or a small molecule.[4] Dendrimers are used as a platform to create multivalent systems; the end-groups can be easily functionalized with different molecules using synthetic strategies such as Diels-Alder, Click chemistry, and Michael reactions.[5]

Dendrimers have gained importance since emerging applications such as antibacterial agents, anticancer drugs[6] and imaging reports were found.[7,8] Due to their external sphere functionalization the hydrophilic surface make them water-soluble, while the hydrophobic core can encapsulate small molecules, either to enhance their solubility and to act as a carrier for drug delivery.[9,10]

Davis, B. G. in 2001, demonstrated that glycodendrons could mimic carbohydrates presents in glycoproteins and glycodendrons chemically attached to a protein in a specific place generating a new family of glycoconjugates called glycodendriproteins (protein platforms coated with glycan ligands). Due to the importance of protein glycosylation (see Chapter 1), access to well-defined scaffolds to obtain models for studying

glycoprotein interactions are necessary. In this sense, glycoproteins bearing branched multivalent glycans were constructed in order to study their binding interactions (Figure 5.1).[11]

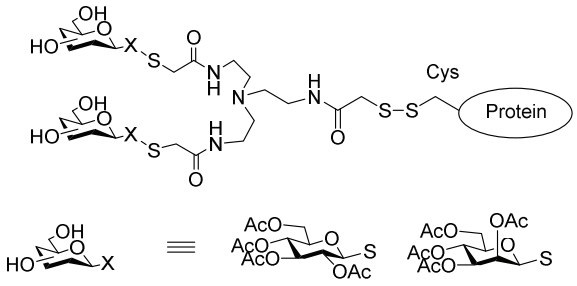


Figure 5.1. Site selective modification of subtilisin *Bacillus lentus* (SBL) (adapted from Davis, B. G.).[11]

In 2004, Davis and co-workers creates a new glycodendriprotein which was prepared by ligation of a chemically synthesized glycodendron and a protein-degrading enzyme (protease). The resulting glycodendriprotein was able to reduce the binding ability of pathogens (Figure 5.2).[12] This need to develop new strategies to inhibit infection was introduced derived from the continue ability of pathogens to resist different treatments.

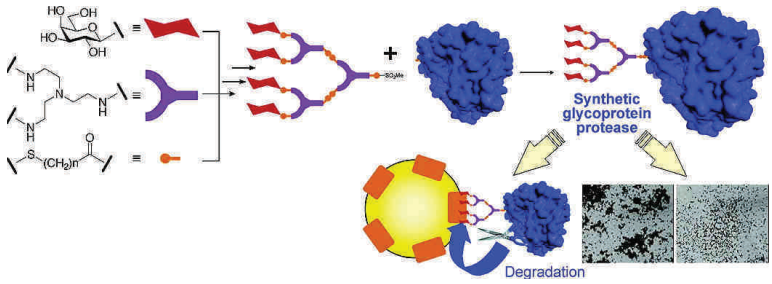


Figure 5.2. Examples of glycodendriproteins (adapted from Rendle, P. M. *et al.*)[12]

Moreover, glycodendriproteins were also constructed in the field of lectin-directed enzyme-activated prodrug therapy (LEAPT). LEAPT consist of a bipartite delivery system. A glycosylated enzyme together with a

prodrug are delivered to cells. This prodrug can also be cleaved by the delivered glycosylated enzyme. In this sense, Robinson *et al.* studied the construction of glycosylated enzymes, based on Naringinase (N) from *Penicillium decumbens* (Figure 5.3) together with the administration of a prodrug resulting in higher concentration of active drug through the use of glycosylated enzymes.[13]

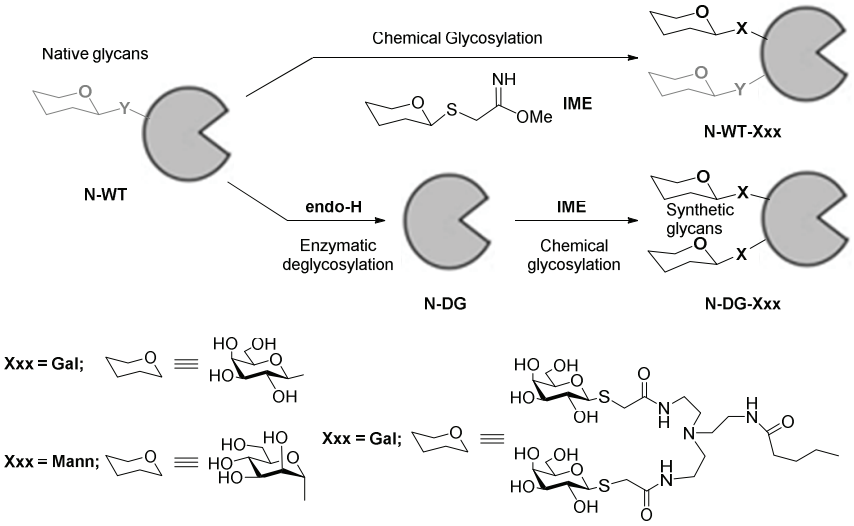


Figure 5.3. LEAPT strategy. Chemical glycosylation of N-WT and N-DG (deglycosylated N-WT enzyme) enzymes for the construction of a series of mixed glycodendriproteins (adapted from Robinson, M. A. *et al.*).[13]

Sato *et al.* found that a glycosylated (sialic acid) insulin using TGase served as a soluble long-term acting insulin for the treatment of type 1 and type 2 diabetes.[14] This sialic acid moiety protects insulin from decomposition. In an attempt to prolongate the insulin biological potency, they created a divalent- and trivalent sialyloligosaccharide-displaying insulin (Figure 5.4).[15] They created a new type of glycodendriprotein showing prolongated blood-glucose-lowering activity, due to the multivalent effect of the sialyloligosaccharides. They can stabilize insulin in the blood stream and lower the affinity for insulin receptors.

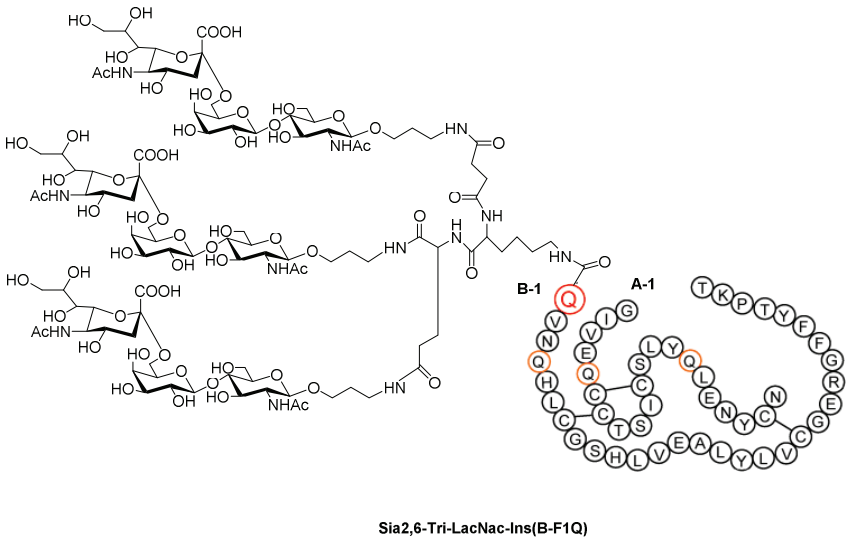


Figure 5.4. Trivalent-sialyloligosaccharide-displaying insulin (adapted from Sato, M. *et al.*).[15]

Whereas the strategy of the current examples is limited to display only a determined carbohydrate valency on a single protein, Ribero-Viana, R. *et al.* found that a high multivalent display of sugars could be achieved by a "nested polyvalency",[16] (Figure 5.5). This novel strategy allows the multivalent assembly of protein monomers carrying polyvalent glycodendrons. The synthetic virus-like glycodendrinanoparticles prevent mammalian cell infection in the nanomolar to picomolar range against Ebola pseudotyped virus.

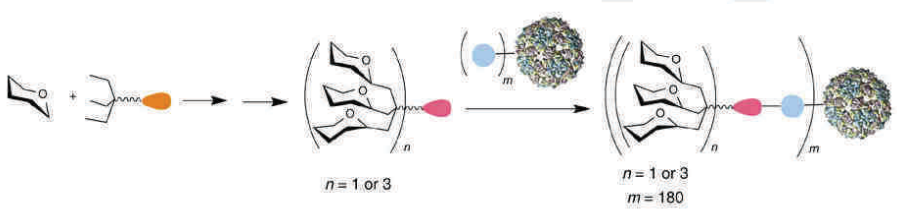


Figure 5.5. Nested polyvalency strategy of virus-like glycodendrinanoparticles (adapted from Ribeiro-Viana, R. *et al.*).[16]

This strategy allows presenting a high number of glycans in a homogeneous manner.

5.1.1. Bio-Layer Interferometry

Bio-layer Interferometry (BLI), is a label-free techniques, used to determine kinetic and affinity constant for protein interactions in real-time. BLI is an emerging tool for small molecules drug discovery. The most considered advantage of BLI compared to SPR is the daily high-throughput screening.[17]

BLI technique is based on the principles of optical interferometry. The instrument emits a white light to the biosensor and the light reflected back is collected. The wavelength of the reflected light that is collected is affected by the thickness on the biosensor.[18]

The shift between solid line (reference) and the dotted line (sample) is due to the changes in the thickness of the coating molecules on the optical layer. This shift is a direct measurement of the change in optical thickness of the biological layer. As more molecules bind to the surface the interferometric profile shifts further to the right, conversely, as molecules dissociate from the surface, it shifts back to the left to its initial position. The data output is a spectrum, captured by a spectrometer (Figure 5.6).

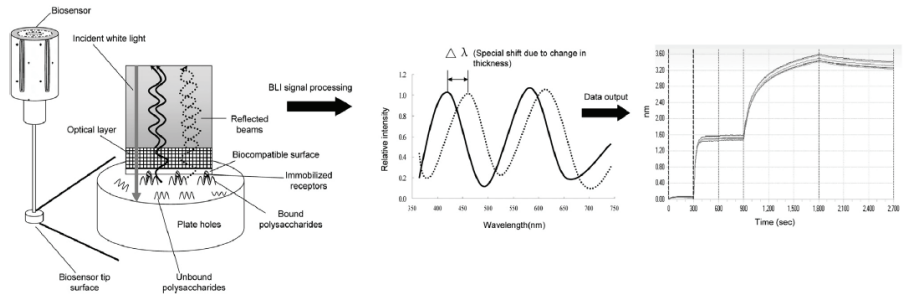


Figure 5.6. BLI-based biosensor technology, real-time binding measurement (adapted from Li, H. *et al.*).[19]

In summary, BLI is a technique that allows the measurement of a target-binding through the analysis of the white light reflected from an immobilized protein on the biosensor tip.

5.1.2. Analytical Ultracentrifugation (AUC)

Analytical ultracentrifugation (AUC) allows the characterization of molecular mass, shape and interactions of biological molecules in solution. Based on the principle property of mass and the laws of gravitation gave AUC a broad applicability. As during AUC analysis samples are characterized in their native state and experiments are performed in free solutions, it is a non-destructive technique.[20,21] When a centrifuge force is applied to a macromolecules solution, each macromolecule begins to settle at a certain velocity. The distance the boundary layer travels each time point correlates to the size, shape and mass of the sedimented macromolecule. The distribution of the different sized molecules in solution is determined by the shape of the boundary layer (Figure 5.7).

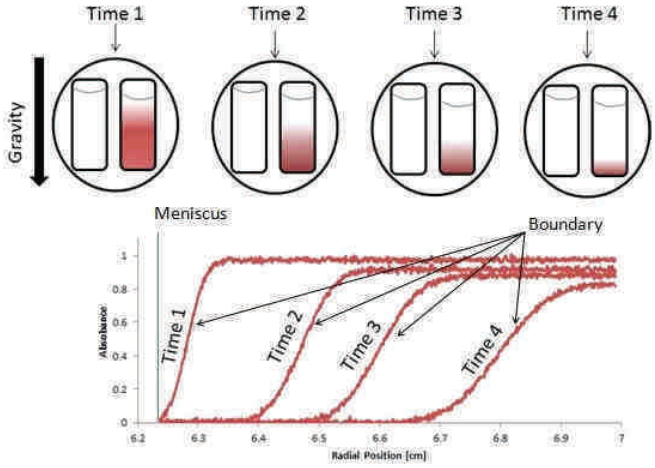


Figure 5.7. Evolution of a sample over time when subjected to centrifugation, cross section of a 2 sector AUC center piece, filled with a blank (left) and the macromolecule solution (right) at different times (adapted from Coriolis Pharma).[22]

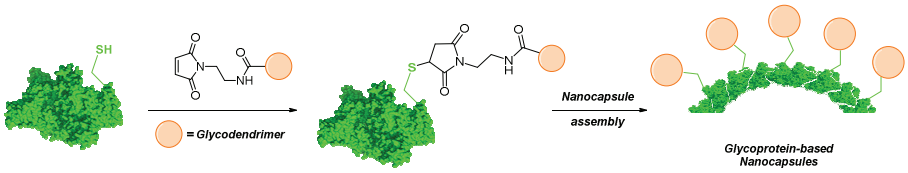
5.2. OUTLOOK AND OBJECTIVES

In order to explore how the different glycodendron architecture affects the binding properties of the glycodendriproteins, our group explored the development of more useful glycodendron systems derived from 3,5-bis and 3,4,5-tris(2-aminoethoxy)benzoic acid backbone. Similar structures have been previously prepared by Liskamp and co-workers[23] and are members of an easily variable class with significant rigidity and considerable distances[24] between the end groups, features that might be favourable for multivalent binding of biological receptors. With this background, in this chapter, our main goal is to prepare a series of glycodendriprotein-based nanocapsules that represents a promising novel strategy for the design of multivalent inhibitors of carbohydrate-lectin interactions with enhanced potency. In this context, the goals of this chapter are:

(i) To determine the binding interaction of a series of synthesized glycodendrons with different architectures based on gallic and pyrogallic scaffolds toward a model lectin (RCA_{120}) using Bio-layer interferometry (BLI), in order to determine the best carbohydrate substitution pattern (densities, conformation, shapes, etc.) and the best dendron scaffold (gallic or pyrogallic acid, or mixed gallic-pyrogallic architectures).

(ii) Once we know which structure is the one that gives the best binding affinities, our second objective is to synthesize the corresponding homogeneous glycodendriprotein. The synthetic glycodendriprotein would be created from the synthesized glycodendrons liked to a protein by using site-selective chemical modifications protocols. Importantly, such ligation strategies will be chosen to afford a stable protein-glycodendron linkage.

(iii) Glycodendriprotein-based nanocapsules would be obtained from those glycodendriproteins using different techniques. Ready access to such glycodendriprotein-based nanocapsules[25] represents a promising novel strategy for the design of multivalent inhibitors of carbohydrate-lectin interactions with enhanced potency. Finally, inhibition studies will be also performed using the synthesized glycodendriprotein-based nanocapsules.



5.3. RESULTS AND DISCUSSION

5.3.1. [G0]/[G1]-Glycodendron synthesis

The present study was focused in determining the binding affinity of different [G0]/[G1]-glycodendrons derived from gallic and/or pyrogallic acid central cores that present D-galactose/D-galabiose in different orientation and valences (Figure 5.8). The [G0]-glycodendrons were constructed both presenting D-galactose (**5.1**, **5.2** and **5.3**) and D-galabiose (**5.4** and **5.5**), whereas [G1]-glycodendrons were just synthesized using D-galactose as the presenting carbohydrate (**5.6**, **5.7**, **5.8** and **5.9**). The different glycodendrimers used in the present study were synthesized by Dr. Omar Boutureira and Dr. Isidro Cobo, for that reason instead of a detailed synthesis of these compounds, a brief summary of the synthetic strategy used to prepare the glycodendrons derivatives is presented in order to introduce them.

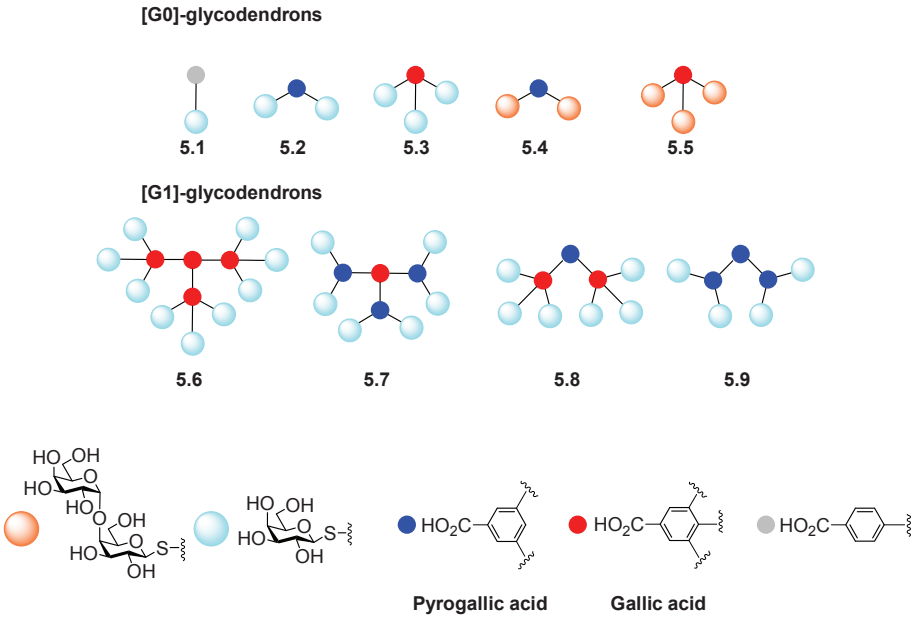
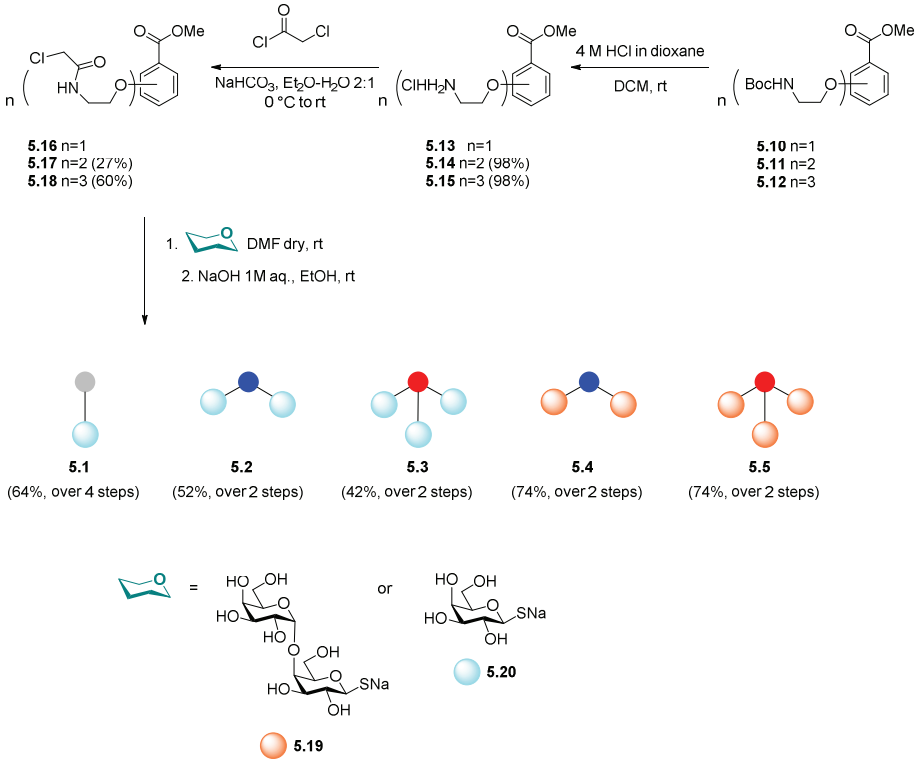
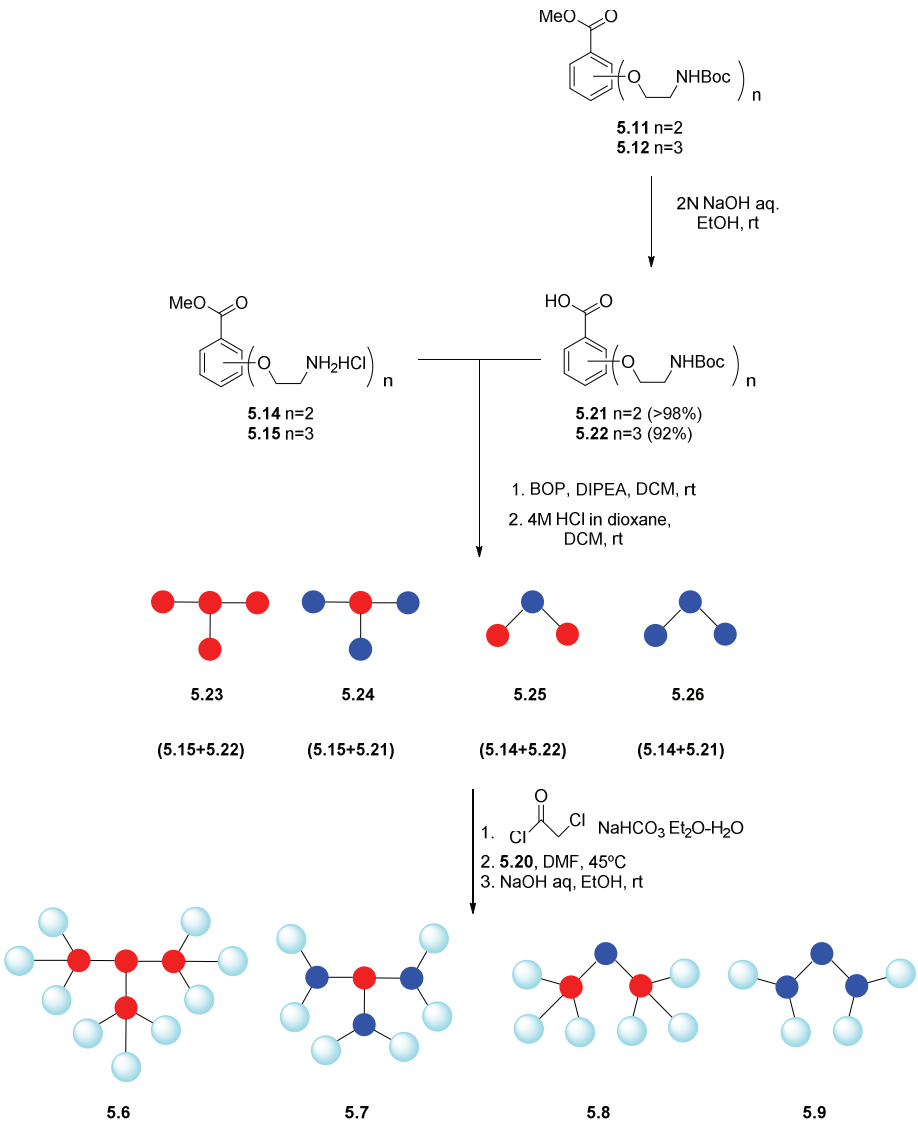


