CARBOHYDRATE MULTIVALENT SYSTEMS TO FUNCTIONALIZE PROTEINS AND SURFACES FOR LECTIN INTERACTION STUDIES

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Doctoral thesis presented by

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"….Science, for me, gives a partial explanation of life. In my view, all that is necessary for faith is the belief that by doing our best, we shall come nearer to success, and that success in our aims (the improvement of the lot of mankind, present and future) is worth attaining..."

Rosalind Franklin

µL	Microliter
µM	Micromolar
Ac	Acetyl
AcCN	Acetonitrile
Ac_2O	Acetic anhydride
AFM	Atomic force microscopy
AIDS	Acquired immune deficiency syndrome
Asc	Ascorbate
Asn	Asparagine
Asp	Aspartic acid
APC	Antigen presenting cell
B-cell	Bone-marrow derived lymphocytes
BAPAD	BisAminoalkylPolyAmide Dendrimers
Bis-MPA	2,2-Bis(hydroxymethyl)propionic acid
BCN	(*N*-[(1*R*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-yn-9 ylmethyloxycarbonyl]-1,8-diamino3,6 dioxa-octane)
Boc	*tert*-Butyloxycarbonyl
BSA	Bovine serum albumin
BzCN	Benzoyl cyanide
ConA	Concanavalin A
CBP	Carbohydrate binding protein
Cbz	Carboxybenzyl
CLR	C-type lectin receptor
CLEC	C-type lectin-like receptor
COSY	Correlation spectroscopy
CRD	Carbohydrate recognition domain

CSA	(1*S*)-(+)-10-Camphorsulfonic acid
CTL	C-type lectin
CTLD	C-type lectin-like domain
CuAAC	Copper(I)-catalyzed Azide Alkyne cycloaddition
δ	Chemical shift
d	doublet
Da	dalton
DAMPs	Damage Associated Molecular Patterns
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	*N,N'*-diciclohexylcarbodiimmide
DCM	Methylene chloride
DC SIGN	Dendritic cell specific intercellular adhesion molecule-3-grabbing non integrin
DC SIGN R	Or L-SIGN is an homologue of DC-SIGN
DCs	Dendritic cells
DCAL	Dendritic-cell-associated C-type lectin
DEC205	Or CD205 is a macrophage mannose receptor family of C-type lectin endocytic receptors
dd	doublet of doublets
DHB	2,5-Dihydroxybenzoic acid
DIPEA	*N,N*-Diisopropylethylamine

DMA	Dimethylacetamide
DMAP	4-(Dimethylamino)pyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DPTS	4-(dimethylamino)pyridinium 4-toluenesulfonate
DSS	Disuccinimidyl suberate
DTAF	5-([4,6-Dichlorotriazin-2-yl]amino) fluorescein
ECD	Extracellular domain
EDTA	Ethylenediaminetetraacetic acid
ETN	European training network
eq	equivalent
EtOAc	Ethyl acetate
EtOH	Ethanol
Et_3N	Triethylamine
ESI	Electrospray ionization
Fmoc	Fluorenylmethyloxycarbonyl
Fuc	Fucose
Gal	Galactose
GalNAc	*N*-Acetylgalactosamine
GBP	Glycan binding protein
GDP	Glycodendriprotein
Glc	Glucose
Glu	Gluctamic acid
Gln	Glutamine
GNA	Galanthus nivalis agglutinin lectin

HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
Hex	Hexane
HIV	Human immunodeficiency virus
HSA	Human serum albumin
HSQC	Heteronuclear single quantum coherence spectroscopy
HSV	Herpes simplex virus
IgG	Immunoglobuline G
ITO	Indium tin oxide
J	Coupling constant
m	multiplet
MALDI	Matrix assisted laser desorption ionization
Man	Mannose
MeOH	Methanol
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MGL	Macrophage galactose lectin
MR	Mannose recptor
MS	Mass spectrometry
MW	Microwave
m/z	mass to charge ratio
NaOMe	Sodium methoxide
NGL	Neoglycolipid
NGP	Neoglycoprotein or neoglycopeptide
NHS	*N*-Hydroxysuccinimide
NIS	N-Iodosuccinimide

NLR	Nod-like receptor
NMR	Nuclear magnetic resonance
Ns	2-Nosyl
ODPA	Octadecylphosphonic acid
OVA	Ovalbumin
PAMAM	Polyamidoamine
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
ppm	Parts per million
PPR	Pattern recognition receptor
Pro	Prolin
Py	Pyridine
q	quartet
RIP	Relative inhibitory potency
RLR	RIG-I-like receptor
rt	Room temperature
s	singlet
SAR	Structure activity relationship
SDS-Page	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SPAAC	Strain-promoted alkyne azide cycloaddition
SPR	Surface plasmon resonance
t	Triplet
T-cell	Thymus derived lymphocytes
TBAI	Tetrabutylammonium iodide
TBTA	Tris(benzyltriazolylmethyl)amine
***t*-BuOOH**	*tert*-Butyl hydroperoxide

TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLR	Toll like receptors
TMSOTf	Trimethylsilyl trifluoromethanesulphonate
TOF	Time-Of-Flight
UV	Ultraviolet

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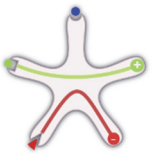
CHAPTER 1
Introduction



1. Introduction

1.1. The human immune system – Our *Aegis* against pathogens

The *Aegis*[1] that protects the mankind against pathogens is the immune system. The human immune system is a complex structure of cells and tissues whose main functions are the recognition and the subsequent elimination of foreign antigens, the formation of an immunologic memory, and the development of tolerance to self-antigens. The immune response could be divided in two types: the *innate* and the *adaptive* immune response.[2]

- The *innate* immune system consists on the cells that firstly recognize and respond to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) releasing cytokines, chemokines and antimicrobial proteins which drive inflammation and ensuing immune response.[3]
- The *adaptive* immune system, in contrast with the innate one, requires more time to respond to the threat and is mediated by two type of lymphocyte cell populations, the bone-marrow derived (B lymphocytes or B-cells) and thymus derived lymphocytes (T-cells). It is highly antigen-specific[4] and develops an immunological memory that provides protection against reinfection of a wide range of antigens presented on virus, bacteria, parasites and fungus.[5]

[1] The *Aegis* in Latin, as cited in the Iliad, is a shield or an armor used by Zeus and his daughter Athena.
[2] a) Palm, N.W.; Medzhitov, R.; *Immunol. Rev.*, **2009**, *227*, 221–233; b) Medzhitov, R.; Janeway, C.A. Jr.; *Curr. Opin. Immunol.*, **1997,** *9*, 4–9.
[3] Janeway, C.A. Jr; Medzhitov, R.; *Annu. Rev. Immunol.*, **2002**, *20*, 197–216.
[4] a) Iwasaki, A.; Medzhitov, R.; *Science*, **2010**, *327*, 291–295; b) Litman, G.W. *et al.*; *Nat. Rev. Immunol.*, **2010**, *10*, 543–553.
[5] Walsh, K.P.; Mills, K.H.; *Trends Immunol.*, **2013**, *34*, 521-30.

These immune responses are induced, coordinated, and regulated[6] by an important class of antigen presenting cells (APCs), the dendritic cells (DCs).[7] These cells have the ability to bridge innate and adaptive immunity via pattern-recognition receptors (PRRs), which recognize and generate a response to the PAMPs or DAMPs. Hence, depending on the pathogen, APCs regulate and drive the function of the B and T cells,[8] and start the adaptive immune response.[9]

1.2. Dendritic cells

The name of these cells "*Dendritic*" derives from the Greek word "*dendros*" that means tree, this is due to the typical stellate shape similar to the branches of a tree.[10] (Figure 1.1)

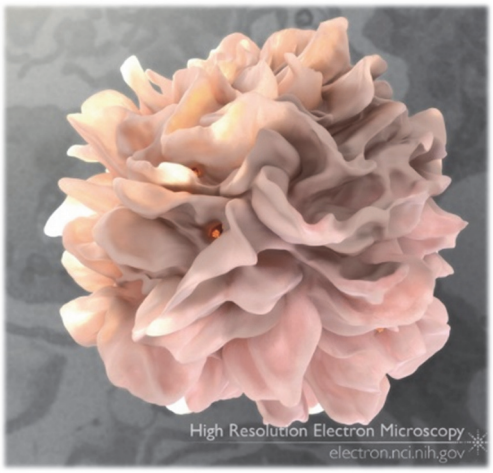


Figure 1.1. A 3D representation of a DC interacting with the HIV-1 (in red).[11] Picture from reference 11.

[6] Steinman, R. M.; *Annu. Rev. Immunol.*, **1991**, *9*, 271–296
[7] Niess, J. H. *et al*., *Science*, **2005**, *307*, 254–258.
[8] a) Marshall, N.A. *et al., Cancer Res.*, **2012**, *72*, 581–591; b) Wilkinson, K.A.; Wilkinson, R.J.; *Eur. J. Immunol.*, **2010**, *40*, 2139–2142; c) Banchereau, J.; Steinman, R.M.; *Nature*, **1998**, *392*, 245-252.
[9] *Carbohydrate-based vaccines methods and protocols*, Ed. Bernd Lepenies, *Humana Press*, **2015**, *Chapter 1*, Hütter, J.; Lepenies, 1-10.
[10] Steinman, R. M.; Cohn, Z. A.; *J. Exp. Med.*, **1973**, *137*, 1142–1162.
[11] Image created by Donald Bliss, National Library of Medicine, Sriram Subramaniam, National Cancer Institute; Felts, R.L. *et al., Proc Natl Acad Sci USA*, **2010**, *107*, 13336-13341.

DCs are population of white blood cells strategically placed in the peripheral tissues, (skin, pharynx, internal and external mucosal surface, respiratory and gastro intestinal treats, etc.) derived from a precursor called hematopoietic stem cells, present in the bone marrow.[12] These stem cells, after the differentiation into immature DCs, migrate to the peripheral tissues and monitor their environment in a continuos manner, capturing pathogens or antigens and inducing the tolerance or the immune response:[13]

- *Tolerance response.* In this mechanism, immature DCs capture antigens and migrate to the draining lymph nodes, subsequently inducing the action of the T cells.[14]
- *Immune response*. Instead, if there is an inflammation of the tissue, DCs migrate and pass from immature to mature, stay with a series of stimulations and co-stimulations that leading to the activation of B cells, and initiating the adaptive immune response.[15,16,17] All the later responses that generate the attack to the PAMPs is controlled by regulatory T cells (T_{Reg}) supported by other lymphocyte cells. [13, 18]

Therefore, DCs have the role of coordinator of all the elements of the immune orchestra and is commonly considered to be one of the most important APCs. This is due to their crucial role in the modulation of the immune reactions, calibrating the balance between tolerance and the induction of an inflammatory response. [19]

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[12] Banchereau, J. *et al*., *Annu. Rev. Immunol.*, **2000**, *18*, 767–811.
[13] Mellman, I.; Steinman, R.M.; *Cell*, **2001**, *106*, 255–258.
[14] a) Randolph, G.J.; Angeli, V.; Swartz, M.A.; *Nat. Rev. Immunol.*, **2005**, *5*, 617–628; b) Cyster, J.G.; *J. Exp. Med.*, **1999**, *189*, 447–450.
[15] Banchereau, J.; Palucka, A.K.; *Nat. Rev. Immunol.*, **2005**, *5*, 296-306.
[16] Lennon-Dumenil, A.M.; Bakker, A.H.; Wolf-Bryant, P.; Ploegh, H.L.; Lagaudriere-Gesbert, C.; *Curr. Opin. Immunol.*, **2002**, *14*, 15–21.
[17] *Fundamental Immunology (7th edition)*, Eds. Paul, W. E., Steinman, R. M. in *Lippincott-Raven, Philadelphia*, **2012**, ISBN 9781451117837.
[18] a) Kadowaki, N. *et al*., *J. Exp. Med.*, **2001**, *193*, 1221–1226; b) Fernandez, N. C. *et al*., *Nat. Med*., **1999**, *5*, 405–411.
[19] Laffont, S.; Powrie, F.; *Nature*, **2009**, *462*, 732-733.

This prominence in the immune mechanisms converts DCs into the target of several pathogenic agents and consequently, represents a relevant target for developing therapeutic treatments and manipulation of the immune system.[13]

There are many examples of the pathogenic action on DCs, some microbial agents *Salmonella typhi*, *Plasmodia*, and viruses such as herpes simplex virus (HSV), human immunodeficiency virus (HIV), varicella zoster, Ebola/Marburg/Lassa fever virus and Measles virus, etc. have the capacity to block the DCs maturation in order to keep the infection.[20]
Moreover, several pathogens are able to affect other steps of DCs life, with the aim to evade the immune response. Indeed, plagues as *Yersinia pestis*, *typhoid* fever, *Salmonella typhi*, etc. inject toxins in DCs, inducing apoptosis.[21]
Additionally, other pathogens can utilize DCs for their purposes of replication and spread into the organism such HIV, Ebola and Dengue virus. In particular, in the case of HIV-1, when the DCs bind the viral particle, the virus remains intact into the cells during the migration to the lymph nodes and transmit the infection to T cells.[22]

1.3. Carbohydrate epitopes and protein receptors

The mammalian cells and the pathogens are characterized by a distinct matrix of glycoconjugates which make the glycomic profile extremely precise for each cell type, like a glycan signature.[23] The glycoconjugates could be oligosaccharide moieties displayed on protein (glycoproteins) or on lipid (glycolipids) scaffolds. The carbohydrates are, on these scaffolds, well exposed on the cell surface and play the role of sensors, as well as biochemical signals for several cell functions.[24]

[20] Querec, T. *et al*., *J. Exp. Med.*, **2006**, *203*, 413–424.
[21] Albert, M. L.; Sauter, B.; Bhardwaj, N.; *Nature*, **1998**, *392*, 86–89.
[22] Geijtenbeek, T.B.H. *et al*., *Cell*, **2000**, *100*, 587–597.
[23] Reuter, G.; Gabius, H-J.; *Cell. Mol. Life Sci.*, **1999**, *55*, 368–422.
[24] Gabius, H.J.; Andre, S.; Kaltner, H.; Siebert, H-C.; *Biochim. Biophys. Acta*, **2002**, *1572*, 165–177.

In this respect, the mammalian organisms have evolved to develop recognition systems to monitor self and pathogenic glycan profiles by the use of the PRRs.[25] The pathogen agents act binding PRRs by specific glycoconjugates that cover the pathogen surface and give them the infectious potential.[9] Consequently, the recognition of carbohydrate-containing PAMPs by immune system cells is mediated by several carbohydrate binding proteins (CBP) that have the function of PRRs.[3] The PRR families expressed by DCs can be classified principally in Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type Lectin Receptors (CLRs).[26] (Figure 1.2)

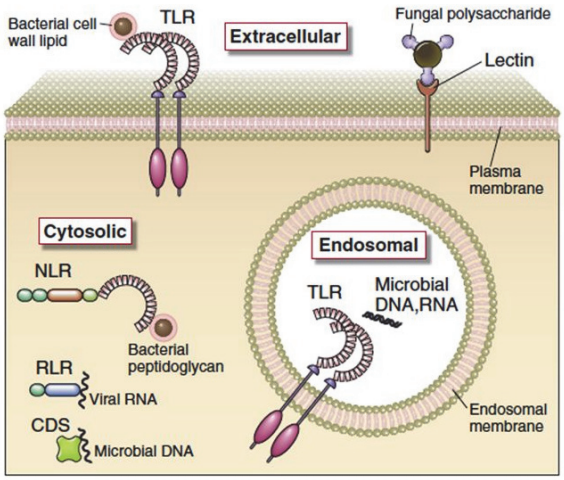

Figure 1.2. Cellular PRRs and their location in the cells.[27] Picture modified from reference 27.

The base of these interactions relay on the recognition of displaying carbohydrate epitopes and the corresponding carbohydrate-receptors. (Figure 1.3)

[25] Gabius, H.J.; Siebert, H.C.; Andre, S.; Jimenez-Barbero, J.; Rudiger, H.; *ChemBioChem*, **2004**, *5*, 740–764.
[26] a) Broz, P.; Monack, D.M.; *Nat. Rev. Immunol.*, **2013**, *13*, 551–565; b) Figdor, C.G.; van Kooyk, Y.; Adema, G.J.; *Nat. Rev. Immunol.*, **2002**, *2*, 77–84.
[27] *Cellular and Molecular Immunology (9th edition)*, Eds. Abbas. A.; Lichtman, A.H.; Pillai, S.; *Elsevier*, **2017**, ISBN: 9780323479783.

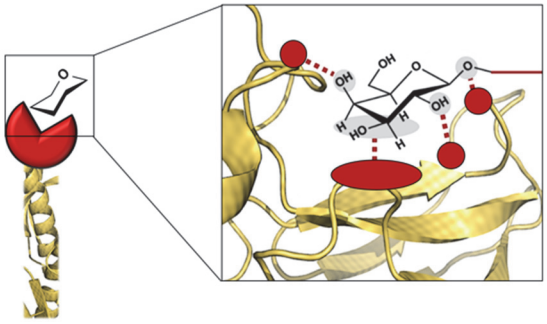

Figure 1.3. Representation of a carbohydrate interacting with the binding site of a CBP. Picture modified from reference 75.

Hence, this interaction becomes of important relevance to study and develop new drugs that inhibit these carbohydrate-protein interactions where the pathogens are involved. At the present, more than 80 human CBPs have been identified,[28] but only few of them have been thoroughly studied and therefore have been investigated and validated as potential drug targets.

The following section will be focused on the protein side of these interactions and then, the rest of this chapter will be focused on the role that multivalency plays in these recognition events and the tools developed to study them.

1.4. Lectins

The research work described in this thesis involves the study of one of these specialized receptors utilized by the myeloid cells to orchestrate the immune response, the lectins.

In 1888, Peter Hermann Stillmark isolated from the *Ricinus communis* the first lectin, the toxic hemagglutinin, which he called Ricin. In 1954, in order to name this new class of carbohydrate recognition proteins able to distinguish between erythrocytes of different blood types, Boyd and Shapleigh proposed the Latin term "*legere*" that

[28] Ernst, B.; Magnani, J.L.; *Nat. Rev. Drug Discover.*, **2009**, *8*, 661-677.

means “read” or “select”. This term has been extended to include not only the plant agglutinins but also other plant and human proteins with a similar behavior.[29]

Lectins can be isolated not only from plant and mammalians, but also from microorganisms. In fact, some lectins on the cell surface act as attaching point of different kind of cells as well as viruses, bacteria or other organisms via the recognition of carbohydrates present at the surface of these systems. However, in other cases, are the glycolipids or the glycoproteins on the cell surface those that interact with lectins of other cells, driving internal phenomena such as gene regulation, differentiation, adhesion, migration, and pathogen infection.[29] (Figure 1.4)

Hence, the lectins represent a superfamily of specialized receptors with a key role in a wide range of cell-cell and cell-pathogens interactions.[29]

[29] Sharon, N.; Lis, H.; *Glycobiology*, **2004**, *14*, 53R-62R.

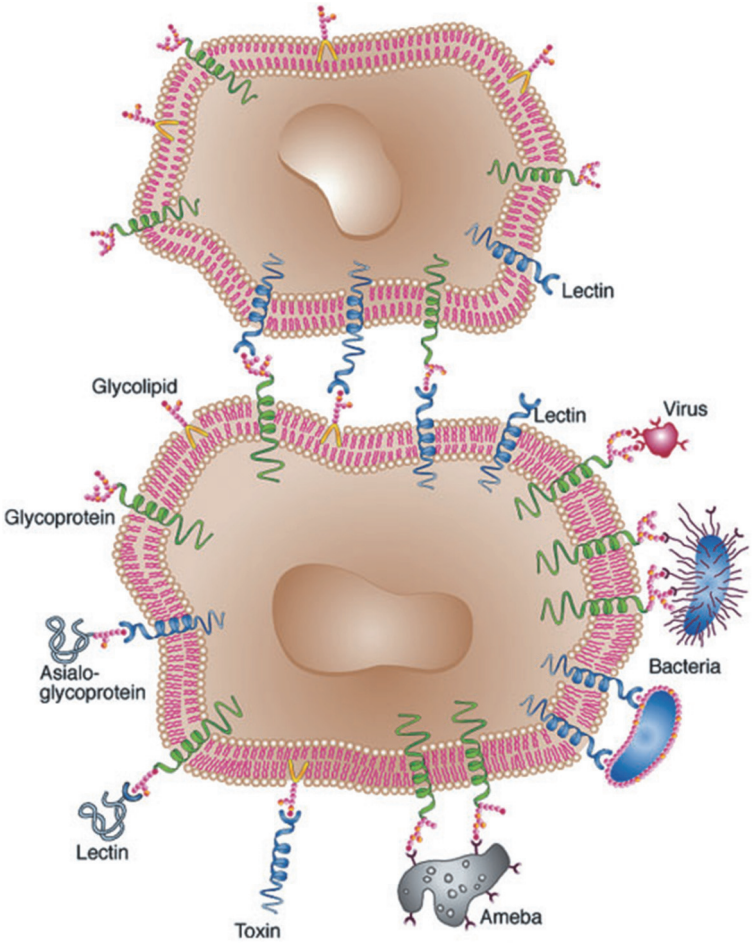

Figure 1.4. Cell surface intercommunication by lectin–carbohydrate interactions with cells or pathogens. Picture from reference 29.

Lectins are divided into specific types, like C-, H-, I-, L-, P-, R-, and S-type; the most promising from a drug targeting point of view among these lectin types are: C-type lectins (CTLs), S-type lectins (or galectins), I-type lectins (in particular siglec that are a subclass of I-type lectins that bind sialic acid).

The work described in this thesis has been focused on the study of the carbohydrate ligand interactions of integral membrane C-type lectins.

1.4.1. C-type Lectins

The immune system, through the use of multiple CTLs, is able to react to a broad spectrum of pathogens.[30] This relays on the ability of CTLs to recognize and engage different glycan pathogen ligands, control the microbicidal activity, modulate the gene transcription and above all, to regulate the function of the cells of the immune system.[31]

Some CTLs are able to recognize the molecular signatures of pathogens, others to detect cell damage, recognizing molecular elements indicative of abnormality on their environment, such as oxidized lipids or heat shock proteins, signals of apoptotic or necrosis cell processes. Then, CTLs are able to start a biomolecular pathway that lead to engage the endocytic and phagocytic cleaner machinery and start the immune response against microbes, viruses, cancer cells or other abnormality in the human body.[30]

In vertebrates, CTLs have the relevant "on first line" role of: interact and engage the pathogen carbohydrate ligands, modulate signals from other receptors and therefore, fine-tuning the adaptive response to the infection or damage. Indeed, CTLs can be soluble or membrane bound and are expressed by myeloid cells APCs such as DCs, granulocytes and macrophages.[32] (Figure 1.5) They are literally the first molecular contact among the PAMPs and the cells of the immune system. As explained before, when the DCs (or other APCs) sense a pathogen, a molecular recognition takes place through the CTL (Figure 1.5 A). The APC internalizes the pathogen by endocytosis, (Figure 1.5 B) processes the pathogen antigens and exposes the pathogen-derived peptides as Major Histocompatibility Complexes (MHCs) in order to engage the T-cell and start the adaptive response.[33] (Figure 1.5 C-D)

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[30] Sancho, D.; Reis e Sousa, C.; *Annu. Rev. Immunol.,* **2012**, *30*, 491-529.
[31] a) Klaver, E.J.; *et al., Int. J. Parasitol.*, **2013**, *43*, 191–200; b) Vautier, S,; MacCallum, D.M.; Brown, G.D.; *Cytokine*, **2012**, *58*, 89–99; c) Lozach, P.Y.; Burleigh. L.; Staropoli, I.; Amara, A.; *Methods Mol. Biol.*, **2007**, *379*, 51–68.
[32] Geijtenbeek T.B.; Gringhuis, S.I.; *Nat. Rev. Immunol.*, **2009**, *9*, 465–479.
[33] Geijtenbeek, T.B.; Gringhuis, S.I.; *Nat. Rev. Immunol.*, **2016**, *16*, 433–448.

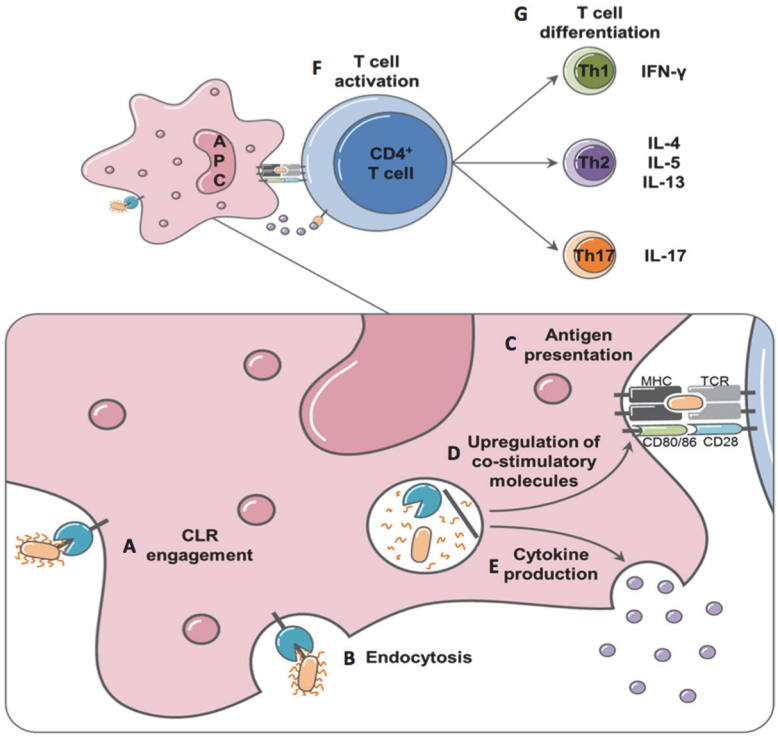

Figure 1.5. Schematic pathogen recognition and role of CTLs in the engagement of the PAMPs. Picture from reference 34a.

In addition, CTLs act, in an indirect way, generating signals with various effector functions such as the production of pro-inflammatory cytokines, which lead the activation of T cells but also interacting with other PRR like TLRs.[34] (Figure 1.5 E)

1.4.2. Lectin classification

In 1988, Kurt Drickamer suggested the idea to organize animal lectins into several categories.[35] The term "C-type lectin" was introduced with the aim to distinguish the group of the Ca^{2+} dependent from the other types that do not require Ca^{2+} ions for

[34] a) Mayer, S.; Raulf, M.K.; Lepenies, B.; *Histochem. Cell. Biol.*, **2017**, *147*, 223-237; b) Kingeter, L.M.; Lin, X.; *Cell. Mol. Immunol.*, **2012**, *9*, 105–112.
[35] Drickamer, K.; *J. Biol. Chem.*, **1988**, *263*, 9557–9560.

binding. The latter are named C-type lectin-like molecules, missing the ability to bind the carbohydrate ligand.[36]

The membrane-bound CTLs are classified into two large groups:[32]

- Type I CTLs. These are part of the mannose receptor family.
- Type II CTLs. These are part of the asialoglycoproteins.

The II type of CTLs includes several lectins associated with DCs immunoreceptors that seems to preferentially recognize mannose, fucose and glucans. This "promiscuity" allows the DCs to recognize most types of pathogens, including bacteria, fungi, viruses and parasites.[37]

The lectins of the group II are type II transmembrane CTLs containing a transmembrane domain, a short cytoplasmatic tail and an extracellular stalk region; and a single CTLD containing the Ca^{2+} ion, involved in the recognition. The subgroups II, V and VI are expressed by the myeloid cells.[38]

The superfamily of the CTL is composed by more than 1000 proteins, which are further classified in 17 subgroups (I-XVII) base on the domain organization and the phylogeny.[38] (Figure 1.6)

[36] Drickamer, K.; *Curr. Opin. Struct. Biol.*, **1999**, *9*, 585-590.
[37] Drummond, R.A.; Brown, G.D., *PLoS Pathog.*, **2013**, *9*, e1003417
[38] Zelensky, A.N.; Gready, J.E.; *FEBS J.*, **2005**, *272*, 6179-6217.

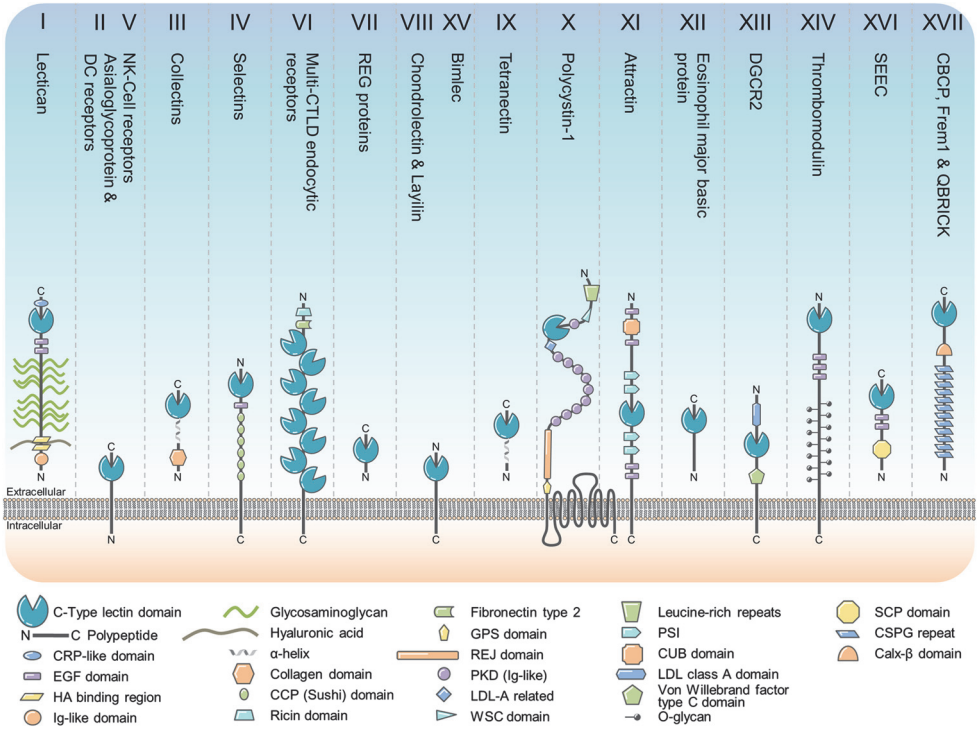

Figure 1.6. Classification of the superfamily of CTLD-containing proteins. These are soluble as well as transmembrane C-type lectin receptors grouped on the basis of their structural composition and effector functions. Picture from reference 34a.