Figure 5.8. Schematic representation of [G0]/[G1]-glycodendrons

Methyl 4-hydroxybenzoate, methyl 3,5-dihydroxybenzoate (pyrogallic) and methyl 3,4,5-trihydroxybenzoate (gallic) were reacted with 2-(*tert*-butoxycarbonylamino)ethyl bromide to obtain **5.10**, **5.11** and **5.12**. The hydrochloride salts **5.13**, **5.14** and **5.15** were obtained in quantitative yield from the Boc group deprotection of benzoate monomers using 4M HCl in dioxane/DCM. Consecutively, salts without further purification were transformed into the *N*-chloroacetylated derivative using a biphasic system (Et_2O/H_2O) in the presence of chloroacetyl chloride and $NaHCO_3$ to form **5.16**, **5.17** and **5.18**. These prebuilt dendritic structures with *N*-chloroacetylated end groups where reacted with either 1-thio-β-D-galabiose **5.19** or 1-thio-β-D-galactopyranose **5.20** to obtain [G0]-glycodendrons **5.1**, **5.2**, **5.3**, **5.4** and **5.5** after ester hydrolysis (Scheme 5.1).



Scheme 5.1. Schematic synthesis to obtain [G0]-glycodendrons. *[G0]-Glycodendrons were prepared by Dr. Omar Boutureira and Dr. Isidro Cobo and they are the starting material for this project.*



Scheme 5.2. Schematic synthesis to obtain [G1]-glycodendrons. *[G1]-Glycodendrons were prepared by Dr. Omar Boutureira and Dr. Isidro Cobo and they are the starting material for this project.*

To increase the library of hypervalent glycoconjugates with varying carbohydrate densities, presentation and shapes, a divergent synthetic strategy was employed to obtain [G1]-glycodendrons (Scheme 5.2). Benzoic acid derivative **5.21** and **5.22** were synthesized from **5.11** and **5.12** using an

aqueous solution of 2 N NaOH.[26] Coupling these acids with the corresponding hydrochloride salts **5.14** or **5.15** using BOP coupling chemistry afforded first generation dendrons in good to moderate yields. With those compounds in hand, it was considered that [G1]-glycodendrons could be obtained from [G1]-cores following a protocol similar to that employed for [G0]-glycodendimers. After Boc deprotection (**5.23**, **5.24**, **5.25** and **5.26**), the free amino groups of the core were acylated with chloracetyl chloride. Then, chloroacylated cores were treated with an excess of sodium 1-thio-β-D-galactopyranose **5.20** to obtain the desired [G1]-glycodendrons (**5.6**, **5.7**, **5.8** and **5.9**).

To measure the binding affinity of the synthetic [G(n)]-glycodendrons towards a model carbohydrate-binding protein (RCA_{120}) Bio-layer interferometry was used. Various attempts to measure the direct binding of the different glycodendrons against RCA_{120} were carried out. Synthetic glycodendrons were immobilized in the biosensor using different conditions. Due to its low molecular weight the immobilization procedure could only be tested by checking the binding with the protein but no binding was observed. For that reason, indirect binding (competition experiments) was measured.

Briefly, 4-aminobutyl-β-galactopyranoside was attached to the biosensor tip surface by an amine coupling protocol. The indirect binding of **5.1** $(Gal)_1$, **5.2** $P(Gal)_2$, **5.3** $G(Gal)_3$, **5.4** $P(Galabiose)_2$, **5.5** $G(Galabiose)_3$, **5.6** $GG(Gal)_9$, **5.7** $GP(Gal)_6$, **5.8** $PG(Gal)_6$, and **5.9** $PP(Gal)_4$ at a concentration range in HBS-EP buffer towards RCA_{120} was measured.

5.3.2. Evaluation of the different carbohydrate presentation in [G0]-glycodendrons against RCA_{120}

The results obtained are shown in Table 5.1 and the inhibition curves are represented in Figure 5.9. When $P(Galabiose)_2$ (**5.4**) and $P(Gal)_2$ (**5.2**) were compared to the monomer, an enhancement of inhibition was found for $P(Gal)_2$ (**5.2**) analyte. Whereas, when central scaffolds presenting 3 carbohydrate residues were compared a 9.5-fold increase in inhibition potency was found in analyte $G(Galabiose)_3$ (**5.5**). As it was mentioned before, this results gave us another example of the importance of carbohydrate presentantion in binding studies.

Table 5.1. BLI Competitve assay for **5.1** $(Gal)_1$, **5.2** $P(Gal)_2$, **5.3** $G(Gal)_3$, **5.4** $P(Galabiose)_2$, **5.5** $G(Galabiose)_3$ against RCA_{120}.

Analyte	N	IC_{50}[a] (μM)	β[b]	β/N[c]
$(Gal)_1$ **(5.1)**	1	248.7 ± 1.73	1	1
$P(Gal)_2$ **(5.2)**	2	66.91 ± 1.16	3.72	1.86
$P(Galabiose)_2$ **(5.4)**	2	119.4 ± 1.16	2.08	1.04
$G(Gal)_3$ **(5.3)**	3	66.74 ± 1.17	3.72	1.24
$G(Galabiose)_3$ **(5.5)**	3	26.07 ± 1.21	9.54	3.18

[a]IC_{50} was obtained for the duplicates of each experiment with double referencing. [b]Enhancement value $\beta=IC_{50}$(monomer)/ IC_{50}(cluster), measured relative to $(Gal)_1$. [c]Relative enhancement per sugar unit β/N.

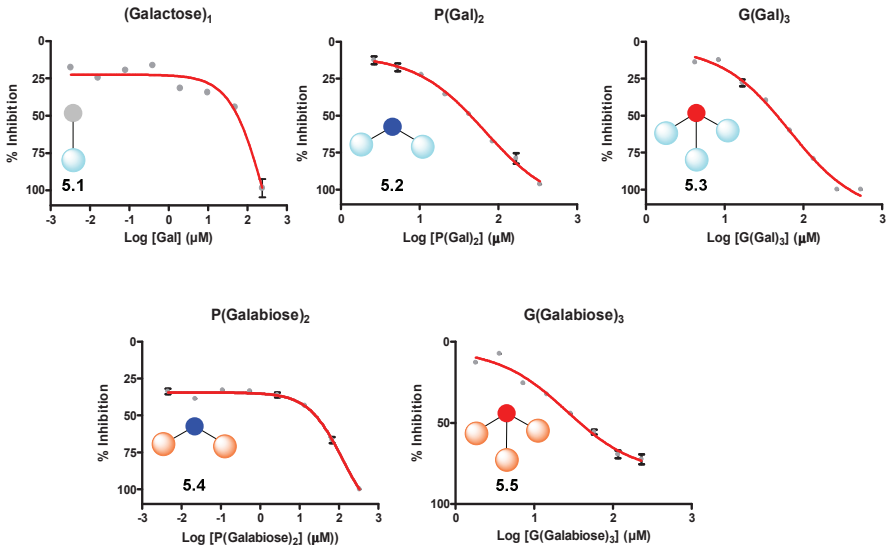


Figure 5.9. Dose-response curves of the competition assay between immobilized 4-aminobutyl-β-galactose and $(Gal)_1$ (**5.1**), $P(Gal)_2$ (**5.2**), $G(Gal)_3$ (**5.3**), $P(Galabiose)_2$ (**5.4**) and $G(Galabiose)_3$ (**5.5**) in solution for RCA_{120} binding. Statistical programm Prism (GraphPad, San Diego, CA, USA) was used for curve fitting and IC_{50} calculations. Non-linear fitting, to dose-response curve model was employed.

To discard that IC_{50} values could be affected by aggregate formation analytical ultracentrifugation (AUC) technique[27] was used to study the macromolecule complex formation. Hence, solutions of RCA_{120} with $P(Gal)_2$ (**5.2**), $P(Galabiose)_2$ (**5.4**), $G(Gal)_3$ (**5.3**) and $G(Galabiose)_3$ (**5.5**) were analysed and only one type of macromolecule complex showing minimal variability from RCA_{120} solution were detected. Thus, we can discard the binding/cross-linking of one glycodendron to more than one protein concluding that the protein binds to the analyte in a 1 to 1 fashion (Figure 5.10).

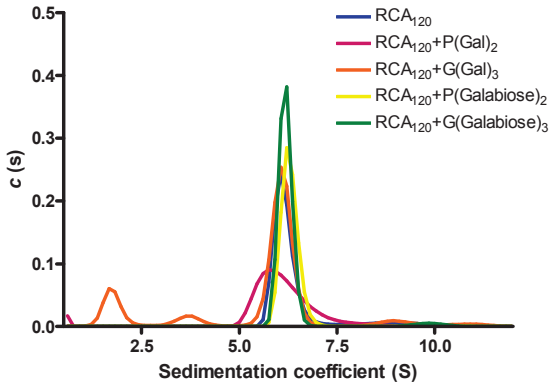


Figure 5.10. Sedimentation coefficient distribution of aggregates.

5.3.3. Evaluation of the different D-galactose presentation in [G0]/[G1]-glycodendrons against RCA_{120}

The results obtained are shown in Table 5.2 and the inhibition curves are represented in Figure 5.11. IC_{50} in the micromolar range were found for all glycodendrons tested. When $P(Gal)_2$ (**5.2**) and $G(Gal)_3$ (**5.3**) were compared with the monomer $(Gal)_1$ (**5.1**) 3.7-fold increase in inhibition was found in both cases. Thus, a 1.86 enhancement per sugar unit was found in $P(Gal)_2$ (**5.2**) and this value decrease due to the increase of sugar units in $G(Gal)_3$ (**5.3**). When four D-galactoses were presented to the protein a 4.4-enhance was observed with a 1.12-enhancement per sugar unit.

As it was mentioned, the central cores of the synthetic glycoclusters allow us to present D-galactose with different densities and shapes. Although the carbohydrate density could allow us to end up with a high ligand valence the carbohydrate presentation (shape) is somehow even more important for the binding to a protein (glycocluster effect). When comparing the same carbohydrate density, $GP(Gal)_6$ (**5.7**) and $PG(Gal)_6$ (**5.8**), 2.8-fold increase in inhibition was observed for $PG(Gal)_6$ (**5.8**) compared to (**5.7**) and a 3.7-fold compared with the monomer (**5.1**). This is a clear example that the same

carbohydrate density presented to the protein in different ways could affect its binding. Moreover, when the highest density glycoclusters $GG(Gal)_9$ (**5.6**) was tested, IC_{50} = 19.1 μM was obtained resulting in the more potent inhibitor.

With these results in hands, our next attempt was to increase the inhibitor effect using multivalent glycodendriproteins-based nanocapsules.

Table 5.2. BLI competitve assay for [Gn]-galactodendrons **5.1** $(Gal)_1$, **5.2** $P(Gal)_2$, **5.3** $G(Gal)_3$, **5.6** $GG(Gal)_9$, **5.7** $GP(Gal)_6$, **5.8** $PG(Gal)_6$ and **5.9** $PP(Gal)_4$ towards RCA_{120}.

Analyte	N	IC_{50}[a] (μM)	β[b]	β/N[c]
$(Gal)_1$ **(5.1)**	1	248.7 ± 1.73	1	1
$P(Gal)_2$ **(5.2)**	2	66.91 ± 1.16	3.72	1.86
$G(Gal)_3$ **(5.3)**	3	66.74 ± 1.17	3.72	1.24
$GG(Gal)_9$ **(5.6)**	9	19.11 ± 1.11	13.01	1.44
$GP(Gal)_6$ **(5.7)**	6	188.0 ± 1.86	1.32	0.22
$PG(Gal)_6$ **(5.8)**	6	65.88 ± 1.19	3.77	0.63
$PP(Gal)_4$ **(5.9)**	4	55.49 ± 1.17	4.48	1.12

[a]IC_{50} was obtained for the duplicates of each experiment with double referencing. [b]Enhancement value $\beta = IC_{50}(monomer)/ IC_{50}(cluster)$, mesured relative to $(Gal)_1$. [c]Relative enhacement per sugar unit β/N.

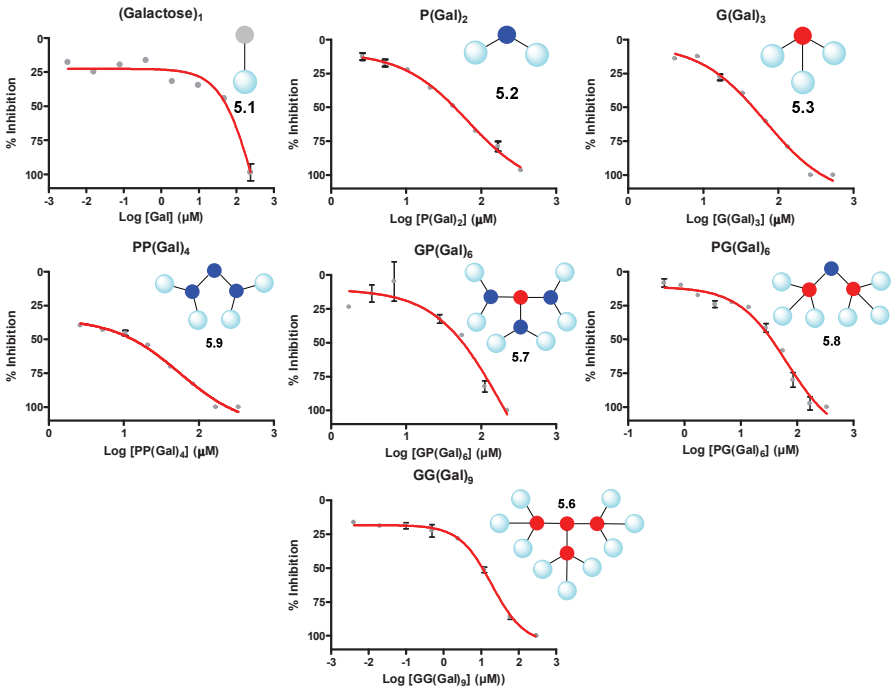
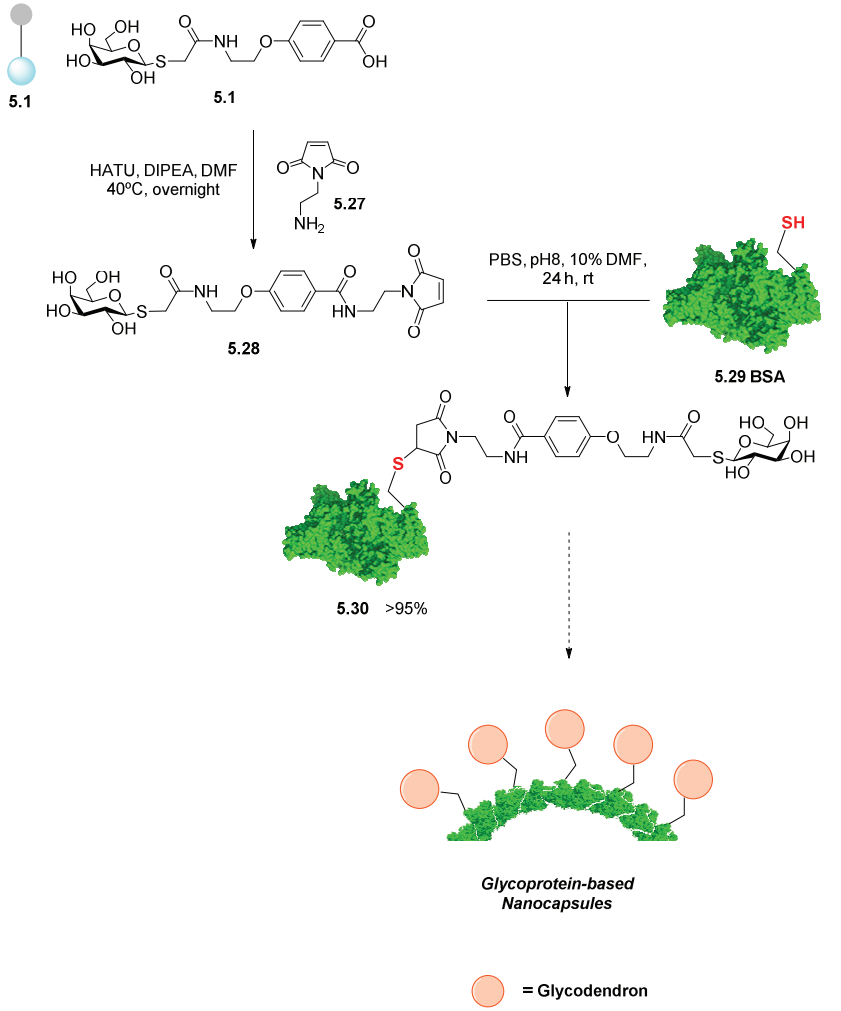


Figure 5.11. Dose-response curves of the competition assay between immobilized 4-aminobutyl-β-galactose and $(Gal)_1$ (5.1), $P(Gal)_2$ (5.2), $G(Gal)_3$ (5.3), $GG(Gal)_9$ (5.6), $GP(Gal)_6$ (5.7), $PG(Gal)_6$ (5.8) and $PP(Gal)_4$ (5.9), in solution for RCA_{120} binding. Statistical programm Prism (GraphPad, San Diego, CA, USA) was used for curve fitting and IC_{50} calculations. Non-linear fitting, to dose-response curve model was employed.

5.3.4. Protein Modification

In order to obtain the desired glycodendriprotein-based nanocapsules the reactivity of our glycodendrons with a selected protein (BSA) was initially studied. Site-selective protein modification is widely studied. The free cysteine residue of BSA (Cys^{34}) allows us to perform multiple chemical modifications.[28] Reaction of cysteine with electrophiles such as maleimides, are extensively studied due to its availability and easy use and synthesis.[29,30] BSA was also studied as a central scaffold for multivalent presentation.[31] Neo-glycoproteins based on BSA with the oligomers LacNAc-LacNAc and



Scheme 5.3. $(Gal)_1$ **5.1** coupling with maleimide and selective chemical protein modification of BSA.

LacDiNAc-LacNAc were synthesized and their binding to galectins were evaluated.[32] Moreover, BSA was also employed in the synthesis of BSA-NPs as drug carriers.[33] Thus, for its multiple benefits BSA was chosen as a model protein for constructing the desired glycodendriprotein-based nanocapsules.

Our first attempt was to focus on the study of the conjugation of monomer $(Gal)_1$-maleimide (**5.28**) with BSA (Scheme 5.3). $(Gal)_1$ (**5.1**) was reacted with maleimide (**5.27**) using HATU as the coupling reagent. BSA was subjected to reduction using DTT before conjugation[34] and free cysteine

was quantified using Ellman's method.[35] Reaction of **5.28** with BSA (**5.29**) in PBS buffer pH 8 and 10% DMF, used to ensure the complete solubility of reagents, afforded **5.30** in >95% conversion after 24 h at RT[36] as monitored by LC–MS.

5.4. CONCLUSIONS AND FUTURE PERSPECTIVES

In summary, a series of glycodendrons with different shape and carbohydrate density were evaluated against lectin RCA_{120} using Bio-layer Inerferometry (BLI) and IC_{50} values were obtained. BSA was functionalized at Cys[34]. This will provide a novel strategy for the designs of multivalent inhibitors based on glycodendriprotein-based nanocapsules with enhance potency. The following conclusions can be extracted:

(i) When comparing [G0]-glycodendrons presenting 2 or 3 D-galactose residues a 1.86 and 1.24-fold enhance in potency per sugar was found compared to the monomer ligand.

(ii) The best inhibition potency compared to monomer 5.1 was found when presenting 3 galabiose residues (**5.5**). A 3.18-fold enhancement per sugar unit was obtained.

(iii) The importance of sugar presentation was evidenced when comparing **5.7** and **5.8** [G1]-glycodendrons. The glycodendron that presented D-galactose in a Pyrogallic-Gallic fashion (**5.8**) results in a 3.7-fold enhance in inhibition.

(iv) The increase in carbohydrate density (**5.6**) results in a 13.0-fold enhance in inhibition compared to the monomer (**5.1**).

(v) AUC experiments reveals that no cross-linking occurred during the binding of [G0]-glycodendrons with RCA_{120} in solution.

(vi) BSA was modified with **5.28** glycodendrons at Cys34 using selective-chemical protein modification with a >95% conversion obtaining the corresponding sulphur-linked protein.

Further experiments are required to obtain the desired glycodendriprotein-based nanocapsules. Coupling of **5.28** with BSA (**5.29**) using different linkages are under investigation. BSA-NP formation based on the glycodendriprotein monomer and its inhibitor potency against RCA_{120} will be studied.

5.5. EXPERIMENTAL PART

General Remarks

Proton (^{1}H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance spectra were recorded on a Varian Mercury spectrometer (400 MHz for ^{1}H), (100.6 MHz for ^{13}C). Spectra were fully assigned using COSY, HSQC, HMBC and NOESY. All chemical shifts are quoted on the δ scale in ppm using either Me_3Si (^{1}H NMR: $CDCl_3$ = 0.00) or the residual solvent as internal standard (^{1}H NMR: D_2O = 4.79). Coupling constants (*J*) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet and app = apparent. Infrared (IR) spectra were recorded on a Jasco FT/IR-600 Plus ATR Specac Golden Gate spectrophotometer. Absorption maxima (νmax) are reported in wavenumbers (cm^{-1}). High resolution mass spectra (HRMS) were recorded on an Agilent 1100 Series LC/MSD mass spectrometer with electrospray ionization (ESI). Nominal and exact *m/z* values are reported in Daltons. Thin layer chromatography (TLC) was carried out using commercial aluminium backed sheets coated with 60F254 silica gel. Visualization of the silica plates was achieved using a UV lamp (λmax = 254 nm) and/or 6% H_2SO_4 in EtOH and/or 2% $PdCl_2$ and 15% H_2SO_4 in water. Flash column chromatography was carried out using C18 silica gel (32–63 μm). HPLC grade

dimethylformamide (DMF) were dried using a solvent purification system (Pure SOLV system-400). All reagents were used as received from commercial suppliers. All reactions using anhydrous conditions were performed using flame-dried apparatus under an atmosphere of argon. RCA_{120} was purchased from Sigma Aldrich, 4-aminobutyl-β-D-galactopyranoside was purchased from Carbosynth. All binding studies were performed at 25 ºC on an Octet Red96 system (*forté*BIO).

Protein Mass Spectrometry

Liquid chromatography-mass spectrometry (LC–MS) was performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 μm, 2.1 × 50 mm). Water with 0.1% formic acid (solvent A) and 70% acetonitrile and 29% water with 0.075% formic acid (solvent B), were used as the mobile phase at a flow rate of 0.2 mL min^{-1}. The gradient was programmed as follows: From 72% A to 100% B for 25 min then 100% B for 2 minutes and 72% A for 18 min. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V. Nitrogen was used as the desolvation gas at a total flow of 850 L hr^{-1}. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions.

$(Gal)_1$-maleimide (**5.28**): **5.1** (3.9 mg, 8.99 μmol) was diluted in dry DMF (250 μL), **5.27** (1.90 mg, 0.01 mmol), HATU (4.4 mg, 0.011 mmol) and DIPEA (3.9 μL) were added. The reaction mixture was stirred overnight at 40ºC. The solvent was then removed *in vacuo* and the crued was purified by flash column chromatography (C18, H_2O). Finally, lyophilization afforded **5.28** as a white solid in quantitative yield. R_f (EtOAc/MeOH/H_2O 7:2:1): 0.56; $[\alpha]_D^{20}$: –48.4 (0.01, H_2O); ^1H NMR (D_2O, 400 MHz) δ in ppm: 7.60 (d, J = 8.9 Hz, 2H,

Ar), 7.00 (d, J = 8.9 Hz, 2H, Ar), 6.79 (s, 2H, CH=CH), 4.27 (d, $J_{1,2}$ =9.8 Hz, 1H, H-1), 4.15 (t, J = 5.0 Hz, 2H, OCH_2), 3.73-3.51 (m, 7H, H-4, H-6a, H-6b, $2CH_2NH$), 3.45 (d, $J_{1,2}$ = 9.8 Hz, 1H, H-2), 3.37 (m, 1H, H-5), 3.21 (m, 3H, H-3, CH_2NH); ^{13}C NMR (D_2O, 100.6 MHz) δ in ppm: 172.8 (2C=O, Maleimide), 172.5, 170.2 (2C=O, NHCO), 161.1 (Ar), 134.2, 134.1 (C=C, Maleimide), 128.1, 126.0, 114.5, 109.9 (Ar), 84.6 (C-1), 78.9 (C-5), 73.5 (C-3), 69.3 (C-2), 68.4 (C-4), 66.5 (OCH_2), 60.8 (C-6), 37.8, 37.6, 37.0 ($2CH_2NH$, NCH_2), 32.7 (CH_2SGal); ATR-IR: 3327, 2982, 2696, 1698, 1402, 1251, 845, 767; HRMS (TOF ES^+) for $(M+H)^+$ $C_{23}H_{30}N_2O_{10}S^+$ (*m/z*): calc. 540.1646; found 540.1643.

5.5.1. Bio-layer Interferometry (BLI)

Binding assays were performed on an Octet Red Instrument (*forté*BIO). Black polypropylene 96-well microplates were used for ligand immobilization, binding reactions, regeneration and washes. 4-aminobutyl-β-galactopyranoside was immobilized in a concentration range (1000 µg/mL – 0.01 µg/mL) on amine-reactive biosensors (AR2G biosensors) in 10 mM NaAc pH 4.0 buffer, using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-hydroxysuccinimide for 5 min at 1000 rpm at 25ºC. All biosensors were subsequently modified by a solution of ethanolamine hydrochloride (1M, pH 8.5), followed by regeneration and wash. Preliminary studies reveled the best binding to RCA_{120} at 0.1 µg/mL for that reason the biosensor immobilized with 0.1 µg/mL was choosen to perform the competitive assays. For indirect binding measurement, solutions of **5.1** $(Gal)_1$, **5.2** $P(Gal)_2$, **5.3** $G(Gal)_3$, **5.4** $P(Galabiose)_2$, **5.5** $G(Galabiose)_3$, **5.6** $GG(Gal)_9$, **5.7** $GP(Gal)_6$, **5.8** $PG(Gal)_6$, and **5.9** $PP(Gal)_4$ at various concentrations in the range 239.07–0.003 µM, 337–2.63 µM, 536.9–4.19 µM, 332.6–0.004 µM, 231.9–1.80 µM, 298.7–0.004 µM, 223.89–1.75 µM, 337–0.43 µM, and 337–2.63 µM, respectively, were tested. 2 µL of RCA_{120} (1mg/mL) was added to 198 µL of analyte solution. Biosensor was washed with buffer (140 s) and placed for 3

minutes in the wells containing the analyte + RCA_{120} mixture, after a dissociation period in HBS-EP buffer (180 s) the biosensor was regenerated using 10mM Glycine-HCl buffer (pH 3.0). All the experiments were done in duplicates**.** Experimental data was corrected from artefacts by double referencing to a control biosensor and buffer injections using Data Analysis (*forté*BIO), with Savitzky-Golay filtering.

Using the data obtained for the indirect binding of RCA_{120} with the different studied Galacose-based ligands the percentatge of inhibition at different concentrations employed was calculated. This percentatge was ploted *vs.* the logarithm of the concentration for all different ligands tested.

5.5.2. Analytical Ultracentrifugation (AUC)

Analytical Ultracentrifugation experiments were performed on a BeckmanCoulter Optima XL–1 instrument. RCA_{120} was subjected to a buffer exchange with HBS-EP buffer using dialisis at 4 ºC overnight. Sedimentation velocity experiments were performed at an angular velocity of 4000 rpm, at 20 ºC for 19 h. All experiments were carried out at 20 ºC.

5.5.3. Protein Modification

Sequence of Bovine Serum Albumin (BSA) (PDB code: 4F5S)

DTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELTE
FAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQ
EPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEI
ARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREK
VLTSSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLV
TDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKP
LLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSF
LYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKL
KHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTRKVPQVSTPTLV

EVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEK
VTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTE
KQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEA
CFAVEGPKLVVSTQTALA

Calculated average isotopic mass = 66462.98

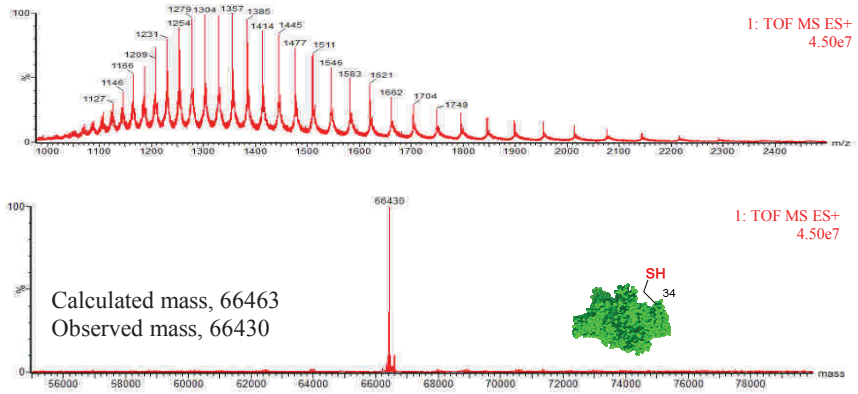
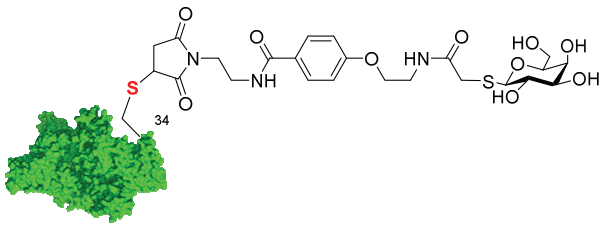


Figure 5.12. ESI–MS spectrum of BSA.

Bovine Serum Albumin-(Gal)$_1$-Maleimide (5.30): BSA was prepared as



1mg/ml in solution in PBS buffer (pH 8.0). To 900 µL of BSA **5.29** solution, **5.28** in 100 µL DMF was added and the reaction was vortexed for a few seconds. After 24 h of additional shaking at room temperature a 2 µL aliquot diluted with 8 µL of the same buffer was analyzed directly by LC–MS and complete conversion was observed (calculated mass, 66971; observed mass, 66966). The sample was then concentrated and purified by ultrafiltration, frozen with liquid nitrogen and stored at –80ºC.

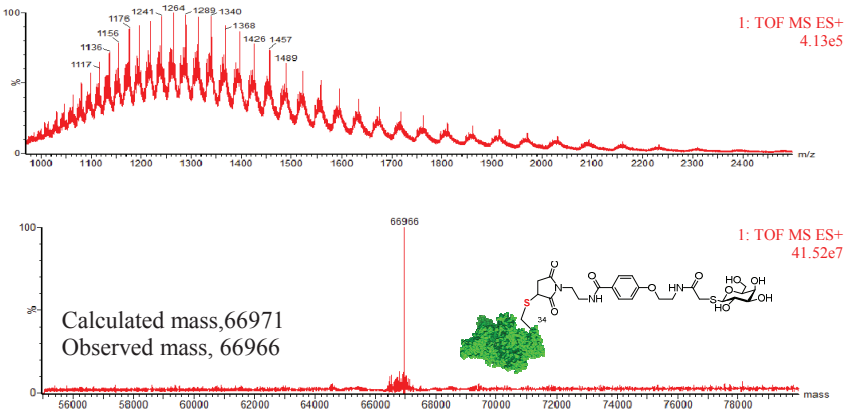


Figure 5.13. ESI–MS spectrum of **5.30**.

6.6. REFERENCES

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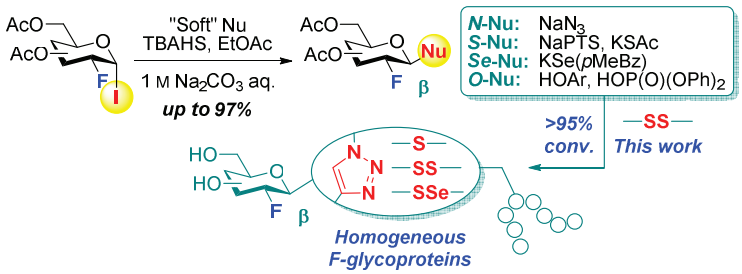
CHAPTER 6

SYNTHESIS OF FLUOROSUGAR REAGENTS FOR THE CONSTRUCTION OF WELL-DEFINED FLUOROGLYCOPROTEINS

2-Deoxy-2-fluoroglycosyl iodides are privileged glycosyl donors for the stereoselective preparation of β-fluorosugars, which are useful reagents for chemical site-selective protein glycosylation. Ready access to β-fluorosugars enables the mild and efficient construction of well-defined fluoroglycoproteins.



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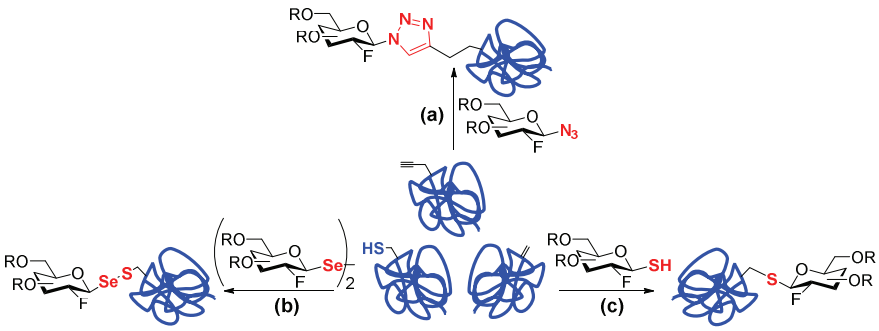
6.1. INTRODUCTION

The incorporation of fluorine into glycoconjugates allows for the simultaneous modulation of electronic, lipophilic and steric parameters, all of which can influence their biological function. The van der Waals radius of fluorine (1.47 Å) falls between that of oxygen (1.52 Å) and that of hydrogen (1.20 Å), making it a versatile element for bioisosteric replacement. The C–F moiety can be involved in polar interactions with strongly positively charged polarized centers, such as lateral chains of basic amino acids. Moreover, F is the smallest substituent that can replace H with minimal structural disruption. F-glycopeptides[1] and the more recently disclosed F-glycoproteins[2] are promising candidates for the preparation of synthetic carbohydrate vaccines[3] and hold great potential as a new generation of [^{18}F]-(glyco)radiopharmaceuticals[4,5] and tracers for the non-invasive imaging techniques positron emission tomography (^{18}F-PET)[6,7] and magnetic resonance/magnetic resonance imaging (^{19}F-NMR/MRI).[8] In addition to the development of more efficient methods for protein/peptide modification,[9] it is also pivotal to access pure F-sugar reagents and building blocks for such transformations.

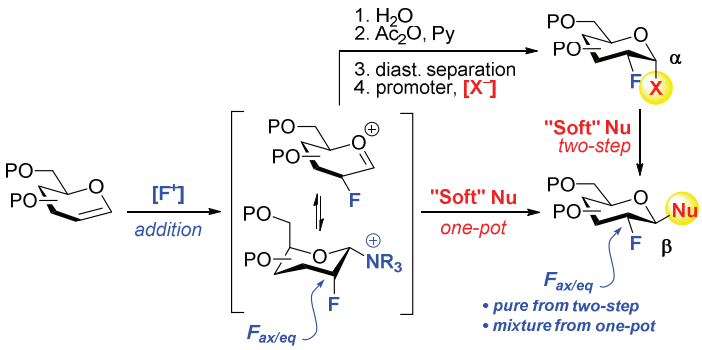
These glycosyl units typically include in their structure reactive handles such as azides (for copper(I) – catalyzed azide – alkyne cycloaddition – CuAAC and Staudinger ligations)[2h] or chalcogens in the form of sulfhydryl groups or diselenide moieties (for exchange reactions with Cys or additions to dehydroalanine),[2e,f] which upon reaction generate a new carbohydrate-protein conjugate (Scheme 6.1).