1.4.3. The CRD

The CBPs of the large family of CTLs are characterized by very different structures; however, all of them have a common domain involved in the recognizing of the carbohydrate ligand, called C-type lectin-like domain (CTLD) or carbohydrate recognition domain (CRD). This is a compact globular structure responsible for the selective binding of the terminal units of the carbohydrate epitopes.[38]

From a structural point of view, the CTLD is characterized by two protein loops,[38] where the second more flexible loop generally contains the protein domain involved in the carbohydrate binding.[36]Four Ca^{2+} binding sites are present in the CTLD structure but only the site 2 is mainly involved in the carbohydrate recognition.[38] (Figure 1.7)

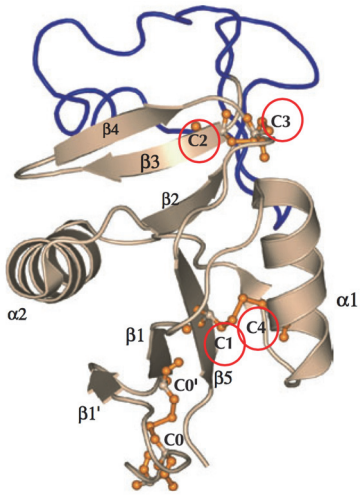

Figure 1.7. A cartoon representing a typical CTLD structure. In blue, the loop region; C1-4 (in red circles), represents the four sites involved in the binding. Picture from reference 38.

The CRD is composed by two amino acids with long carbonyl side chains separated by a *cis* proline. This carbonyl side chains coordinate Ca^{2+}, forming hydrogen bonds with the carbohydrates and determining the binding specificity. Two principal motifs have been identified:

- EPN. Amino acid sequence Glu-Pro-Asn, specific for mannose-ligands
- QPD. Amino acid sequence Gln-Pro-Asp, specific for galactose-ligands

However, the versatility of the CTLD is demonstrated by the fact that in several cases, it was observed that the Ca^{2+} binding sites can interact with ligands which are not carbohydrates, like proteins, lipids or inorganic ligands.[38]

1.4.4. Human C-type lectins involved in the immune response

Mannose-type lectins which are part of the group II and therefore have an EPN sequence, mediate many biological events such as cell-cell or cell-matrix adhesion, and glycoprotein turnover.

These CTLs have a remarkable contribution to the innate immune response through pathogen recognition and internalization of antigens. Furthermore, some CLTs

possess intrinsic signaling properties that enable them to modulate the immune reactions.[39]

In particular, DCs express al large variety of CTLs which are often downregulated upon maturation, but that give the essential ability to immature DCs to bind and internalize glycosylated antigens.[40] It is important to highlight that several CTLs expressed by DCs (MR, DCAL-2, CLEC-2, DEC205, MGL, Dectin-1, Mincle, Langerin and DC-SIGN) have demonstrated to do not function merely as antigen uptake receptors but they have the ability to facilitate the efficient loading of antigens and the T cell induction.[41] (Figure 1.8)

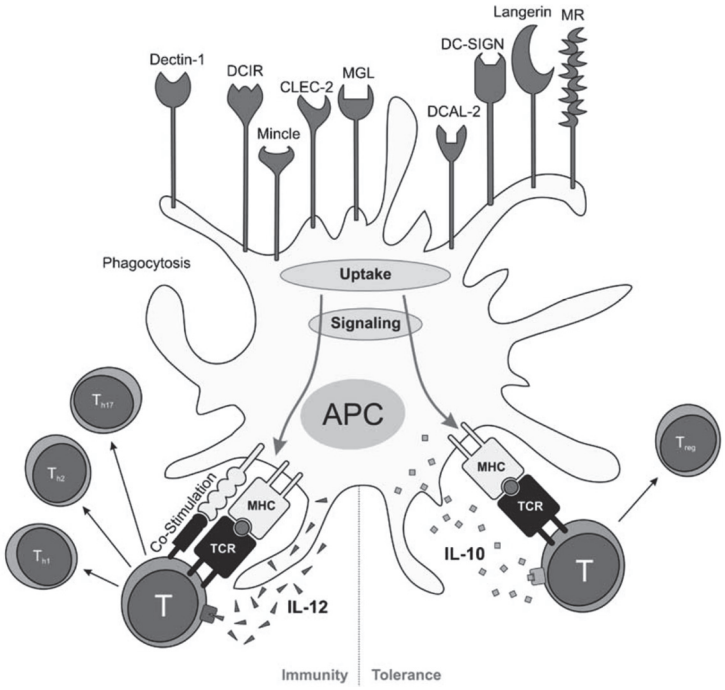

Figure 1.8. APCs express CTLs involved in uptake and signaling processes. Picture from reference 39.

[39] van Vliet, S.J.; Garcia-Vallejo, J.J.; van Kooyk, Y.; *Immunol. Cell Biol.*, **2008**, *86*, 580-587.
[40] a) Wells, C.A. *et al., J. Immunol.*, **2008**, *180*, 7404-7413; b) Weck, M.M. *et al., Blood*, **2008**, *111*, 4264-4272; c) Engering, A. *et al., J. Immunol.*, **2002**, *168*, 2118-2126.
[41] a) Tacken, P.J. *et al*., *Blood*, **2005,** *106*,1278-1285; b) Bonifaz, L.C. *et al*., *J. Exp. Med*., **2004**, *199*, 815-824.

In this thesis, we describe two of the most important and studied Human CTLs, Langerin and DC-SIGN. (Figure 1.8)

It has been demonstrated that both CTLs are involved in different important pathways from the pathogen recognition to the cell-cell interaction, involving the recognition of specific glycans exposed on pathogens or pathogenic structures, such as mannose structures, Lewis-type antigens, GalNAc or β-glucan.[42]

1.4.5. Langerin

Langerin is a type II transmembrane CTL expressed exclusively by Langerhans cells which are DCs present in the epithelial layer and epidermis. Langerin is localized in Birbeck granules in the cytoplasm of Langerhans cells.[43] The structure of Langerin is characterized by an extracellular domain that consist of a CRD and a neck region that stabilizes the formation of trimers of CRDs.[44] (Figure 1.9)

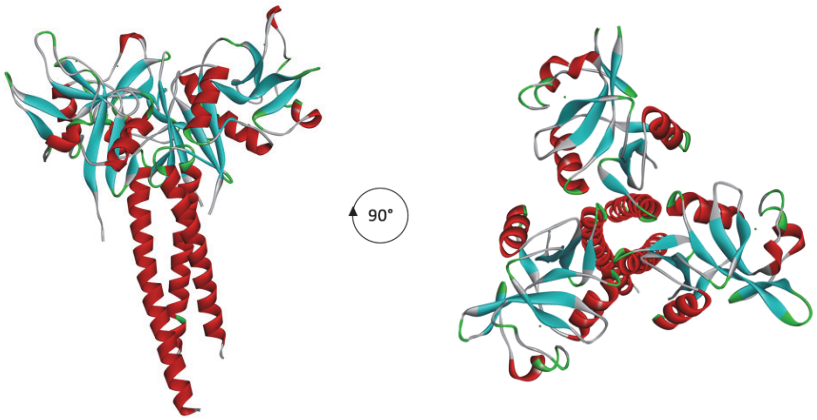

Figure 1.9. Two views of the trimeric structure of Langerin. Picture from the reference 44.

The CRD of Langerin has affinity for high mannose, fucose, GlcNAc and β-glucans and sulfated carbohydrates. These affinities make Langerin a protein binder of glycoproteins and glycolipids present on several microorganisms and lead to the

[42] a) de Witte, L. *et al*., *Nat. Med.*, **2007**, *13*, 367-371; b) Tassaneetrithep, B. *et al*., *J. Exp. Med.*, **2003**, *197*, 823-829.

[43] Valladeau, J. *et al., Immunity*, **2000**, *12*, 71–81.

[44] Chatwell, L.; Holla, A.; Kaufer, B.B.; Skerra, A.; *Mol. Immunol.*, **2008**, *45*, 1981-1994; Image obtained by PDB: 3KQG in "BIOVIA Discovery Studio 4.5©" program.

internalization of the pathogen antigens into Birbeck granules introducing them in processing pathways. Langerin ligands are mainly expressed in different pathogens.[43, 45, 46] (Table 1.1.)

Ligand	Ligand origin
High mannose, fucose(Le^y and Le^B) and GlcNAc	- Viruses: HIV-1. - Bacteria: *M. leprae*. - Fungi: *Candida* spp, *Saccharomyces* spp, *Malassezia furfur*. - Type I procollagen

Table 1.1. Selective ligands for Langerin and their origin.

In the particular case of HIV-1, the binding of the high mannose of gp120 facilitates the degradation of the virus, in contrast to DC-SIGN, that binds the gp120 facilitating the HIV transmission to T cells.[42a] Langerin is able to directly capture HIV-1 through gp120 binding and sequentially degrading it into the Birbeck granules to promote T cell elimination of HIV-1 infection. This suggest that Langerin plays a pleiotropic role in HIV infection.[47,48]

Besides all this information, many pathways in which Langerin is involved, like its role in the regulation of DC activation, remains unknown.[30]

1.4.6. Human DC-SIGN

Human Dendritic Cell-Specific Intercellular adhesion molecule-3 Grabbing Non-integrin (DC-SIGN) is mainly expressed by myeloid DCs but also in alveolar, lymph nodes, sinus endothelial cells, Hofbauer cells in the placenta by dermal, interstitial and monocyte-derived DCs,[22] and macrophages.[48] A closely related receptor is the L-

[45] Liu, Y.; Liu, J.; Pang, X.; Liu, T.; Ning, Z.; Cheng, G.; *Molecules*, **2015**, *20*, 2272–2295.
[46] a) de Jong, M.A. *et al., Mol. Immunol.*, **2010**, *47*, 1216–25; b) de Witte, L. *et al*., *Nat. Med.*, **2007**, *13*, 367–371; c) Hunger, R.E. *et al., J. Clin. Investig.*, **2004**, *113*, 701–708.
[47] a) Schwartz, O.; *Nat. Med.*, **2007**, *13*, 245–246; b) Turville, S.G.; Cameron, P.U.; Handley, A.; Lin, G.; Pöhlmann, S.; Doms, R.W.; Cunningham, A.L.; *Nat. Immunol.*, **2002**, *3*, 975–983.
[48] Granelli-Piperno, A. *et al., J. Immunol.*, **2005**, *175*, 4265–4273.

SIGN or DC-SIGN-related (DC-SIGN R) which is found on endothelial cells, liver, lymph nodes and placenta.[49]

They share the same structure formed by a single CRD globular structure consisting of twelve β-strands (in blue, Figure 1.10 A), two α helices (in red, Figure 1.10 A), and three disulphide bridges. On the CRD, there is a loop that projects beyond the protein surface and forms part of two Ca^{2+} binding sites that are responsible for the coordination of carbohydrates at the CRD. (Figure 1.10 A) The CRD is characterized by a highly conserved EPN motif which defines the ligand specificity of the receptor. Besides the CRD, there is a neck region, responsible of the tetramerization of the lectin, constituted by seven complete and one incomplete tandem repeats, and a transmembrane region followed by a short cytoplasmic tail, which is responsible for signaling and internalization. [50,51,52] (Figure 1.10 B)

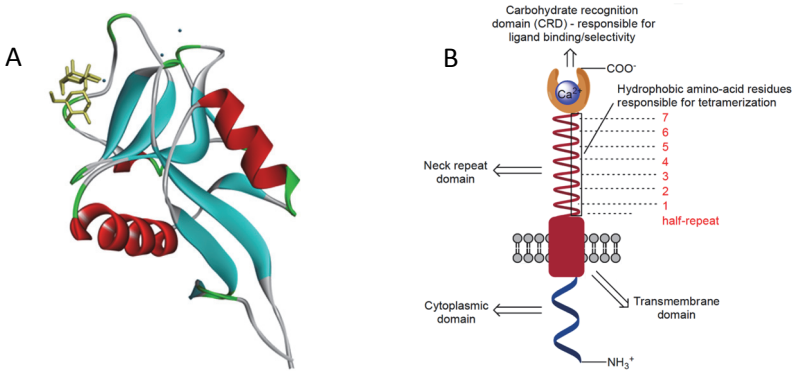

Figure 1.10. A) CRD of DC-SIGN with a di-mannose (yellow). The blue spheres are the Ca^{2+} atoms. Picture from reference 51. B) DC-SIGN schematic structure, Picture modified from reference 50.

However, DC-SIGN is not a totally rigid macromolecule and shows a degree of flexibility that allow it to adapt to several ligands.[53] This lectin oligomerizes in a

[49] Soilleux, E.J.; *Clinical Sci.*, **2003**, *104*, 437-46.
[50] Feinberg, H.; Castelli, R.; Drickamer, K.; Seeberger, P.H.; Weis, W.I.; *J.Biol.Chem.*, **2007**, *282*, 4202-4209.
[51] Image obtained by PDB:2IT6 in "BIOVIA Discovery Studio 4.5©" program.
[52] Anderluh, M.; Jug, G.; Svajger, U.; Obermajer, N.; *Cur. Med. Chem.*, **2012**, *19*, 992-1007.
[53] Menon, S. *et al., Proc. Natl. Acad. Sci. USA*, **2009**, *106*, 11524-11529.

tetrameric form in which the CRD domains are spaced from the membrane by an extended region containing α helices.[48,54] (Figure 1.11)

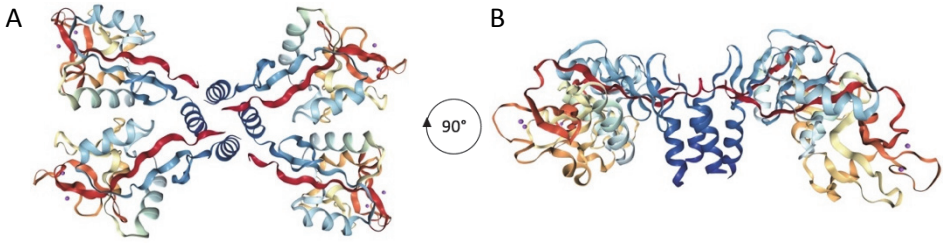

Figure 1.11. Two views of the crystal structure of a fragment of DC-SIGN R, containing the carbohydrate recognition domain and two repeats of the neck. Image from reference 54.

The oligomerization status of the DC-SIGN and the other CTLs depends on the number of helical repeats of the neck region. Indeed, at least 6 repeats are needed for tetramerization.[50]

The CRD recognizes and binds mainly internal mannose branched structures and the terminal D-mannoses, in particular the High Mannose ($Man_9GlcNAc_2$) oligosaccharide 130- and 17-fold more tightly than the single mannose. In addition, CRD recognizes fucose-bearing glycans, L-fucose and Lewis antigens and GlcNAc.[55] DC-SIGN ligands are principally expressed in pathogens.[22, 56] (Table 1.2)

[54] Feinberg, H.; Guo, Y.; Mitchell, D.A.; Drickamer, K.; Weis, W.I.; *J. Biol. Chem.*, **2005**, *280*, 1327-1335. Image obtained from PDB:1XAR by NGL Viewer (dio:10.1093/bioinformatics/bty419).

[55] a) Lai, W.K. *et al., Am. J. Pathol.*, **2006**, *169*, 200-208; b) Geijtenbeek, T.B. *et al., Cell*, **2000**, *100*, 575–585.

[56] a) Carroll, M.V.; Sim, R.B.; Bigi, F.; Jakel, A.; Antrobus, R.; Mitchell, D.A.; *Protein Cell*, **2010**, *1*, 859–870; b) Gringhuis, S,I,; den Dunnen, J.; Litjens, M.; van der Vlist, M.; Geijtenbeek, T.B.H.; *Nat. Immunol.*, **2009**, *10*, 1081–1088; c) Hodges *et al*., *Nat. Immunol.*, **2007**, *8*, 569–77; d) Zhang, P. *et al*., *J. Leukoc. Biol.*, **2006**, *79*, 731–738; e) Barreiro, L.B. *et al*., *Hum. Immunol.*, **2006**, *67*, 102–107; f) Kwon, D.S.; Gregorio, G.; Bitton, N.; Hendrickson, W.A.; Littman, D.R.; *Immunity*, **2002**, *16*, 135–144; g) Alvarez, C.P. *et al., J. Virol.*, **2002**, *76*, 6841–6844.

Ligand	Ligand origin
High mannose, fucose and Lewis^X	- Viruses: HIV-1, measles, Dengue, SARS, CMV, filoviruses, Ebola. - Bacteria: *Mycobacterium spp., Lactobacilli spp., H. pylori, E. coli, M. bovis, M. leprae.* - Fungi: *C. albicans* - Parasites: *Leishmania spp.* - Parasitic worms: *Ixodes saliva Salp15, Schistosoma* egg antigen - *ICAM -2, ICAM-3*

Table 1.2. Specific ligands for DC-SIGN and their origin.

In the Table 1.2, it is possible to see that DC-SIGN binds endogenous ligands such as intercellular adhesion molecules (ICAMs) ICAM-2 and ICAM-3.[57] This demonstrates that DC-SIGN mediates binding and trans-endothelial migration of DCs[58] and in particular, the binding to ICAM 3, enables the essential clustering among the DC and the T cell in the phases of the immune responses.[55b]

Therefore, it is possible to affirm that DC-SIGN has two distinct roles, to mediate the adhesion of T cells and to start the recognition of pathogens.[59] In fact, DC-SIGN acts mainly as a PRR, recognizing and binding a wide range of ligands expressed by pathogens such as viruses, bacteria, fungi and parasites. (Table 1.1) DC-SIGN interacts with various mycobacterial species [57] such as *M. tubercolisis*, [56b] *E. coli,* [56d] *M. bovis,*[56a] and *M. leprae* [56e] etc. (Table 1.2) Some of this wide spectrum of microbial species are able to exploit the strong binding to DC-SIGN, entering into the DCs but escaping the "normal" lytic processing in the DCs endosomes.[52]
In fact, DC-SIGN was originally identified as a receptor for the HIV-1 that lead to the Acquired Immune Deficiency Syndrome (AIDS). [55b] This is due to the fact that it binds the mannose glycans exposed on the gp120, an envelope glycoprotein of the HIV-1.[60] DC-SIGN is involved in the HIV-1 infection through a common mechanism also used by other pathogens that deceive and take advantage of the host cells for their purposes. HIV-1 deceives DC-SIGN receptor in DCs, which capture it and uptake the virus,[55b] subsequently passed to the lymph node. The virus particles are distributed

[57]Geijtenbeek, T.B.H. *et al*., *J. Exp. Med.*, **2003**, *197*, 7–17.
[58] Geijtenbeek, T.B.H. *et al., Nat. Immunol.*, **2000**, *1*, 353–357.
[59] Cambi, A.C.; Figdor, C.G.; *Curr. Opin. Cell. Biol.*, **2003**, *15*, 539-546.
[60] Doores, K.J. *et al*., *Proc. Natl. Acad. Sci. USA*, **2010**, *107*, 17107-17112.

within DCs and assemble at the interface among DCs and the T cells which are the real target of the infection.[61] The trans-infection is then possible even with a low load of virus transmitted to the T cell infected to other not infected.[62] This allows the virus, at the same, time to keep the infection and to escape antigen processing.

1.5. C-type Lectins as therapeutic target

From the analysis of the CBPs, a large number of pathogens bind or interact with the CTLs to start or spread the infection. Hence, it is reasonable to think that CTLs could be good candidates to study and to investigate the pathogenic and the immune response mechanisms.

The glycobiology and in particular the glycochemistry have converted CTLs, as a preferential target to develop new therapeutic tools with the aim to modulate the immune responses and in some cases, to inhibit the action of the pathogens involved in several diseases.[34a]

In this context, DC-SIGN and Langerin have a key role in the immunological response which makes them a receptors of interest in the design of new anti-infective compounds and vaccines. [52] Indeed, these two CTLs are involved both, in microbial and viral diseases and therefore, a potential drug that target these lectins could have the positive effect of being both antibacterial and antiviral. These pontential candidates could prevent, not only localized infection of DCs, but also pathogen dissemination.[63]

Nevertheless, until now, there are not clinical proofs that blocking the DC-SIGN or Langerin- mediated pathogen infection pathways can be a therapy, but at the same time, a large number of publications have involved the study of these two CTLs and

[61] McDonald, D.; Wu, L.; Bohks, S.M.; KewalRamani, V.N.; Unutmaz, D.; Hope, T.J. *Science*, **2003**, *300*, 1295–1297.
[62] van Kooyk, Y.; Engering, A.; Lekkerkerker, A.N.; Ludwig, I.S.; Geijtenbeek, T.B.H.; *Curr. Opin. Immunol.*, **2004**, *16*, 488–493.
[63] Khoo, U.S.; Chan, K.Y.; Chan, V.S.; Lin, C.L.; *J. Mol. Med.*, **2008**, *86*, 861-874.

in particular, focusing on DC-SIGN, due its involvement in a greater number of pathogen infections.

In fact, it was demonstrated how DC-SIGN may be exploited to inhibit pathogen infections by several distinct strategies involving:

- Specific ligands like small molecules which acts like DC-SIGN antagonists.[57,64]
- Carbohydrate-specific ligands that bind DC-SIGN, inhibiting its interaction with pathogens.[65]
- Specific ligands/antibodies of PAMP that bind DC-SIGN.[57]
- Use of specific DC-SIGN targeted vectors that encode pathogen proteins to induce immunization.[66]

Probably, the most straightforward methodology to block pathogen binding to DC-SIGN is the use of small molecules as DC-SIGN antagonists. However, as reported in an interesting review by Lepenies on the importance of the glycan tools in the modulation of the immune responses,[67] still numerous questions regarding the application of glycans for CTLs targeting in innate immunity and their utility to modulate immune response remain, and at the same time, numerous mechanisms of CTLs are still unclarified or unknown.[30]

For these reasons, it is mandatory to keep on the way to pursue the research work on better tools in order to reproduce, as close as possible, the natural carbohydrate-protein interactions.

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[64] a) Sattin, S. *et al., ACS Chem. Biol.*, **2010**, *5*, 301-312; b) Requena, M. *et al., Immunology*, **2008**, *123*, 508-518.
[65] Alen, M.M.F.; Kaptein, S.J.F.; De Burghgraeve, T.; Balzarini, J.; Neyts, J.; Schols, D.; *Virology*, **2009**, *387*, 67-75.
[66] a) Dai, B.; Yang, L.; Yang, H.; Hu, B.; Baltimore, D.; Wang, P.; *Proc. Natl. Acad. Sci. USA*, **2009**, *106*, 20382-20387; b) Yang, L. *et al*., *Nat. Biotechnol.*, **2008**, *26*, 326-334.
[67] Johannssen, T; Lepenies, B.; *Trends Biotechnol.,* **2017**, *35*, 334-346.

1.6. Multivalency in CTLs

Langerin and DC-SIGN tend to oligomerize the CRDs, forming a trimer and a tetramer, respectively. In fact, CTLs oligomerize in order to promote a high avidity (refers to the accumulated strength of multiple affinities of individual non-covalent binding interactions) for specific glycans.[68] The formation of clusters by the CRDs has a great impact not only on the avidity but above all, on the lectin selectivity. Indeed, every single CRD can act independently from the other and bind the final part of the oligosaccharide moiety.[69] Moreover, the affinity (the strength of the single interaction) is also influenced by whether the ligand is presented in a monomeric or in an oligomeric form, which affects how the ligand is presented to the CLR.[70]

This is due to the fact that the single carbohydrate-CRD interaction is very weak, with limited specificity and selectivity per saccharide unit.[71] Taking DC-SIGN as an example, this lectin recognizes and binds monosaccharides like mannose and fucose with a K_i 13.1 mM and 6.7 mM, respectively.[72] In order to compensate this paradoxically weak protein-carbohydrate interaction, Nature plays the card of the multivalency by a polyvalent exposition of the CRDs and of the carbohydrate moieties as well. Indeed, the stacked CRDs of DC-SIGN, in its tetramerized form, lead to an increase of the selectivity for the multiple repeating oligosaccharide units on the host molecules, intensifying the recognition ability of this receptor for its ligands. In the same way, the carbohydrate ligands such as glycoproteins or glycolipids, bearing numerous mannose or fucose oligosaccharides, and/or glycoconjugates containing a fucose or mannose moieties exposed in a multimeric form, bind to DC-SIGN with high affinity.[73]

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[68] Teillet, F.; Dublet, B.; Andrieu, J.P.; Gaboriaud, C.; Arland, G.J.; Thielens, N.M.; *J. Immunol.*, **2005**, *174*, 2870-2877.

[69] Rini, J.M.; *Biophys. Biomol. Struct.*, **1995**, *24,* 551-577.

[70] Iborra, S.; Sancho, D.; *Immunobiology*, **2014**, *220*, 175-184.

[71] a) Dam, T.K.; Roy, R.; Page, D.; Brewer, C.F.; *Biochemistry*, **2002**, *41*, 1359–1363; b) Mann, D.A.; Kanai, M.; Maly, D.J.; Kiessling, L.L.; *J. Am. Chem. Soc.*, **1998**, *120*, 10575-10582.

[72] Mitchell, D.A.; Fadden, A.J.; Drickamer, K.; *J. Biol. Chem.*, **2001**, *276*, 28939-28945.

[73] Appelmelk, B.J.; van Die, I.; van Vliet, S.J.; Vandenbroucke-Grauls, C.M.; Geijtenbeek, T.B.; van Kooyk, Y.; *J. Immunol.*, **2003**, *170*, 1635-1639.

An example of DC-SIGN native ligands is the HIV-1 gp120, which is a highly glycosylated protein that exploit a multivalent presentation of the high mannose ligand to increase binding affinity. At the same time, the tetramerization of the DC-SIGN and its CRDs clustering, increases the avidity for the PAMP and influences pathogen binding of the DCs.

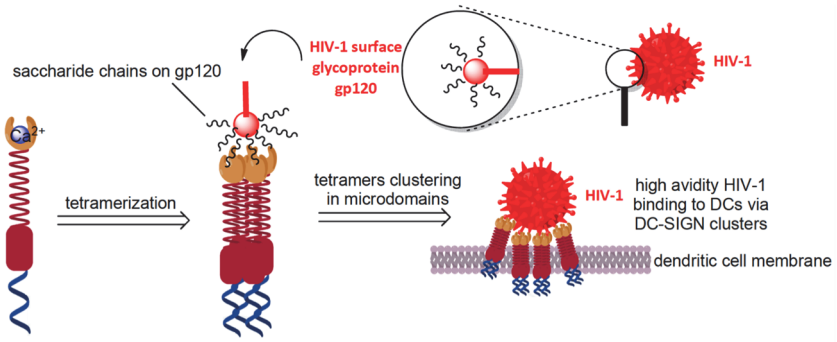


Figure 1.12. Tetramerization of DC-SIGN and binding of gp120 HIV-1. Picture from reference 52.

The survival of the organism depends on the ability of its immune system to recognize and respond, as rapid as possible, to the dangerous invaders that attack it. Consequently, the immune system needs to have the tools to quickly identify and neutralize the pathogens. This increase in the ability to recognize these external agents is precisely due to the multivalency. Therefore, the evolution has selected this strategy to provide the immune system of living beings with a powerful weapon against infections.

For this reason, only polyvalent ligands could efficiently mimic the natural CTLs ligands and only multivalent carbohydrate systems can generate efficient tools to study these carbohydrate receptors.[74] In fact, most of the lectins possess multimeric quaternary structures; however, the physiological relevance of this multivalent presentation of binding sites is still not complete clarified such is the case of Langerin. [30]

[74] Rojo, J.; Delgado, R.; *J. Antimicrob. Chemother.*, **2004**, *54,* 579-581.

Nonetheless, an important issue is assumed: a large part of biological functions such as adhesion events, modulation, and regulation processes happen involving multivalent interactions.[75]

1.7. The Multivalency

It is important to highlight that many biological systems, from viruses to mammals, have evolved using both monovalent, as well as multivalent interactions.[75]

In the past, the relevance of these kind of interactions, and in particular, the participation of carbohydrates in complex biological processes, has been largely underestimated. Currently, this trend has mainly changed and multivalent carbohydrates-lectin interactions have, at the moment, an important position in the field of chemical biology.

In these studies, it was described their relevance for a fundamental understanding of non-pathological as well as pathological processes. Therefore, the synthesis of multivalent glycomimetic architectures has been broadly developed in the past decades. This has allowed to better understand the processes in which multivalency is involved, as well as the protein receptors, *in primis* CTLs.[75,76]

What are the mechanisms that mediate the multivalent carbohydrate-protein recognition? The vision of a monovalent protein–carbohydrate interaction, in a ratio 1:1, is useful but not sufficient. Typically, a monovalent ligand binds a single protein (Figure 1.13 a) or mediate the heterodimerization. [77] (Figure 1.13 b)

[75] Cecioni, S.; Imberty, A.; Vidal, S.; *Chem. Rev.*, **2015**, *115*, 525-561.
[76] a) Chabre, Y. M.; Roy, R.; *Chem. Soc. Rev.*, **2013**, *42*, 4657-4708; b) Pieters, R.J.; *Org. Biomol. Chem.*, **2009**, *7*, 2013-2025; c) Deniaud, D.; Julienne, K.; Gouin, S. G.; *Org. Biomol. Chem.*, **2011**, *9*, 966-979; d) Jayaraman, N.; *Chem. Soc. Rev.*, **2009**, *38*, 3463-3483; e) Lahmann, M.; *Top. Curr. Chem.*, **2009**, *288*, 17-65; f) Lundquist, J.J.; Toone, E.J.; *Chem. Rev.*; **2002**, *102*, 555-578.
[77] Gestwicki, J.E.; Cairo, C.W.; Strong, L.E.; Oetjen, K.A.; Kiessling, L.L.; *J. Am. Chem. Soc.,* **2002**, *124*, 14922-14933.