Scheme 6.1. Chemical protein modification with Fsugars. (a) Cu(I)-promoted azide-alkyne cycloaddition (CuAAC), (b) SeS formation and (c) reaction of dehydroalanine (Dha) with thiols.

Retrosynthetic analysis revealed that 2-F-glycosyl halides are suitable glycosyl donors for the preparation of such reagents *via* biphasic glycosylations/phase-transfer catalyzed reactions (PTC). These moieties enable exquisite control of β-anomeric selectivity (*via* S_N2-like mechanism) during the introduction of the reactive handle, which is typically a "soft" nucleophilic moiety.[10] There are two general strategies for the preparation of 1-β-"soft Nu"-2-deoxy-2-fluoroglycosides using electrophilic F^+ reagents (Scheme 6.2)[11]; the first one is a "one-pot" strategy from glycals while the other one is a two-step reaction from 2-F-pyranoses.

While the one-pot strategy works well for certain configurations (*e.g.* Gal) and nucleophiles (Nu), the two-step approach is more general and thus preferred for the preparation of pure F-sugar reagents in a more efficient and homogeneous manner (giving a better overall yield and controlled $F_{ax/eq}$ diastereoselectivity).



Scheme 6.2. General strategies for the preparation of 1-β-"soft Nu"-2-deoxy-2-fluoroglycosides using F^+.

Among glycosyl halides, iodides[12] have been recently utilized in several glycosylation strategies and provide a robust platform for PTC reactions, perhaps leading to higher yields compared to chlorides or bromides due to their superior leaving group properties under S_N2-like reaction conditions. There are very few examples of 2-F- and 2,2-di-F-glycosyl iodides. This rare class of glycosyl donors include some isolated examples of 2-F- and 2,2-diF-pyranosides[13] and our 2-F-galactopyranosyl iodide and more recently 2,2-diF-furanosides[14] (Figure 6.1).

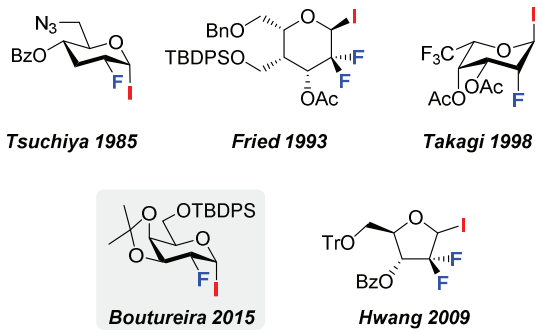


Figure 6.1. Examples of 2-deoxy-2-fluoro and 2-deoxy-2,2-difluoroglycosyl iodides.

Despite their great potential, low preparative yields in the pyranose series and inseparable α/β mixtures in the case of furanoses has so far hampered their wide utilization as β-selective glycosyl donors in PTC protocols.

6.2. OUTLOOK AND OBJECTIVES

With this background, in this chapter, we envisioned a "two-step" strategy for the preparation of 2-F-glycosyl iodides from ready available 1-*O*-Ac that may overcome such limitations enabling a more efficient preparation of this class of highly reactive glycosyl donors. We then sought to explore their reactivity for the preparation of fluorosugar reagents that are useful moieties to achieve site-selective chemical glycosylation of proteins. In this context, the goals of this chapter are:

i) To optimize the halogenation conditions to obtain 2-F-galactopyranose halides from the corresponding 1-*O*-Ac derivatives and expand the scope to other configurations (*Glc* and *Man*).

ii) To study the reactivity of the obtained 2-F-glycopyranose halides with "soft Nucleophiles" (*O*-, *N*-, *S*- and *Se*-nucleophiles) to obtain 1-β-"soft Nu"-2-deoxy-2-fluoroglycosides employing phase-transfer catalysed reactions (PTC).

iii) To explore the reactivity of some F-sugar derivatives with a model single-Cys protein (Annexin V) to provide an useful platform to disulfide-linked Fglycoproteins.

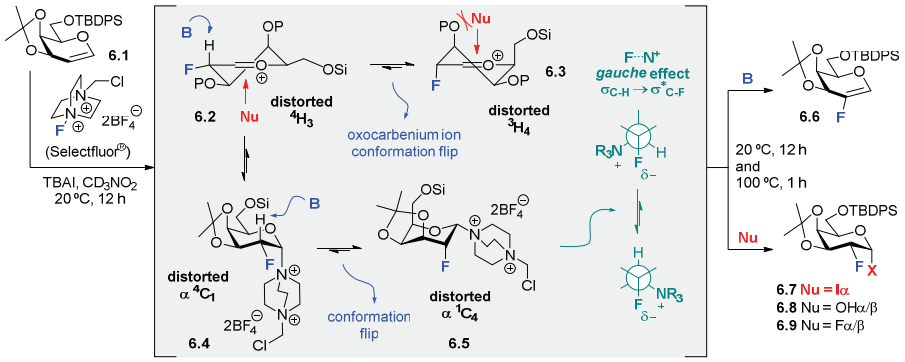
6.3. RESULTS AND DISCUSSION

6.3.1. Synthesis of 2-F-α-glycopyranosyl halides *via* one-pot reaction

As it has been mentioned before, the preparation of 2-F-glycopyranosyl halides are crucial for the preparation of glycosyl units to further obtain a new F-carbohydrate-protein conjugate.

Preliminary studies *via* "one-pot" strategy from glycals were performed using Selectfluor® as the fluorinating agent and TBAI to obtain the desired 2-F-galactopyranosyl iodide (Scheme 6.3).

The analysis of the signals obtained in the ^{1}H and ^{19}F NMR spectra were tentatively assigned to compounds **6.6**, **6.7**, **6.8** and **6.9**. Despite an exhaustive conformational study has not been performed, their hypothetic conformations are shown in Scheme 6.3.



Scheme 6.3. Preliminary "one-pot" synthesis of 2-dexy-2-fluoroglycopyranosyl iodides.

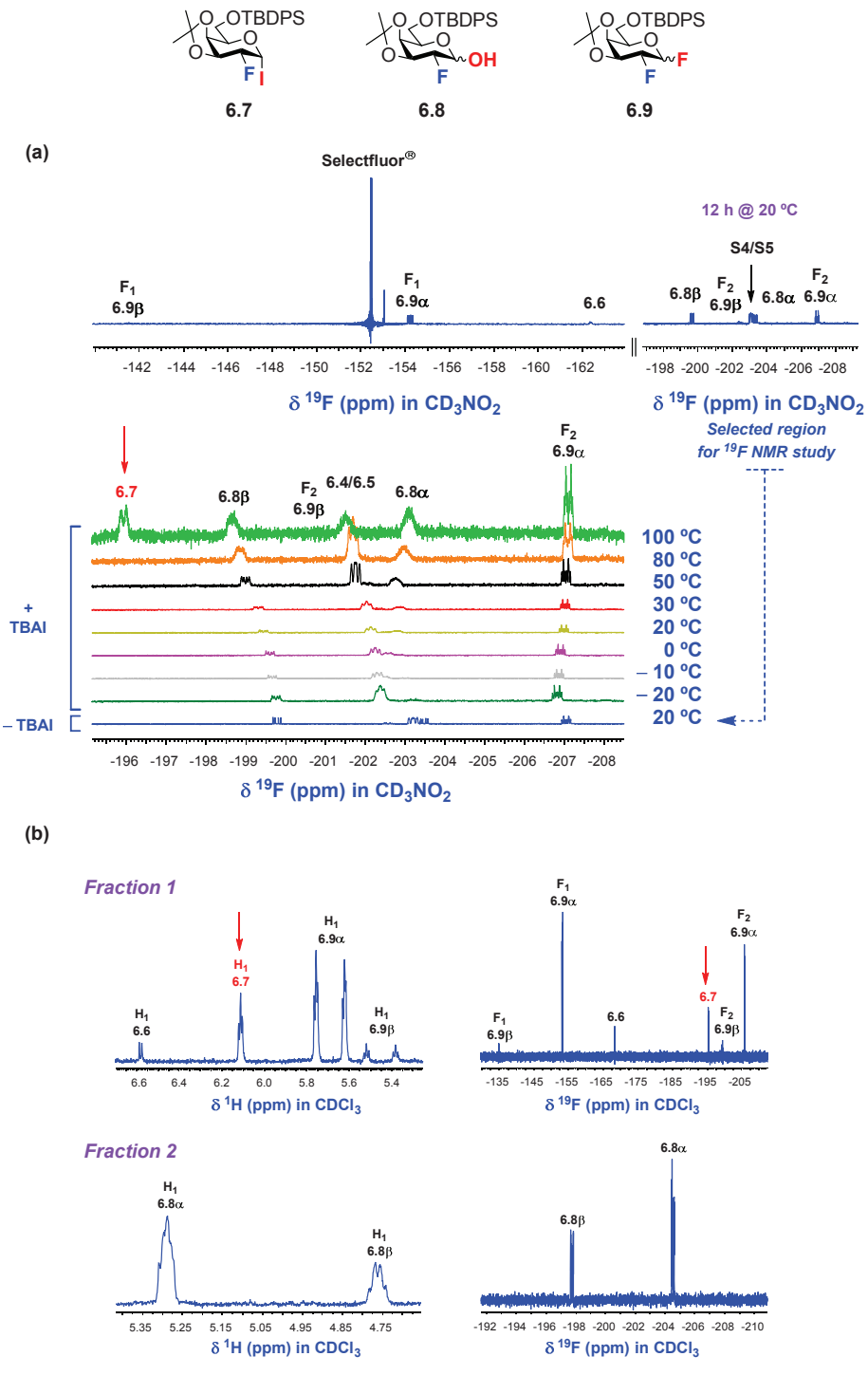
Two different conformations, a distorted $^{4}H_3$ (**6.2**) and a distorted $^{3}H_4$ (**6.3**), could be formed after the attack of the glycal to the electrophilic fluorine. The fluorine-axial conformation **6.3** is likely to be more stable than the fluorine equatorial conformer **6.2** due to stabilizing hyperconjugative

interactions between σ_{C-F} and π^*_{C-O} of the oxocarbenium. The attack of the nucleophile to finally obtain a chair conformation (more stable intermediate than a boat conformation) has to be performed from the bottom face of the oxocarbenium **6.2** or from the top face of the oxocarbenium **6.3**. In this last conformation the attack of the nucleophile is hindered due to steric effects. Moreover, $^4H_3$ conformation (**6.2**) can undergo E1 elimination to form the corresponding 2-F-glycal. In an E1 elimination the new double bond can only be formed if the vacant *p* orbital of the carbocation of the breaking C–H bond have a parallel alignment.

After nucleophilic attack, a distorted $^4C_1$ (**6.4**) in equilibrium with a distorted $^1C_4$ (**6.5**) conformations were observed. The distorted α-$^4C_1$ conformation (**6.4**) can undergo an E2 by a base-proton abstraction and the subsequent elimination of the group in an anti position to afford the corresponding 2-F-glycal. This distorted α-$^4C_1$ (**6.4**) is in equilibrium with the more stable $^1C_4$ conformation (**6.5**) due to the gauche effect, due to hyperconjugative interaction between the σ_{C-F} and the σ^*_{C-F}. This occurs when the fluorine atom and the hydrogen atom are both in axial position.

In order to confirm the different by-products obtained formed when the glycal **6.1** was treated with Selectfluor® and TBAI during 12h at 20ºC, VT-NMR experiments were performed (Scheme 6.4a).[15]

After 12h reaction with Selectfluor® at 20 ºC it was observed in ^{19}F NMR, signals corresponding to 2-F-glycal **6.6**, hydrolyzed product **6.8** and the fluorine signals corresponding to compound **6.9** (**α**/**β**). In order to study the evolution of the intermediates and the formation of the desired iodinated product **6.9** *via* ^{19}F NMR, TBAI was added to the NMR tube and a variable temperature NMR experiment was performed from –20 to 100 ºC. It was observed that signals corresponding to intermediates **6.4** and **6.5**, disappeared, whereas signals corresponding to **6.7** appeared just when the temperature reached 100 ºC. When the reaction mixture was purified, two



Scheme 6.4. Preliminary "one-pot" (a) VT-NMR experiments and (b) ^{1}H and ^{19}F NMR spectra of the fractions obtained.

different fractions were obtained. The desired 2-F-galactosyl iodide **6.7**, together with 2-F-galactal **6.6** and 2-F-galacto fluorine **6.9α/β** were obtained in the first fraction. Whereas hydrolyzed 2-F-galactose **6.8α/β** was obtained in the second fraction.

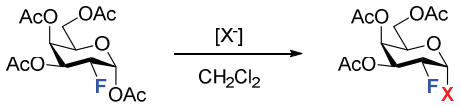
As this "one-pot" strategy from glycals was not successful enough in our case, we decided to change the strategy to obtain 2-F-glycopyranosyl halides *via* the two-step reaction from 2-F-pyranoses.

6.3.2. Synthesis of 2-F-α-glycopyranosyl halides *via* two-step reaction

We first evaluated the reaction conditions using 2-F-α/β-galactopyranose **6.10** (Table 6.1). Initial experiments revealed TMSI[16] as an effective promoter for the preparation of glycosyl iodide **6.11a** although the reaction was completed (57%) after two consecutive reaction cycles (Table 6.1, entry 1).

We found that addition of MgO[17] that acts as a scavenger for the *in situ* generated TMSOAc avoids the reversible formation of starting **6.10** while increasing the yields up to 78% (Table 6.1, entry 2). Good yields (up to 85% in a single reaction cycle) were finally obtained by increasing the amount of both TMSI (2.2 equiv.) and MgO (4 equiv.) as well as reducing the reaction temperature (from reflux to RT) in order to avoid detrimental side reactions (Table 6.1, entry 3). Other promoters were also evaluated (Table 6.1, entries 4–7). While the use of HMDS/I_2 proved ineffective and complex mixtures of products were observed (Table 6.1, entry 7),[18] the *in situ* formation of highly reactive HI by refluxing AcSH and I_2 allowed the formation of desired iodide **6.11a** albeit in lower yields compared to TMSI (up to 54%) (Table 6.1, entries 4–6).[19] Interestingly, the use of MgO was found costly (24%) when using AcSH and I_2 propably due to its incompatibility with AcSH and/or HI (Table 6.1, entry 4). 2-F-galactosyl bromide **6.11b** was also prepared following a reported procedure in 95% yield (Table 6.1, entry 8).[20]

Table 6.1. Optimization of reaction conditions for the halogenation of **6.10**.[a]



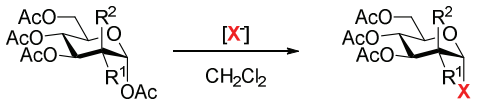
6.10 **6.11a** X=I **6.11b** X=Br

Entry	Conditions (equiv)	Product	Yield (%)
1	TMSI (1), RT, 8 h	**6.11a**	57[b]
2	TMSI (1.1), MgO (2), reflux, 12 h	**6.11a**	78[b]
3	TMSI (2.2), MgO (4), RT, 24 h	**6.11a**	85
4	AcSH (1.7), I_2 (0.8), MgO (2), reflux, 30 h	**6.11a**	24[b]
5	AcSH (4.4), I_2 (2), reflux, 12 h	**6.11a**	34[b]
6	AcSH (2.2), I_2 (1), reflux, 12 h	**6.11a**	54[b]
7	HMDS (0.6), I_2 (0.6), RT, 24 h	**6.11a**	mixture
8	33% HBr in AcOH, RT, 8 h	**6.11b**	95

[a]*General conditions*: 2-F-α/β-galactopyranose **6.10** (2:1 α/β) (1 equiv.) in dry CH_2Cl_2 (0.1 M) unless otherwise indicated. [b]Isolated yield after two consecutive reaction cycles. TMS=trimethylsilyl, HMDS=hexamethyldisilazane.

With the optimized conditions in hand we set to evaluate the scope of the iodination with other configurations (Table 6.2).

Table 6.2. Scope of halogenation.[a]



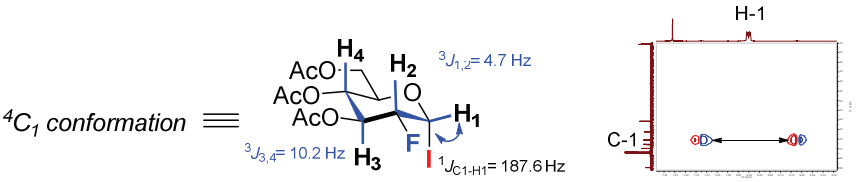
6.12 R^1=F, R^2=H (*Glc*)
6.13 R^1=H, R^2=F (*Man*)

6.14a R^1=F, R^2=H, X=I; **6.14b** X=Br
6.15a R^1=H, R^2=F, X=I; **6.15b** X=Br

Entry	2-F-Pyranose	Conditions	Product	Yield (%)
1	**6.12**	A	**6.14a**	38[b]
2	**6.12**	B	**6.14a**	82[b]
3	**6.12**	C	**6.14b**	98
4	**6.13**	A	**6.15a**	50[b]
5	**6.13**	B	**6.15a**	75[b]
6	**6.13**	C	**6.15b**	94

[a]*General conditions*: 2-F-α/β-pyranose (1:0–1:3 α/β) (1 equiv.) in dry CH_2Cl_2 (0.1 M) unless otherwise indicated. [b]Isolated yield after two consecutive reaction cycles. Conditions A: TMSI (2.2 equiv.), MgO (4 equiv.), RT, 30 h. Conditions B: AcSH (2.2), I_2 (1), reflux, 5 h. Conditions C: 33% HBr in AcOH, RT, 3–8 h. TMS=trimethylsilyl.

Conditions A (with TMSI) where utilized with 2-F-α/β-pyranoses of *Glc* **6.12** and *Man* **6.13** configuration affording desired glycosyl iodides **6.14a** and **6.15a** in moderate yields (Table 6.2, entries 1 and 4). To our delight, conditions B (with AcSH and I_2) improved the yield to 82% for **6.14a** and 75% for 6.15a (Table 6.2, entries 2 and 5). 2-F-glycosyl bromides **6.14b** and **6.15b** were also prepared in excellent yields (up to 98%) using the standard conditions (Table 6.2, entries 3 and 6). Practical yields of the corresponding α-glycosyl iodides as sole anomers (*e.g.* $^1J_{C1,H1}$ = 187.6 Hz, $^3J_{1,2}$ = 4.7 Hz and $^3J_{3,4}$ = 10.2 Hz in **6.14a** – $^4C_1$ conformation, Figure 6.2)[21] were obtained regardless of the pyranoside configuration (*Gal*, *Glc* and *Man*).



6.14a

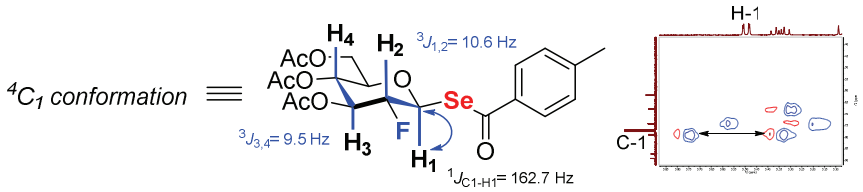
Figure 6.2. Conformational analysis of glycosyl iodide **6.14a**.

2-F-glycosyl iodides proved stable and were purified by column chromatography with no decomposition observed. They are also UV visible (at 254 nm) providing an easy experimental follow-up. Similar to other protocols that use strong acid activators (*e.g.* HBr), base sensitive acetyl groups are the more appropriate protecting groups for the overall synthetic route (Selectfluor®–halogenation–PTC–deprotection). Acetyl protecting groups are easily introduced, prove stable under the sequence of transformations and are easily removed under Zemplén conditions to afford the final unprotected and ready-to-conjugate fluorosugar reagents. Other acid sensitive groups such as *tert*-butyldiphenylsilyl (TBDPSi) were not tolerated under the optimized acid-based reaction conditions and extensive deprotection was observed.