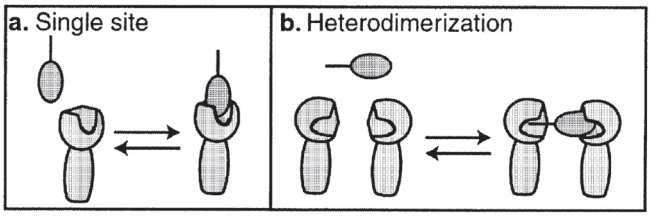


Figure 1.13. Monovalent interaction mechanisms. Picture from reference 78.

For a multivalent interaction, it can be considered different mechanisms that contribute to the binding events, increasing the complexity of the interaction between a multivalent ligand and a multivalent receptor. In addition, one major problem is the formation of an equilibrium involving all these mechanisms and the formation of a dynamic competition among them. [77]

Taking into account that a natural multivalent ligand and a receptor interact in their natural biological environment, the real interaction can be the result of a sum of mechanisms which produce different processes and biological responses.[78]

First of all, the *n* ligands from a multivalent glycomolecule can simultaneously interact with the *m* binding sites of the homomultimeric lectin, in a "chelate association" mechanism. This mechanism, one of the most studied, decrease the off-rate and increase the functional affinity in a remarkable way.[79] (Figure 1.14 A) A second mechanism called "receptor clustering" happens in the situation in which a monovalent lectin is anchored to the cell membrane. In this case, the presence of a multivalent ligand causes the diffusion of other receptors through the dynamic lipidic bilayer, leading to a clustering among the single multivalent ligand and the receptors. This can happen due to the extreme flexibility of many CRDs. In this case,

[78] a) Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E.; *Curr. Opin. Chem. Biol.*, **2000**, *4*, 696-703; b) Mammen, M.; Choi, S.-K.; Whitesides, G. M.; *Angew. Chem., Int. Ed.*, **1998**, *37*, 2754-2794.
[79] Wittmann, V.; Pieters, R.J.; *Chem. Soc. Rev.*, **2013**, *42*, 4492-4503.

the proximity of the CTLs recruited can generate specific signal transduction events through the membrane.[80] (Figure 1.14 B)

A third type is the "subsite binding" in which a secondary binding interaction is involved in another region of the protein, with a different affinity and specificity. This can also involve a different ligand from the primary, typically present on hetero divalent ligands.[81] (Figure 1.14 C)

In addition, it is necessary to take into account the statistical effects of the re-binding of the multivalent ligands, favored by a high density of epitopes in proximity to the binding site. This could happen for a receptor of both, a homomultimeric lectin or a monomeric lectin.[80a, 82] (Figure 1.14 D)

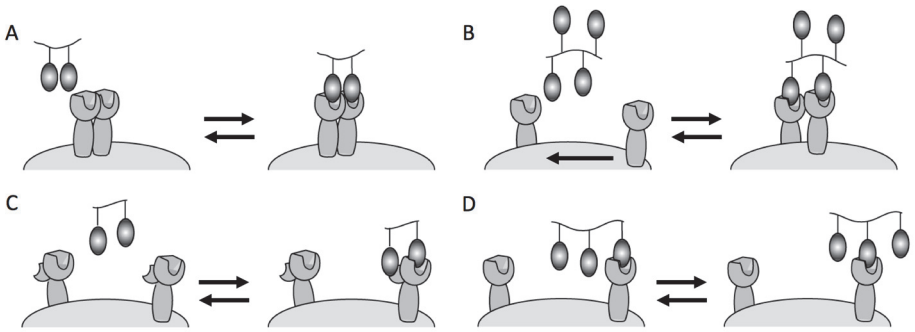


Figure 1.14. Mechanisms of interaction between multivalent ligands and multivalent receptors. Picture modified from reference 80a.

The binding affinity is the result not only of the mere sum of the number of single ligand-receptor interactions, but also an additional value due to all these mechanisms that synergically act between the multivalent ligand and the multivalent receptor.

[80] a) Kiessling, L.L.; Gestwicki, J.E.; Strong, L.E.; *Angew. Chem. Int. Ed.*, **2006**, *45*, 2348-2368; b) Bertozzi, C. R.; Kiessling, L.L.; *Science*, **2001**, *291*, 2357-2364; c) Gestwicki, J.E.; Kiessling, L.L.; *Nature*, **2002**, *415*, 81-84; d) Heldin, C.H.; *Cell*, **1995**, *80*, 213-223

[81] a) Guiard, J.; Fiege, B.; Kitov, P.I.; Peters, T.; Bundle, D.R.; *Chem. Eur. J.*, **2011**, *17*, 7438-7441; b) Rademacher, C. *et al., Chem. Eur. J.*, **2011**, *17*, 7442-7453.

[82] Dam, T.K.; Gerken, T.A.; Brewer, C.F.; *Biochemistry*, **2009**, *48*, 3822-3827.

The consequence is that the usually weak monovalent carbohydrate-protein interactions are transformed into very potent attractive forces, naturally reinforced when several copies of the ligands are closely presented on a multivalent scaffold. This phenomenon is known as the "*Glycocluster effect*".[76f]

This multivalent effect gives to the cellular receptors the ability to easilyr discriminate among structurally similar recognition epitopes when monovalent binding events presents are of low affinity. Finally, it is mandatory to highlight how the multivalent systems allow a high functional affinity but at the same time, are characterized by a unique kinetics. In monovalent binding events, the dissociation constant is exclusively determined by the rate constant for dissociation, without interference by the presence of a competing ligand. Instead, multivalent binding events exhibit a greater reversibility in the binding, in the presence of competing ligands, due to the presence of a multiple number of copies.[83]

From a structural point of view, it is fundamental to consider that architectural changes in the spacing or orientation of the carbohydrates epitopes could alter the nature of an interaction but at the same time, have not any influence in the recognition elements themselves. In fact, it has been shown that the architecture of the multivalent scaffold determines whether a multivalent display acts as an inhibitor or an effector in a carbohydrate-protein interaction.[77]

This is extremely relevant in the design phase of artificial multivalent glycosystems, since they allow certain "degrees of liberty" into the structure disposition without, at the same time, negatively affect the binding phenomenon to study.

[83] Rao, J.H.; Lahiri, J.; Isaacs, L.; Weis, R.M.; Whitesides, G.M.; *Science*, **1998**, *280*, 708-711.

1.8. Development of tools to modulate Carbohydrate-Lectin interactions

With the aim to study, characterize, understand, and interfer the mechanism that governing the multivalent carbohydrate-protein interactions, and the processes where they are involved, interesting and useful tools have been generated.[76a] (Figure 1.15)

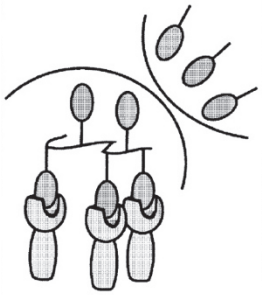


Figure 1.15. Mechanism of inhibition by a multivalent glycoconjugate. Picture modified from reference 77.

The goal is to mimic, as good as possible, the natural displaying of the oligosaccharides present on the surface of the host cells. The spatial proximity of the single sugar terminal epitopes, involved in the binding with the multivalent lectins, is essential for the generation of the appropriate structural requirements that lead to the multivalent action, therefore, leading to high avidities.

The idea is to create a tool able to compete with a particular pathogen and the priority is to incorporate into the structure, the characteristics that allow to have high affinity and a good selectivity to specifically block the lectin adhesion on the target tissues. Therefore, the design has to be strongly influenced by the natural ligand of the target lectin in terms of valency, topology and density of carbohydrates. The designed system could be also able to perturb the normal association of a lectin with its natural ligand and also to modulate the signaling processes associated with the lectin.[75]

The election of both, a monovalent glycomimetic and a multivalent glycosylated structure approaches, are valid depending on the individual situation and both of them have advantages, specific features, and benefits.

Nonetheless, monovalent synthetic glycoconjugates would not reproduce exactly the valency and the topology of a natural ligand that, as described above, is usually displayed in a multivalent form. However, the binding properties obtained with the monovalent glycomimetic approach are, in most of the cases, of remarkable impact and this has made possible their application as therapeutics.

Two widely general approaches are currently adopted to design a multivalent artificial ligand: [75]

- *Lectin-based design*. After a deep study of the multivalent lectin, the multiglycosylated structure that would best fit the lectin topology is built.
- *Ligand-based design*. This approach starts from the study of the natural multivalent ligand to design a simplified and optimized structure taking into mind the natural valency, topology and density of the ligand. (Figure 1.16)

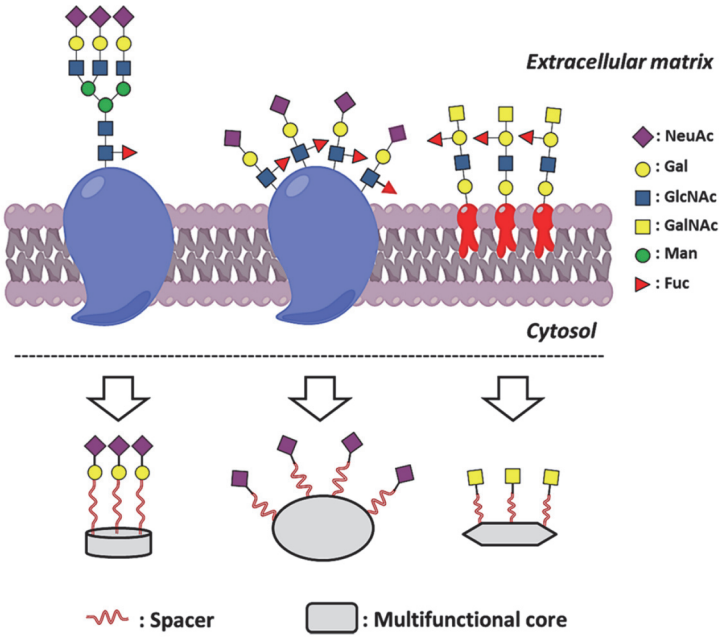


Figure 1.16. Schematic presentation of the natural carbohydrate ligands mimicked by artificial carbohydrate multivalent scaffolds. Picture from reference 75.

Both approaches have been demonstrated to be valid for addressing lectin studies. Nevertheless, in our research group, the ligand-based approach is the one widely explored and applied.

The ligand-based approach involves the simplification of the complex oligosaccharidic structure, and its presentation, using a multivalent core appropriately selected to mimic the natural scaffold. At the same time, the structure has to provide the desired valency and the correct displaying of the epitopes to the target receptor.

The first problem is the selection of the multivalent core. Along the past 25 years, chemists have designed a large number of scaffolds for this aim. It has not only to display multiple copies of the sugar epitopes but above all, to create a multivalent presentation of these epitopes that can efficiently mimic the natural mode of affinity that arises from several multivalent mechanisms involved between the binding proteins and the carbohydrate ligands.

The range of possible scaffolds to generate multivalent glycoconjugates is quite large and all of these have demonstrated to be excellent core skeletons to display the sugar moiety in a good way.[84] Depending on the scaffold and the conjugation chemistry used, the resulting glycoconjugates can be divided in polydisperse and monodisperse glycoconjugates.

The multivalent glycomimetic structures derived from a scaffold that presents the carbohydrates in a polidispersive form, are probably the closest mimetic to the natural ligand of the lectin. This is due to the fact that in the organism, the glycosilation of proteins and lipids, is usually a polidispersive modification. Popular

[84] Bernardi, A. *et al., Chem Soc Rev.*, **2013**, *42*, 4709-4727.

examples are neoglycoproteins, glycopolymers,[85] glyconanoparticles[86] or glyconanotubes.[87] (Figure 1.17)

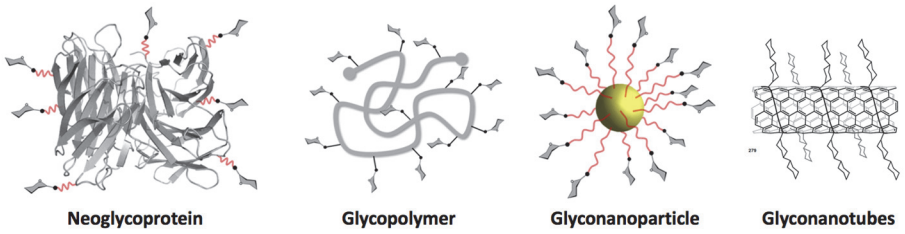


Figure 1.17. Some polydisperse multivalent glycomimetics. Picture modified from reference 75.

On the contrary, the multivalent glycomimetic structures derived from a scaffold that presents them in a monodisperse and well-defined form, mimic probably less faithfully the natural ligands, but this does not mean with less effectiveness. The major examples are glycofullerenes,[88] glycoclusters[89] and in particular glycodendrimers (see section 1.9). (Figure 1.18)

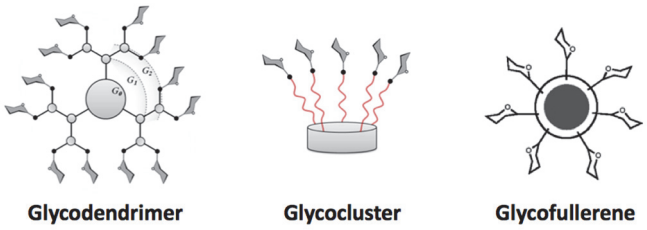


Figure 1.18. Monodisperse multivalent glycomimetics. Picture modified from reference 75.

From a quantitative point of view, one evident advantage of a multivalent exposition is the increasing of the binding affinity properties of monovalent CTLs antagonists.

[85] Spain, S.G.; Gibson, M.I.; Cameron, N.R.; *J. Polym. Sci. Part A: Polym. Chem.*, **2007**, *45* 2059–2072.
[86] a) de la Fuente, J.M.; Penades, S.; *Biochim. Biophys. Acta*, **2006**, *1760*, 636–651. b) de la Fuente, J.M. *et al., Angew. Chem. Int. Ed.*, **2001**, *41*, 1554–1557.
[87] Bianco, A.; Kostarelos, K.; Partidos, C.D.; Prato, M.; *Chem. Commun.*, **2005**, 571–577.
[88] Bosi, S.; Da Ros, T.; Spalluto, G.; Prato, M.; *Eur. J. Med. Chem.*, **2003**, *38* 913–923.
[89] Patel, A.; Lindhorst, T.B.; *Carbohydr. Res.*, **2006**, *341*, 1657–1668.

Usually, monovalent glycomimetics are characterized by a poor or modest affinity for the lectin, which can be considerably increased when they are conjugated to a scaffold for a polyvalent presentation.

1.9. Dendrimers and Dendrons

Dendrimer derives from the join of two Greek words, "*dendros*" (tree or branch) and "*meros*" (part). This name was given in 1985, when Tomalia's group published the synthesis of a poly(amidoamine) compound, that is currently famous with its acronym PAMAM.[90]

However, the history of the "parts of a tree" is a bit older, and starts in 1941 when Flory[91] proposed the idea, supported by statistical calculations, to prepare trivalent branched molecules using a polymeric approach. But at that time, the synthetic and analytical technologies were not sufficient to experimentally support his intriguing idea. Thirty-seven years passed to see the first paper that described the synthesis of low molecular weight dendritic polyamines by Vögtle, in 1978.[92] During the following 40 years, thanks also to the pioneering work of Tomalia[93] and Newcome,[94] dendritic structures have been well-established as a new class of branched monodisperse polymers with a great value for material and medicinal technologies.[95a]

Indeed, dendrimers and dendrons were quite early recognized as very useful molecules to be excellent scaffold for a multivalent presentation and also for a wide variety of different applications. At the moment, there is a remarkably high number of patent applications as well as publications on scientific journals, which highlight

[90] Tomalia, D.A. *et al., Polym. J.*, **1985**, *17*, 117–132.
[91] Flory, P.J.; *J. Am. Chem. Soc.*, **1941**, *63*, 3091–3096.
[92] Buhleier, E.; Wehner, W.; Vögtle, F.; *Synthesis*, **1978**, 155–158.
[93] *Dendrimer-Based Drug Delivery Systems: From Theory to Practice*, Eds. Cheng, Y.; Tomalia, D.A., Wiley, **2012**, ISBN: 978-0-470-46005-4.
[94] a) *Dendrimers and Dendrons: Concepts, Syntheses, Applications*, Eds. Newcome, G.R.; Moorefield, C.N.; Vögtle, F., Wiley, **2001**, ISBN: 3527299971 / 9783527299973: b) Newcome, G.R.; Zhong-qi, Y.; Baker, G.R.; Gupta, V.K.; *J. Org. Chem.*, **1985**, *50*, 2003–2004.

their applications in different fields such as chemistry, medicine, biology, physics and material engineering.[95]

From a structural point of view, the dendrons are highly branched monodisperse and polyfunctional macromolecules, constituted by three distinct elements:

- Core or focal point
- Branching point or generation
- End points or termini

The core is generally a small molecule with a precise number of functional groups which defines the number of branches of the dendrimer. The single branch is called "dendron". In the case of dendrons there is not a core, but a "focal point" which can consist of a functional group that has the aim to allow the anchorage of the dendron to another molecule (such as a fluorescent probe) or to a scaffold (a protein, a surface etc.). The branching points are composed of repeating monomeric units that define the generation (G_X). The size of the dendrimer/dendron increases with the number of generation and the growth process is mainly regulate by the chemistry used. The end points of the dendrimers are the functional groups that markedly characterize the properties of the entire structure and they can be manipulated for the desired application.[96] In the case of glycodendrons, the termini are the carbohydrate structures. (Figure 1.19)

[95] a) Müller, C.; Despras, G.; Lindhorst, T.K.; *Chem. Soc. Rev.*, **2016**, *45*, 3275-3302; b) *Carbohydrate chemistry*, *Chemical and Biological Approaches vol 41*, Eds Rauter, A.P.; Lindhorst, T.K.; Albertina, C.; Queneau,Y.; *The Royal Society of Chemistry*, **2015**, Chapter 1, Sattin, S.; Bernardi, A. pg 1-25 and L'Haridon, L.; Mallet, J.M. pg 257-269. c) Inoue, K.; *Prog. Polym. Sci.*, **2000**, *25*, 453–571; b) Vögtle, F.; Gestermann, S.; Hesse, R.; Schwierz, H.; Windisch, B.; *Prog. Polym. Sci.*, **2000**, *25*, 987–1041; d) Astruc, D.; Boisselier, E.; Ornelas, C.; *Chem. Rev.*, **2010**, *110*, 1857–1959.

[96] *Frontiers of Nanoscience, Vol 4*; Chapter 5, Sanchez-Navarro, M.; Rojo, J.; **2012**, 143-156.

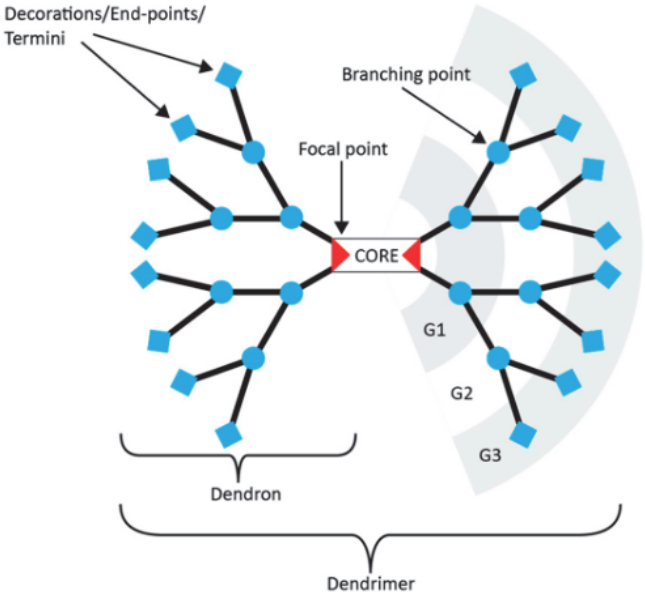


Figure 1.19. Structural elements of dendrimer and dendron.

1.9.1. Glycodendrons

The nano- or picomolar binding affinities which usually characterize the therapeutic compounds are in most of the cases difficult to reach using glycomimetics in a monovalent form. Hence, a successful strategy has to consider a synthetic approach that could be conceived in this way:

A) The design of a monovalent glycomimetic based on a natural lectin binding oligosaccharide.
B) Multivalent presentation of the monosaccharide/oligosaccharide or glycomimetic on a multivalent scaffold. (Figure 1.20)

Moreover, the two sequential steps can be independently applied from each other. In fact, it is possible to focus the research energies on developing a small monovalent glycomimetic with a strong affinity, or developing a multivalent macromolecule with an excellent presentation of the mono/oligosaccharides. (Figure 1.20)

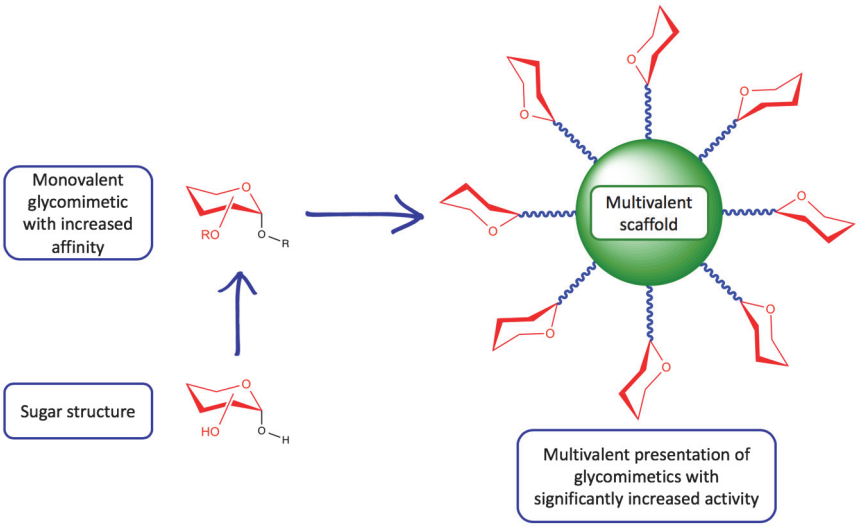


Figure 1.20. Strategy for increasing binding affinity/avidity of CTLs ligand.

In the literature, it is possible to find a lot of applications of this approach. For instance, the inhibition of the interactions between DC-SIGN and pathogen surface glycans using DC-SIGN ligands/antagonists is currently considered a promising way in order to create efficient and powerful antimicrobial drugs.[63]

In particular, focusing on glycodendrons, the creativity in the glycochemistry during these years developed a wide library of dendronic platforms to expose the sugar moieties. This is demonstrated by numerous pubblications and reviews on the topic.[64a,75,76a,84, *97*,98]

The first glycodendron in a scientific publication was developed by Roy's group in 1993, using solid state synthesis, a hyperbranched L-lysine and multiple copies of sialic acid in the termini; that demonstrate its activity as inhibitors of influenza A virus haemagglutinin. (Figure 1.21)[99]

[97] Roy, R.; Shiao. T.C.; *Chem. Soc. Rev.*, **2015**, *44*, 3924.
[98] a) Appelhans, D. *et al., Chem. Soc. Rev.*, **2015**, *44*, 3968; b) Reina, J.J. *et al., Braz. J. Pharm. Sci.*, **2015**, *49*, 109-124; c) Malkoch, M. *et al., Macromolecules*, **2005**, *38*, 3663–3678; d) Wu, P.; *Chem. Commun.*, **2005**, 5775–5777.
[99] Roy, R.; Zanini, D.; Meunier, S. J.; Romanowska, A.; *Chem. Comm.*, **1993**, *24*, 1869-1872.

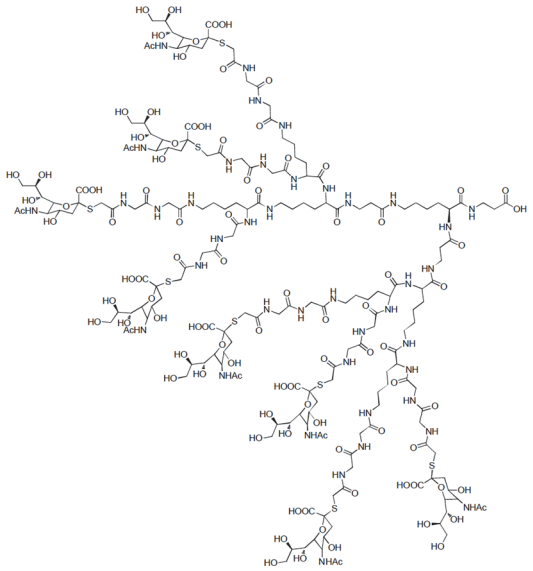


Figure 1.21. First glycodendron synthetized by Roy *et al*.

Focusing in particular on glycodendrons which shown an effectively action on CTLs, an interesting example was reported by Wang *et al*.[100] They synthetized glycondendrons as inhibitors of recombinant dimeric gp120 binding to DC-SIGN, displaying 3, 9 and 27 copies of the sugar moiety. The oligosaccharides were a linear tetramannosyl ligand and an oligosaccharide having nine copies of mannose. (Figure 1.22) In competition experiments using gp120 as a ligand for DC-SIGN, the second generation of dendron having nine copies of Man9 showed an IC_{50} of 8nM.

[100] Wang, S.K.; Liang, P.H.; Astronomo, R.D.; Hsu, T.L.; Hsieh, S.L.; Burton, D.R.; Wong, C.H.; *Proc. Natl. Acad. Sci. USA*, **2008**, *105*, 3690-3695.

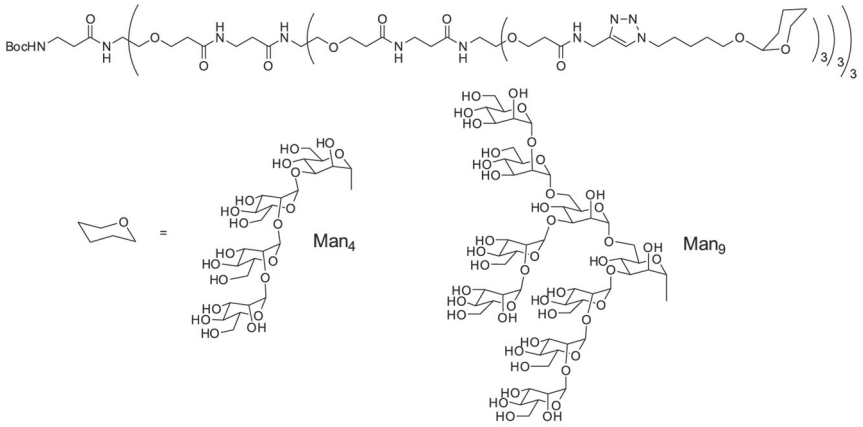


Figure 1.22. Oligomannose glycodendrons synthetized by Wang *et al.* in 2008.

Another interesting application on the study of CTL, and in particular on the multivalence was reported by Pieters' group.[101] They synthetized mono to octavalent mannose glycodendrons (Figure 1.23) with the aim to prepare a glycodendrimer microarray by the attachment of the dendrons on an aluminum oxide surface. In this work, Pieters *et al.* were able to perform binding experiment on fluorescently labeled lectins ConA and GNA, observing in particular a distinct multivalent binding of the higher generation mannose dendrimers towards the GNA lectin.

[101] Branderhorst, H. M.; Ruijtenbeek, R.; Liskamp, R. M. J.; Pieters, R. J.; *ChemBioChem*, **2008**, *9*, 1836–1844.

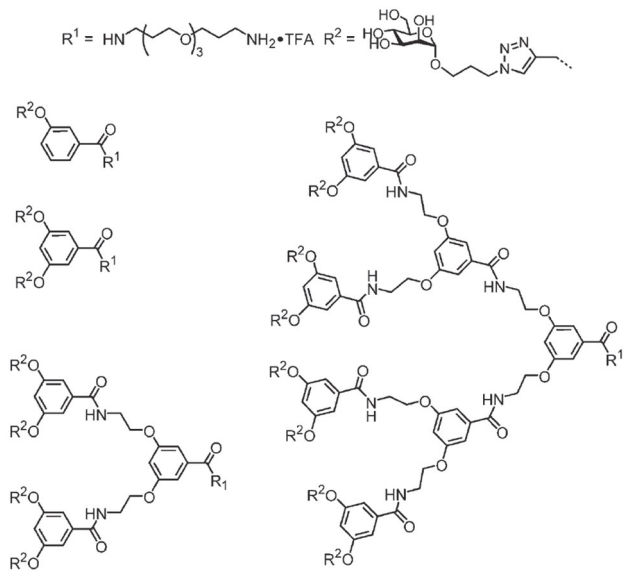


Figure 1.23. Mannose glycodendrons mono to octavalent synthetized by Pieters *et al.*

In 2012 Wagner *et al*.,[102] reported the synthesis of amphiphilic compounds and ther application as HIV-1 *trans*-infection inhibitors. These were composed of mannose or di-mannose termini which maked the head of the compound, a hydrophilic linker essential for solubility in aqueous media, and a lipid chain of variable length. (Figure 1.24) These molecules presented unusual properties based on a cooperation between the mannoside head and the lipid chain, which enhanced the affinity and decreased the need for multivalency. In particular, the trivalent conjugate was able to inhibit the interaction of the HIV-1 envelope with DCs by DC-SIGN, and to drastically reduce *trans*-infection of HIV-1 mediated by DCs (IC_{50}s in the low micromolar range).

[102] Dehuyser, L.; Schaeffer, E.; Chaloin, O.; Mueller, C.G.; Baati, R.; Wagner, A.; *Bioconjug. Chem.*, **2012**, *23*, 1731–1739.

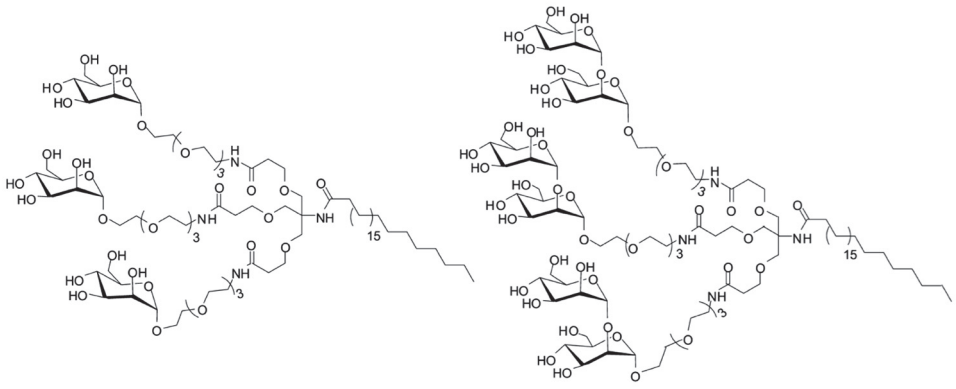


Figure 1.24. Amphiphilic glycodendrons having HIV-1 *trans*-infection inhibitorial activity.

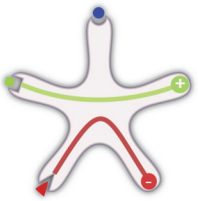
These examples are necessary to understand all the creativity that scientific research can bring in a vast field like glycoscience. In particular, into the design and the preparation of multivalent tools able of interacting with CTLs, in order to study these receptors or inhibit pathogen infection.

It is important to highlight, that a strong contribution has been done by our research group since 2003. We have designed and prepared several multivalent glycostructures, with the aim to study binding properties of human lectins like DC-SIGN and Langerin and at the same time trying to find always better ligands for these two CBPs. This will be discussed in more detail in Chapter 2.

In this context, the research work that constitutes this doctoral thesis is placed. The work has been developed as part of a Marie Skłodowska Curie European Training Network "IMMUNOSHAPE" composed by 14 European partners in several multidisciplinary and multi-sectorial fields focused to biomedical glycoscience and industrial applications. The network was created with the aim to develop selective multivalent carbohydrate immunomodulators targeting CTLs receptors on APCs.

CHAPTER 2

The antecedents and objectives



2. The antecedents and objectives

2.1 The antecedents

In the last 15 years, our research group has developed novel multivalent glycosystems, mainly dendrimer-based. In particular, They have developed the generation of innovative tools to modulate and better understand the action of CTLs like DC-SIGN.

In this context, several type of dendrimers and dendrons have been used as scaffold to have a multivalent presentation of the selected glycoepitopes. Indeed, there are several examples where the glycosystems, created in our group, have had applications on the study of carbohydrate-lectin interactions, or have been used as vectors to internalize into DCs, molecules of therapeutic interest such as peptides with an antiallergenic activity and potential vaccine action.

At the beginning of 2000, our research group designed and prepared useful glycodendrimers based on glycodendritic structures with 16 and 32 copies of mannose residues conjugated to the commercial available hyperbranched dendritic polymer BoltornH20 and BoltornH30, respectively. The Boltorn-type compounds are constructed using 2,2-Bis(hydroxymethyl)propionic acid (Bis-MPA) as branching unit and pentaerythritol as a core. The corresponding glycodendritic polymers of 2nd and 3rd generation were perfectly soluble in physiological conditions.[103] The glycodendrimer of 3rd generation, having 32 mannose residues, (Figure 2.1) was able to block DC-SIGN mediated Ebola virus infection resulting in an IC_{50}=337 nM.

[103] Lasala, F.; Arce, E.; Otero, J. R.; Rojo, J.; Delgado, R.; *Antimicrob. Agents Chemother.*, **2003**, *47*, 3970-3972.

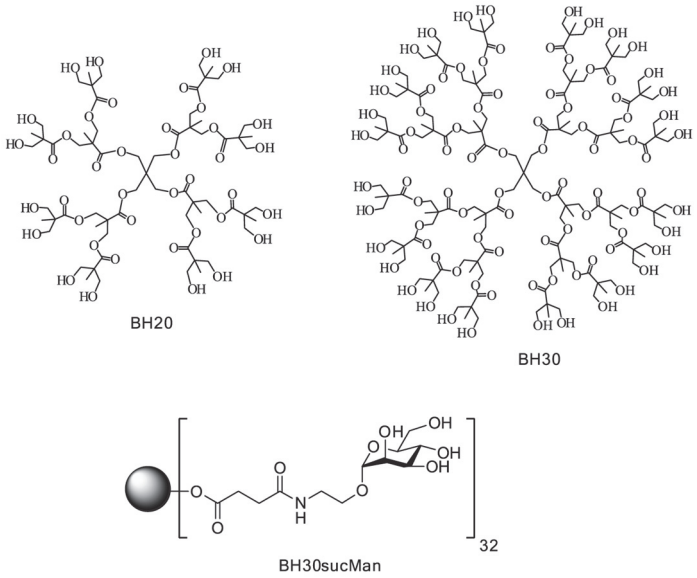


Figure 2.1. Boltorn hyperbranched structures of 2nd (BH20) and 3rd (BH30) generation and the corresponding glycodendrimers having 30 mannose units.

Afterwards, these glycodendrimers were also tested by surface plasmon resonance (SPR) in competition experiments in order to measure the ability of these compounds to inhibit the interaction of HIV-1 gp120 with DC-SIGN, resulting in a sub-micromolar range activity for the compound with 32 copies.[104]

Following these interesting results, it was established a collaboration with Bernardi's research group, in order to generate a multivalent presentation of DC-SIGN antagonists glycostructures. These mannose mimetics had already demonstrated, in a monovalent form, a good inhibitory activity on Ebola virus entry into Jurkat cells expressing DC-SIGN.[64a,105]

In particular, the pseudo disaccharidic and pseudo trisaccharidic compounds, (Figure 2.2) were installed on a tetravalent platform based in Bis-MPA, with an inhibition

[104] Tabarani, G.; Reina, J.J.; Ebel, C.; Vives, C.; Lortat-Jacob, H.; Rojo, J.; Fieschi, F.; *FEBS Lett.*, **2006**, *580*, 2402-2408.
[105] a) Sutkeviciute, I. *et al., ACS Chem. Biol.*, **2014**, *9*, 1377−1385; b) Luczkowiak, J. *et al., Bioconjug. Chem.*, **2011**, *22*, 1354–1365.

action in the low micromolar range.[105a-b] The tetravalent glycodendrons showed to inhibited the DC-SIGN gp120 interaction, and in particular the pseudo-trisaccharide glycodendron blocked the HIV-1 transfection to $CD4^+$ T lymphocytes with a percentage >94%.[64a] (Figure 2.2)

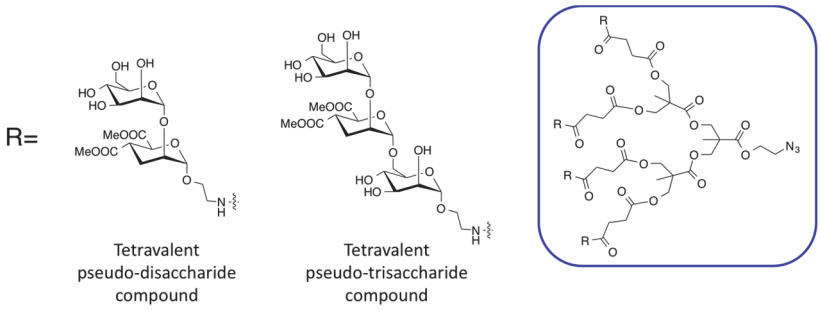


Figure 2.2. Tetrameric presentation on bis-MPA dendrons of glycomimetics compounds.

These examples show the actually efficacy of a multivalent presentation of a glycomimetic compound, in order to obtain a high affinity for a protein receptor like DC-SIGN.

Taking advantage of the potential of the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC), also called "click" reaction,[106] the research group has developed several monodisperse glycodendron and glycodendritic systems with different valences: from three to eighteen, fully characterized by NMR and mass spectrometry. These dendrimers are composed by mannose units at the end points and a pentaerythritol core.[107] (Figure 2.3)

[106] a) Tiwari, V. K.; Mishra, B. B.; Mishra, K. B.; Mishra, N.; Singh, A. S.; Chen, X.; *Chem. Soc. Rev.*, **2016**, *116*, 3086 - 3240; b) Tabacaru, A.; Furdui, B.; Ghinea, I.O.; Cârâc, G.; Dinicâ, R. M.; *Inorg. Chim. Acta*, **2016**, 455, 329-349; c) Singh, M. S.; Chowdhury, S.; Koley, S.; *Tetrahedron*, **2016**, *72*, 5257-5283; d) Liang, L.; Astruc, D.; *Coord. Chem. Rev.*, **2011**, *255*, 2933– 2945.
[107] Varga, N. *et al., Biomaterials*, **2014**, *35*, 4175-4184.

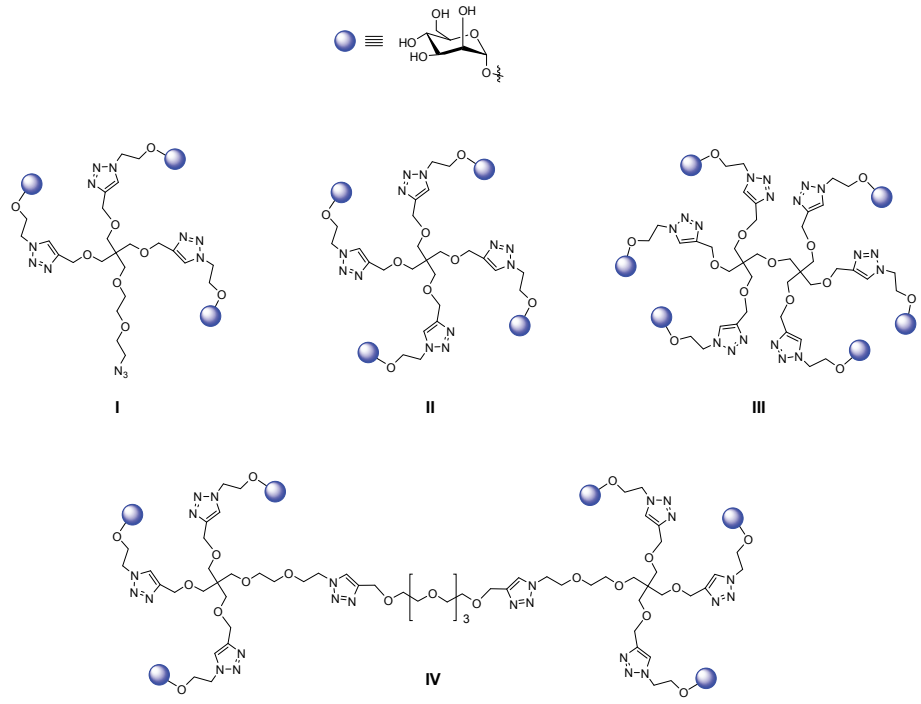
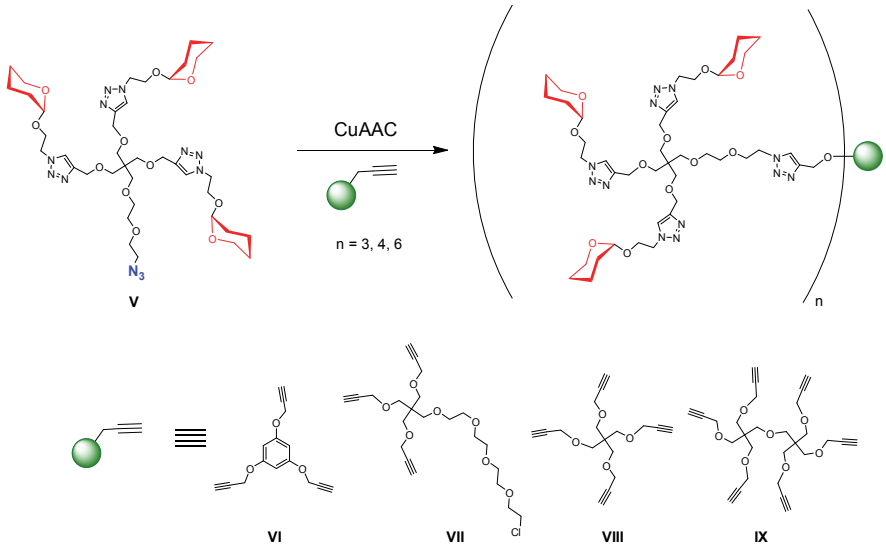


Figure 2.3. Mannose glycodendrimers of first generation previously synthetized in our laboratories.

However, it was possible to increase the dendrimer generation, taking advantage by the possibility to have an azido group at the focal position of the glycodendron **V**, and by a second click reaction on different cores (**VI**-**IX**). (Scheme 2.1)



Scheme 2.1. Click reaction among the glycodendron and several cores.

With this strategy adopted, several glycodendrons of second generatuion with different carbohydrate epitopes were prepared, such as those **X**-**XII** described in Figure 2.4.[107]

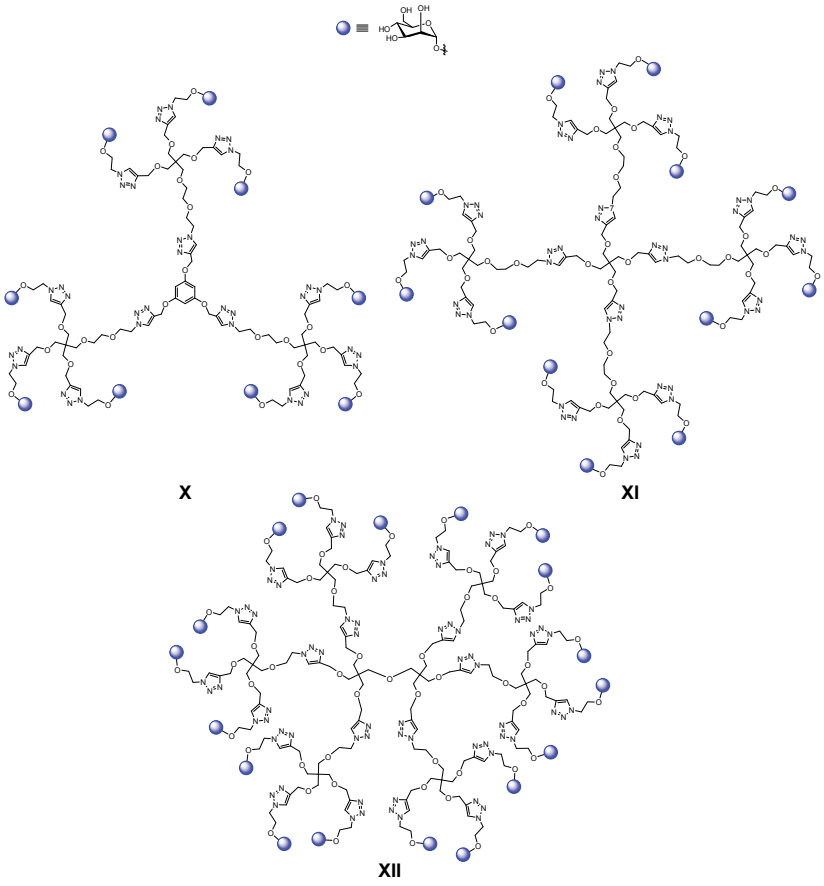


Figure 2.4. Mannose glycodendrons of second generation previously prepared in our laboratory.

The possibility and the versatility to have an azido or a chloride group at the focal position have allowed the conjugation of trivalent (**V**) and nonavalent glycodendrons (derived from scaffold **VII**) on other scaffolds such as:

- Graphene. Demonstrating a selective interaction with the plant lectin ConA, tested by AFM, Fluorescence and UV-Vis studies.[108] (Figure 2.5 A)

[108] Ragoussi, M.E.; Casado, S.; Ribeiro-Viana, R.; de la Torre, G.; Rojo, J.; Torres, T.; *Chem. Sci.*, **2013**, *4*, 4035-4041.

- Fullerene. Demonstrating for the first time that a fullerene decorated by monovalent or trivalent glycodendrons of mannose is able to inhibit efficiently the Ebola infection process.[109] Figure 2.5 B
- Gold nanoclusters. Demonstrating that a gold nanoparticle with trivalent mannose glycodendrons on the surface, show an increasing in the uptaking on human-monocyte-derived DCs, in comparison with non-carbohydrate gold nanoclusters.[110] Figure 2.5 C
- With a fluorophore marker. Demonstrating the uptake process into DCs via a receptor-dependent mechanism of glycodendrons of mannose, fucose and maltotriose.[111] (Figure 2.5 D) Proving the actually efficacy of glycodendrons of mannose, fucose, or *N*-acetyl-galactosamine in the study of the microalgae interaction mechanisms.[112] Figure 2.5 E

[109] Luczkowiak, J. *et al., Biomacromolecules*, **2013**, *14*, 431-437.
[110] Le Guével, X. *et al*., *ACS Appl. Mater. Interfaces*, **2015**, *7*, 20945−20956.
[111] Ribeiro-Viana, R; Garcia-Vallejo, J.J.; Collado, D.; Perez-Inestrosa, E.; Bloem, K.; van Kooyk, Y.; Rojo, J.; *Biomacromolecules*, **2012**, *13*, 3209−3219.
[112] de Almeida Gonçalves, C.; Cunha Figueiredo, R.; Giani, A.; Collado, D.; Pérez-Inestrosa, E.; Rojo, J.; Cunha Figueredo, C.; *J. Appl. Psychol.*, **2018**, 1-11.

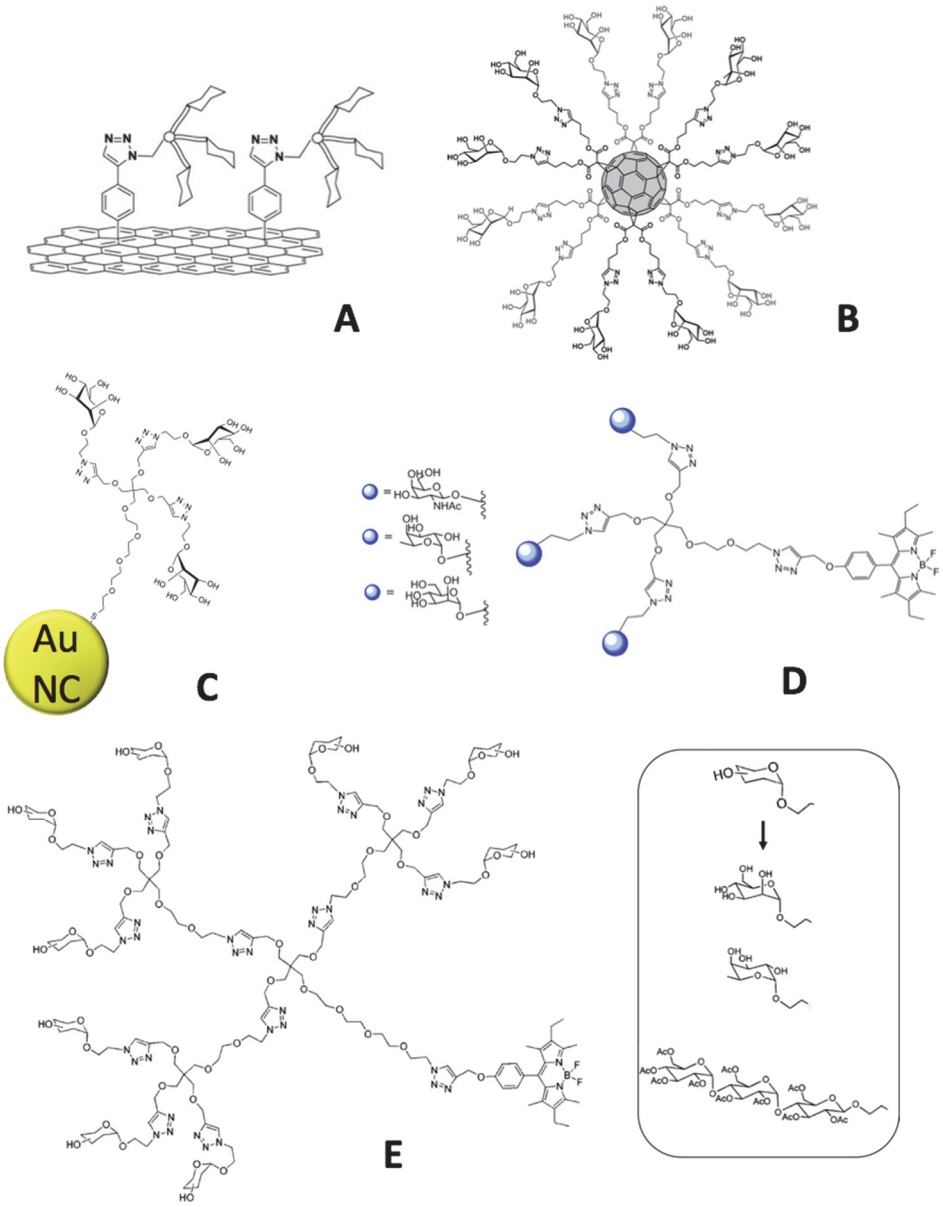


Figure 2.5. Several constructs generated by the conjugation of glycodendrons.

Finally, mannose trivalent and nonavalent glycodendrons were used to functionalized a protein, with the aim to generate glycodendriproteins.[113] In particular, this application will be explained in detail in the chapter dedicated to the neoglycoproteins (chapter 4).[114]

This wide range of scaffold's functionalizations demonstrates the great versatility of this approach. Moreover, it is possible to change the functional group at the focal position of the glycodendron for other possible application of this type of compounds.

According to the extreme versatility of applications of the glycodendron structures and the background of the research group, the following objectives, which are the topic of this doctoral thesis, are proposed.

[113] Ribeiro-Viana, R.; Sánchez-Navarro, M.; Luczkowiak, J.; Koeppe, J. R.; Delgado, R.; Rojo, R.; Davis, B. G.; *Nat. Commun.*, **2012**, *3*, 1303-1310.
[114] See Chapter 4, section 4.2.4.

2.2 Objectives

The primary objective of this doctoral thesis is the development and the preparation of multivalent systems in order to address carbohydrate-protein interaction studies on plant and human CTLs. The aim will be to better understand the mechanisms that drive the interactions of these CTLs, which play a crucial role in the immune system. A great effort has been focused on the necessity to generate efficient tools, based on a quickly and easily reproducible preparation approach.

In order to achieve this general aim, the following specific objectives are proposed:

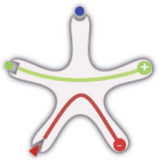
- Preparation of microarray surfaces, functionalized with a novel class of activated cyclooctyne-dendrons to employ in SPAAC reactions, with the aim to generate on-chip a library of glycodendrons of different valences with the desired ligands. These surfaces will be applied to estimate how dendron's presentation affects the ligand-protein interactions on human lectins by fluorecence screening.

- Design and set-up synthetic methods which allow to quickly functionalize proteins' surface with glycoconjugates (also glycodendrons) with the aim to generate powerful CTLs ligands, evaluating them by lectin arrays.

- Developing of a new and rapid synthetic strategy to obtain α1,2 mannobioside derivatives in gram-scale, not only with a high yield but also in a rapid, straightforward and versatile manner.

CHAPTER 3

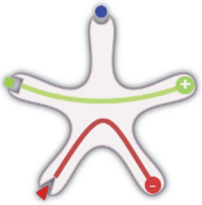
On-chip synthesis of glycodendrons to study carbohydrate-protein interactions.



Esta parte de la tesis se publicará con un retraso de 12 meses desde su aprobación y defensa.

CHAPTER 4

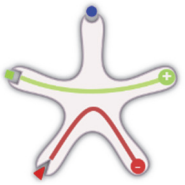
Synthesis of novel glycodendriproteins: interaction studies on lectins arrays and evaluation of T cell activation through by DCs.



Esta parte de la tesis se publicará con un retraso de 12 meses desde su aprobación y defensa.

CHAPTER 5

Glycodendron structures based on α1,2 mannobioside disaccharide.



5. Glycodendron structures based on α1,2 mannobioside disaccharide.

5.1 Mannose disaccharide, an important epitope in glycoscience

A great effort has been invested in the glycoscience field concerning the design and the synthesis of efficient tools able to mimic the natural structures present in living organisms. In particular, the development of synthetic strategies enabling to obtain the desired glycans in good quantity and, above all, as similar as possible to the natural systems.

One of the principal targets of these studies are the *N*-glycan structures, due to their ability to affect in a tremendous way, many properties of the glycoproteins in which they are attached, like solubility, conformation, activity, antigenicity, and especially, the recognition by glycan-binding proteins (GBPs).

In eukaryotes, all the *N*-glycans contain a common core sequence, $Man_3GlcNAc_2Asn$, and they are classified into three types[316] (Figure 5.1):

- *Oligomannose or High-Mannose*. The core is extended only by mannose residues.
- *Hybrid*. The core is extended by mannose through the Manα1-6 arm of the core and one or two GlcNAc extend the Manα1-3 arm.
- *Complex*. The antennae initiated by GlcNAc, extend the core.

[316] *Essentials in Glycobiology*, Ed Varki, A.; Cummings, R. D.; Esko, J. D.; Freeze, H. H.; Stanley, P.; Bertozzi, C. R.; Hart, G. W.; Etzler, M. E.; *Cold Spring Harbor Laboratory Press, Plainview*, **2009**.

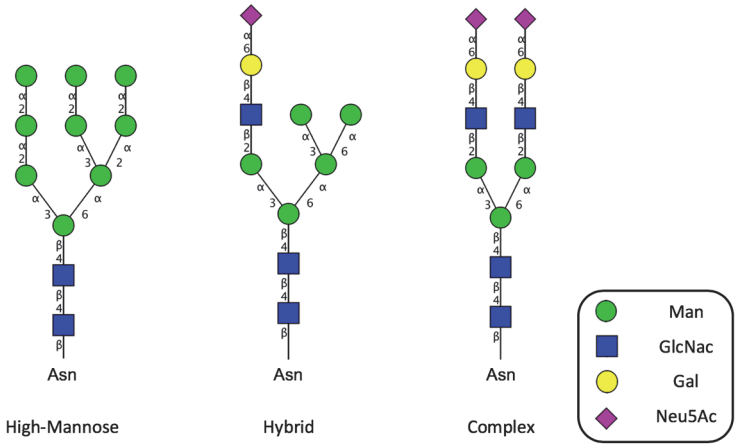


Figure 5.1. Types of *N*-glycans.

Among these types of *N*-glycans, a great attention has been focused on the oligomannose glycans; due to the fact that many important classes of lectins recognize ligands containing mannose, or higher mannose structures.[317] The high mannose oligosaccharides are usually characterized by two to six α-mannose residues bound to the core. Thus, the largest high-mannose, discovered for the first time in the 1977,[318] contains nine mannose and two *N*-acetylglucosamine. (Figure 5.1) It turn out to be one the main carbohydrate ligand recognized by DC-SIGN and Langerin. For this reason, the structure of high mannose has been one of the principal sources of inspiration for the design of high affinity ligands for these important lectins.[319]

Hence, in the past two decades one of the targets for the developing of GBP ligands has been the total synthesis of the $(Man)_9(GlcNAc)_2$.[225c,320] However, the high complexity of this class of glycan structures has required the use of alternative

[317] Kwon, D.S., *et al., Immunity*, **2002**, *16*, 135–144.
[318] Ito, S.; Yamashita, K.; Spiro, R.G.; Kobata, A.; *J. Biochem.*, **1977**, *81*, 1621-1631.
[319] Guo, Y. e*t al., Nat. Struct. Mol. Biol.*, **2004**, *11*, 591-598.
[320] a) Lee, H.K., *et al., Angew. Chem. Int. Ed.*, **2004**, *43*, 1000 –1003; b) Geng, X.; Dudkin, V.Y.; Mandal, M.; Danishefsky, S.J.; *Angew. Chem. Int. Ed.*, **2004**, *43*, 2562-2565; c) Grice, P.; Ley, S.V.; Pietruszka, J.; Priepke, H.W.M.; *Angew. Chem. Int. Ed.*, **1996**, *35*, 197–200.; d) Merritt, J.R.; Naisang, E.; Fraser-Reid, B.; *J. Org. Chem.*, **1994**, *59*, 4443-4449.

synthetic structures in order to offset the possibility to have this ligand in large amount and address biological studies.[321]

In fact, synthetic glycan mimetics has been widely adopted in the study of the relevant biological interactions that involve high-mannose ligands and its natural receptor, with the aim to better understand the role and the structure-activity relationships of the carbohydrates in biological processes.[322] One strategy was the use of the single antennas alternatively to the full Man_9 molecule. This disaccharidic or trisaccharidic epitopes could be actually installed on multivalent scaffold in order to mimic the natural multivalent presentation of these glycans. (Figure 5.2)

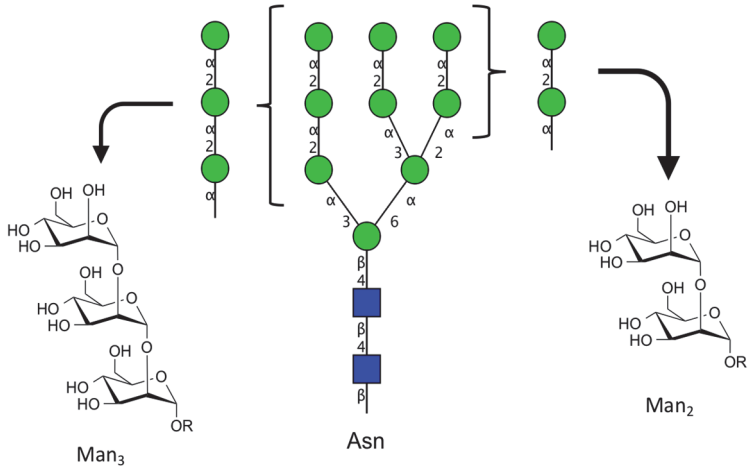


Figure 5.2. High-mannose and its principal antennas.