6.3.3. Preparation of 1-β-"soft Nu"-2-deoxy-2-fluoroglycosides employing phase-transfer catalyzed reactions (PTC)

Next, the synthesis of 1-β-"soft Nu"-2-deoxy-2-fluoroglycosides was attempted using 2-F-glycosyl halides **6.11a,b** and **6.14–6.15a,b** (Scheme 6.5). A wide range of *N*-, *S*- and *Se*-nucleophiles leading to β-fluorosugar reagents **6.16–6.21** used in chemical protein glycosylation strategies[2e-2h] were evaluated, even including those with challenging 1-β-*cis*-Man[22] disposition **6.18a–c**. Excellent yields (up to 98%) and pure β-selectivity ($^1J_{C1,H1}$ = 162.7 Hz, $^3J_{1,2}$ = 10.6 Hz and $^3J_{3,4}$ = 9.5 Hz in **6.17c** – $^4C_1$ conformation, Figure 6.3)[21] were always obtained. Iodides proved slightly better in terms of yield

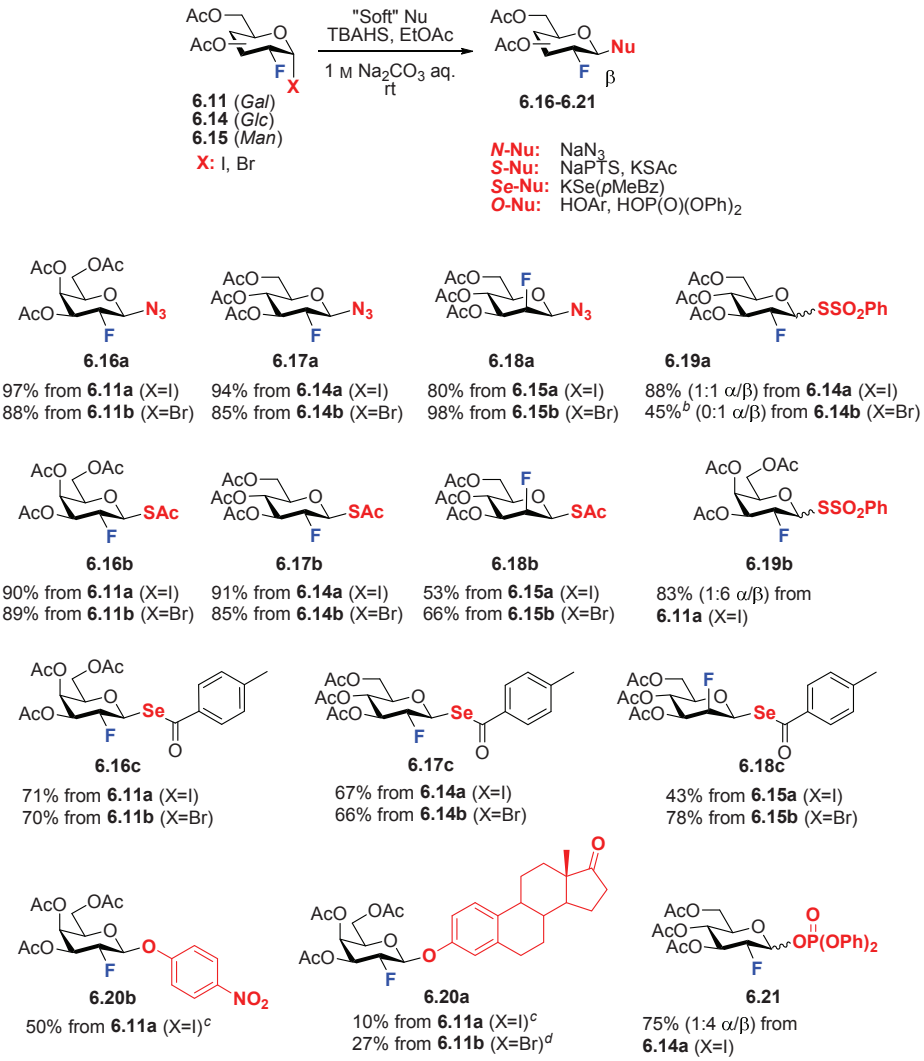
(*ca.* 10–15%) over their bromide counterparts using a better Nu in a "match" scenario of reactivity with the exception of mannosyl iodides. We believe the low reactivity of mannosyl iodides to be probably due to disfavored dipolar interactions between the donor and the incoming Nu and/or conformational issues that reduced the reaction rate and enabled the operation of otherwise negligible side reactions that reduced the overall yield.[23]



6.17c

Figure 6.3. Conformational analysis of glycosyl selenide **6.17c**.

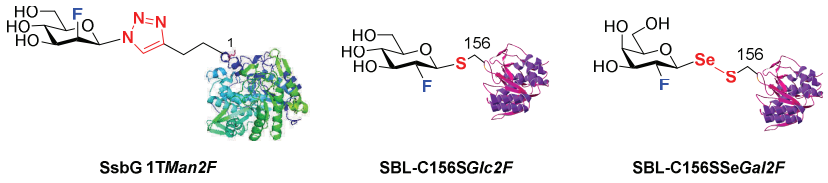
Weaker Nu such as sodium benzenethiosulfonate (NaPTS)[24] necessitated the more reactive iodides to afford moderate-to-good yields (88% for I *vs.* 45% for Br in **6.19a**). Our data indicates that 2-F-glycosyl iodides are needed when using weaker nucleophiles. The reported 2-F-glycosyl iodides may also find broader utility for example in the preparation of advanced fluorinated building blocks such as *O*-linked natural product mimetics[25] **6.20a** and covalent inhibitors[26,27] of carbohydrate processing enzymes **6.20b** and **6.21** where the use of "soft" nucleophilic acceptors are required.



Scheme 6.5. Phase-transfer catalyzed reactions (PTC) for the preparation of **6.16**–**6.21**. [a]*General conditions*: 2-F-α-pyranosyl halide (1 equiv.), Nu (2 equiv.), TBAHS (2 equiv.) in 5:1 EtOAc–1 M Na_2CO_3 aq. (0.04 M), rt, from 1 h up to 7 days unless otherwise indicated. [b]Determined by ^{19}F NMR of the crude reaction mixture. [c]2-F-α-galactosyl bromide **6.11b** (1 equiv.), Nu (2 equiv.), TBAB (2 equiv.) in 3:2 CH_2Cl_2–5% NaOH aq. (0.02 M), RT, 14–22 h. [d]2-F-α-galactosyl bromide **6.11b** (2 equiv.), estrone (1 equiv.), 60% NaH in mineral oil (1.5 equiv.) in dry 1,4-dioxane (0.08 M), rt, 24 h.

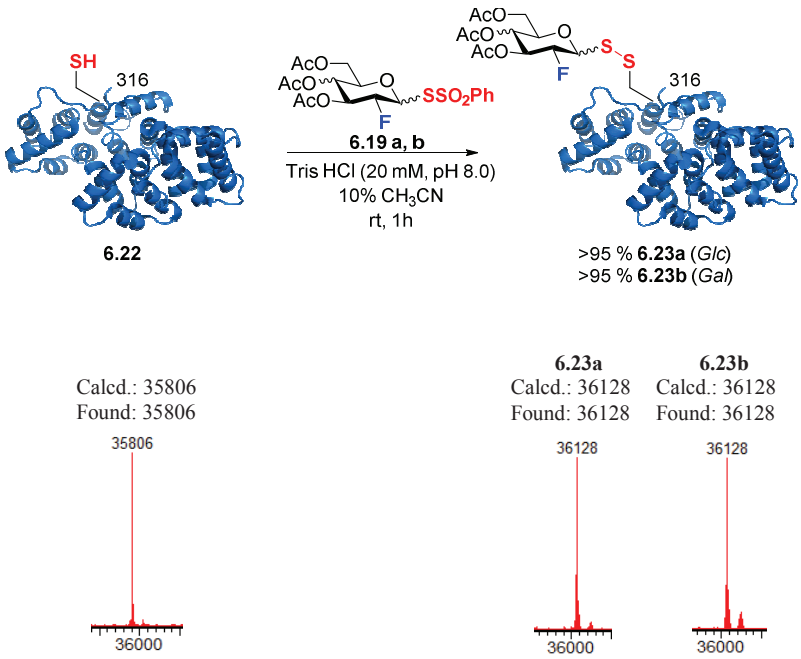
6.3.4. Selective chemical protein modification

Finally, to further complement previous reports on the preparation of a variety of well-defined triazole,[2h] thioether[2f,2i] and *SeS*-linked[2e,2i] fluorinated glycoproteins including their [^{18}F]-counterparts (Scheme 6.6), site selective chemical protein modification was evaluated using the reagent previously prepared.



Scheme 6.6. Structures of well-defined fluorinated glycoproteins.

To illustrate the application of such reagents we used to evaluate the reaction between Cys-specific Ac_3*Glc*[1i] and *Gal*F-1-PTS reagents **6.19a,b** with Annexin V **6.22**. This protein displays a unique naturally occurring surface exposed Cys and is widely utilized as a marker of apoptosis (Scheme 6.7).[28] Indeed, reaction of an excess of **6.19a** or **6.19b** with Annexin V **6.22** in 20 mM Tris HCl buffer pH 8 and 10% CH_3CN, that was used to ensure reagent's solubility, gave the expected homogeneous disulfide-linked F-glycoproteins **6.23a,b** in >95% conversion after 1 h at room temperature. LC-MS was used to monitor the reaction and complete conversion of **6.23a** and **6.23b** was observed after just 1 hour. This is demonstrative of the utility of the reagents that can now be easily prepared from the 2-F-glycosyl iodide reagents we report in this work.



Scheme 6.7. Selective chemical protein modification of Annexin V **6.22**. [a]*General conditions*: Annexin V **6.22** (1 mg mL^{-1}) (1 equiv.), 2-F-sugar reagent **6.19a,b** (250 equiv.) in 20 mM Tris HCl buffer (pH 8.0) and 10% CH_3CN, RT, 1 h.

6.4. CONCLUSIONS

In summary, we disclosed a general strategy to access a wide range of fluorosugars that are useful reagents for chemical-site selective protein glycosylation. The glycosyl iodide intermediates prepared here possess a seemly balance between stability and reactivity that facilitates their preparation, purification and storage. Importantly, these intermediates also ensure product homogeneity by their exquisite β-control during stereoselective glycosylations with "soft" Nu (*via* S_N2-like reactions). The reported F-sugar reagents will find broad utility not only for building homogeneous ^{19}F– but also [^{18}F]-glycoprobes that are valuable tools in the fields of chemical biology and biomedical imaging.

6.5. EXPERIMENTAL PART

General Remarks

Proton (^{1}H NMR), carbon (^{13}C NMR), fluor (^{19}F NMR), and phosphorus (^{31}P NMR) nuclear magnetic resonance spectra were recorded on a Varian Mercury spectrometer (400 MHz for ^{1}H), (100.6 MHz for ^{13}C), (376.5 MHz for ^{19}F), and (162 MHz for ^{31}P). Spectra were fully assigned using COSY, HSQC, HMBC and NOESY. All chemical shifts are quoted on the δ scale in ppm using either Me_3Si (^{1}H NMR: $CDCl_3$ = 0.00) or the residual solvent as internal standard (^{1}H NMR: $CDCl_3$ = 7.26 and ^{13}C NMR: $CDCl_3$ = 77.00) and $CFCl_3$ and 85% H_3PO_4 (^{19}F NMR: $CDCl_3$ = 0.00 and ^{31}P NMR: $CDCl_3$ = 0.00) as external standards, respectively. Coupling constants (*J*) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet and app = apparent. Infrared (IR) spectra were recorded on a Jasco FT/IR-600 Plus ATR Specac Golden Gate spectrophotometer. Absorption maxima (vmax) are reported in wavenumbers (cm^{-1}). Elemental analyses (C, H, N, and S) were performed with a Carlo Erba EA 1108 Analyzer. Optical rotations were measured on a Perkin–Elmer 241 polarimeter with a path length of 1.0 dm and are reported with implied units of 10^{-1} deg cm^2 g^{-1}. Concentrations (c) are given in g/100 mL. High resolution mass spectra (HRMS) were recorded on a Agilent 1100 Series LC/MSD mass spectrometer with electrospray ionization (ESI) by the Servei de Recursos Científics (URV). Nominal and exact *m/z* values are reported in Daltons. Thin layer chromatography (TLC) was carried out using commercial aluminium backed sheets coated with 60F254 silica gel. Visualization of the silica plates was achieved using a UV lamp (λmax = 254 nm) and/or 6% H_2SO_4 in EtOH and/or 2% $PdCl_2$ and 15% H_2SO_4 in water. Flash column chromatography was carried out using silica gel 60 A CC (230–400 mesh). Radial chromatography was performed on 1, 2, or 4 mm plates of Kieselgel 60 PF254 silica gel, depending on the amount of product. Mobile phases are reported in relative composition (e.g. 1:1 EtOAc/hexane v/v). HPLC grade

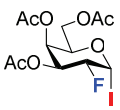
dichloromethane, tetrahydrofuran (THF) and dimethylformamide (DMF) were dried using a solvent purification system (Pure SOLV system-400). All other solvents were used as supplied (Analytical or HPLC grade), without prior purification. Distilled water was used for chemical reactions and Milli–QR purified water for protein manipulations. All reagents were used as received from commercial suppliers. All reactions using anhydrous conditions were performed using flame-dried apparatus under an atmosphere of argon. Brine refers to a saturated solution of sodium chloride. Anhydrous magnesium sulfate ($MgSO_4$) was used as drying agent after reaction work-up, as indicated.

Protein Mass Spectrometry

Liquid chromatography-mass spectrometry (LC–MS) was performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 μm, 2.1 × 50 mm). Water with 0.1% formic acid (solvent A) and 70% acetonitrile and 29% water with 0.075% formic acid (solvent B), were used as the mobile phase at a flow rate of 0.2 mL min^{-1}. The gradient was programmed as follows: From 72% A to 100% B for 25 min then 100% B for 2 minutes and 72% A for 18 min. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V. Nitrogen was used as the desolvation gas at a total flow of 850 L hr^{-1}. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions.

6.5.1. Synthesis of peracetylated 2-deoxy-2-fluoroglycopyranosyl halides

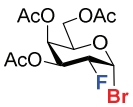
3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl iodide (6.11a):



TMSI (87 μL, 0.629 mmol) was added to a mixture of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-galactopyranose **6.10**[29] (2:1 α/β) (100 mg, 0.285 mmol) and MgO (47 mg, 1.14 mmol) in

dry CH_2Cl_2 (2.4 mL) at room temperature. The reaction mixture was stirred at the same temperature for 24 h. The crude was then diluted with CH_2Cl_2 and washed with saturated aqueous $Na_2S_2O_3$. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by radial chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.11a** (101.5 mg, 85%) as a yellowish oil. R_f (1:1 EtOAc/hexane): 0.44; $[\alpha]_D^{20}$: +183.6 (0.445, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.01 (dd, $J_{1,2}$ = 4.4 Hz, $J_{F,1}$ = 1.6 Hz, 1H, H-1), 5.51 (m, 1H, H-4), 5.36 (dt, $J_{F,3}$ = $J_{2,3}$ = 8.8 Hz, $J_{3,4}$ = 3.2 Hz, 1H, H-3), 4.30–4.03 (m, 4H, H-2, H-5, H-6a, H-6b), 2.15, 2.07, 2.06 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.0, 169.6 (3C=O, Ac), 83.6 (d, $J_{2,F}$ = 194.5 Hz, C-2), 73.6 (C-5), 72.7 (d, $J_{1,F}$ = 26.7 Hz, C-1), 70.8 (d, $J_{3,F}$ = 17.5 Hz, C-3), 68.9 (d, $J_{4,F}$ = 7.6 Hz, C-4), 60.4 (C-6), 20.5, 20.4 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –188.4 (ddd, $J_{F,2}$ = 50.4 Hz, $J_{F,3}$ = 8.8, $J_{F,1}$ = 1.6 Hz, F-2); FT–IR (neat) ν in cm^{-1}: 2966, 1733, 1362, 901; Anal. Calcd for $C_{12}H_{16}FIO_7$: C, 34.47; H, 3.86. Found: C, 34.45; H, 3.88; HRMS (TOF ES$^+$) for $(M+Na)^+$ $C_{12}H_{16}FINaO_7^+$ (*m/z*): calc. 440.9817; found 440.9806.

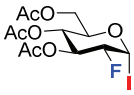
3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl bromide



(6.11b): 33% HBr in AcOH (3.7 mL) was added to a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-galactopyranose **6.10**[29] (2:1 α/β) (185.1 mg, 0.528 mmol) in dry CH_2Cl_2 (3.7 mL) at room temperature. The reaction mixture was stirred at the same temperature for 8 h. The crude was then diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford **6.11b** (193.5 mg, 95%) as a yellowish oil. Used in the next step without further purification. R_f (1:1 EtOAc/hexane): 0.56; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 6.60 (d, $J_{1,2}$ = 4.1 Hz, 1H, H-1), 5.52 (ddd, $J_{F,4}$ = 3.4 Hz, $J_{3,4}$ = 3.5 Hz, $J_{4,5}$ = 1.4 Hz, 1H, H-4), 5.45 (ddd, $J_{F,3}$ = 10.3 Hz, $J_{2,3}$ = 10.1 Hz, $J_{3,4}$ = 3.5 Hz, 1H, H-3), 4.74 (ddd, $J_{F,2}$ = 50.2 Hz, $J_{2,3}$ = 10.1 Hz, $J_{1,2}$ = 4.1 Hz,

1H, H-2), 4.50 (appt, $J_{5,6a} = J_{5,6b} = 6.4$ Hz, $J_{4,5} = 1.4$ Hz, 1H, H-5), 4.16 (dd, $J_{6a,b} = 11.4$ Hz, $J_{5,6a} = 6.4$ Hz, 1H, H-6a), 4.10 (dd, $J_{6a,b} = 11.4$ Hz, $J_{5,6b} = 6.4$ Hz, 1H, H-6b), 2.13, 2.05, 2.04 (s, 9H, $3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –195.0 (ddd, $J_{F,2} = 50.2$ Hz, $J_{F,3} = 10.3$ Hz, $J_{F,4} = 3.4$ Hz, F-2); spectroscopic data was identical to that previously reported.[29]

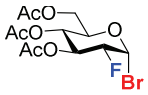
3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl iodide (6.14a):



AcSH (141 μL, 1.88 mmol) was added to a mixture of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-glucopyranose **6.12**[30] (1:3 α/β) (300 mg, 0.86 mmol) and I_2 (217 mg, 0.86 mmol) in dry CH_2Cl_2 (8.6 mL) at room temperature. The reaction mixture was heated under reflux for 5 h. The crude was then diluted with CH_2Cl_2 and washed with saturated aqueous $Na_2S_2O_3$. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was filtered through a short path of silica (1:1 EtOAc/hexane) and the crude subsequently treated with AcSH (141 μL, 1.88 mmol) and I_2 (217 mg, 0.86 mmol) in dry CH_2Cl_2 (8.6 mL) at room temperature. The reaction mixture was heated under reflux for 5 h. The crude was then diluted with CH_2Cl_2 and washed with saturated aqueous $Na_2S_2O_3$. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by radial chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.14a** (293.6 mg, 82%) as a yellowish syrup. R_f (1:1 EtOAc/hexane): 0.60; $[\alpha]_D^{20}$: +210.0 (0.58, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 6.91 (d, $J_{1,2} = 4.7$ Hz, 1H, H-1), 5.55 (ddd, $J_{F,3} = 11.5$ Hz, $J_{3,4} = 10.2$ Hz, $J_{2,3} = 9.4$ Hz, 1H, H-3), 5.14 (appt, $J_{3,4} = J_{4,5} = 10.2$ Hz, 1H, H-4), 4.35 (dd, $J_{6a,b} = 12.5$ Hz, $J_{5,6a} = 4.5$ Hz, 1H, H-6a), 4.12 (appd, $J_{6a,b} = 12.5$ Hz, 1H, H-6b), 4.07 (m, 1H, H-5), 3.94 (ddd, $J_{F,2} = 49.2$ Hz, $J_{2,3} = 9.4$ Hz, $J_{1,2} = 4.7$ Hz, 1H, H-2), 2.09, 2.07 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.4, 169.8, 169.5 (3C=O, Ac), 85.8 (d, $J_{2,F} = 197.6$ Hz, C-2), 74.8 (C-5), 72.8 (d, $J_{3,F} = 18.5$ Hz, C-3), 70.5 (d, $J_{1,F} = 26.1$ Hz, C-1), 66.2 (d, $J_{4,F} = 6.9$ Hz, C-4), 60.7 (C-6), 20.7, 20.6, 20.5 ($3CH_3$, Ac); ^{19}F NMR

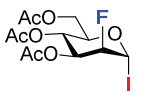
($CDCl_3$, 376.5 MHz) δ in ppm: –182.0 (dd, $J_{F,2}$ = 49.2 Hz, $J_{F,3}$ = 11.5 Hz, F-2); FT–IR (neat) ν in cm^{-1}: 2958, 1742, 1365, 1219, 1065, 1033, 903; HRMS (TOF ES^+) for $(M+Na)^+$ $C_{12}H_{16}FINaO_7^+$ (*m/z*): calc. 440.9817; found 440.9803.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl bromide (6.14b):



33% HBr in AcOH (1.6 mL) was added to a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-glucopyranose **6.12**[29] (3:2 α/β) (79.2 mg, 0.226 mmol) in dry CH_2Cl_2 (1.6 mL) at room temperature. The reaction mixture was stirred at the same temperature for 3 h. The crude was then diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford **6.14b** (82.5 mg, 98%) as a yellowish oil. Used in the next step without further purification. R_f (1:1 EtOAc/hexane): 0.63; ^1H NMR ($CDCl_3$, 400 MHz) δ in ppm: 6.51 (dd, $J_{1,2}$ = 4.6 Hz, $J_{F,1}$ = 1.1 Hz, 1H, H-1), 5.59 (ddd, $J_{F,3}$ = 11.3 Hz, $J_{3,4}$ = 9.8 Hz, $J_{2,3}$ = 9.4 Hz, 1H, H-3), 5.09 (appt, $J_{3,4}$ = $J_{4,5}$ = 9.8 Hz, 1H, H-4), 4.52 (ddd, $J_{F,2}$ = 49.3 Hz, $J_{2,3}$ = 9.4 Hz, $J_{1,2}$ = 4.6 Hz, 1H, H-2), 4.37–4.21 (m, 2H, H-5, H-6a), 4.09 (dd, $J_{6a,b}$ = 10.6 Hz, $J_{5,6b}$ = 3.0 Hz, 1H, H-6b), 2.07, 2.06, 2.03 (s, 9H, $3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –188.5 (ddd, $J_{F,2}$ = 49.3 Hz, $J_{F,3}$ = 11.3 Hz, $J_{F,1}$ = 1.1 Hz, F-2); spectroscopic data was identical to that previously reported.[31]

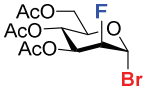
3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-mannopyranosyl iodide (6.15a):



AcSH (139 µL, 1.95 mmol) was added to a mixture of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoro-α-D-mannopyranose **6.13**[32] (311 mg, 0.89 mmol) and I_2 (225 mg, 0.89 mmol) in dry CH_2Cl_2 (8.9 mL) at room temperature. The reaction mixture was heated under reflux for 5 h. The crude was then diluted with CH_2Cl_2 and washed with saturated aqueous $Na_2S_2O_3$. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was filtered

through a short path of silica (1:1 EtOAc/hexane) and the crude subsequently treated with AcSH (139 μL, 1.95 mmol) and I_2 (225 mg, 0.89 mmol) in dry CH_2Cl_2 (8.9 mL) at room temperature. The reaction mixture was heated under reflux for 5 h. The crude was then diluted with CH_2Cl_2 and washed with saturated aqueous $Na_2S_2O_3$. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by radial chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.15a** (275.4 mg, 75%) as a colourless oil. R_f (1:1 EtOAc/hexane): 0.44; $[\alpha]_D^{20}$: +186.2 (0.42, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 6.84 (d, $J_{F,1}$ = 12.1 Hz, 1H, H-1), 5.73 (ddd, $J_{F,3}$ = 26.0 Hz, $J_{3,4}$ = 10.3 Hz, $J_{2,3}$ = 2.6 Hz, 1H, H-3), 5.44 (appt, $J_{3,4}$ = $J_{4,5}$ = 10.3 Hz, 1H, H-4), 5.03 (dd, $J_{F,2}$ = 50.4 Hz, $J_{2,3}$ = 2.6 Hz, 1H, H-2), 4.33 (dd, $J_{6a,b}$ = 12.5 Hz, $J_{5,6a}$ = 4.4 Hz, 1H, H-6a), 4.13 (dd, $J_{6a,b}$ = 12.5 Hz, $J_{5,6b}$ = 2.2 Hz, 1H, H-6b), 3.93 (ddd, $J_{4,5}$ = 10.3 Hz, $J_{5,6a}$ = 4.4 Hz, $J_{5,6b}$ = 2.2 Hz, $J_{4,5}$ = 2.2 Hz, 1H, H-5), 2.10, 2.09, 2.07 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.5, 170.0, 169.3 (3C=O, Ac), 89.8 (d, $J_{2,F}$ = 190.7 Hz, C-2), 75.1 (C-5), 69.0 (d, $J_{3,F}$ = 17.6 Hz, C-3), 65.7 (d, $J_{1,F}$ = 23.7 Hz, C-1), 64.9 (d, $J_{4,F}$ = 1.5 Hz, C-4), 60.9 (C-6), 20.63, 20.57 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –174.4 (ddd, $J_{F,2}$ = 50.4 Hz, $J_{F,3}$ = 26.0 Hz, $J_{F,1}$ = 12.1 Hz, F-2); FT–IR (neat) ν in cm^{-1}: 2960, 1741, 1364, 1040, 900; HRMS (TOF ES$^+$) for $(M+Na)^+$ $C_{12}H_{16}FINaO_7^+$ (*m/z*): calc. 440.9817; found 440.9860.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-mannopyranosyl bromide



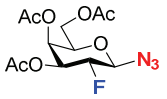
(6.15b): 33% HBr in AcOH (4 mL) was added to a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoro-α-D-mannopyranose **6.13**[30] (200 mg, 0.571 mmol) in dry CH_2Cl_2 (4 mL) at room temperature. The reaction mixture was stirred at the same temperature for 5 h. The crude was then diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford **6.15b** (199.2 mg, 94%) as a yellowish oil. Used in the next step without further purification. R_f

(1:1 EtOAc/hexane): 0.50; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 6.44 (dd, $J_{F,1}$ = 9.2 Hz, $J_{1,2}$ = 1.2 Hz, 1H, H-1), 5.63 (ddd, $J_{F,3}$ = 26.3 Hz, $J_{3,4}$ = 10.4 Hz, $J_{2,3}$ = 2.5 Hz, 1H, H-3), 5.45 (appt, $J_{3,4}$ = $J_{4,5}$ = 10.4 Hz, 1H, H-4), 4.99 (ddd, $J_{F,2}$ = 49.1 Hz, $J_{2,3}$ = 2.5 Hz, $J_{1,2}$ = 1.2 Hz, 1H, H-2), 4.34 (dd, $J_{6a,b}$ = 12.3 Hz, $J_{5,6a}$ = 4.3 Hz, 1H, H-6a), 4.24–4.21 (m, 1H, H-5), 4.18 (dd, $J_{6a,b}$ = 12.3 Hz, $J_{5,6b}$ = 2.3 Hz, 1H, H-6b), 2.12, 2.11, 2.09 (s, 9H, $3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –181.6 (ddd, $J_{F,2}$ = 49.1 Hz, $J_{F,3}$ = 26.3 Hz, $J_{F,1}$ = 9.2 Hz, F-2); spectroscopic data was identical to that previously reported.[33]

6.5.2. Synthesis of 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoroglycopyranosyl reagents

General procedure for the synthesis of 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoroglycosyl reagents with anomeric N_3, SAc, PTS and Se(4-MeBz) groups: The corresponding nucleophile salt (2 mmol) was added to a mixture of the corresponding peracetylated 2-deoxy-2-fluoroglycosyl halide (1 mmol), TBAHS (2 mmol), and 1 M aqueous Na_2CO_3 (4 mL) in EtOAc (21 mL) at room temperature. The reaction mixture was stirred at the same temperature and the progress monitored by TLC. The crude was then diluted with EtOAc and extracted. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by chromatographic techniques.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-galactopyranosyl azide (6.16a):



The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl iodide **6.11a** (43.5 mg, 0.104 mmol), NaN_3 (13.5 mg, 0.208 mmol), TBAHS (72.8 mg, 0.208 mmol) and 1 M aqueous Na_2CO_3 (0.4 mL) in EtOAc (2.2 mL). The reaction mixture was stirred at room temperature for 1 h. After standard work-up, the residue was

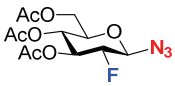
purified by column chromatography (1:2 EtOAc/hexane) to afford **6.16a** (33.6 mg, 97%) as a white solid. R_f (1:1 EtOAc/hexane): 0.45; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.45 (m, 1H, H-4), 5.14 (ddd, $J_{F,3}$ = 12.9 Hz, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 3.2 Hz, 1H, H-3), 4.84 (dd, $J_{1,2}$ = 8.4 Hz, $J_{F,1}$ = 3.3 Hz, 1H, H-1), 4.46 (ddd, $J_{F,2}$ = 51.0 Hz, $J_{2,3}$ = 9.6 Hz, $J_{1,2}$ = 8.4 Hz, 1H, H-2), 4.20–4.03 (m, 3H, H-5,6a,b), 2.16, 2.07 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.4, 169.9, 169.8 (3C=O, Ac), 88.0 (d, $J_{1,F}$ = 23.6 Hz, C-1), 87.5 (d, $J_{2,F}$ = 188.4 Hz, C-2), 72.7 (C-5), 70.8 (d, $J_{3,F}$ = 19.1 Hz, C-3), 67.4 (d, $J_{4,F}$ = 7.6 Hz, C-4), 61.0 (C-6), 20.6, 20.51, 20.49 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –205.6 (dddd, $J_{F,2}$ = 51.0 Hz, $J_{F,3}$ = 12.9 Hz, $J_{F,1}$ = $J_{F,4}$ = 3.3 Hz, F-2); spectroscopic data was identical to that previously reported.[33]

3,4,6-Tri-*O*-acetyl-1-*S*-acetyl-2-deoxy-2-fluoro-1-thio-β-D-galactopyranose (6.16b): The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl iodide **6.11a** (100 mg, 0.239 mmol), KSAc (56.0 mg, 0.478 mmol), TBAHS (167.3 mg, 0.478 mmol) and 1 M aqueous Na_2CO_3 (0.96 mL) in EtOAc (5 mL). The reaction mixture was stirred at room temperature for 1 h. After standard work-up, the residue was purified by column chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.16b** (79 mg, 90%) as a yellowish foam. R_f (1:1 EtOAc/hexane): 0.59; $[\alpha]_D^{20}$: +67.5 (0.90, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.49 (appt, $J_{3,4}$ = $J_{4,5}$ = 3.3 Hz, 1H, H-4), 5.33 (dd, $J_{1,2}$ = 9.5 Hz, $J_{F,1}$ = 2.6 Hz, 1H, H-1), 5.20 (ddd, $J_{F,3}$ = 13.2 Hz, $J_{2,3}$ = 9.5 Hz, $J_{3,4}$ = 3.3 Hz, 1H, H-3), 4.64 (dt, $J_{F,2}$ = 50.2 Hz, $J_{1,2}$ = $J_{2,3}$ = 9.5 Hz, 1H, H-2), 4.12–4.06 (m, 3H, H-5,6a,b), 2.44 (s, 3H, CH_3, SAc), 2.44, 2.06, 2.05 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 191.6 (C=O, SAc), 170.3, 170.0, 169.8 (3C=O, Ac), 85.5 (d, $J_{2,F}$ = 188.4 Hz, C-2), 79.9 (d, $J_{1,F}$ = 25.2 Hz, C-1), 75.1 (C-5), 72.1 (d, $J_{3,F}$ = 19.8 Hz, C-3), 67.9 (d, $J_{4,F}$ = 8.3 Hz, C-4), 61.0 (C-6), 30.9 (CH_3, SAc), 20.7, 20.6, 20.5 ($3CH_3$, Ac); ^{19}F NMR

($CDCl_3$, 376.5 MHz) δ in ppm: –200.0 (ddd, $J_{F,2}$ = 50.2 Hz, $J_{F,3}$ = 13.2 Hz, $J_{F,1}$ = 2.6 Hz, F-2); FT–IR (neat) ν in cm^{-1}: 2960, 2919, 1740, 1700, 1360, 1221; Anal. Calcd for $C_{14}H_{19}FO_8S$: C, 45.90; H, 5.23; S, 8.75. Found: C, 45.84; H, 5.26; S, 8.70; HRMS (TOF ES^+) for $(M+Na)^+$ $C_{14}H_{19}FNaO_8S^+$ (*m/z*): calc. 389.0677; found 389.0678.

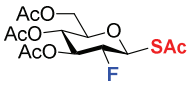
3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-1-*Se*-(4-methylbenzoyl)-1-seleno-β-D-mannopyranose (6.16c): The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl iodide **6.11a** (24.5 mg, 0.059 mmol), potassium 4-methylselenobenzoate (27.8 mg, 0.117 mmol), TBAHS (41 mg, 0.117 mmol) and 1 M aqueous Na_2CO_3 (0.24 mL) in EtOAc (1.2 mL). The reaction mixture was stirred at room temperature for 2 h. After standard work-up, the residue was purified by radial chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.16c** (20.5 mg, 71%) as a yellowish oil. R_f (1:1 EtOAc/hexane): 0.44; $[\alpha]_D^{20}$: +65.9 (0.275, CH_2Cl_2); ^1H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.78 (d, *J* = 8.0 Hz, 2H, Ar), 7.28 (d, *J* = 8.0 Hz, 2H, Ar), 5.58 (dd, $J_{1,2}$ = 10 Hz, $J_{F,1}$ = 1.2 Hz, 1H, H-1), 5.54 (appt, $J_{3,4}$ = $J_{4,5}$ = 2.8 Hz, 1H, H-4), 5.24 (ddd, $J_{F,3}$ = 13.2 Hz, $J_{2,3}$ = 9.2 Hz, $J_{3,4}$ = 2.8 Hz, 1H, H-3), 4.85 (ddd, $J_{F,2}$ = 60.0 Hz, $J_{1,2}$ = 10 Hz, $J_{2,3}$ = 9.2 Hz, 1H, H-2), 4.15–4.09 (m, 3H, H-5, H-6a, H-6b), 2.42 (s, 3H, CH_3, Ar), 2.16, 2.07, 2.04 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 190.2 (C=O, SeBz), 170.4, 169.9 (3C=O, Ac), 145.6, 135.4 (C, Ar), 129.7, 127.7 (CH, Ar), 86.4 (d, $J_{2,F}$ = 186.9 Hz, C-2), 79.3 (d, $J_{1,F}$ = 26.7 Hz, C-1), 76.1 (C-5), 72.1 (d, $J_{3,F}$ = 19.8 Hz, C-3), 68.1 (d, $J_{4,F}$ = 8.3 Hz, C-4), 61.1 (C-6), 21.8 (CH_3, Ar), 20.7, 20.6 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –197.3 (ddd, $J_{F,2}$ = 60.0 Hz, $J_{F,3}$ = 13.2 Hz, $J_{F,1}$ = 1.2 Hz, F-2); FT–IR (neat) ν in cm^{-1}: 2960, 2919, 2847, 1744, 1030; Anal. Calcd for $C_{20}H_{23}FO_8Se$: C, 49.09; H, 4.74. Found: C, 49.20; H, 4.90; HRMS (TOF ES^+) for $(M+Na)^+$ $C_{20}H_{23}FNaO_8Se^+$ (*m/z*): calc. 513.0434; found 513.0430.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl azide (6.17a):



The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl iodide **6.14a** (42.8 mg, 0.102 mmol), NaN_3 (13.3 mg, 0.205 mmol), TBAHS (72.0 mg, 0.205 mmol) and 1 M aqueous Na_2CO_3 (0.4 mL) in EtOAc (2.1 mL). The reaction mixture was stirred at room temperature for 1 h. After standard work-up, the residue was purified by column chromatography (1:2 EtOAc/hexane) to afford **6.17a** (32.1 mg, 94%) as a white solid. R_f (1:1 EtOAc/hexane): 0.48; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.34 (dt, $J_{F,3}$ = 15.3 Hz, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, 1H, H-3), 5.06 (t, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, 1H, H-4), 4.84 (dd, $J_{1,2}$ = 8.4 Hz, $J_{F,1}$ = 3.1 Hz, 1H, H-1), 4.34–4.12 (m, 3H, H-2, H-6a, H-6b), 3.82 (m, 1H, H-5), 2.11, 2.10, 2.05 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.5, 169.9, 169.4 (3C=O, Ac), 88.9 (d, $J_{2,F}$ = 193.0 Hz, C-2), 87.7 (d, $J_{1,F}$ = 22.1 Hz, C-1), 73.9 (C-5), 72.5 (d, $J_{3,F}$ = 19.8 Hz, C-3), 67.5 (d, $J_{4,F}$ = 7.6 Hz, C-4), 61.5 (C-6), 20.7, 20.6, 20.5 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –198.5 (ddd, $J_{F,2}$ = 48.8 Hz, $J_{F,3}$ = 15.3 Hz, $J_{F,1}$ = 3.1 Hz, F-2); spectroscopic data was identical to that previously reported.[34]

3,4,6-Tri-*O*-acetyl-1-*S*-acetyl-2-deoxy-2-fluoro-1-thio-β-D-glucopyranose



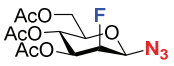
(6.17b): The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl iodide **6.14a** (33.9 mg, 0.081 mmol), KSAc (19.1 mg, 0.16 mmol), TBAHS (56.6 mg, 0.16 mmol), and 1 M aqueous Na_2CO_3 (0.3 mL) in EtOAc (1.7 mL). The reaction mixture was stirred at room temperature for 1 h. After standard work-up, the residue was purified by column chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.17b** (27.0 mg, 91%) as a yellowish solid. R_f (1:1 EtOAc/hexane): 0.52; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.40 (ddd, $J_{F,3}$ = 14.8 Hz, $J_{3,4}$ = 9.9 Hz, $J_{2,3}$ = 8.8 Hz, 1H, H-3), 5.30 (dd, $J_{1,2}$ = 10.4 Hz, $J_{F,1}$ = 2.2 Hz, 1H, H-1), 5.05 (dt, $J_{3,4}$ = $J_{4,5}$ = 9.9 Hz, 1H, H-4), 4.42 (ddd, $J_{F,2}$ = 49.7

Hz, $J_{1,2}$ = 10.4 Hz, $J_{2,3}$ = 8.8 Hz, 1H, H-2), 4.25 (dd, $J_{6a,b}$ = 12.5 Hz, $J_{5,6a}$ = 4.5 Hz, 1H, H-6a), 4.09 (dd, $J_{6a,b}$ = 12.5 Hz, $J_{5,6b}$ = 2.4 Hz, 1H, H-6b), 3.84 (ddd, $J_{4,5}$ = 9.9 Hz, $J_{5,6a}$ = 4.5 Hz, $J_{5,6b}$ = 2.4 Hz, 1H, H-5), 2.42 (s, 3H, CH_3, SAc), 2.10, 2.08, 2.06 (s, 9H, $3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –192.1 (ddd, $J_{F,2}$ = 49.7 Hz, $J_{F,3}$ = 14.8 Hz, $J_{F,1}$ = 2.2 Hz, F-2); spectroscopic data was identical to that previously reported.[35]