The use of this approach is supported by several studies performed in the past two decades, which have demonstrated how the use of the disaccharide Man_2 or the trisaccharide Man_3, can be efficiently adopted instead of the whole structure.[50] First of all, an structural study on the crystal of a complex including the high mannose $(Man)_9(GlcNAc)_2$ and a Fab fragment of the gp120 antibody 2G12 shown that the 85%

[321] *Carbohydrate Chemistry: State of the Art and Challenges for Drug Development*, Ed Cipolla, L., *Imperial College Press*, **2016**; Chapter 15, Sattin, S.; Fieschi, F., Bernardi, A.; 379-394.
[322] a) Reina, J.; Bernardi, A.; *Mini Rev. Med. Chem.,* **2012**, *12*, 1434-1442.; b) Reina, J.; Bernardi, A.; Clerici, M.; Rojo, J.; *Fut. Med. Chem.*, **2010**, *2*, 1141–1159.

of the protein contact were constituted by the disaccharide Manα1,2Man.[323] In addition, Seeberger's group demonstrated, by a microarray study using several oligomannose ligands (Man_9 included), that a high density of Manα1-2Man was able to bind DC-SIGN as effective as the entire high mannose structure.[324] Therefore, the use of the Manα1,2Man fragment can be considered as an interesting starting point at the moment to design new mimetic systems with the aim to study interaction processes with protein receptors. This is precisely the case of mimicking high-mannose interactions, involving mannose compounds as epitopes.

The preparation of multivalent systems using different scaffolds such as proteins or surfaces to perform the desired application with this disaccharidic epitope required an efficient synthesis of the Manα1,2Man epitope. In this respect, several approaches have been described in the literature to prepare α1,2 mannobioside derivatives.[325] The synthesis of these compounds is complicated by the fact that the hydroxyl group at the C-2 position of the mannose moiety is not directly accessible by a regioselective glycosylation.[326] Thus, the synthesis required the use of many reaction steps of selective protections and deprotections with several and tedious purification steps of the intermediate products.

Therefore, these classical procedures require a large time consumption in term of synthesis and purification, high cost in term of reagents and solvents, and in

[323] Calarese, D.A., *et al., Science*, **2003**, *300*, 2065-2071.
[324] Adams, E.W.; Ratner, D. M.; Bokesch, H.R.; MacMahon, J. B.; O'Keefe, B.R.; Seeberger, P.H.; *Chem. Biol.*, **2004**, *11*, 875-881.
[325] a) Patel, M. K.; Vijayakrishnan, B.; Koeppe, J.R.; Chalker, J.M.; Doores, K.J.; Davis, B.G.; *Chem. Commun.*, **2010**, 9119–9121; b) Jaipuri, F.A.; Pohl, N.L.; *Org. Biomol. Chem.*, **2008**, *6*, 2686–2691; c) Mathew, F.; Mach, M.; Hazen, K.C.; Fraser-Reid, B.; *Synlett*, **2003**, *9*, 1319-1322; d) Ogawa, T.; Nukada, T.; *Carbohydr. Res.*, **1985**, *136*, 135–152.
[326] a) Pratt, M. R.; Bertozzi, C. R.; *J. Am. Chem. Soc.*, **2003**, *125*, 6149-6159; b) Matsuo, I.; Wada, M.; Manabe, S.; Yamaguchi, Y.; Otake, K.; Kato, K.; Ito, Y.; *J. Am. Chem. Soc.*, **2003**, *125*, 3402-3403; c) Ogawa, T.; Sasajima, K.; *Carbohydr. Res.*, **1981**, *93*, 67-81; d) Ogawa, T.; Nakabayashi, S.; *Agric. Biol. Chem.*, **1981**, *45*, 2329-2335; d) Arnarp, J.; Haraldsson, M.; Lönngren, J. *J. Chem. Soc., Perkin Trans. 1,* **1985**, 535-539.

particular, the several purification steps affect in a tremendous manner the global yield of the final product.

In fact, a one-pot procedure could overcome these problems such as purification and the numerous steps and could maximize the final yield, diminishing the time required for the synthesis. This is the case of the work done by the groups of Wang[327] and Davis[328] more recently. In the later case, there was a polymerization strategy very efficient; however, it is important to highlight that these protocols result in very good overall yield only in same cases. Indeed, only good yields were achieved in this polymerization procedure when at the reducing terminus there was an alcohol with a particular length and substituents. These chemical constraints are an evident disadvantage which makes difficult the application of this strategy to the synthesis of Manα1,2Man with a desired group at the anomeric position.

With this in mind, our research group planned the development of a new strategy that allowed to obtain α1,2 mannobioside derivatives in gram-scale, not only with a high yield but also in a rapid, straightforward and versatile manner. In our approach, a 2-azidoethyl group was introduced at the α-anomeric position to perform later the coupling of this disaccharide with alkyne or cyclooctyne groups in a click reaction procedure, in order to afford the preparation of multivalent structures.

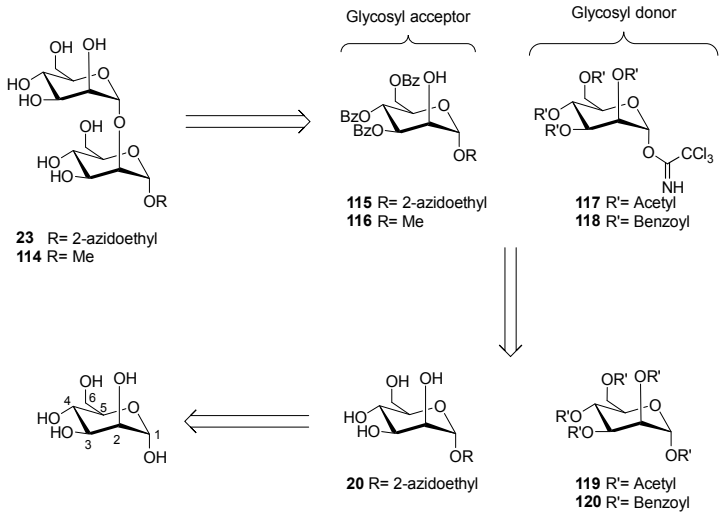
In particular, in this chapter it will be described the synthesis of α1,2 mannobioside compounds and with one of these disaccharides, the synthesis of glycondendrons structures having a biotin at the focal position.

5.2. Synthesis of Manα1,2Man disaccharides

In this synthetic strategy, two glycosyl acceptor have been selected, one having a methoxy group at the anomeric position, and the other with an azido ethyl spacer for a further conjugation. These two different groups can be used to demonstrate that the efficacy of the strategy is not influenced by the group at the reducing end.

[327] Wang, C.C.; Zuluetab, M.M.L.; Hung, S.C.; *Chimia*, **2011**, *65*, 54-58.
[328] Schuster, H.J.; Vijayakrishnan, B.; Davis, B.G.; *Carbohydr. Res.*, **2015**, *403*, 135-141.

In addition, to further verify the versatility of the strategy, two glycosyl donors, per-acetylated (**117**) and per-benzoylated, (**118**) were selected. (Scheme 5.1)

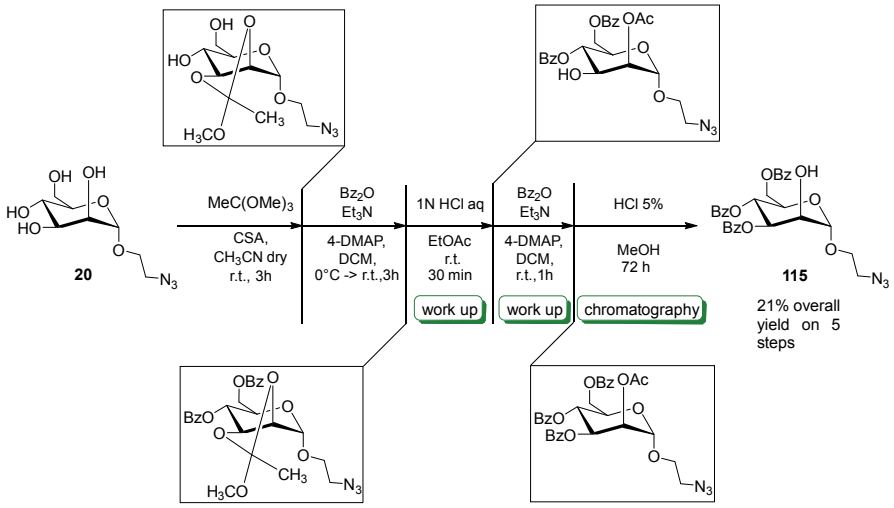


Scheme 5.1. Retrosynthetic scheme for the synthesis of Manα1,2Man disaccharides.

First of all, it has been necessary to select the orthogonal protective group strategy on the hydroxyl groups of the glycosyl donor and the glycosyl acceptor. (Scheme 5.1) The acetate protecting group was selected at the position 2 of the glycosyl acceptor and benzoates groups were selected as permanent protecting groups in the other hydroxyl groups. These two orthogonal groups are cleavable under different conditions,[191] allowing the stability of the benzoates in all the steps of the synthesis until the final total deprotection. All these protective groups are compatible with the presence of the azido group on the chain at the anomeric position.

In this approach, the limiting factor, in terms of time and yield, is the synthesis of the glycosyl acceptors (**115**-**116**). This is due to the fact that the production of a mannose orthogonally protected with an acetate in position C2 and benzoate groups in the other positions may be relatively complicated. Nevertheless, the synthesis of the glycosyl donors armed with a trichloroacetimidate group at the anomeric position (**117**-**118**) is relative simple and straightforward.

The strategy has as starting product the 2-azido ethyl mannopyranoside (**20**) which was used to prepare the glycosyl acceptor **115**. The advantage of the designed approach relays on the development of a consecutive synthesis. All the reaction conditions for the protection and deprotection steps were compatible to be carried out in a sequential manner, using only a simple work-up and removing the solvent after each step. In this way, it was possible to avoid the chromatographic purification of the intermediates. In scheme 5.2 is depicted the consecutive synthesis of the mannose derivative **115**. There was an initial mono protection of the C2 position by an orthoester formation and later, the per-benzoylation of the remaining hydroxyl groups.



Scheme 5.2. Consecutive synthesis of the compound **115**.

Mannose **20** was treated with trimethyl orthoacetate and a catalytic amount of camphorsulphonic acid (CSA) with the aim to allow the formation of an intra-cyclic acetyl orthoester between the hydroxyl groups at the positions C2 and C3. (Scheme 5.2) Several trials to isolate the orthoester compound were fruitless. Unfortunately, it was impossible the isolation due to the fact that a chromatographic column produced the ring opening and the deprotection of the hydroxyl at position C3. Therefore, without performing any purification procedure and simply removing the solvent, the crude was directly prepared for the second step, the benzoylation of the hydroxyl groups at C4 and C5 positions. The crude was treated with benzoic

anhydride, triethylamine and a catalytic amount of 4-dimethylaminopyridine (DMAP). After three hours of reaction, the solvent was removed under vacuum and the selective opening of the orthoester with a solution of HCl 5% was carried out, leaving an acetate group at position 2. The HCl was removed from the solution and $NaHCO_3$ and water were added to restore a neutral pH. The remaining free hydroxyl group at position C3 was protected with a benzoate group, using the same procedure described before. Finally, the selective deprotection of the acetyl group was performed by a reaction with HCl 5% in methanol for 72 hours. The purification of the final product was performed by column chromatography on silica gel, affording compound **115** with an overall yield of 21%. This means an average of 46% for every single step. (Scheme 5.2)

The analysis by ^{1}H NMR allowed to characterize compound **115**; indeed, in the spectrum is clearly visible that the protons of positions C3, C4 and C6 have a chemical shift values typical of their protected form, except the hydrogen at the position C2 that resonates in a region not typical of a C2 hydroxyl group protected. (Figure 5.3)

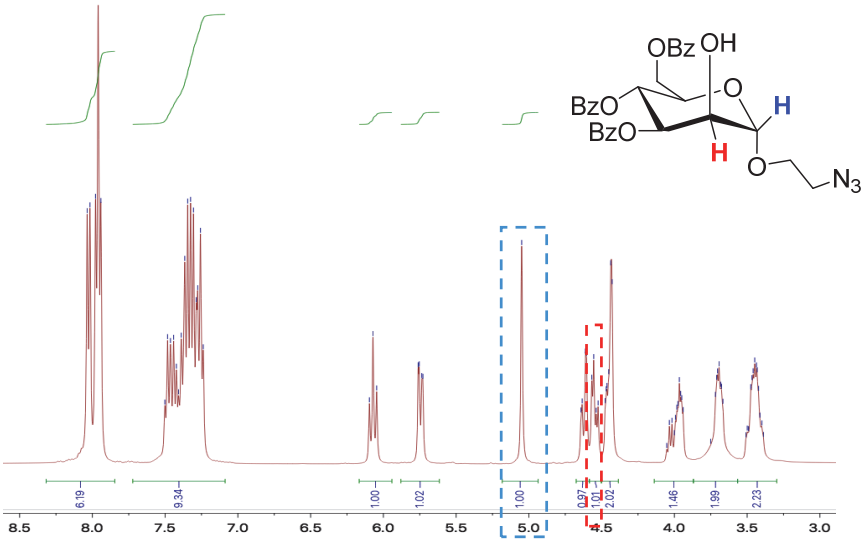


Figure 5.3. ^{1}H NMR spectrum of compound **117**.

Investigating the sub-products formed in this reaction by TLC, it was possible to detect two spots from the first step of reaction. This couple of spots persisted in the

following steps until the final reaction. The isolation of this sub-product corresponded with a mannose having the hydroxyl groups at positions C2 and C4 deprotected. In the ^{1}H NMR was clear the absence of the triplet signal of the hydrogen at position C4 with the hydroxyl group protected. (Figure 5.4)

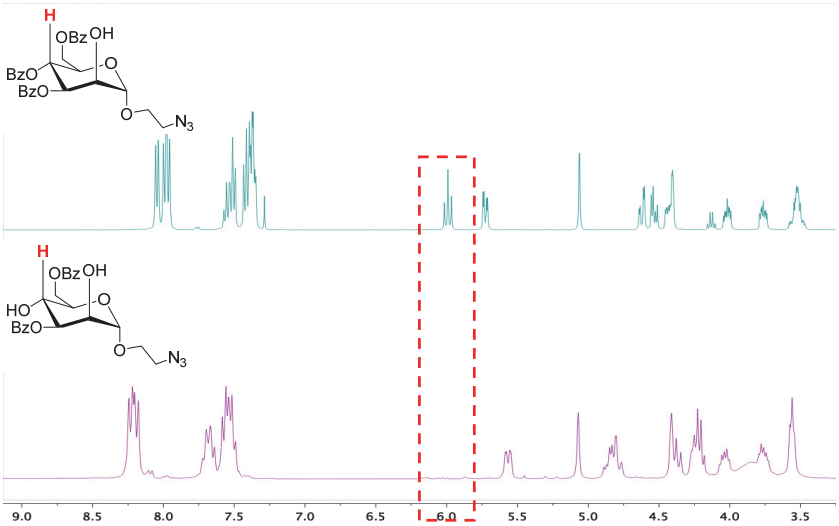
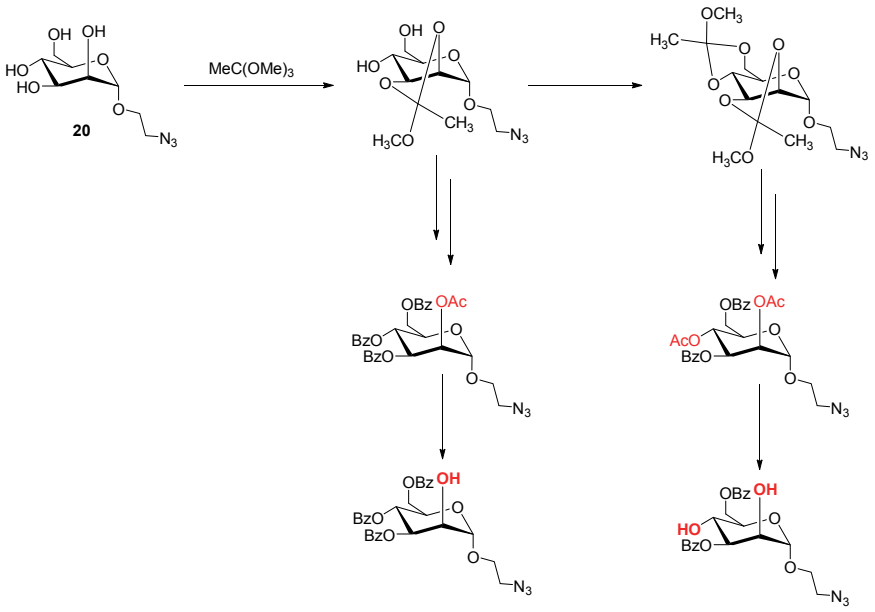


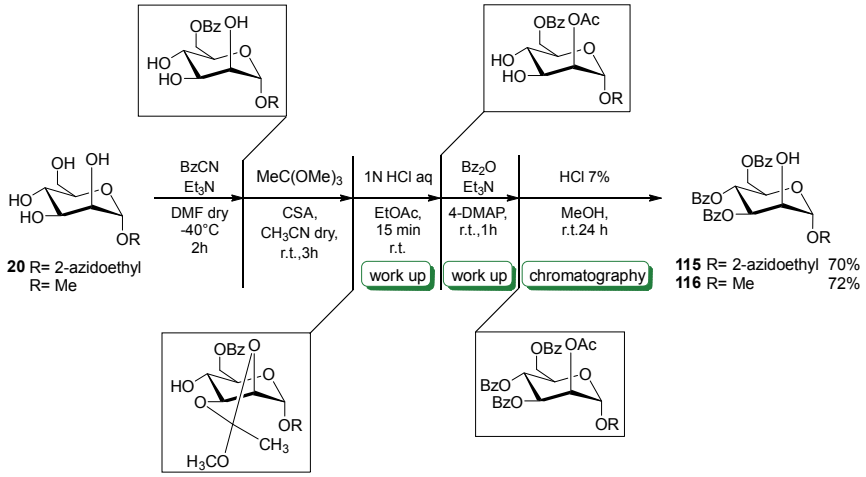
Figure 5.4. ^{1}H NMR spectrum of compound **115** and sub-product.

This fact could be explained by the formation of a second orthoester between the C4 and C5 positions in the first step of the consecutive synthesis which is reflected in a formation of an acetyl group in the position C4 as well as in position C2. (Scheme 5.3)



Scheme 5.3. Possible pathway for the sub-product formation.

The formation of this sub-product in the first step of the synthesis has had a tremendous impact on the final yield of the compound **115**. For this reason, it was decided to eliminate the possible formation of this intermediate, performing a regioselective mono benzoylation on the primary alcohol of the position C6. (Scheme 5.4) This selective benzoylation was performed using a solution of Benzoyl cyanide dropped slowly in a mixture of **20** dissolved in DMF in dry conditions at -40°C. By TLC was possible to see only a little percentage of other benzoyl derivatives, probably reacted at the position C3 or C2. Having the primary alcohol protected, the rest of the steps were addressed in the same way than previously. Only a change was included in the last deprotection step. Indeed after four steps, the fully protected mannose compound was finally treated with a solution of HCl 7% in methanol to assure the complete eliminatio of the acetyl protection. The use of HCl 7% in methanol was the best compromise both, to remove the acetyl in 24 hours and avoiding the possible partial hydrolysis of the bezoyl groups. In this case, after the final and unique column chromatography, the azidoethyl mannose **115** was obtained with an overall yield of 70%. The same procedure was adopted with the methyl α-D-mannopyranose affording the protected compound **116** in a 72% yield. (Scheme 5.4)



Scheme 5.4. Consecutive synthesis of glycosyl acceptor **115** and **116**.

The regioselective mono-benzoylation of the C6 alcohol is probably the most important step that allowed to increase the yield in a remarkable way. In addition, there are not other examples in the literature in which the selective protection of the primary alcohol has been performed on a fully deprotected mannose. Usually, the protection of this group in the presence of secondary alcohols has been addressed using a high sterical hindrance group like the *tert*-butyldiphenylsilyl ether (TBDPS).[329] However, in this case the use of an acetyl protective group on C2 lead incompatible the application of a silylether group, due to the fact that the deprotection conditions to remove the acetyl group could lead to a simultaneous removal of the silyl protective group at position C6.

In summary, the synthetic approach allowed the production of the glycosyl acceptors **115** and **116** in excellent yields in 5 steps and in only 28 hours with a single chromathographic purification. The same synthetic procedure was performed step by step, in our laboratory, for both compounds, **115** and **116**. In this case, performing the 5 reaction steps with 4 purifications by silica gel flash chromatography have resulted in an overall yield of 40-42% This means that a consecutive strategy has

[329] a) Reina, J.J., *et al., Org. Biomol. Chem.*, **2008**, *6*, 2743-2754; b) Reina, J.J.; Rojo, J.; *Tetrahedron Lett.*, **2006**, *47*, 2475-2478.

increased the yield around 30% over the stepwise synthesis, demonstrating in an unequivocally manner that purifications have a remarkable impact on the global yield.

Once the glycosyl acceptors have been prepared in gram-scale, it was only necessary to prepare the glycosyl donor to complete the disaccharide synthesis. As shown in Scheme 5.1, to afford the synthesis of Manα1,2Man, two different glycosyl donors were selected, one peracetylated, **117** and one per-benzoylated, **118**. (Figure 5.5) To obtain the α-1,2 linkage, the trichloroacetimidate was selected as activating group, because with this group the glycosylation conditions are soft and the reaction can be processed easily. The stereochemistry of the reaction is determined by the presence of a participant acyl group on position C2 of the glycosyl donor that move the reaction to a formation of the α product.[330]

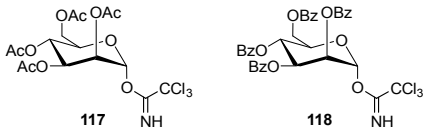
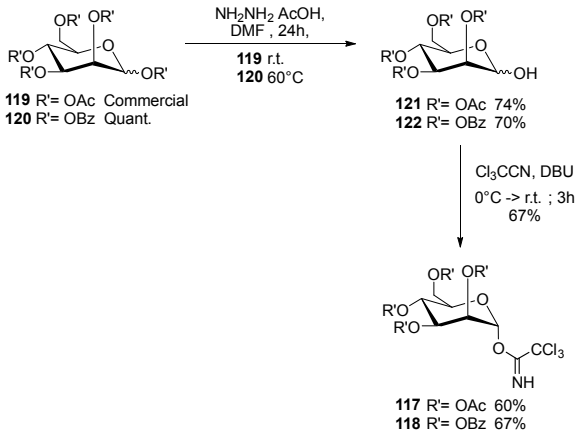


Figure 5.5. Glycosyl donors, peracetylated **117**, and perbenzoylated **118**.

The synthesis of compounds **117** and **118** started from the commercially available per-acetylated and perbenzoylated mannose. Having the per-acetylated **119** and per-benzoylated **120** mannoses, the regioselective deprotection of the anomeric position was performed using a classical procedure that involve the use of hydrazine acetate in DMF. (Scheme 5.5) It was necessary to perform the reaction at room temperature for the acetyl mannose **121**[330b] and warming at 60°C for the benzoyl derivative **120**.[330a] At this step, the anomeric hydroxyl group was functionalized using trichloroacetonitrile in dry atmosphere and using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as activating agent. After a purification by silica gel column chromatography, the flycosyl donors **117** and **118** were obtained with a yield around 60-67% (Scheme 5.5) due to the extreme reactivity of the trichloroacetimidate

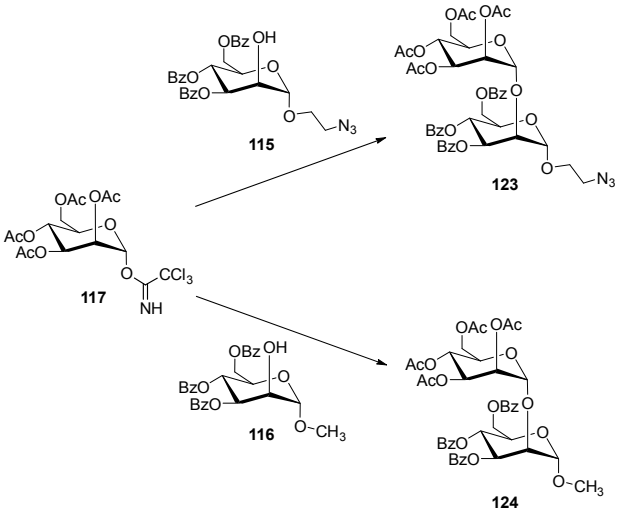
[330] a) Lee, D.; Kowalczyk, J. R.; Muir, V. J.; Rendle, P. M.; Brimble, M. A.; *Carbohydr. Res.,* **2007**, *342*, 2628; b) Excoffier, G.; Gagnaire, D.; Utille, J.P.; *Carbohydr. Res.*, **1975**, *39*, 368-373.

group, being very sensible to the hydrolysis degradation. By ^{1}H-NMR, the appearance of the typical singlet at 8.50-9.00 ppm corresponding to the NH group of the trichloroacetimidate confirmed without doubts the formation of the desired products.



Scheme 5.5. Synthesis of the glycosyl donors **117** and **118**.

The mannose derivatives so armed were ready to react with the glycosyl acceptors **115** and **116**. For this aim, acetyl compound **117** was use as a donor, in order to obtain the disaccharides **123** and **124**.



Scheme 5.6. Scheme of the preparation of protected disaccharides **123** and **124**.

To set-up the optimized conditions for this reaction, first trials were carried out with 2-azido ethyl mannose derivative **115** as glycosyl acceptor, using as promoter the trimethylsilyl triflate (TMSOTf).[331] A varying range of temperatures were examined, in dichloromethane under argon atmosphere and using molecular sieves 4Å. (Table 5.1)

	Glycosyl donor	*Glycosyl acceptor*	*TMSOTf (eq)*	*T (°C)*	*Yield (%)*
1	**117**	**115**	0.2	-41	25
2				-20	47
3				0	59
4				r.t.	13

Table 5.1. Reaction conditions for the glycosylation to obtain the compound **123**.

By ^{1}H NMR, it was possible to determinate the correct glycosylation of the product through the detection of two peaks at 5.31 ppm corresponding to the anomeric proton of the glycosyl donor moiety and at 5.24 ppm corresponding to the corresponding glycosyl acceptor. (Figure 5.6)

[331] a) Francesconi, O; Nativi, C.; Gabrielli, G.; Gentili, M.; Palchetti, M.; Bonora, B.; Roelens, s.; *Chem. Eur. J.*, **2013**, *339*, 11742-11752; b) Depras, G.; Robert, R.; Sendid, B.; Machez, E.; Poulain, D.; Mallet, J.M.; *Bioorg. Med. Chem.*, **2012**, *20*, 1817-1823; c) Heng, L.; Ning, J.; Kong, F.; *J. Carbohydr. Chem.*, **2001**, *20*, 285-296.

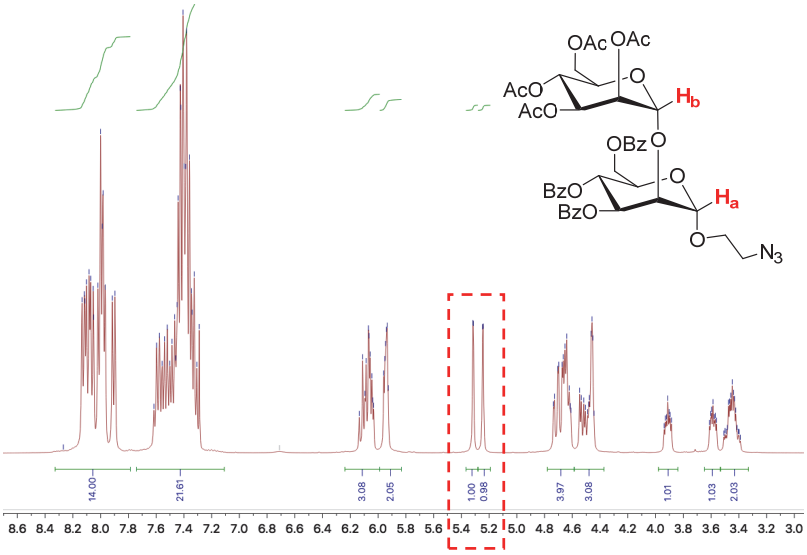
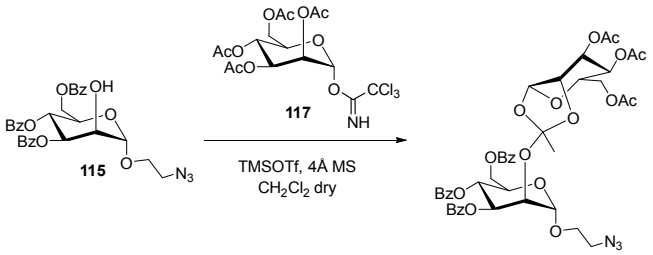


Figure 5.6. ^{1}H-NMR spectrum of the disaccharide **123**.

From these trials, it was clear that the use of room temperature (entry 4 Table 5.1), could cause the rapid degradation of the glycosyl donor. Indeed, in the TLC, at this temperature, appeared a large spot corresponding to the hydrolysis of the donor. The use of a very low temperature (-41°C entry 1 Table 5.1) resulted in a yield of 25% and using a higher temperature (-20°C entry 2 Table 5.1) the yield increased up to 47%. The best results were obtained at 0°C (entry 3 Table 5.1), using also 0.2 equivalents of TMSOTf, in comparison with the glycosyl acceptor, that was in defect. In this case, the yield of the glycosylation reaction was 59%.