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-1-*Se*-(4-methylbenzoyl)-1-seleno-β-D-glucopyranose (6.17c): The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl iodide **6.16a** (80.4 mg, 0.192 mmol), potassium 4-methylselenobenzoate (91.0 mg, 0.384 mmol), TBAHS (130.0 mg, 0.388 mmol) and 1 M aqueous Na_2CO_3 (0.7 mL) in EtOAc (3.8 mL). The reaction mixture was stirred at room temperature for 2 h. After standard work-up, the residue was purified by column chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.17c** (63.4mg, 67%) as a yellowish foam. R_f (1:1 EtOAc/hexane): 0.45; $[\alpha]_D^{20}$: –4.5 (0.595, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.77 (d, J = 8.2 Hz, 2H, Ar), 7.28 (d, J = 8.2 Hz, 2H, Ar), 5.57 (dd, $J_{1,2}$ = 10.6 Hz, $J_{F,1}$ = 1.6 Hz, 1H, H-1), 5.42 (ddd, $J_{F,3}$ = 10.6 Hz, $J_{3,4}$ = 9.5 Hz, $J_{2,3}$ = 9.0 Hz, 1H, H-3), 5.14 (appt, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, 1H, H-4), 4.66 (ddd, $J_{F,2}$ = 49.3 Hz, $J_{1,2}$ = 10.6 Hz, $J_{2,3}$ = 9.0 Hz, 1H, H-2), 4.31 (dd, $J_{6a,b}$ = 12.5 Hz, $J_{5,6a}$ = 4.7 Hz, 1H, H-6a), 4.12 (dd, $J_{6a,b}$ = 12.5 Hz, $J_{5,6b}$ = 2.0 Hz, 1H, H-6b), 3.91 (ddd, $J_{4,5}$ = 9.5 Hz, $J_{5,6a}$ = 4.7 Hz, $J_{5,6b}$ = 2.0 Hz, 1H, H-5), 2.42 (s, 3H, CH_3, Ar), 2.10, 2.08, 2.06 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 190.2 (C=O, SeBz), 170.6, 169.9, 169.6 (3C=O, Ac), 145.6, 135.3 (C, Ar), 129.7, 127.7 (CH, Ar), 88.1 (d, $J_{2,F}$ = 191.5 Hz, C-2), 78.8 (d, $J_{1,F}$ = 25.9 Hz, C-1), 77.4 (C-5), 74.1 (d, $J_{3,F}$ = 20.6 Hz, C-3), 67.8 (d, $J_{4,F}$ = 6.7 Hz, C-4), 61.7 (C-6), 21.80 (CH_3, Ar), 20.73, 20.70, 20.6 ($3CH_3$, Ac); NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –190.0 (dd, $J_{F,2}$ = 49.3 Hz, $J_{F,3}$ = 10.6 Hz, F-2); FT–IR (neat) ν in cm^{-1}: 2952, 1742, 1688, 1365, 1203, 1172,

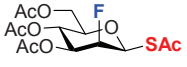
1035, 878, 783; HRMS (TOF ES$^+$) for $(M+Na)^+$ $C_{20}H_{23}FNaO_8Se^+$ (*m/z*): calc. 513.0434; found 513.0427.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-mannopyranosyl azide (6.18a):



The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-mannopyranosyl bromide **6.15b** (100 mg, 0.269 mmol), NaN_3 (35 mg, 0.539 mmol), TBAHS (188.6 mg, 0.539 mmol) and 1 M aqueous Na_2CO_3 (1.1 mL) in EtOAc (5.6 mL). The reaction mixture was stirred at room temperature for 2 h. After standard work-up, the residue was purified by column chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.18a** (87.8 mg, 98%) as a white solid. R_f (1:1 EtOAc/hexane): 0.56; $[\alpha]_D^{20}$: –44.6 (0.125, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.36 (appt, $J_{3,4} = J_{4,5} = 10.2$ Hz, 1H, H-4), 5.01 (ddd, $J_{F,3} = 27.9$ Hz, $J_{3,4} = 10.2$ Hz, $J_{2,3} = 2.7$ Hz, 1H, H-3), 4.89 (dd, $J_{F,2} = 50.5$ Hz, $J_{2,3} = 2.7$ Hz, 1H, H-2), 4.58 (d, $J_{F,1} = 20.7$ Hz, 1H, H-1), 4.27 (dd, $J_{6a,b} = 12.5$ Hz, $J_{5,6a} = 4.7$ Hz, 1H, H-6a), 4.23 (dd, $J_{6a,b} = 12.5$ Hz, $J_{5,6b} = 2.7$ Hz, 1H, H-6b), 3.77 (ddd, $J_{4,5} = 10.2$ Hz, $J_{5,6a} = 4.7$ Hz, $J_{5,6b} = 2.7$ Hz, 1H, H-5), 2.12, 2.11, 2.06 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.7, 170.2, 169.3 (3C=O, Ac), 88.2 (d, $J_{2,F} = 190.6$ Hz, C-2), 84.8 (d, $J_{1,F} = 16.1$ Hz, C-1), 74.4 (C-5), 71.4 (d, $J_{3,F} = 16.9$ Hz, C-3), 64.9 (C-4), 61.9 (C-6), 20.7, 20.63, 20.59 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –217.7 (ddd, $J_{F,2} = 50.5$ Hz, $J_{F,3} = 27.9$ Hz, $J_{F,1} = 20.7$ Hz, F-2); FT–IR (neat) ν in cm^{-1}: 2627, 2119, 1739, 1368, 1054, 904, 795; HRMS (TOF ES$^+$) for $(M+Na)^+$ $C_{12}H_{16}FN_3NaO_7^+$ (*m/z*): calc. 356.0864; found 356.0861.

3,4,6-Tri-*O*-acetyl-1-*S*-acetyl-2-deoxy-2-fluoro-1-thio-β-D-



mannopyranose (6.18b): The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-mannopyranosyl bromide **6.15b** (19.7 mg, 0.047 mmol), KSAc (11.5 mg, 0.1 mmol), TBAHS (33.9 mg, 0.1 mmol) and

1 M aqueous Na_2CO_3 (0.4 mL) in EtOAc (2 mL). The reaction mixture was stirred at room temperature for 15 h. After standard work-up, the residue was purified by column chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.18b** (11.4 mg, 66%) as a yellowish foam. R_f (1:1 EtOAc/hexane): 0.56; $[\alpha]_D^{20}$: –38.1 (0.44, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 5.44 (d, $J_{F,1}$ = 28.8 Hz, 1H, H-1), 5.37 (appt, $J_{3,4}$ = $J_{4,5}$ = 10.1 Hz, 1H, H-4), 5.08 (ddd, $J_{F,3}$ = 26.9 Hz, $J_{3,4}$ = 10.1 Hz, $J_{2,3}$ = 2.7 Hz, 1H, H-3), 4.92 (dd, $J_{F,2}$ = 49.34 Hz, $J_{2,3}$ = 2.7 Hz, 1H, H-2), 4.27 (dd, $J_{6a,b}$ = 12.5 Hz, $J_{5,6a}$ = 4.7 Hz, 1H, H-6a), 4.13 (dd, $J_{6a,b}$ = 12.5 Hz, $J_{5,6b}$ = 2.0 Hz, 1H, H-6b), 3.82 (ddd, $J_{4,5}$ = 10.1 Hz, $J_{5,6a}$ = 4.7 Hz, $J_{5,6b}$ = 2.0 Hz, 1H, H-5), 2.42 (s, 3H, CH_3, SAc), 2.11, 2.09, 2.06 (s, 9H, 3CH_3, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 191.9 (C=O, SAc), 170.8, 170.2, 169.4 (3C=O, Ac), 89.0 (d, $J_{2,F}$ = 186.9 Hz, C-2), 79.0 (d, $J_{1,F}$ = 17.6 Hz, C-1), 76.8 (C-5), 72.3 (d, $J_{3,F}$ = 17.6 Hz, C-3), 64.9 (C-4), 61.9 (C-6), 30.6 (CH_3, SAc), 20.8, 20.7, 20.6 (4CH_3, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –206.8 (ddd, $J_{F,2}$ = 49.4 Hz, $J_{F,1}$ = 28.8 Hz, $J_{F,3}$ = 26.9 Hz, F-2); FT–IR (neat) ν in cm^{-1}: 2961, 2920, 1743, 1700, 1365, 1220; HRMS (TOF ES$^+$) for $(M+Na)^+$ $C_{14}H_{19}FNaO_8S^+$ (*m/z*): calc. 389.0677; found 389.0678.

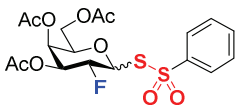
3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-1-*Se*-(4-methylbenzoyl)-1-seleno-β-D-mannopyranose (6.18c): The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-mannopyranosyl bromide **6.15b** (10.9 mg, 0.029 mmol), potassium 4-methylselenobenzoate (14.1 mg, 0.059 mmol), TBAHS (20.7 mg, 0.059 mmol) and 1 M aqueous Na_2CO_3 (0.1 mL) in EtOAc (0.6 mL). The reaction mixture was stirred at room temperature for 7 days. After standard work-up, the residue was purified by column chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.18c** (10.0 mg, 78%) as a yellowish foam. R_f (1:1 EtOAc/hexane): 0.59; $[\alpha]_D^{20}$: –29.7 (0.22, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.77 (d, *J* = 8.2 Hz, 2H, Ar), 7.29 (d, *J* = 8.2 Hz, 2H, Ar),

5.70 (d, $J_{F,1}$ = 31.0 Hz, 1H, H-1), 5.43 (appt, $J_{3,4}$ = $J_{4,5}$ = 10.1 Hz, 1H, H-4), 5.16 (ddd, $J_{F,3}$ = 26.4 Hz, $J_{3,4}$ = 10.1 Hz, $J_{2,3}$ = 2.8 Hz, 1H, H-3), 5.09 (dd, $J_{F,2}$ = 48.2 Hz, $J_{2,3}$ = 2.8 Hz, 1H, H-2), 4.31 (dd, $J_{6a,b}$ = 12.3 Hz, $J_{5,6a}$ = 4.7 Hz, 1H, H-6a), 4.15 (dd, $J_{6a,b}$ = 12.3 Hz, $J_{5,6b}$ = 2.2 Hz, 1H, H-6b), 3.87 (ddd, $J_{4,5}$ = 10.1 Hz, $J_{5,6a}$ = 4.7 Hz, $J_{5,6b}$ = 2.2 Hz, 1H, H-5), 2.43 (s, 3H, CH_3, Ar), 2.13, 2.09, 2.08 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 190.7 (C=O, SeBz), 170.8, 170.2, 169.4 (3C=O, Ac), 145.7, 135.3 (C, Ar), 129.7, 127.6 (CH, Ar), 90.1 (d, $J_{2,F}$ = 185.1 Hz, C-2), 78.2 (d, $J_{1,F}$ = 19.0 Hz, C-1), 77.8 (C-5), 72.4 (d, $J_{3,F}$ = 18.0 Hz, C-3), 65.1 (C-4), 62.1 (C-6), 21.80 (CH_3, Ar), 20.80, 20.68, 20.65 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –200.8 (ddd, $J_{F,2}$ = 48.2 Hz, $J_{F,1}$ = 31.0 Hz, $J_{F,3}$ = 26.4 Hz, F-2); FT–IR (neat) ν in cm^{-1}: 2962, 2920, 2847, 1745, 1050; HRMS (TOF ES^+) for $(M+Na)^+$ $C_{20}H_{23}FNaO_8Se^+$ (*m/z*): calc. 513.0434; found 513.0429.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-glucopyranosyl phenylthiosulfonate (6.19a): The title compound was prepared following the general procedure above, starting from starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl iodide **6.14a** (32.3 mg, 0.077 mmol), NaPTS (30.2 mg, 0.154 mmol), TBAHS (54 mg, 0.154 mmol) and 1 M aqueous Na_2CO_3 (0.3 mL) in EtOAc (1.6 mL). The reaction mixture was stirred at room temperature for 24 h. After standard work-up, the residue was purified by radial chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.19a** (31.6 mg, 88%) as an inseparable 1:1 α/β mixture as an orange syrup. R_f (1:1 EtOAc/hexane): 0.48. Data for **6.19aα**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.99–7.56 (m, 5H, Ar), 6.26 (d, $J_{1,2}$ = 6.0 Hz, 1H, H-1), 5.34–5.24 (m, 1H, H-3), 5.02 (appt, $J_{3,4}$ = $J_{4,5}$ = 7.2 Hz, 1H, H-4), 4.54–4.37 (m, 1H, H-2), 4.30–3.93 (m, 2H, H-6a, H-6b), 3.82–3.71 (m, 1H, H-5), 2.10, 2.05 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.6, 169.9, 169.4 (3C=O, Ac), 141.9 (C, Ar), 134.6–127.0 (CH, Ar), 86.9 (d, $J_{2,F}$ = 153.3 Hz, C-2), 85.6 (d, $J_{1,F}$ = 27.5 Hz, C-1), 76.5 (C-5), 73.5 (d, $J_{3,F}$ = 19.8

Hz, C-3), 67.3 (d, $J_{4,F}$ = 6.9 Hz, C-4), 61.5 (C-6), 20.68, 20.58, 20.58 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –192.4 (dd, $J_{F,2}$ = 49.8 Hz, $J_{F,3}$ = 14.9 Hz, F-2). Data for **6.19aβ**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.99–7.56 (m, 5H, Ar), 5.34–5.24 (m, 2H, H-1, H-3), 5.04 (appt, $J_{3,4}$ = $J_{4,5}$ = 10.0 Hz, 1H, H-4), 4.45 (dd, $J_{F,2}$ = 50.0 Hz, $J_{2,3}$ = 9.7 Hz, $J_{1,2}$ = 9.4 Hz, 1H, H-2), 4.30–3.93 (m, 2H, H-6a, H-6b), 3.82–3.71 (m, 1H, H-5), 2.08, 2.06, 2.04 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.6, 169.8, 169.4 (3C=O, Ac), 145.8 (C, Ar), 134.6–127.0 (CH, Ar), 86.8 (d, $J_{2,F}$ = 193.8 Hz, C-2), 85.8 (d, $J_{1,F}$ = 22.1 Hz, C-1), 76.3 (C-5), 73.5 (d, $J_{3,F}$ = 19.8 Hz, C-3), 67.5 (d, $J_{4,F}$ = 6.9 Hz, C-4), 61.4 (C-6), 20.73, 20.63, 20.55 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –191.9 (ddd, $J_{F,2}$ = 50.0 Hz, $J_{F,3}$ = 14.8 Hz, $J_{F,1}$ = 2.0 Hz, F-2); spectroscopic data was identical to that previously reported.[36]

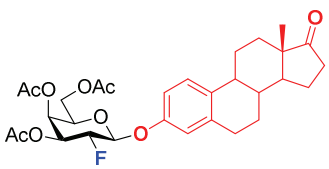
3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-galactopyranosyl



phenylthiosulfonate (6.19b): The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl iodide **6.11a** (24.6 mg, 0.059 mmol), NaPTS (23.1 mg, 0.118 mmol), TBAHS (41.2 mg, 0.118 mmol) and 1 M aqueous Na_2CO_3 (0.24 mL) in EtOAc (1.2 mL). The reaction mixture was stirred at room temperature for 24 h. After standard work-up, the residue was purified by radial chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.19b** (22.6 mg, 83%) as an inseparable 1:6 α/β mixture as a yellowish syrup. R_f (1:1 EtOAc/hexane): 0.38; FT–IR (neat) ν in cm^{-1}: 2990, 2920, 2860, 1744; Anal. Calcd for $C_{18}H_{21}FO_9S_2$: C, 46.54; H, 4.56; S, 13.81. Found: C, 46.62; H, 4.64; S, 13.77; HRMS (TOF ES+) for (M+Na$^+$) $C_{18}H_{21}FNaO_9S_2^+$ (*m/z*): calc. 487.0503; found 487.0512. Data for **6.19bα**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.99–7.55 (m, 5H, Ar), 6.29 (d, $J_{1,2}$ = 6.0 Hz, 1H, H-1), 5.50–5.41 (m, 1H, H-4), 5.12 (ddd, $J_{F,3}$ = 12.8 Hz, $J_{2,3}$ = 8.8 Hz, $J_{3,4}$ = 3.2 Hz, 1H, H-3), 4.91 (ddd, $J_{F,2}$ = 51.6 Hz, $J_{2,3}$ = 8.8 Hz, $J_{1,2}$ = 6.0 Hz, 1H, H-2),

4.19–3.84 (m, 3H, H-5, H-6a, H-6b), 2.11, 2.07, 2.05 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 170.4, 169.9, 169.7 (3C=O, Ac), 146.0 (C, Ar), 134.6–127.1 (CH, Ar), 86.1 (d, $J_{1,F}$ = 24.3 Hz, C-1), 85.9 (d, $J_{2,F}$ = 189.2 Hz, C-2), 75.2 (C-5), 71.7 (d, $J_{3,F}$ = 19.1 Hz, C-3), 67.4 (d, $J_{4,F}$ = 8.4 Hz, C-4), 61.0 (C-6), 20.7, 20.6 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –199.4 (dd, $J_{F,2}$ = 51.6 Hz, $J_{F,3}$ = 12.8 Hz, F-2). Data for **6.19bβ**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.99–7.55 (m, 5H, Ar), 5.50–5.41 (m, 1H, H-4), 5.30 (dd, $J_{1,2}$ = 10.8 Hz, $J_{F,1}$ = 2.4 Hz, 1H, H-1), 5.17 (ddd, $J_{F,3}$ = 13.2 Hz, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 3.6 Hz, 1H, H-3), 4.66 (ddd, $J_{F,2}$ = 50.4 Hz, $J_{1,2}$ = 10.8 Hz, $J_{2,3}$ = 9.6 Hz, 1H, H-2), 4.19–3.84 (m, 3H, H-5, H-6a, H-6b), 2.11, 2.06, 2.05 (s, 9H, $3CH_3$, Ac); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 169.9, 169.8, 169.7 (3C=O, Ac), 141.6 (C, Ar), 134.6–127.1 (CH, Ar), 86.6 (d, $J_{1,F}$ = 24.3 Hz, C-1), 84.7 (d, $J_{2,F}$ = 191.4 Hz, C-2), 75.1 (C-5), 71.8 (d, $J_{3,F}$ = 19.1 Hz, C-3), 67.6 (d, $J_{4,F}$ = 8.4 Hz, C-4), 60.6 (C-6), 20.7, 20.6 ($3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –198.9 (ddd, $J_{F,2}$ = 50.4 Hz, $J_{F,3}$ = 13.2 Hz, $J_{F,1}$ = 2.4 Hz, F-2).