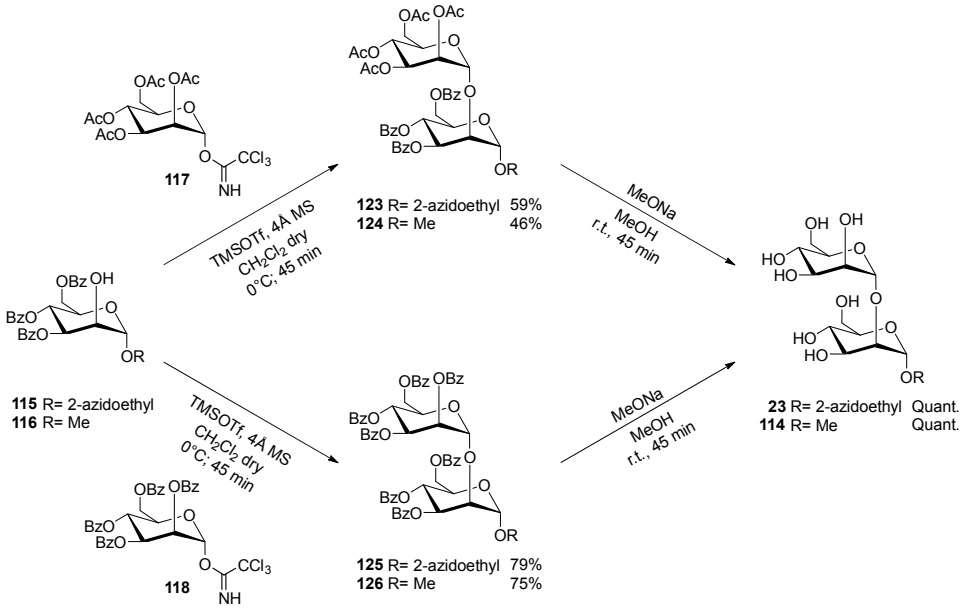
With the aim to minimize the formation of the orthoester compound, a typical sub-product of this reaction, (Figure 5.7) a small amount of TMSOTf was used. This subproduct comes out from an intramolecular reaction of the glycosyl donor, in which there is a participation of the acetyl group in position C2. (Scheme 5.7)



Scheme 5.7. Possible sub-product of the glycosylation.

These reaction conditions were applied also to the synthesis of the disaccharide having the methoxy group at the anomeric position **124**, reaching a yield of the 46%. (Scheme 5.8) Once the preparation of the disaccharide derived from per-acetylated donor **117** was optimized, it was set up the preparation of the disaccharide using the per-benzoylated **118** as glycosyl donor. In this case, the orthoester formation is not possible or at least it is reduced to a minimum, due to the fact that the benzoyl group has a major steric hindrance in comparison with the acetyl group.

Taking advantage from the experience obtained with the disaccharide **123**, the same conditions were used to prepare the disaccharides **125** and **126**. In this case, as expected, there was a strong increasing of the glycosylation yield which confirm the hypotheses described above. In fact, changing the protective group of the glycosyl donor, from acetates to benzoates, the yield increased from 46-59% to 79% for the disaccharide **125** and to 75% for the disaccharide **126**. (Scheme 5.8)



Scheme 5.8. Synthesis and deprotection of disaccharides compounds **23** and **114**.

Deprotection of disaccharides having the 2 azido ethyl group, and the methoxy group, were achieved using the classical Zemplen conditions,[193] sodium methoxide in methanol. The neutral pH was restored using an Amberlite IRA 120-H+ resin and after filtration, the deprotection afforded the final disaccharides in quantitative yield. (Scheme 5.8) This procedure allowed a rapid synthesis to produce the disaccharide **23** in gram scale.

5.3. Synthesis of glycodendrons with biotin at the focal position

Once the Manα1-2Man disaccharide has been prepared, we afforded the preparation of multivalent dendritic compounds using this carbohydrate as a ligand. For this, we have synthesized a family of glycodendrons of valence nine and tri and the corresponding monovalent compound, all having a biotin at the focal position of the dendron scaffolds. (Figure 5.7)

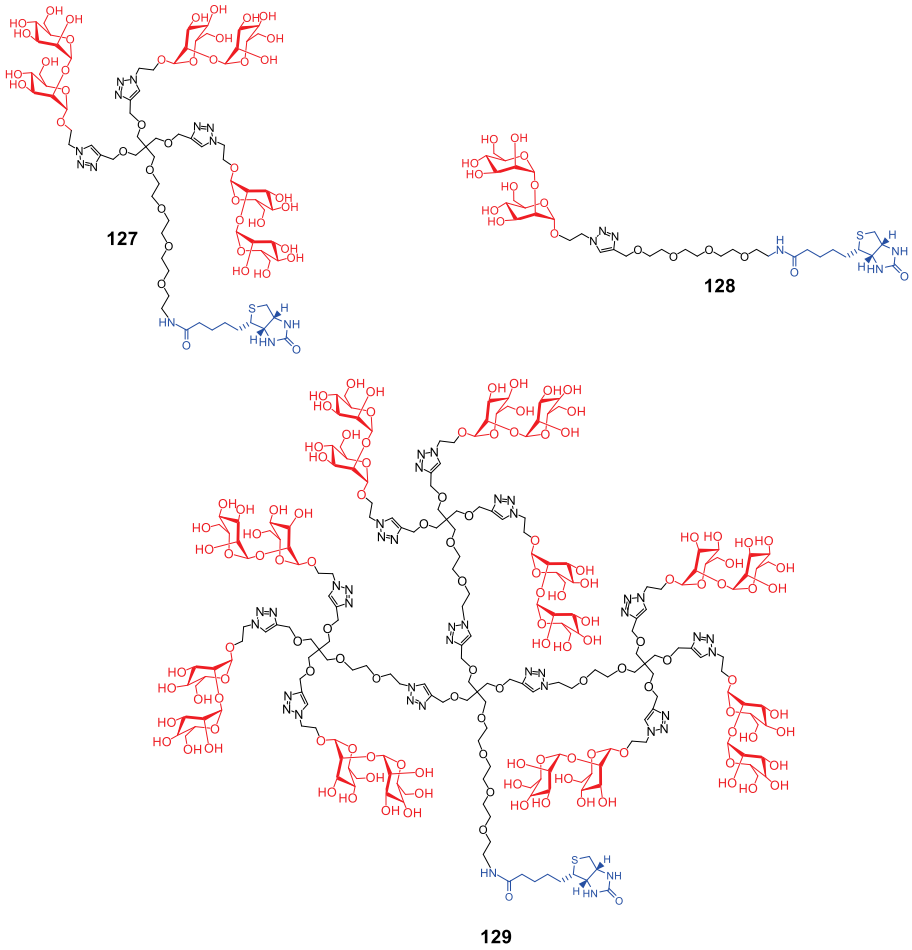
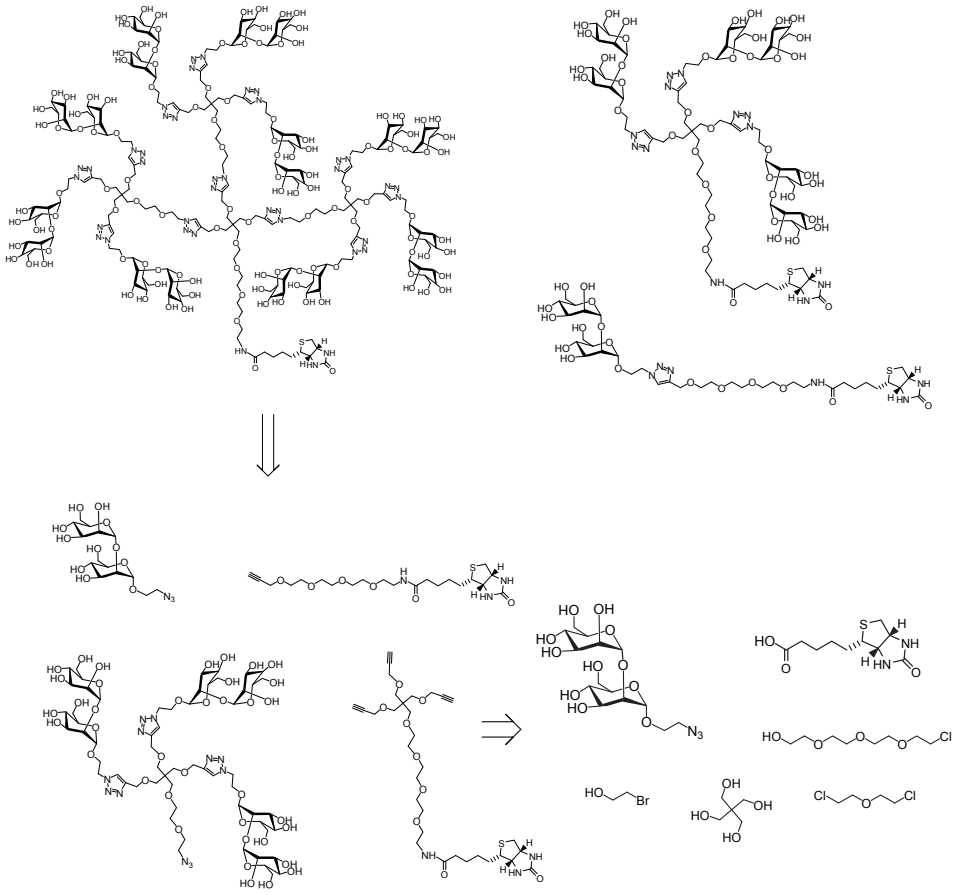


Figure 5.7. Glycodendrons with a biotin group at the focal position **127**-**129**.

The synthetic strategy to address the preparation of these glycodendrons was based on the previous experience of our research group for the preparation of first and second generation mannosylated glycodendrons and glycodendrimers using the CuAAC coupling. [111, 113,292]

The retrosynthetic strategy for the preparation of these glycodendrons is depicted in Scheme 5.9



Scheme 5.9. Retrosynthetic route for the preparation of glycodendrons **127-129**.

5.3.1. Synthesis of building blocks

The preparation of the building blocks to achieve the synthesis of the glycodendrons needed the Manα(1,2)Man with an azido group at the anomeric position (**23**) and the alkynylated scaffolds necessary to perform the click reactions. Respect to the alkyne building blocks, it was selected a trialkyne pentaerythritol functionalized with a short diethylene glycol linker with a terminal chloride **76**, a trialkyne pentaerythritol functionalized with a long tetraethylene glycol with a terminal biotin **130**, and a linear tetraethylene glycol, functionalized with a biotin and a propargyl group at the ends **131**. (Figure 5.8) The synthesis of these compounds were

performed using a strategy, appropriately modified, developed in our group [113, 332] from a precedent work of Roy *et al.*[294]

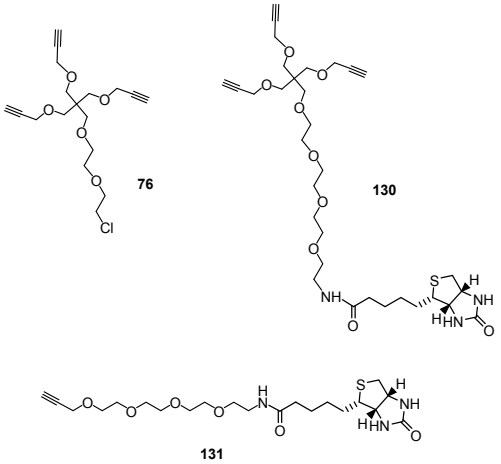
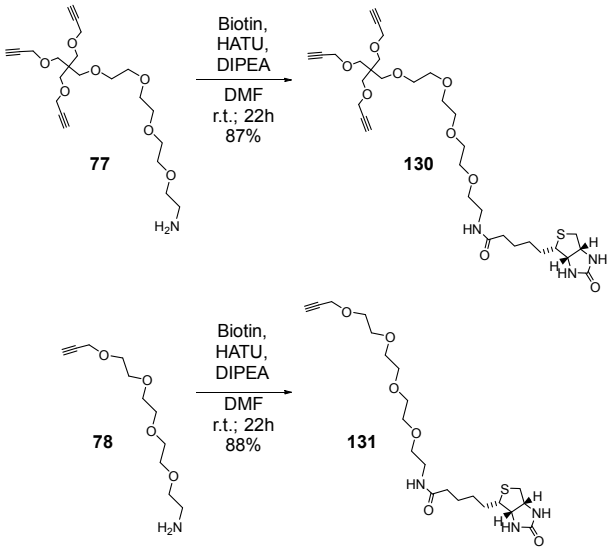


Figure 5.8. Alkyne building blocks **76**, **130** and **131**.

The synthesis of the scaffold **76** was described in the previously Chapter 4, section 4.4.3. The preparation of compounds **130** and **131** derived from the biotinylation of the compounds **77** and **78** are depitected in Scheme 5.10.



Scheme 5.10. Synthetic route for the preparation of the building blocks **130** and **131.**

[332] Sanchez-Navarro, M. *et al., Chem. Eur. J.*, **2011**, *17*, 766-769.

The coupling of the amino group at the focal position with biotin, was performed using hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU),[333] to promote the amide formation. After the purification by silica gel column chromatography, the building block **130** was obtained with a 87%, yield. (Scheme 5.10) The formation of the important building block was confirmed both, by NMR analysis and mass spectrometry (Figure 5.9).

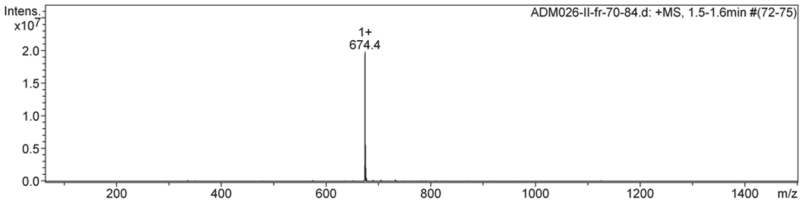


Figure 5.9. ESI-MS of the amino compound **130**.

In order to obtain the mono alkyne building block **131**, the same procedure was applied, obtaining the mono alkyne compound with a good yield. (Scheme 3.10)

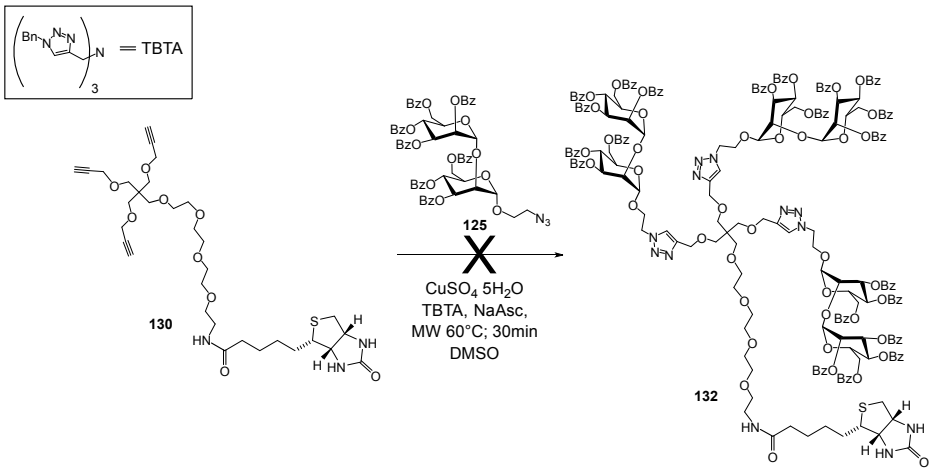
5.3.2. Synthesis of the glycodendrons with biotin

With the carbohydrates and the alkyne building blocks on hands, the conjugation of both entities was performed using click chemistry reaction to prepare the corresponding Manα1,2Man glycodendron structures.

The 1,3 dipolar cycloaddiction, catalyzed by copper, between the trialkyne **130** and the Manα1,2Man disaccharide **23** was performed using the experimental conditions explained in the section 4.4.4; except for the use of microwave heating in order to speed up the reaction reducing the time normally required for this type of reaction.[334] This microwave warming was recently applied by our research group with a remarkable success in the synthesis of mannosylated glycodendrons with different valency and dendritic generation. [292]

[333] Zhou, Y., *et al., Analyst,* **2017**, *142*, 345–350.
[334] Bock, V.D.; Hiemstra, H.; van Maarseveen, J.H.; *Eur. J. Org. Chem*., **2006**, 51–68.

The same conditions were applied, using the protected form of the disaccharide **23**, the benzoylated disaccharide **125**,[334] with the idea to remove the protection after the purification phase. To carry on the reaction, $CuSO_4 \cdot 5H_2O$ (0.3 equivalents) were used, this means, 0.1 equivalent per alkyne group. TBTA (0.6 equivalents) and sodium ascorbate (0.6 equivalents to reduce the Cu(II) to Cu(I)), were added. The reaction was heated under microwave irradiation at 60°C for 30 min in DMSO. After this time, the TLC revealed the complete absence of any reaction product. (Scheme 5.11)

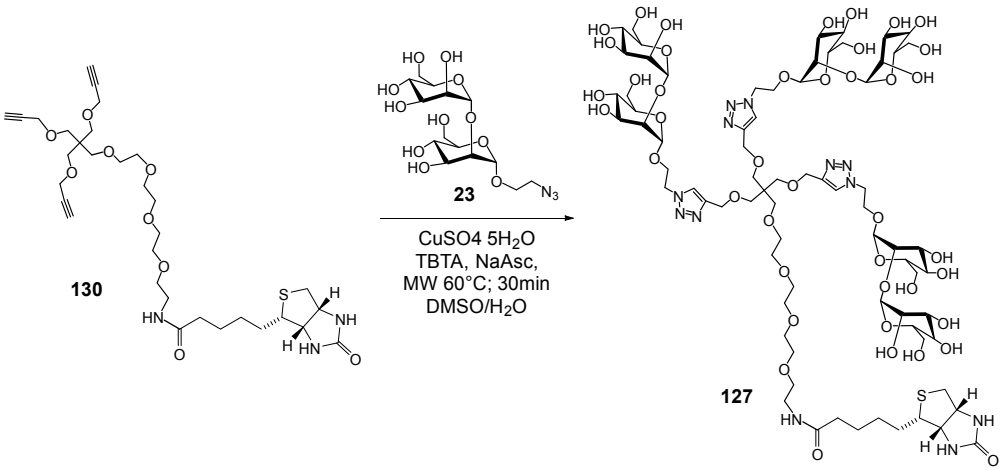


Scheme 5.11 A first approach to the preparation of the protected trivalent glycodendron **132**.

A second trial was performed doubling the number of equivalents of the reagents and also, a double cycle of microwave heating after the addition of more catalyst. None of these variations in the synthesis allowed to observe changes on the negative final result. Studying the click reactions with microwave reported in our research group in the recent past, one of the difference was the use of carbohydrates protected with acetyl group instead of the benzoyls. Probably, the aromatic ring of the benzoyl protecting group has the capacity to chelate the copper, decreasing the available amount of catalyst in the reaction.

For this reason, it was decided to use the deprotected disaccharide **23**, in order to avoid the possible chelation of the Cu. The same reaction conditions used before

were applied. (Scheme 5.12) In the TLC, it was possible to observe the formation of several spots. More copper catalyst, TBTA and ascorbate were added, in a ratio 0.3/0.6/0.3 equivalents in correlation with the scaffold **130** and one more microwave warming cycle of 30 min. Then, from the TLC, it was possible to observe only small differences in comparison with the previous TLC. Therefore, it was decided to purify the reaction in order to verify the nature of the compounds coming out for the reaction. Indeed, taking advantage from the difference in the molecular weight among the hypothetic final compound and the reagents, a size exclusion chromatography was selected as purification method. First, the copper catalyst was removed by adding a metal scavenger, the resin QuadraSil® Mercaptopropyl. After filtration, the crude was purified by Sephadex LH20.



Scheme 5.12. Synthesis of the trivalent glycodendron **127** using microwaves.

The Sephadex purification did not allow to separate two spots, close each other, but easy to recognize. An ESI MS was performed to analyze the molecular weigth of this mixture of compounds, discovering that corresponded to two of the three possible combinations deriving from the uncomplete click on the trivalent building block **130**. (Figure 5.10) Analyzing the mass spectrum, it was clear the presence of two distinct product, one having a molecular weight coincident with the complete clicked final product **127**, (A in Figure 5.10) and another one having a molecular weight coincindent with a molecule with only two coupled ligands (B in Figure 5.10).

Moreover, the compounds A and B are similar in terms of molecular weight and for this reason, it was not possible to separate them by a Sephadex LH20.

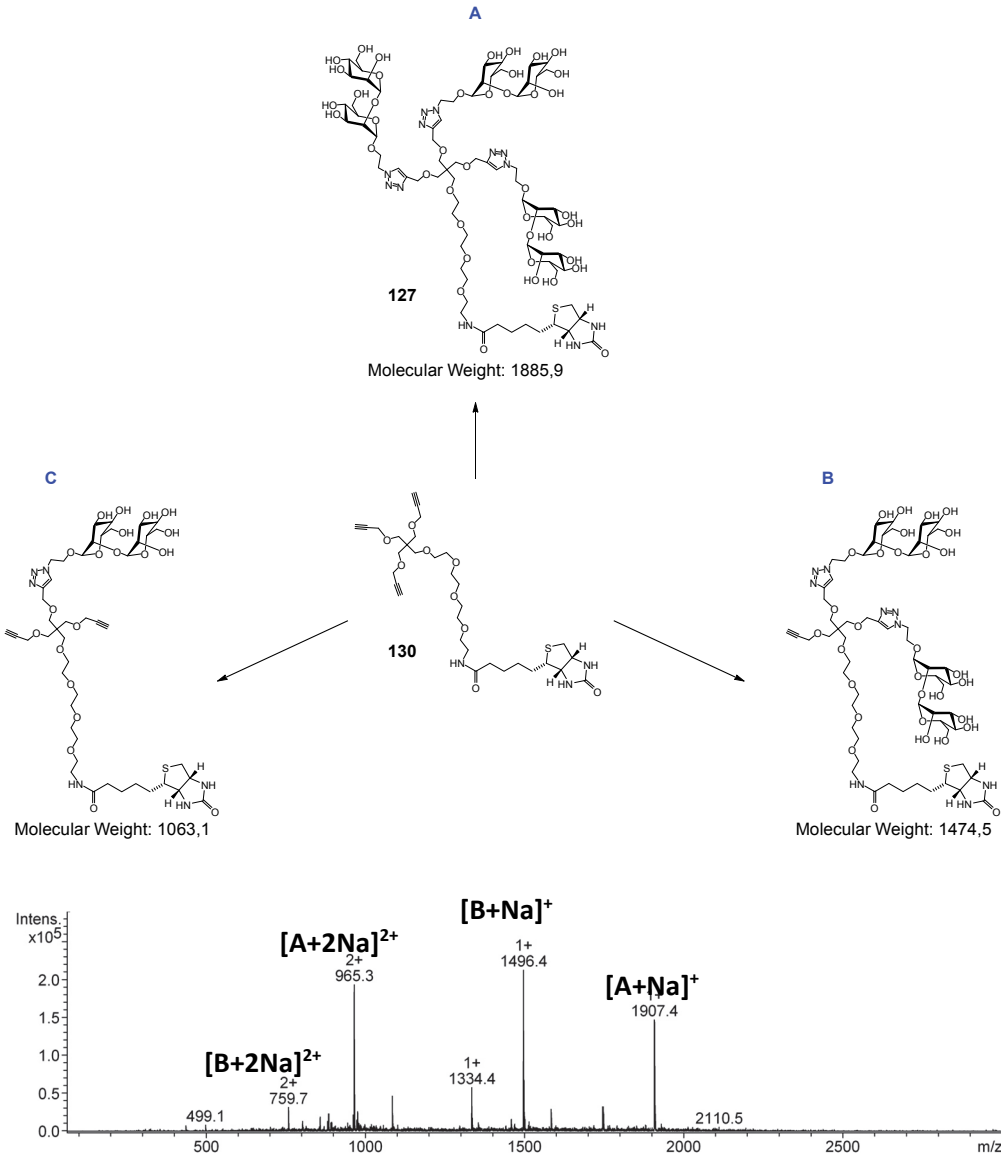
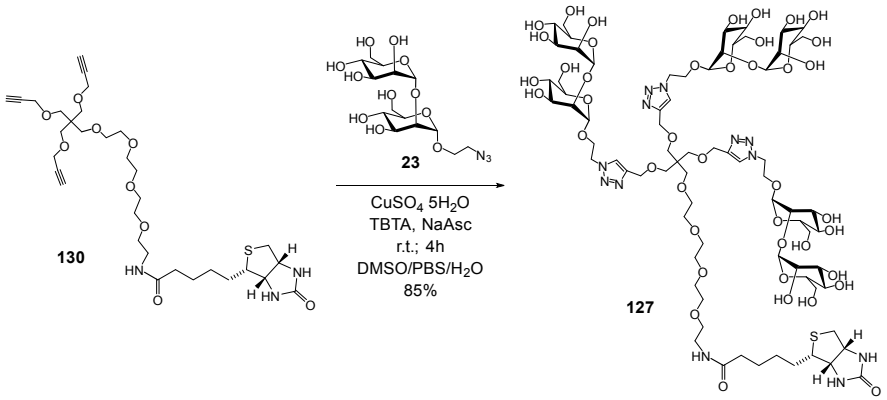


Figure 5.10. ESI-MS of the click reaction of **130** with **23**.

A different conditions for the CuAAC reaction were tried using a mixture having the buffer phoshpate (PBS) at pH 7.4 and avoiding the microwave warming. In fact, it

was demonstrated that a basic/neutral buffer[334] promotes the loss of the proton at the alkyne group, thus improving the CuAAC (the mixture DMSO/H_2O in fact resulted in a pH less than 6.0).

The new conditions were 1.0 eq of trialkyne **130**, 4.0 eq of the azide compound **23**, 0.3 eq of $CuSO_4$ $5H_20$, 0.6 eq of TBTA and 0.3 eq of sodium ascorbate. The solvent of the reaction was in this case, a mixture of DMSO, PBS, and water in a ratio 1/5/2.5. (Scheme 5.13) This mixture allowed on one hand, the complete solubilization of the reagents (TBTA is only partially soluble in aqueous solvents) and on the other hand, a basic pH media to promote the CuAAC. After two hour of reaction, one more addition of catalyst was carried out. After 4 hour of reaction, the TLC showed only one spot.



Scheme 5.13. Synthesis of glycodendron **127** using as solvent a mixture of DMSO/PBS/water.

To purify the reaction, the copper catalyst was first removed with the scavenger resin and then, a size exclusion chromatography was performed. NMR and ESI-MS confirmed the formation of the final product **127**, without sub-products, and with a 85% yield. In particular, the integration of the signal in the ^1H NMR corresponding to the triazole proton at δ 8.04 ppm *versus* the rest of the signals of the molecule as well as the presence of the two carbon signals of the triazole at δ 144.8 (quaternary carbon) and 124.4 ppm (CH) in the ^{13}C NMR spectrum, indicated clearly the formation of compound **127**. (Figure 5.11)

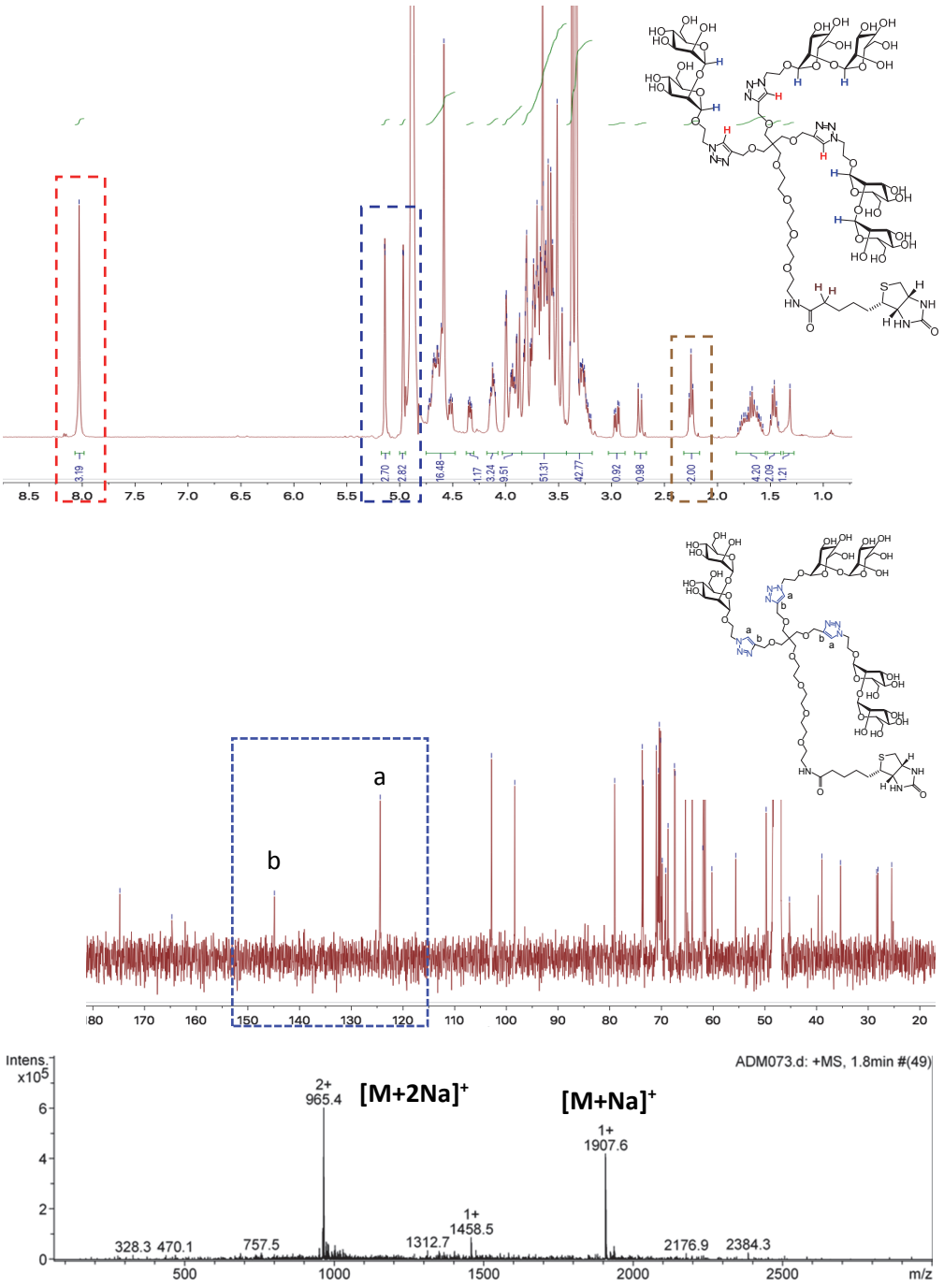
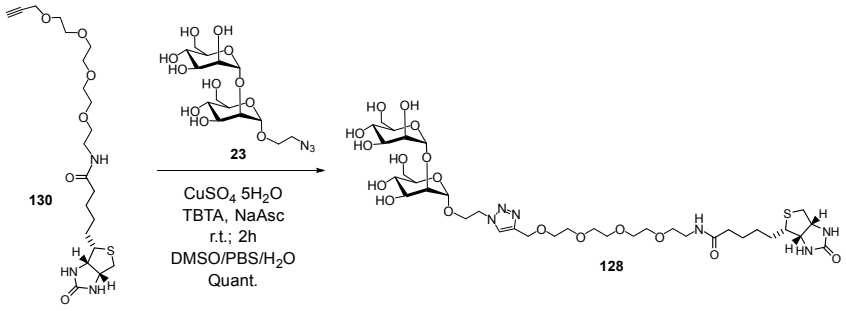


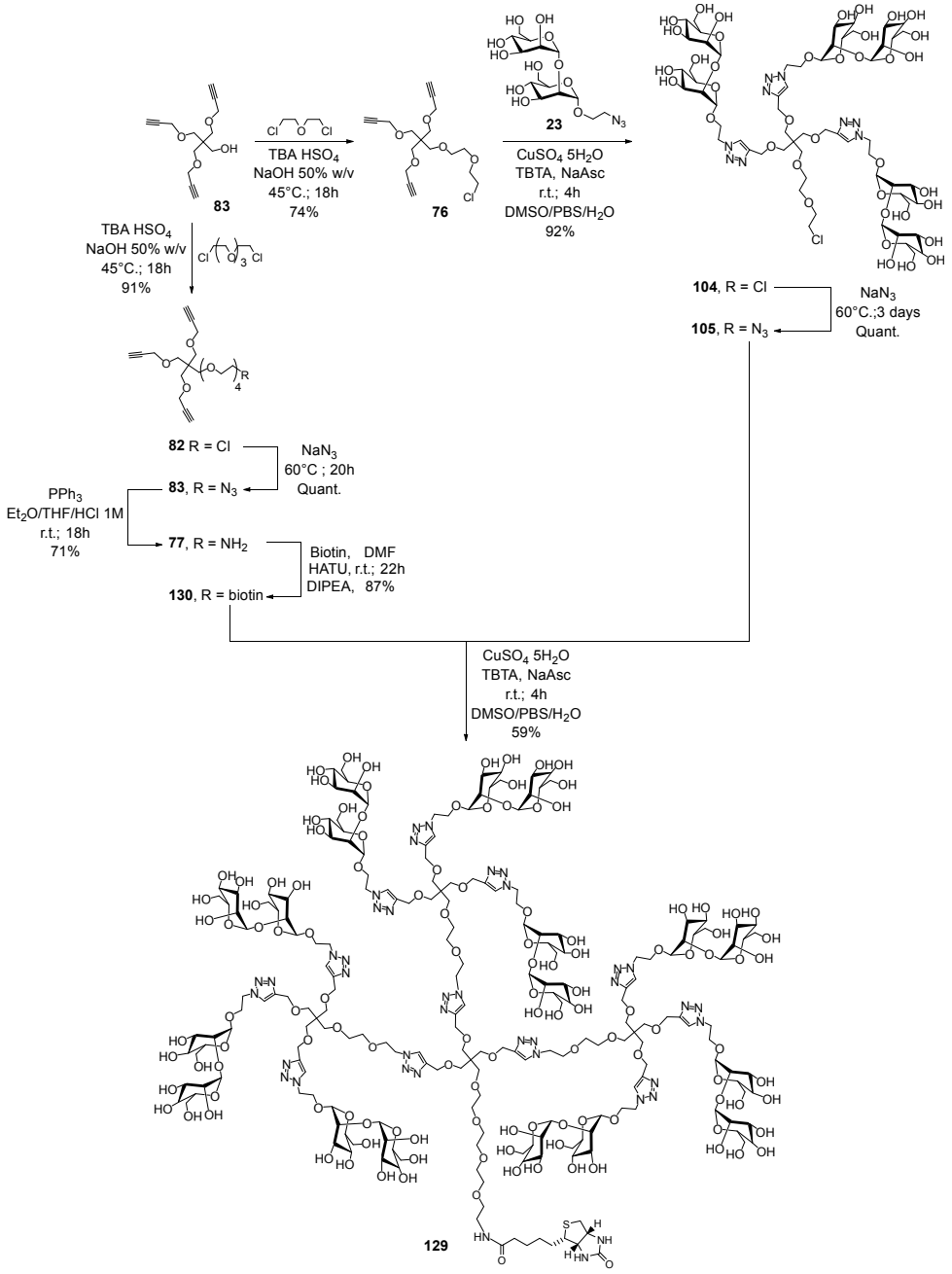
Figure 5.11. ^{1}H and ^{13}C NMR spectra and ESI mass spectrum of the glycodendron **127**.

With these conditions mono valent compound was synthetized. The conditions for the coupling were the same that those used for the trivalent compound but reducing the number of equivalents of copper sulfate, TBTA and sodium ascorbate, to a third part. After 2h of reaction, it was possible to verify the complete conversion of the alkyne substrate by TLC. Purification by scavenger resin and Sephadex LH 20 column chromatography allowed to obtain the compound **128** with quantitative yield. (Scheme 5.14) The formation of the final compound was confirmed by NMR and ESI-MS.



Scheme 5.14. Synthesis of the monovalent compound ***128***.

To synthetize the nonavalent glycodendron, a convergent synthetic route was proposed as described in the following Scheme 5.15. The synthesis started with a first click reaction of the alkyne compound **76** with the mannose disaccharide **23**, followed by a functionalization of the focal point position with an azido group. Then, a second click reaction on the biotynilated alkyne core **130** was performed with the trivalent glycodendron **105** described previously in chapter 4 of this Thesis. (Scheme 5.15)



Scheme 5.15. Synthetic route to achieve the synthesis of a glycodendron of second generations **129**.

Glycodendron **105** constitutes the branches of the nonavalent structure that are displayed on a central pentaerhytritol core, having three alkynes groups and a long linker with the biotin at the focal position (compound **130**). Using a second click reaction, it was possible to increase the generation by coupling **105** on this scaffold. (Scheme 5.15)

The conjugation conditions were similar to the previous ones with the exception that in this case, the large number of free hydroxyl groups present in the trivalent **105** required an excess of the Cu(I) catalyst, added in small portions with an interval of 1h. The ESI-MS of the crude allowed to verify the complete formation of the nonavalent product. After removing the excess of copper by adding a scavenger resin, the crude was purified by size exclusion chromatography.

The characterization of the compound **129** has been done by the NMR analysis due to its structural characteristics. Indeed, the nonavalent glycodendrons have two kind of triazole rings, an internal one corresponding to the core, and an external one corresponding to the three branches. In the ^{1}H NMR spectrum, it was possible to observe, around 8 ppm, two singlets corresponding to the protons of the internal triazole rings (indicated in blue, Figure 5.12) and of the external ones (indicated in red, Figure 5.12) of the structure with a ratio of 3:9, respectively. In this way, it was possible to verify the complete functionalization of the core by integrating these signals and also by their multiplicity, because the incomplete products would give different signals (loss of symmetry). Moreover, in the ^{13}C NMR spectrum were visible two peaks corresponding to the carbon atoms of the triazole ring, very close one to each other. In Figure 5.12, it is possible to observe two peaks. Around 125 ppm, there are the peaks corresponding to the CH carbons of the triazole, and around 144 ppm, those corresponding to the quaternary carbons. In blue, are indicated the peak of the internal triazole rings and in red, the corresponding to the external ones. The HSQC spectrum was used to confirm this correlation.

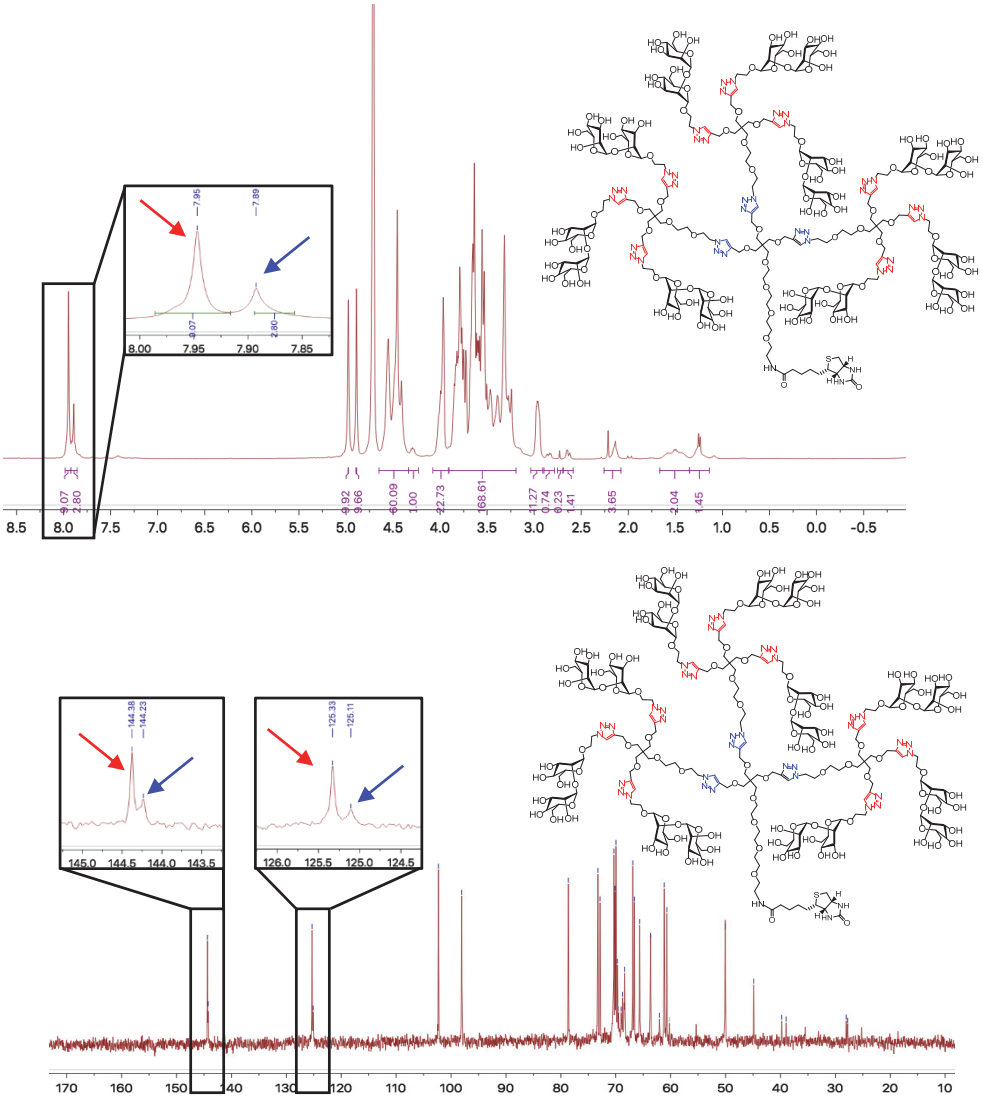


Figure 5.12. ^{1}H and ^{13}C NMR spectra of the nonavalent glycodendron **129**.

Using ESI-MS, it was possible to confirm the formation of the product, identifying the peaks having triple and quadruple charge. Figure 5.13

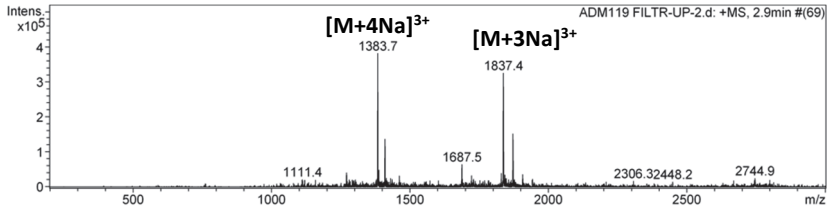


Figure 5.13. ESI-MS spectrum of the nonavalent glycodendron **129**.

5.4. Discussion

Adopting a consecutive synthesis of the glycosyl acceptor, the total cost and the time of preparation of the final deprotected disaccharide has been reduced in a significant manner. This approach allowed the increase of the yield and the decrease of the time the cost was also reduced due to the minimizing of the purification procedures to only one column chromatography after five consecutive steps.

In this way, the Manα1,2Man has been prepared in a gram-scale and in less than 2 days, in a very easy and reproducible way. The versatility of this approach was demonstrated using as aglycon group the methyl and the 2-azido ethyl. The availability of this disaccharide in large quantity has allowed the easy set up of the synthesis of other compounds such as the glycodendrons.[335]

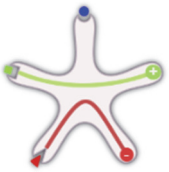
Manα1,2Man glycodendrons of valence nine and tri and the monovalent compound were designed and synthetized with a biotin at the focal position. In fact, taking advantage from the strong non-covalent bond of biotin-streptavidin, one of the application of these compounds, in the future, will be the study of the importance of the multivalency on several plant lectins using the surface plasmon resonance imaging, conjugating these biotynilated glycodendrons on a streptavidin surface.

Moreover, these glycodendrons have been used to start a collaboration with Dr. Frank Momburg and his PhD student Márcia Gonçalves, at the German Cancer Research Center (DFKZ), Germany. The goal will be to measure the binding of the

[335] Reina, J.J.; Di Maio, A.; Ramos-Soriano, J.; Figueiredo, R.-C.; Rojo, J.; *Org. Biomol. Chem.*, **2016**, *14*, 2873-2882.

glycodendrons on native human blood leukocytes (peripheral blood mononuclear cells, “buffy coat” preparations) by flow cytometry. Cell surface binding of the glycans will be measured by biotin-streptavidin interaction, the types of cells within the lymphocyte preparation will then be further defined by their specific (CD) markers, so we can differentiate binding to T-(CD4/CD8), B-,NK cells and monocytes. These experiments should give us a first estimation about the capacity of these glycodendrons to bind to certain types of blood lymphocytes and their possible impact on the cellular activation stage.

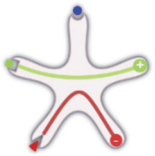
CHAPTER 6
Conclusions



Esta parte de la tesis se publicará con un retraso de 12 meses desde su aprobación y defensa.

CHAPTER 7

Experimental Section



7. Experimental section

7.1. Reagents, solvents and analytical techniques

Chemicals were purchased from Sigma-Aldrich, Merck, Dextra or CarboSynth and used without further purification, unless otherwise indicated. Solvents were purchased from Fisher scientific, ScharLab and Carlo Erba. Anhydrous solvents were purchased from Sigma-Aldrich® with a content of water ≤ 0.005 %. H_2O was purified with a Milli-Q purification system from Millipore (18.3 Ω). Microplates HB8 96 well were purchased by Biomat, Italy.

All reactions that needed dry conditions were carried out using standard techniques under an inert atmosphere of oxygen-free Argon or Nitrogen, unless otherwise stated. TLC were performed using pre-coated aluminum chromate-plates Silica Gel 60 F254 Merck of 0.25 mm thick. Compounds were visualized by using UV light (254 nm) and revealed by immersion into solutions of molybdenum blue (20 g of ammonium molybdate (VI) tetrahydrate, 0.4 g of sulphate of hydrated cerium and 10% sulfuric acid in 400 mL of H_2O) and heating at 100 ºC, or 0.1% ninhydrin in ethanol, or a basic solution (10 % w/w K_2CO_3 in water) of $KMnO_4$.

Purifications by Flash column chromatography were performed using Silica gel 60 (particle size 0,063-0,200 nm or 0,015-0,040 mm),from Merck, eluting by gravity or subjecting it to light pressure. Purifications by Size-Exclusion Chromatography has been carried out in columns filled with Sephadex LH-20 or G-25 (GE Healthcare Life Science), eluting by gravity.

NMR spectra, ^{1}H and ^{13}C, were recorded at 298 K on a Bruker DRX300, DRX400 and DRX500 spectrometers. All chemical shifts were reported in ppm (δ) using the residual proton solvent peaks as internal standards. These abbreviations were used to indicate the multiplicities: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, td = triplet of doublets, m = multplet.

ESI Mass spectra were obtained with an Esquire 6000 ESI-Ion Trap (Bruker Daltonics) at the Centro de investigacion isla de la Cartuja, CICCartuja.

Microwave reactions were performed on a Biotage© Initiator EXP US Microwave Reactor Synthesis System w Robot Sixty/Eight.

The following analysis were performed at the GLYcoDIAG laboratories (Orléans, France): Absorbance measurements were performed by a FLUOstar© Omega multimode plate reader spectrometer; SDS-PAGE electrophoresis was carried out using the Invitrogen system (XCell-Cell, NuPAGE Novex Bis-Tris gel, NuPAGE MES buffer). The molecular masses of the proteins have been compared with a protein standard (Perfect Protein Markers 15-150 kDa from Novagen). The gels were visualized by staining with coomassie blue (Instant Blue from Expedeon).

The following analysis were performed at the CIC Biomagune laboratories (San Sebastian, Spain): Mass spectra were recorded on MALDI-TOF Ultraflextreme III time-of-flight mass spectrometer equipped with a pulsed Nd:YAG laser (355 nm) in positive reflector ion mode with pulse duration of 50ns, laser fluence of 40% and laser frequency of 500Hz. 2,5-Dihydroxybenzoic acid (DHB) was used as matrix. Samples were dissolved in MeOH. DHB was dissolved in Acetonitrile at a concentration 0.05 M. Sample (1μL) and matrix (2μL) solutions were mixed and 0.5μL of the resulting mixture was placed on the MALDI plate. High Resolution Mass spectra were recorded on Apex II ICR FTMS. High resolution mass measurements were carried out using the calibration standards of similar mass (Lamivudine m/z 230.0594, Leucine-enkephalin m/z 556.2766, des-Arg- Bradykinin m/z 904.4681, Angiotensin, m/z 1296.6853 and Glu-Fibrinopeptide B m/z 1570.6774) to achieve high mass accuracy.

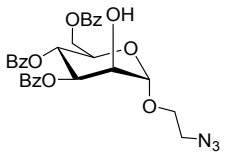
Microarrays were printed employing a robotic piezoelectric SciFLEXARRAYER spotter S11 (Scienion, Berlin, Germany). Indium tin oxide (ITO) coated slides (75 mm x 25 mm) were obtained from Hudson Surface Technology, Inc. (Fort Lee, NJ). The slides have a nominal transmittance of >78 % and an ITO thickness of 130 nm. Modified surfaces were stored under vacuum conditions until its use. Plant lectins were

Esta parte de la tesis se publicará con un retraso de 12 meses desde su aprobación y defensa.

washed. For the fluorescence test, PBS was added in plates and fluorescence was read by a FLUOstar© Omega multimode plate reader spectrometer at λ_{exc} = 485nm and λ_{em} = 530nm.

7.14. Preparation of Manα1,2Man disaccharides

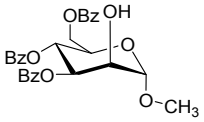
2-azidoethyl-3,4,6-tri-O-benzoyl-α-Dmannopyranoside (115)



To a solution of **20** (1.8 g, 7.23 mmol) in DMF (80 mL) at −40 °C were added dropwise a solution of BzCN (1.14 g, 8.68 mmol) in DMF (20 mL) and a catalytic amount of Et_3N and the reaction was stirred for 2 hours. After that, MeOH (40 mL) was added to quench excess BzCN and the reaction was warmed until room temperature. Then, the solvent was evaporated. After being dissolved in CH_3CN (80 mL), to the resulting mixture containing 2-azidoethyl-6-O-benzoyl-α-D-mannopyranoside were added CSA (335 mg, 1.45 mmol) and trimethyl orthoacetate (2.60 mL, 21.63 mmol) and the reaction was stirred at room temperature for 3 hour. After that, the reaction was quenched with Et_3N (1 mL) and the solvent was evaporated. Then, the residue was dissolved in EtOAc (200 mL) and washed with 1 M HCl (200 mL), the organic phase was dried with anh. $MgSO_4$ and the solvent was evaporated. To the resulting mixture containing 2-azidoethyl 2-O-acetyl-6-O-benzoyl-α-D-mannopyranoside in DCM (100 mL) were subsequently added Bz_2O (6.5 g, 28.92 mmol), Et_3N (4 mL, 28.92 mmol) and DMAP (104 mg, 0.80 mmol) and the reaction was stirred at room temperature for 1 hour. Then, the solvent was evaporated and the residue was dissolved with EtOAc (200 mL). The solution was washed with 1 M HCl (250 mL), sat. $NaHCO_3$ (250 mL) and water (250 mL), the organic phase was dried over anh. $MgSO_4$ and the solvent was evaporated. Finally, to the residue containing 2-azidoethyl 2-O-acetyl-3,4,6-tri-O-benzoyl- α-D-mannopyranoside in CH_3CN (40 mL) was added a solution of 7% HCl in MeOH (200 mL) and the reaction was stirred at room temperature for

24 hours. Then, the solvent was evaporated and the residue was dissolved in EtOAc (250 mL). The solution was washed with a solution of sat. $NaHCO_3$ (2 × 500 mL) and water (500 mL). The organic phase was dried over anh. $MgSO_4$ and the solvent was evaporated. The residue was purified by flash chromatography on silica gel (Hex :EtOAc, 3:1) to obtain compound **115** as a transparent oil (2.84 g, 70%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.03 (d, *J* = 6.9 Hz, 1H, 2HBz), 7.97 (d, *J* = 7.0 Hz, 2H, 2HBz), 7.94 (d, *J* = 7.0 Hz, 2H, 2HBz), 5.98 (t, *J* = 10.0 Hz, 1H, H4Man), 5.71 (dd, *J* = 10.0, 3.1 Hz, 1H, H3Man), 5.04 (d, *J* = 1.9 Hz, 1H, H1Man), 4.60 (dd, *J* = 12.0, 3.1 Hz, 1H, H6Man), 4.52 (dd, *J* = 12.0, 5.2 Hz, 1H, H6Man), 4.47–4.33 (m, 2H, H2Man + H4Man), 4.00 (ddd, *J* = 10.4, 6.2, 3.9 Hz, 1H, H7), 3.74 (ddd, *J*= 10.4, 6.0, 3.7 Hz, 1H, H7), 3.61–3.39 (m, 2H, H8). ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.6 (CvOBz), 166.0 (CvOBz), 133.7 (CHBz), 133.4 (CHBz), 130.2 (CHBz), 130.1 (CHBz), 130.0 (CHBz), 129.5 (CBz), 129.4 (CBz), 128.8 (CHBz), 128.8 (CHBz), 128.7 (CHBz), 100.2 (C1Man), 72.8 (C3Man), 69.5 (C2Man or C5Man), 69.4 (C2Man or C5Man), 67.4 (C4Man or C7), 67.3 (C4Man or C7), 63.9 (C6Man), 50.8 (C8). ESI-MS m/z for $C_{29}H_{27}N_3O_9$: calcd.: 561.2 $[M]^+$; found: 584.2 $[M + Na]^+$; ESI-HRMS m/z calcd for $C_{29}H_{27}N_3O_9$: 584.1645 $[M + Na]^+$ found: 583.1636 $[M + Na]^+$.

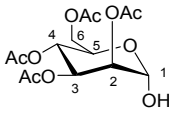
Methyl-3,4,6-tri-O-benzoyl-α-D-mannopyranoside (116)



To a solution of commercial methyl α-D-mannopyranose (1.5 g, 7.72 mmol) in DMF (80 mL) at −40 °C were added dropwise a solution of BzCN (1.21 g, 9.26 mmol) in DMF (20 mL) and a catalytic amount of Et_3N and the reaction was stirred for 2 hours. After that, MeOH (40 mL) was added to quench excess BzCN and the reaction was warmed until room temperature. Then, the solvent was evaporated. After being dissolved in CH_3CN (80 mL), to the resulting mixture containing the methyl 6-O-benzoyl-α-D-mannopyranoside were added CSA (361 mg, 1.55 mmol) and trimethyl orthoacetate (2.95 mL, 23.16 mmol) and the reaction was stirred at room

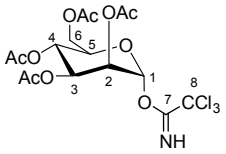
temperature 1 hour. After that, the reaction was quenched with Et_3N (1.5 mL) and the solvent was evaporated. Then, the residue was dissolved in EtOAc (200 mL) and washed with 1 M HCl (200 mL), the organic phase was dried with anh. $MgSO_4$ and the solvent was evaporated. To the resulting mixture containing 2-methyl 2-O-acetyl-6-O-benzoyl-α-D-mannopyranoside in CH_2Cl_2 (100 mL) were subsequently added Bz_2O (7.0 g, 30.88 mmol), Et_3N (4.3 mL, 30.88 mmol) and DMAP (104 mg, 0.80 mmol) and the reaction was stirred at room temperature for 1 hour. Then, the solvent was evaporated and the residue was dissolved in EtOAc (200 mL). The solution was washed with 1 M HCl (250 mL), sat. $NaHCO_3$ (250 mL) and water (250 mL), the organic phase was dried over anh. $MgSO_4$ and the solvent was evaporated. Finally, to the residue containing methyl 2-O-acetyl-3,4,6-tri-O-benzoyl-α-D-mannopyranoside in CH_3CN (40 mL) was added a solution of 7% HCl in MeOH (200 mL) and the reaction was stirred at room temperature for 24 hours. Then the solvent was evaporated and the residue was dissolved in EtOAc (250 mL). The solution was washed with a solution of sat. $NaHCO_3$ (2 × 500 mL) and water (500 mL). The organic phase was dried over anh. $MgSO_4$ and the solvent was evaporated. The residue was purified by flash chromatography on silica gel (Hex : EtOAc, 3:1) to obtain **116** as a colorless oil (2.81 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (d, *J* = 6.9 Hz, 1H, 2HBz), 8.00 (d, J = 8.3, 1.6 Hz, 1H, 2HBz), 7.97 (d, *J* = 8.3, 1.6 Hz, 1H, 2HBz), 7.63–7.47 (m, 2H, 3HBz), 7.48–7.34 (m, 6H, 6HBz), 5.96 (t, *J* = 10.0 Hz, 1H, H4Man), 5.70 (dd, *J* = 10.0, 3.1 Hz, 1H, H3Man), 4.92 (d, *J* = 1.8 Hz, 1H, H1Man), 4.63 (dd, *J* = 12.0, 3.0 Hz, 1H, H6Man), 4.52 (dd, *J* = 12.0, 5.5 Hz, 1H, H6Man), 4.42–4.33 (m, 1H, H2Man + H5Man), 3.54 (s, 3H, –OCH3), 2.26 (d, *J* = 4.7 Hz, 1H, –OH). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.2 (COBz), 165.6 (COBz), 165.5 (COBz), 133.4 (CHBz), 133.3 (CHBz), 133.1 (CHBz), 129.8 (CHBz), 129.8 (CHBz), 129.7 (CHBz), 129.2 (CHBz), 129.1 (CHBz), 128.4 (CHBz), 128.4 (CHBz), 128.3 (CHBz), 100.7 (C1Man), 72.6 (C3Man), 69.41 (C2Man or C5Man), 68.6 (C2Man or C5Man), 67.0 (C4Man), 63.6 (C6Man), 55.38 (C-OCH_3). ESI-MS calcd. for $C_{28}H_{26}O_9$: 506.2 $[M]^+$; found: 529.3 $[M + Na]^+$; ESI-HRMS m/z calcd. for $C_{28}H_{26}O_9Na$: 529.1469 $[M + Na]^+$; found: 529.1452 $[M + Na]^+$.

(2R,3R,4S,5S)-2-(Acetoxymethyl)-6-hydroxytetrahydro-2H-pyran-3,4,5-triyl triacetate (121)[330b]



To a solution of commercial peracetylated mannose (3.0 g, 7.69 mmol) in DMF (30 mL) under nitrogen atmosphere, hydrazine acetate (1.14 g, 12.4 mmol) was added in portions. After 24h of stirring at room temperature, the mixture was cooled at room temperature and the crude was purified by column chromatography on silica gel (EtOAc:Hex 1:1) affording **121** as a fluffy light yellow solid (1.9 g, 74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.38 (dd, *J* = 10.0, 3.4 Hz, 1H, H3), 5.32 – 5.16 (m, 2H, H2 and H4), 4.27 – 4.17 (m, 2H, H6 and H1), 4.14 – 4.03 (m, 2H, H6' and H5), 2.13 (s, 3H, CH_3), 2.07 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 1.97 (s, 3H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$): δ 171.0, 170.3, 170.2, 169.9 (CO), 92.0 (C1), 70.2 (C2), 68.9 (C3), 68.2 (C4), 66.1 (C5), 62.6 (C6), 20.6 (CH3).

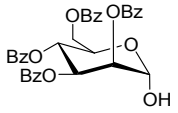
(2R,3R,4S,5S,6R)-2-(Acetoxymethyl)-6-(2,2,2-trichloro-1-iminoethoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (117)[330b]



Under nitrogen atmosphere, trichloroacetonitrile (2.7 g, 18.1 mmol) was dropped slowly to a solution **121** (2.50 g, 4.19 mmol) and DBU (0.78 g, 5.04 mmol) at 0°C. the reaction mixture was warmed slowly at room temperature and stirred for 3 h. Then, it was concentrated under vaccum and the crude product was purified by flash column chromatography on silica gel (EtOAc/Hex, 1:2) to afford per-acetylated mannose trichloroacetimidate **117** (1.31 g, 67%) as a fluffy yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.79 (s, 1H, NH), 6.26 (s, 1H, H1), 5.65 – 5.28 (m, 3H, H3, H2, H4), 4.72

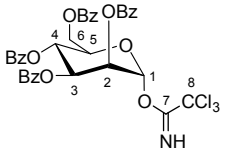
– 3.81 (m, 4H, H6 and H5), 2.37 – 1.79 (m, 12H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.8, 170.1, 170.0, 169.9 (CO), 160.0 (C7), 94.8 (C1), 90.8 (C8), 71.53 (C5), 69.1 (C3), 68.2 (C2), 65.7 (C4), 62.3 (C6), 21.1, 21.0, 20.9 (CH_3).