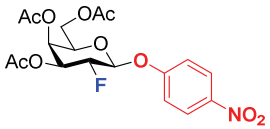
6.5.3. Miscellaneous reactions with 2-deoxy-2-fluoroglycopyranosyl halides

17-Oxoestra-1,3,5(10)-trien-3-yl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-galactopyranoside (6.20a): 

60% NaH in mineral oil (4.14 mg, 0.104 mmol) was added to a solution of estrone (18.6 mg, 0.069 mmol) in dry 1,4-dioxane (552 µL) at room temperature. After 10 minutes, 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl bromide **6.11b** (51.6 mg, 0.139 mmol) in dry 1,4-dioxane (276 µL) was added and the resulting mixture was stirred at the same temperature for 24 h. The crude was concentrated under reduced pressure and treated with pyridine (224 µL) and Ac_2O (104 µL) at room temperature for 12 h. The crude was then diluted with CH_2Cl_2 and washed with saturated

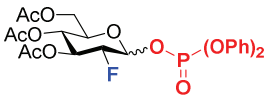
aqueous $CuSO_4$, NH_4Cl and brine. The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.20a** (10.3 mg, 27%) as a white solid. R_f (1:1 EtOAc/hexane): 0.48; $[\alpha]_D^{20}$: +158.7 (0.104, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.23 (d, J = 8.2 Hz, 1H, Ar), 6.88 (dd, J = 8.2 Hz, J = 2.7 Hz, 1H, Ar), 6.83 (d, J = 2.7 Hz, 1H, Ar), 5.48 (ddd, $J_{F,4} = J_{3,4}$ = 3.5 Hz, $J_{4,5}$ = 0.8 Hz, 1H, H-4), 5.19 (ddd, $J_{F,3}$ = 13.6 Hz, $J_{2,3}$ = 9.7 Hz, $J_{3,4}$ = 3.5 Hz, 1H, H-3), 5.10 (dd, $J_{1,2}$ = 7.8 Hz, $J_{F,1}$ = 3.9 Hz, 1H, H-1), 4.77 (ddd, $J_{F,2}$ = 51.4 Hz, $J_{2,3}$ = 9.7 Hz, $J_{1,2}$ = 7.8 Hz, 1H, H-2), 4.22 (dd, $J_{6a,b}$ = 11.3 Hz, $J_{5,6a}$ = 6.6 Hz, 1H, H-6a), 4.15 (dd, $J_{6a,b}$ = 11.3 Hz, $J_{5,6b}$ = 6.2 Hz, 1H, H-6b), 4.06 (ddd, $J_{5,6a}$ = 6.6 Hz, $J_{5,6b}$ = 6.2 Hz, $J_{4,5}$ = 0.8 Hz, 1H, H-5), 2.18, 2.09, 2.07 (s, 9H, $3CH_3$, Ac), 2.93–1.22 (m, 15H, CH, CH_2), 0.92 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ in ppm: 220.8 (C=O), 170.3, 170.04, 169.98 (3C=O, Ac), 154.8, 138.1, 135.1 (C, Ar), 126.5, 117.4, 114.7 (CH, Ar), 99.4 (d, $J_{1,F}$ = 23.8 Hz, C-1), 87.5 (d, $J_{2,F}$ = 188.3 Hz, C-2), 71.1 (C-5), 71.0 (d, $J_{3,F}$ = 19.2 Hz, C-3), 70.9 (C-5), 67.5 (d, $J_{4,F}$ = 8.5 Hz, C-4), 61.2 (C-6), 50.4, 48.0, 44.0, 38.2, 35.8, 31.5, 29.6, 26.4, 25.8, 21.6 (C, CH), 20.7, 20.61, 20.58 ($3CH_3$, Ac), 13.8 (CH_3); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –206.6 (dddd, $J_{F,2}$ = 51.4 Hz, $J_{F,3}$ = 13.6 Hz, $J_{F,1}$ = 3.9 Hz, $J_{F,4}$ = 3.5 Hz, F-2); FT–IR (neat) ν in cm^{-1}: 3270, 2927, 1746, 1736, 1372, 1227, 1075, 1045; HRMS (TOF ES$^+$) for $(M+NH_4)^+$ $C_{30}H_{41}FNO_9^+$ (*m/z*): calc. 578.2760; found 578.2755.

4-Nitrophenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-galactopyranoside



(6.20b): A mixture of 4-nitrophenol (19.2 mg, 0.138 mmol), TBAB (44.9 mg, 0.138 mmol) and 5% aqueous NaOH (1.8 mL) in CH_2Cl_2 (1.8 mL) was stirred at room temperature for 10 minutes. 3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl iodide **6.11a** (28.8 mg, 0.069 mmol) in CH_2Cl_2 (0.9 mL) was then added and the resulting mixture was stirred at the same temperature for 14 h. The crude was then diluted with CH_2Cl_2 and extracted.

The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by radial chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.20b** (14.8 mg, 50%) as a yellowish foam. R_f (1:1 EtOAc/hexane): 0.45; ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 8.25 (d, J = 9.5 Hz, 2H, Ar), 7.16 (d, J = 9.5 Hz, 2H, Ar), 5.52 (appt, $J_{F,4} = J_{3,4}$ = 3.7 Hz, 1H, H-4), 5.27 (dd, $J_{1,2}$ = 7.7 Hz, $J_{F,1}$ = 4.0 Hz, 1H, H-1), 5.23 (ddd, $J_{F,3}$ = 13.2 Hz, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 3.7 Hz, 1H, H-3), 4.83 (ddd, $J_{F,2}$ = 51.3 Hz, $J_{2,3}$ = 9.9 Hz, $J_{1,2}$ = 7.7 Hz, 1H, H-2), 4.26–4.13 (m, 3H, H-5, H-6a, H-6b), 2.19, 2.11, 2.09 (s, 9H, $3CH_3$, Ac); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –207.3 (dddd, $J_{F,2}$ = 51.3 Hz, $J_{F,3}$ = 13.2 Hz, $J_{F,1}$ = 4.0 Hz, $J_{F,4}$ = 3.7 Hz, F-2); spectroscopic data was identical to that previously reported.[33]



Diphenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-glucopyranosyl phosphate (6.21): The title compound was prepared following the general procedure above, starting from 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl iodide **6.14a** (20 mg, 0.048 mmol), diphenyl phosphate (24 mg, 0.096 mmol), TBAHS (33.6 mg, 0.096 mmol) and 1 M aqueous Na_2CO_3 (0.19 mL) in EtOAc (1 mL). The reaction mixture was stirred at room temperature for 80 h. After standard work-up, the residue was purified by radial chromatography (from hexane to 1:1 EtOAc/hexane) to afford **6.21** (19.4 mg, 75%) as an inseparable 1:4 α/β mixture as a colourless oil. R_f (1:1 EtOAc/hexane): 0.35. Data for **6.21α**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.40–7.20 (m, 10H, Ar), 6.16–6.13 (m, 1H, H-1), 5.62–5.57 (m, 1H, H-3), 5.07 (appt, $J_{3,4} = J_{4,5}$ = 9.9 Hz, 1H, H-4), 4.61 (m, 1H, H-2), 4.15 (dd, $J_{6a,b}$ = 12.8 Hz, $J_{5,6a}$ = 4.0 Hz, 1H, H-6a), 4.01 (m, 1H, H-5), 3.80 (m, 1H, H-6b), 2.09, 2.05, 2.04 (s, 9H, $3CH_3$, Ac); ^{31}P NMR ($CDCl_3$, 162.0 MHz) δ in ppm: –12.9 (s, P=O); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –200.9 (dd, $J_{F,2}$ = 48.9 Hz, $J_{F,3}$ = 12.0 Hz, F-2). Data for **6.21β**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ in ppm: 7.40–7.22 (m, 10H, Ar), 5.48–5.54 (m, 1H, H-1), 5.37–5.29 (m, 1H,

H-3), 5.07 (appt, $J_{3,4} = J_{4,5} = 9.9$ Hz, 1H, H-4), 4.45 (ddd, $J_{F,2} = 50.4$ Hz, $J_{2,3} = 9.2$ Hz, $J_{1,2} = 7.7$ Hz, 1H, H-2), 4.23 (dd, $J_{6a,b} = 12.5$ Hz, $J_{5,6a} = 4.4$ Hz, 1H, H-6a), 4.03 (dd, $J_{6a,b} = 12.5$ Hz, $J_{5,6b} = 1.8$ Hz, 1H, H-6b), 3.84–3.81 (m, 1H, H-5), 2.08, 2.05, 2.04 (s, 9H, $3CH_3$, Ac); ^{31}P NMR ($CDCl_3$, 162.0 MHz) δ in ppm: –13.2 (s, P=O); ^{19}F NMR ($CDCl_3$, 376.5 MHz) δ in ppm: –200.3 (ddd, $J_{F,2} = 50.4$ Hz, $J_{F,3} = 13.5$ Hz, $J_{F,1} = 3.1$ Hz, F-2); spectroscopic data was identical to that previously reported.[37]

6.5.4. Protein Modification

Sequence of Annexin V (PDB code: 1AVR)

AQVLRGTVTDFPGFDERADAETLRKAMKGLGTDEESILTLLTSRSNA
QRQEISAAFKTLFGRDLLDDLKSELTGKFEKLIVALMKPSRLYDAYEL
KHALKGAGTNEKVLTEIIASRTPEELRAIKQVYEEEYGSSLEDDVVGD
TSGYYQRMLVVLLQANRDPDAGIDEAQVEQDAQALFQAGELKWGT
DEEKFITIFGTRSVSHLRKVFDKYMTISGFQIEETIDRETSGNLEQLLLA
VVKSIRSIPAYLAETLYYAMKGAGTDDHTLIRVMVSRSEIDLFNIRKE
FRKNFATSLYSMIKGDTSGDYKKALLLLCGEDD

Calculated average isotopic mass = 35805.58 (*N*-terminal Met cleaved)

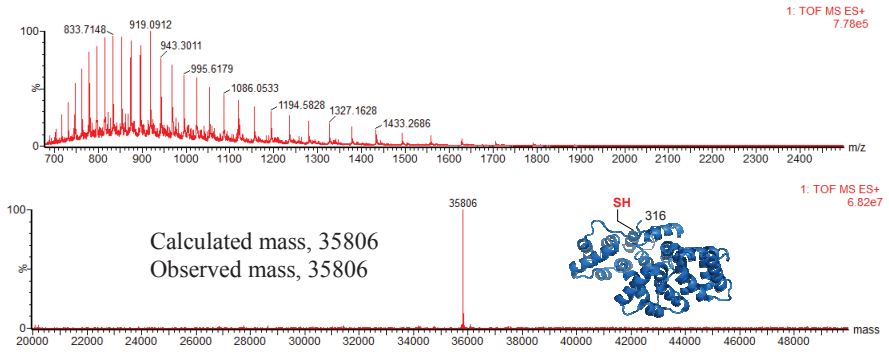


Figure 6.4. ESI–MS spectrum of Annexin V **6.22**.

AnnexinV C316-SS-Ac$_3$GlcF (6.23a): Annexin V **6.22** was prepared as a 1 mg/mL solution in 20 mM Tris HCl buffer (pH 8.0) and 25 µL (0.698 nmol) were added to a 0.5 mL eppendorf. A stock solution of **6.19a** was prepared in a separate eppendorf by dissolving 0.29 mg in 10 µL of acetonitrile. 2.8 µL of this solution (174.6 nmol) was added by micropipette to the protein solution and the reaction vortexed for a few seconds. After 1 h of additional shaking at room temperature, a 2 µL aliquot diluted with 8 µL of the same buffer was analyzed directly by LC–MS and complete conversion to **6.23a** was observed (calculated mass, 36128; observed mass, 36128). The sample was flash frozen with liquid nitrogen and stored at –20 ºC.

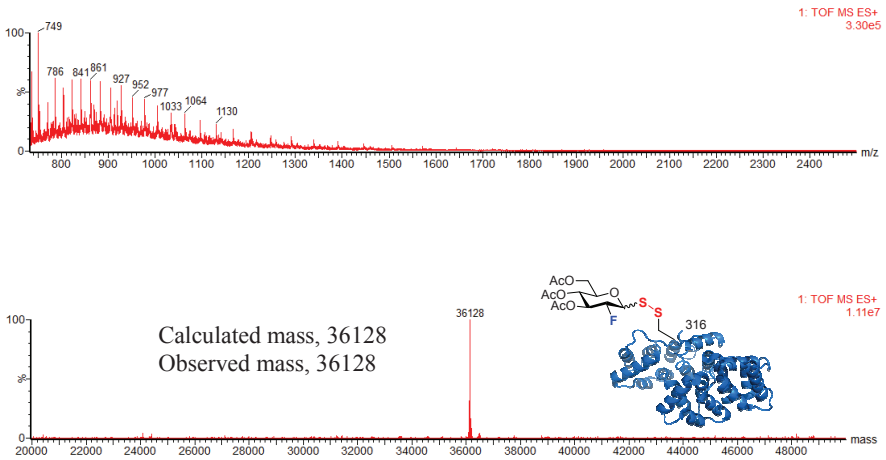


Figure 6.5. ESI–MS spectrum of Annexin V C316-SS-Ac$_3$GlcF **6.23a**.

AnnexinV C316-SS-Ac$_3$GalF (6.23b): Annexin V **6.22** was prepared as a 1 mg/mL solution in 20 mM Tris HCl buffer (pH 8.0) and 25 µL (0.698 nmol) were added to a 0.5 mL eppendorf. A stock solution of **6.19b** was prepared in a separate eppendorf by dissolving 0.29 mg in 10 µL of acetonitrile. 2.8 µL of this solution (174.6 nmol) was added by micropipette to the protein solution and the reaction vortexed for a few seconds. After 1 h of additional shaking at room temperature, a 2 µL aliquot diluted with 8 µL of the same buffer was analyzed directly by LC–MS and complete conversion to **6.23b** was observed (calculated mass, 36128; observed mass, 36128). The sample was flash frozen with liquid nitrogen and stored at –20 ºC.

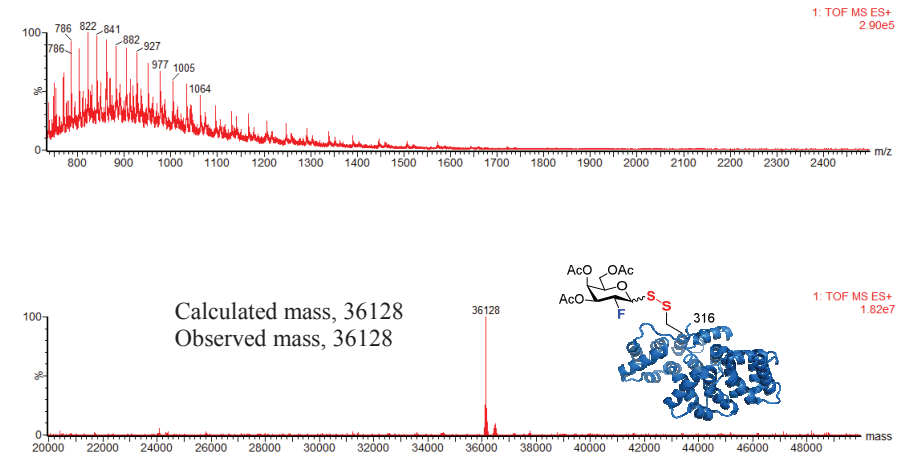


Figure 6.6. ESI–MS spectrum of Annexin V C316-SS-Ac$_3$GalF **6.23b**.

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CHAPTER 7

GENERAL CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
SYNTHETIC GLYCOLIPIDS AS MODULATORS OF CARBOHYDRATEPROTEIN
INTERACTIONS
Míriam Salvadó Molero

This PhD thesis is focused in the study of multivalent systems and protein binding/recognition events. Different aspects were evaluated ranging from carbohydrate structure, presentation and density to the length and polarity of the linker used to attach carbohydrates to a central core. Moreover, different techniques were applied in order to study the benefits of presenting monomers as multivalent systems in protein binding/recognition.

In Chapter 3, it was carried out the synthesis of monomeric glycolipids, glycoclusters and glycopolymers as potential inhibitors of glycosidases and their behaviour against of α-glycosidase (baker's yeast), β-galactosidase (bovine liver), β-galactosidase (*E. coli*), α-galactosidase (green coffee) and β-glucosidase (almonds) was evaluated. The following conclusions were extracted:

(i) A series of glycolipids with different groups at position C-2 (H, OH, NHAc, and F) were synthesized using different strategies. The glycosylation reaction to obtain 2-OH-β-glycolipids was studied using glycosyl donors with 2,6-diFBz and Ac groups at position C-2 and better results were obtained when the group 2,6-diFBz was present in the glycosyl donor. The synthesis of 2-F and 2-NHAc-glycolipids required microwave heating to achieve moderate yields.

The inhibition potency of the family of glycolipids (with modifications in the pyranose ring and modifications in the aglycone moiety) and their corresponding multivalent presentation were evaluated against commercial glycosidases.

(i) When synthetic glycolipids bearing different groups at C-2 were evaluated, it was found that the presence of fluorine is detrimental for inhibition as reflected in the strong increase of K_i values. The presence of this highly electronegative group decreases both glycosylation and deglycosylation rates.

(ii) Comparing glycolipids **5.5** and **5.25** (with and without azide group), it was found that **5.25** was hydrolyzed while **5.5** was not with β-galactosidase (*E.coli*), which indicates that **5.25** is a substrate for this enzyme.

(iii) No inhibition was found for glycoclusters that present 1 and 3 carbohydrate residues (**5.30** and **5.31**), while inhibition was found for the monomer.

(iv) When the corresponding monomers are compared with the hyperbranched structures, higher inhibition was found for the multivalent systems in all cases exhibiting a 1.5-fold for long aliphatic chain, 3-fold for short chain and 9-fold for the two aliphatic chains. However, the relative sugar potency is lower for hyperbranched polymers, and higher for the glycocluster **3.32**.

In Chapter 4, it was studied how the topology of multivalent systems affects the binding to lectin RCA_{120}. The following conclusions were extracted:

(i) Synthetic multivalent systems allowing carbohydrate presentation in a monodisperse and a polydisperse manner based on dendrimers and hyperbranched polymers (Boltorn-H30), respectively, were prepared.

(ii) Their affinity towards a model plant toxin (RCA_{120}) was evaluated using SPR and DLS techniques, giving relevant binding and aggregation differences due to the alternative spatial presentation of sugar epitopes in homogeneous *vs.* heterogeneous central cores. Thus, hyperbranched glycopolymers gave enhanced binding affinities than glycodendrimers of similar size.

(iii) While for hyperbranched glycopolymers protein aggregation in solution with RCA_{120} was observed, no aggregation was found under the same condition for glycoclusters as determined by DLS.

(iv) A thorough study by SPR revealed a complex profile of interactions including chelate and rebinding mechanisms.

In Chapter 5, it was studied a novel strategy for the design of multivalent inhibitors based on glycodendriprotein-based nanocapsules. The following conclusions can be extracted:

(i) Glycodendrons presenting D-galactose or D-galabiose with different shapes and carbohydrate densities were evaluated against a model plant toxin (RCA_{120}) using BLI to obtain the corresponding IC_{50} values.

(ii) It was found that although the carbohydrate density could allow presenting a high carbohydrate valency, their spatial display is even more important. When the surface was coated with the same number of carbohydrates, glycodendrons that presented D-galactose in a pyrogallic-gallic fashion (**5.8**) resulted in a 3.7-fold enhanced inhibition compared to gallic-pyrogallic **5.7** cores.

(iii) Functionalization of BSA at Cys34 was studied with compound **5.28** obtaining the corresponding sulfur-linked protein.

In Chapter 6, it was developed a procedure for synthesizing fluorosugar reagents for the construction of well-defined fluoroglycoproteins. The following conclusions can be extracted:

(i) A general strategy to access a wide range of fluorosugars modified as *O*-, *N*-, *S*- and *Se*-glycosides, as useful reagents for chemical-site selective protein glycosylation was disclosed. These fluorosugar reagents were synthesized *via* S_N2-like reactions, from glycosyl

iodide intermediates, which possess a seemly balance between stability and reactivity that facilitates their preparation. The exquisite β-control achieved during stereoselective glycosylation with soft nucleophiles ensured product homogeneity.

(ii) A platform to construct disulfide-linked F-proteins was obtained during the reactivity studies of some F-sugars derivatives with a model single-Cys protein (Annexin V).

(iii) This strategy could be applied for building homogeneous [^{18}F]-glycoprobes, considered valuable tools in chemical biology and biomedical imaging.