2,3,4,6-Tetra-O-benzoyl-α -D- mannopyranose (122)[330a]



To a solution of α-D-mannopyranose pentabenzoate **120** (5.00 g, 7.14 mmol) in anhydrous DMF (50 mL) under nitrogen atmosphere, hydrazine acetate (0.78 g, 8.57 mmol) was added in portions. After 24h of stirring at 60°C, the mixture was cooled at room temperature and the crude was purified by column chromatography on silica gel (EtOAc:Hex 1:2) affording **122** as a fluffy white solid (2.9 g, 70%). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.13 – 7.84 (m, 8H, HBz), 7.60 – 7.25 (m, 12H, HBz), 6.18 (t, *J* = 10.1 Hz, 1H, H2), 6.01 (dd, *J*= 10.1, 3.3 Hz, 1H, H3), 5.75 (dd, *J* = 3.3, 1.9 Hz, 1H, H4), 5.54 (d *J*= 1.9 Hz, 1H, H1), 4.77 (dd, *J*= 12.2, 2.7 Hz, 1H, H6), 4.68 (m, 1H, H5), 4.46 (dd, *J*= 12.2, 3.7 Hz, 1H, H6'). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 166.5, 165.7, 165.6 (CO), 133.5, 133.3, 133.2, 130.0, 129.4, 129.2, 129.2, 128.7, 128.6, 128.4 (Carom), 92.5 (C1), 71.0 (C5), 70.0 (C3), 69.0 (C2), 67.0 (C4), 62.9 (C6).

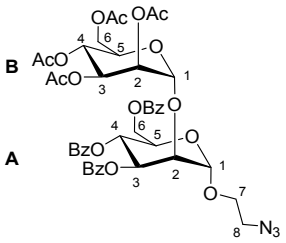
2,3,4,6-Tetra-O-benzoyl-α-D-mannopyranosyl trichloroacetimidate (118)[330a]



Under nitrogen atmosphere, trichloroacetonitrile (2.42 g, 16.8 mmol) was dropped slowly to a solution of **122** (1.4 g, 4.0 mmol) and DBU (0.78 g, 5.04 mmol) at 0°C. The reaction mixture was warmed slowly at room temperature and stirred for 3 h. Then, it was concentrated under vaccum and the crude product was purified by flash column chromatography on silica gel (EtOAc/Hex, 1:2) to afford **118** (2.71 g, 67 %) as

a fluffy colourless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.87 (s, 1H, NH), 8.12-7.83 (m, 8H, HBz), 7.65-7.25 (m, 12H, HBz), 6.58 (d, *J* = 1.8 Hz, 1H, H1), 6.18 (m, *J*= 10.1 Hz, 1H, H2), 6.01 (dd, *J*= 10.1, 3.3 Hz, 1H, H3), 5.75 (dd, *J*= 3.3, 1.9 Hz, 1H, H4), 4.77 (dd, 1H, *J*= 12.2, 2.7 Hz, 1H, H6), 4.68 (m, 1H, H5), 4.46 (dd, *J*= 12.2, 3.7 Hz, 1H, H6'). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 166.0, 165.5, 165.4, 165.1 (CO), 159.9 (C7), 133.6, 133.3, 129.0, 128.8, 128.7, 128.4 (Carom), 94.7 (C1), 90.6 (C8), 71.5 (C5), 69.8 (C3), 68.9 (C2), 66.1 (C4), 62.4 (C6).

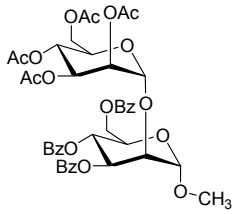
2-Azidoethyl O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-(1-2)-3,4,6-tri-O-benzoyl-α-D-mannopyranoside (123)



A mixture of the acceptor **115** (120 mg, 0.214 mmol) and the donor per-acetylated mannose trichloroacetimidate **117** (158 mg, 0.321 mmol) was co-evaporated from toluene three times. Powdered and activated 4 Å molecular sieves were added, and the mixture was kept under vacuum for few hours and then dissolved in CH_2Cl_2 (8 mL). The mixture was cooled to 0 °C for 15 min, followed by the addition of TMSOTf (8.5 µL, 0.040 mmol), and stirred for 30 min at 0 °C. The reaction was quenched by the addition of Et_3N, filtered over a pad of Celite and dried under vacuum. The crude was purified by flash column chromatography on silica gel (DCM:MeOH, 100:1) to obtain **123** as a white solid (112 mg, 59%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (d, *J* = 7.1 Hz, 2H, HBz), 7.98 (d, *J* = 7.1 Hz, 2H, HBz), 7.95 (d, *J* = 7.0 Hz, 2H, HBz), 7.68–6.99 (m, 9H, HBz), 5.94 (t, *J* = 9.9 Hz, 1H, H4ManA), 5.84 (dd, *J* = 9.9, 3.2 Hz, 1H, H4ManA), 5.49–5.43 (m, 2H, H2ManB + H3ManB), 5.26 (t, *J* = 9.5 Hz, 1H, H4ManB), 5.15 (d, *J* = 1.9 Hz, 1H, H1ManA), 4.98 (d, *J* = 1.5 Hz, 1H, H1ManB), 4.63 (dd, *J* = 12.2, 3.0 Hz, 1H, H6ManA), 4.52 (dd, J = 12.1, 5.4 Hz, 1H, H6ManA), 4.41 (ddd, J = 10.1, 5.4, 2.9 Hz, 1H, H5ManA), 4.34 (dd, *J* = 3.2, 1.9 Hz, 1H, H2ManA), 4.25 (dd, *J* = 11.9, 5.4 Hz, 1H,

H6ManB), 4.17 (ddd, *J* = 12.1, 7.4, 3.3 Hz, 1H, H5ManB), 4.11 (dd, *J* = 11.9, 2.5 Hz, 1H, H6ManB), 4.01 (dt, *J* = 10.3, 4.7 Hz, 1H, H7), 3.82–3.72 (m, 1H, H7), 3.54 (t, *J* = 5.0 Hz, 2H, H8), 2.10 (s, 3H, $COOCH_3$), 2.06 (s, 3H, $COOCH_3$), 2.04 (s, 3H, $COOCH_3$), 2.01 (s, 3H, $COOCH_3$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.51, 169.80, 169.48, 169.42 (COAc), 166.22, 165.50, 165.19 (COBz), 133.43, 133.32, 133.02, 129.92, 129.86, 129.70 (CHBz), 128.97 (CBz), 128.82 (CBz), 128.55, 128.47, 128.36 (CHBz), 99.46 (C1ManB), 98.65 (C1ManA), 76.50 (C2ManA), 70.62 (C3ManA), 69.30 (C2ManB), 69.2 (C5ManB), 69.1 (C5ManA), 68.8 (C3ManB), 67.2 (C7), 67.1 (C4ManA), 66.3 (C4ManB), 63.6 (C6ManA), 62.6 (C6ManB), 50.4 (C8), 20.70 , 20.67 (CH_3). ESI-MS calcd. $C_{43}H_{45}N_3O_{18}$: 891.3 $[M]^+$; found: 914.3 $[M + Na]^+$; ESI-HRMS m/z calcd. for $C_{43}H_{45}N_3O_{18}Na$: 914.2596; found; 914.2590.

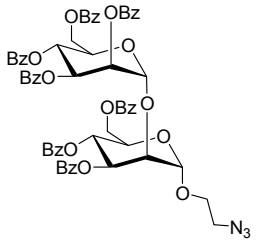
Methyl O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-(1-2)-3,4,6-tri-O-benzoyl-α-D-mannopyranoside (124)



A mixture of the acceptor **116** (150 mg, 0.296 mmol) and the donor per-acetylated mannose trichloroacetimidate **117** (219 mg, 0.445 mmol) was co-evaporated from toluene three times. Powdered and activated 4 Å molecular sieves were added, and the mixture was kept under vacuum for few hours and then dissolved in DCM (10 mL). The mixture was cooled to −0 °C for 15 min, followed by the addition of TMSOTf (13 µL, 0.059 mmol), and stirred for 30 min at 0 °C. The reaction was quenched by the addition of Et_3N, filtered over a pad of Celite and dried under vacuum. The crude was purified by flash column chromatography on silica gel (DCM:MeOH, 100:1) to obtain **124** as an white solid (114 mg, 46%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.15–8.07 (m, 4H, HBz), 8.04 (d, *J* = 7.0 Hz, 2H, HBz), 8.02 (d, *J* = 7.0 Hz, 2H, HBz), 8.00–7.95 (m, 4H, HBz), 7.90 (d, *J* = 6.9 Hz, 2H, HBz), 7.65–7.30 (m, 21H, HBz), 6.12–6.05 (m, 2H, H3ManB + H4ManB), 6.02 (t, *J* = 9.9 Hz, 1H, H4ManA), 5.94 (dd, *J* = 2.3, 1.8 Hz, 1H,

H2ManB), 5.91 (dd, *J* = 9.9, 3.2 Hz, 1H, H3ManA), 5.29 (d, *J* = 1.8 Hz, 1H, H1ManB), 5.09 (d, *J* = 1.8 Hz, 1H, H1ManA), 4.73-4.64 (m, 3H, H5ManB + H6ManB + H6ManA), 4.60 (dd, *J* = 12.2, 5.5 Hz, 1H, H6ManA), 4.50 (dd, *J* = 12.0, 5.3 Hz, 1H, H6ManB), 4.41 (dd, *J* = 2.6, 1.8 Hz, H2ManA), 4.41-4.35(m, 1H, H5ManA), 3.42 (s, 3H, OMe). ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.52, 169.84, 169.48, 169.40 (COAc), 166.26, 165.55, 165.20 (COBz), 133.39, 133.27, 132.98, 129.91, 129.84, 129.70 (CHBz), 128.87, 128.54, 128.45 (CBz), 99.47 (C1ManB), 98.65 (C1ManA), 72.50 (C2ManA), 70.84 (C3ManA), 69.29 (C2ManB), 69.22 (C5ManB), 69.1 (C5ManA), 68.82 (C3ManB), 68.68 (C7), 67.30 (C4ManA), 66.27 (C4ManB), 63.69 (C6ManA), 62.65 (C6ManB), 50.31 (C8), 29.71, 20.72, 20.69 (CH3). ESI-MS m/z calcd. for $C_{42}H_{44}O_{18}$: 836.2 $[M]^+$; found: 859.3 $[M+Na]^+$; ESI-HRMS m/z calcd. for $C_{42}H_{44}O_{18}Na$: 859.2425; found: 859.2413.

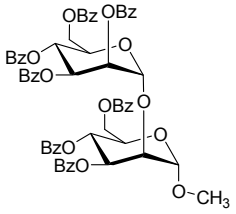
2-Azidoethyl O-(2,3,4,6-tetra-O-benzoyl-α-Dmannopyranosyl)-(1-2)-3,4,6-tri-O-benzoyl-α-D-mannopyranoside (125)



A mixture of the acceptor **115** (250 mg, 0.440 mmol) and the donor **118** (494 mg, 0.670 mmol) was co-evaporated from toluene three times. Powdered and activated 4 Å molecular sieves were added, and the mixture was kept under vacuum for few hours and then dissolved in DCM (12 mL). The mixture was cooled to 0 °C for 15 min, followed by the addition of TMSOTf (20 µL, 0.088 mmol), and stirred for 30 min at 0 °C. The reaction was quenched by the addition of Et3N, filtered over a pad of Celite and dried under vacuum. The crude was purified by flash column chromatography on silica gel (DCM:MeOH, 100:1) to obtain **125** as a white solid (396 mg, 79%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (d, *J* = 7.3 Hz, 2H, HBz), 8.09 (d, *J* = 7.3 Hz, 2H, HBz), 8.06 (d, *J* = 7.3 Hz, 2H, HBz): δ 8.03–7.95 (m, 6H, HBz), 7.91 (d, *J* = 7.6 Hz, 2H, HBz),

7.65–7.29 (m, 21H, HBz), 6.15–6.02 (m, 3H, H4ManA + H3ManB + H4ManB), 5.97–5.90 (m, 2H, H2ManB + H3ManA), 5.31 (d, *J* = 1.8 Hz, 1H, H1ManB), 5.24 (d, J = 1.8 Hz, 1H, H1ManA), 4.74–4.60 (m, 3H, H5ManB + H6ManB + H6ManA), 4.53 (dd, J = 12.0, 4.9 Hz, 1H, H6ManA), 4.49 (m, 2H, H2ManA + H6ManB), 3.91 (ddd, J = 10.5, 6.7, 3.8 Hz 1H, H7), 3.59 (ddd, J = 10.2, 6.0, 3.5 Hz 1H, H7), 3.52–3.37 (m, 2H, H8). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.3, 166.1, 165.6, 165.3, 165.1, 164.9 (COBz), 133.5, 133.4, 133.3, 133.3, 133.1, 133.1, 130.0, 130.0, 129.9, 129.8, 129.8, 129.7 (CHBz), 129.2, 129.0, 128.8, 128.6 (CBz), 128.5, 128.5, 128.4, 128.4, 128.3 (CHBz), 99.7 (C1ManA), 98.7 (C1ManB), 76.9 (C2ManA), 70.6, 70.1, 69.8, 69.7, 69.2, 67.5, 67.0, 66.9, 63.7 (C6ManA), 63.1 (C6ManB), 50.3 (C8). ESI-MS m/z calcd. for $C_{63}H_{53}N3O_{18}$: 1139.3 $[M]^+$; found: 1162.5 $[M + Na]^+$; ESI-HRMS m/z calcd. for $C_{63}H_{53}N_3O_{18}Na$: 1162.3222; found: 1162.3212.

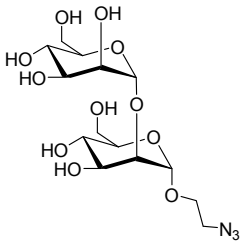
Methyl O-(2,3,4,6-tetra-O-benzoyl-α-Dmannopyranosyl)-(1-2)-3,4,6-tri-O-benzoyl-α-D-mannopyranoside (126)



A mixture of the acceptor **116** (60 mg, 0.119 mmol) and the donor **118** (105 mg, 0.142 mmol) was co-evaporated from toluene three times. Powdered and activated 4 Å molecular sieves were added, and the mixture was kept under vacuum for few hours and then dissolved in DCM (5 mL). The mixture was cooled to 0 ºC for 15 min, followed by the addition of TMSOTf (4,5 µL, mmol, 0.024 mmol), and stirred for 30 min at 0 ºC. The reaction was quenched by the addition of Et_3N, filtered over a pad of celite and dried under vacuum. The crude was purified by flash column chromatography on silica gel (DCM:MeOH, 100:1) to obtain **126** as an white solid (97 mg, 75 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15-8.07 (m, 4H, HBz), 8.04 (d, J = 7.0 Hz, 2H, HBz), 8.02 (d, J = 7.0 Hz, 2H, HBz): δ 8.00-7.95 (m, 4H, HBz), 7.90 (d, J = 6.9 Hz,

2H, HBz), 7.65-7.30 (m, 21H, HBz), 6.12-6.05 (m, 2H, H3ManB + H4ManB), 6.02 (t, J = 9.9 Hz, 1H, H4ManA), 5.94 (dd, J = 2.3, 1.8 Hz, 1H, H2ManB), 5.91 (dd, J = 9.9, 3.2 Hz, 1H, H3ManA), 5.29 (d, J = 1.8 Hz, 1H, H1ManB), 5.09 (d, J = 1.8 Hz, 1H, H1ManA), 4.73-4.64 (m, 3H, H5ManB + H6ManB + H6ManA), 4.60 (dd, J = 12.2, 5.5 Hz, 1H, H6ManA), 4.50 (dd, J = 12.0, 5.3 Hz, 1H, H6ManB), 4.41 (dd, J = 2.6, 1.8 Hz, H2ManA), 4.41-4.35(m, 1H, H5ManA), 3.42 (s, 3H, OMe). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.4, 166.1, 165.6, 165.3, 165.0, 165.0 (COBz), 133.5, 133.4, 133.3, 133., 133.1, 133.0, 130.0, 130.0, 129.9, 129.8, 129.8, 129.7 (CHBz), 129.2, 129.1, 128.9, 128.8 (CBz), 128.5, 128.5, 128.4, 128.4, 128.3 (CHBz), 99.6 (C1ManA), 99.6 (C1ManB), 76.9 (C2ManA), 70.8, 70.1, 69.8, 69.7, 68.8, 67.6, 67.0 (C2,3,4,5ManA-B), 63.8 (C6ManA), 63.1 (C6ManB), 55.2 (CH3). ESI-MS m/z calcd. for $C_{62}H_{52}O_{18}$: 1084.3 $[M]^+$; found: 1107.3 $[M+Na]^+$; ESI-HRMS m/z calcd. for $C_{62}H_{52}O_{18}Na$: 1107.3051; found: 1107.3039.

2-Azidoethyl α-D-mannopyranosyl-(1-2)-α-D mannopyranoside (23)

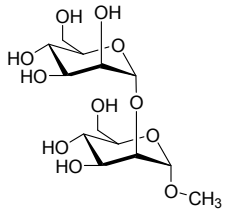


*From acetylated donor **123***. To a solution of **123** (100 mg, 0,112 mmol) in dry methanol, under nitrogen at room temperature, was added a 1 M solution of sodium methoxide in MeOH (2 equiv.) and the reaction was stirred for 1 hour. Then, the reaction mixture was neutralized with Amberlite IRA 120-H^+ resin. The resin was filtered off and the filtrate was concentrated under reduced pressure. The crude was diluted in H_2O (10 mL), washed with DCM (10 mL) and the aqueous phase was lyophilized to obtain **23** as a white solid (45 mg, quant.).

*From benzoylated donor **125***. To a solution of **125** (390 mg, 0.342 mmol) in dry methanol, under nitrogen at room temperature, was added a 1 M solution of sodium methoxide in MeOH (2 equiv.) and the reaction was stirred for 1 hour. Then, the reaction mixture was neutralized with Amberlite IRA 120-H+ resin. The resin was filtered off and the filtrate was concentrated under reduced pressure. The crude was diluted in H_2O (10 mL), washed with CH_2Cl_2 (10 mL) and the aqueous phase was lyophilized to obtain **23** as a white solid (140 mg, quant.).

^{1}H NMR (400 MHz, D_2O): δ 5.08 (d, *J* = 1.8 Hz, 1H, H1ManB), 4.95 (d, *J* = 1.9 Hz, 1H, H1ManA), 3.99 (dd, *J* = 3.4, 1.8 Hz, 1H, H2ManB), 3.92 (dd, *J* = 3.3, 1.8 Hz, 1H, H2ManA), 3.89–3.74 (m, 4H, H3ManA + H3ManB + H6ManA + H4ManA + H7), 3.73–3.50 (m, 7H, H4ManB + H5ManA + H5ManB + H6ManA + 2H6ManB + H7), 3.49–3.35 (m, 2H, H8); ^{13}C NMR (101 MHz, D_2O): δ 102.3 (C1ManB), 98.2 (C1ManA), 78.6 (C2ManA), 73.3 (C5ManB), 72.9 (C5ManA), 70.3 (C3ManB), 70.0 (C3ManA), 69.9 (C2ManB), 66.9 (C4ManB), 66.9 (C4ManA), 66.4 (C7), 61.1(C6ManA), 60.9 (C6ManB), 50.2 (C8); ESI-MS m/z calcd. for $C_{14}H_{25}N_3O_{11}$: 411.1 $[M]^+$; found: 434.2 $[M + Na]^+$; ESI-HRMS m/z calcd. for $C_{14}H_{25}O_{11}N_3Na$: 434.1381; found: 434.1372.

Methyl α-D-mannopyranosyl-(1-2)-α-D mannopyranoside (114)



*From acetylated donor **124***. To a solution of **124** (100 mg, 0.119 mmol) in dry methanol, under nitrogen at room temperature, was added a 1 M solution of sodium methoxide in MeOH (2 equiv.) and the reaction was stirred for 1 hour. Then, the reaction mixture was neutralized with Amberlite IRA 120-H^+ resin. The resin was filtered off and the filtrate was concentrated under reduced pressure. The crude was

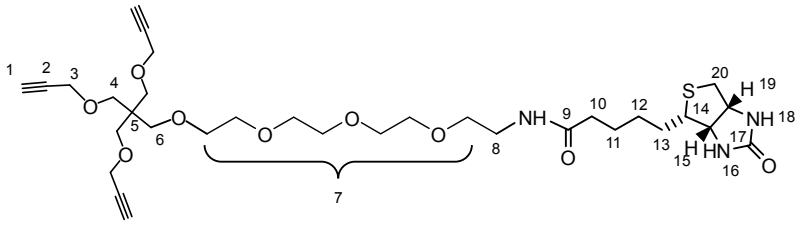
diluted in H_2O (10 mL), washed with DCM (10 mL) and the aqueous phase was lyophilized to obtain **114** as a white solid (42 mg, quant.).

*From benzoylated donor **126***. To a solution of **126** (90 mg, 0.081 mmol) in dry methanol, under nitrogen at room temperature, was added a 1 M solution of sodium methoxide in MeOH (2 equiv.) and the reaction was stirred for 1 hour. Then, the reaction mixture was neutralized with Amberlite IRA 120-H^+ resin. The resin was filtered off and the filtrate was concentrated under reduced pressure. The crude was diluted in H_2O (10 mL), washed with DCM (10 mL) and the aqueous phase was lyophilized to obtain **114** as a white solid (29 mg, quant.).

^{1}H NMR (400 MHz, D_2O): δ 4.95 (d, *J* = 1.8 Hz, 1H, H1ManB), 4.92 (d, *J* = 2.0 Hz, 1H, H1ManB), 3.99 (dd, *J* = 3.3, 1.8 Hz, 1H, H2ManB), 3.88 (dd, *J* = 3.3, 1.8 Hz, 1H, H2ManA), 3,85–375 (m, 4H, H3ManA + H3ManB + H6ManA + H4ManA), 3.74–3.50 (m, 6H, H4ManB + H5ManA + H5ManB + H6ManA + 2H6ManB), 3,33 (s, 3H, OCH_3); ^{13}C NMR (101 MHz, D_2O): δ 102.3 (C1ManB), 99.3 (C1ManA), 78.5 (C2ManA), 73.2 (C5ManB), 72.5 (C5ManA), 70.3 (C3ManB), 70.3 (C3ManA), 69.9 (C2ManB), 66.9 (C4ManB), 66.8 (C4ManA), 61.1 (C6ManA), 60.9 (C6ManB), 54.8 (CH3); ESI-MS m/z calcd. for $C_{13}H_{24}O_{11}$: 356.1 $[M]^+$; found: 379.2 $[M + Na]^+$; ESI-HRMS m/z calcd. for $C_{13}H_{24}O_{11}Na$: 379.1211; found; 379.1204.

7.15. Preparation of Glycodendrons with biotin at the focal position

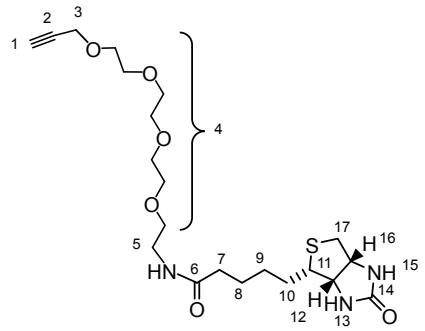
Compound 130



To a solution of D-Biotin (0.103 g, 0.423 mmol) in DMF (2 mL), HATU (0.193 g, 0.51 mmol) and DIPEA (0.131 g, 1.026 mmol) were added. After 10 minutes, the scaffold

77 (0.180 g, 0.423 mmol) in DMF (1 mL) was added, and the reaction mixture was stirred at room temperature for 22h. After checking the complete diseppearence of the starting product, the crude of reaction was dried under vacuum and the purification was afforded by column chromatography on silica gel (DCM:MeOH 99:1) to give **130** like a yellow oil. (0.169 g, 87 %) ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.87 (s, 1H, H16), 6.45 (s, 1H, H18), 4.51 (dd, *J* = 7.9, 4.6 Hz, 1H, H19), 4.32 (dd, *J* = 7.8, 4.6 Hz, 1H, 1H, H15), 4.11 (d, *J* = 2.4 Hz, 6H, H3), 3.75 – 3.36 (m, 24H, H4, H6, H7, H8), 3.23 – 3.08 (m, 1H, H14), 2.90 (dd, *J* = 12.9, 4.8 Hz, 1H, H18), 2.75 (d, *J* = 12.8 Hz, 1H, H18'), 2.43 (t, *J* = 2.4 Hz, 3H, H1), 2.23 (t, *J* = 7.4 Hz, 2H, H10), 1.83 – 1.56 (m, 4H, H11, H13), 1.22 (d, *J* = 11.8 Hz, 2H, H12). ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.2 (C9), 164.1 (C17), 80.1 (C2), 75.0 (C1), 70.2, 70.1, 70.0, 69.9 (C7), 69.7 (C6), 69.0 (C4), 61.7 (C15), 60.2 (C19), 59.1 (C3), 55.5 (C14), 45.0 (C5), 43.4 (C20), 39.1 (C8), 35.8 (C10), 28.2 (C12), 27.9 (C13), 24.3 (C11). ESI-MS m/z calcd. for $C_{32}H_{49}N_3O_9S$: 651.8 $[M]^+$; found: 674.4 $[M + Na]^+$.

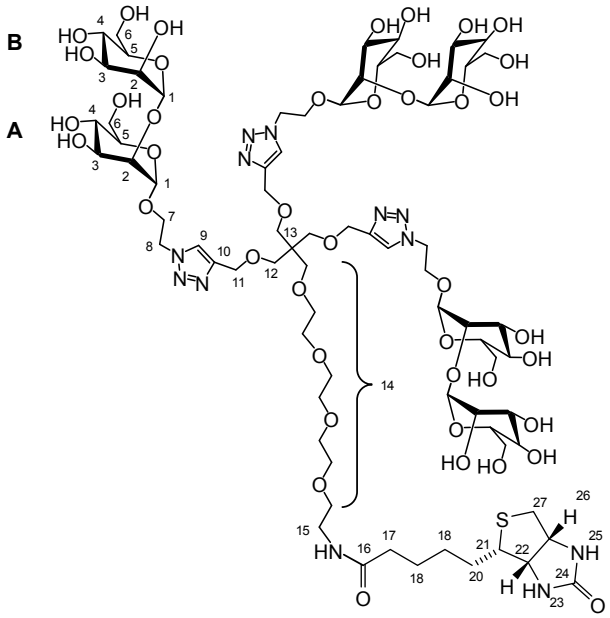
Compound 131



To a solution of D-Biotin (0.458 g, 1.88 mmol) in DMF (8 mL), HATU (0.858 g, 2.26 mmol) and DIPEA (0.485 g, 3.76 mmol) were added. After 10 minutes the scaffold **78** (0.434 g, 1.88 mmol) in DMF (2 mL) was added and the reaction mixture was stirred at room temperature for 22h. After checking of the complete disappearance of the starting product, the crude of reaction was dried under vacuum and the purification was afforded by column chromatography on silica gel (DCM:MeOH 99:1) to give **131** as a yellow oil. (0.757 g, 88 %) ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.97 (m, 1H, H15), 6.83

(s, 1H, H13), 6.04 (s, 1H, NH), 4.48 (dd, *J* = 7.9, 4.7 Hz, 1H, H16), 4.28 (dd, *J* = 8.0, 4.6 Hz, 1H, H12), 4.17 (d, *J* = 2.4 Hz, 2H, H3), 3.79 – 3.48 (m, 14H, CPEG), 3.40 (m, 2H,H5), 3.11 (td, *J* = 7.2, 4.3 Hz, 1H, H11), 2.87 (dd, *J* = 12.8, 4.8 Hz, 1H, H17), 2.72 (d, *J* = 12.7 Hz, 1H, H17'), 2.46 (t, *J* = 2.4 Hz, 1H, H1), 2.20 (t, *J* = 7.5 Hz, 2H, H7), 1.69 (m, 4H, H8 and H10), 1.41 (m, 2H, H9). ^{13}C NMR (101 MHz, $CDCl_3$): δ 173.4 (C6), 164.3 (C14), 79.6 (C2), 74.7 (C1), 70.5, 70.4, 70.3, 70.0, 69.9, 69.0 (C4), 61.7 (C12), 60.2 (C16), 58.4 (C3), 55.7 (C11), 40.5 (C17), 39.1 (C5), 35.9 (C7), 28.3 (C9), 28.1 (C10), 25.6 (C8). ESI-MS m/z calcd. for $C_{21}H_{35}N_3O_6S$: 457.5 $[M]^+$; found: 480.4 $[M + H]^+$.

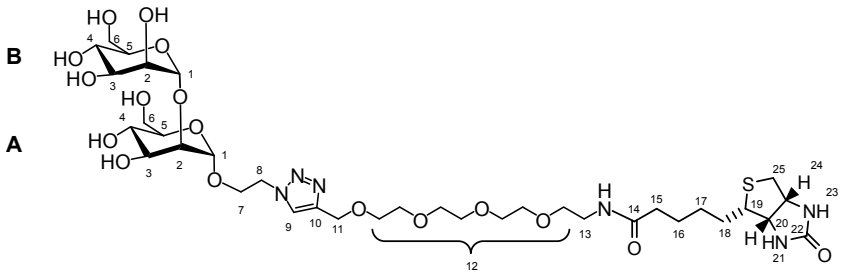
Dimannose Trivalent dendron 127



To a solution of disaccharide **23** (0.026 g, 0.066 mmol) and scaffold **130** (0.010g, 0.0098 mmol) in a mixture of DMSO/H_2O (0.5 mL/0.25 mL) $CuSO_4{\cdot}5H_2O$ (0.003 g, 0.009 mmol), sodium ascorbate (0.005 g, 0.027 mmol) and TBTA (0.009 g, 0.018 mmol), all three dissolved in DMSO (1.0 mL), were added. The reaction was treated under microwave irradiation at 60°C for 30 min. Then one portion more of $CuSO_4{\cdot}5H_2O$ (0.3 eq) was added and the reaction was irradiated again with MW for

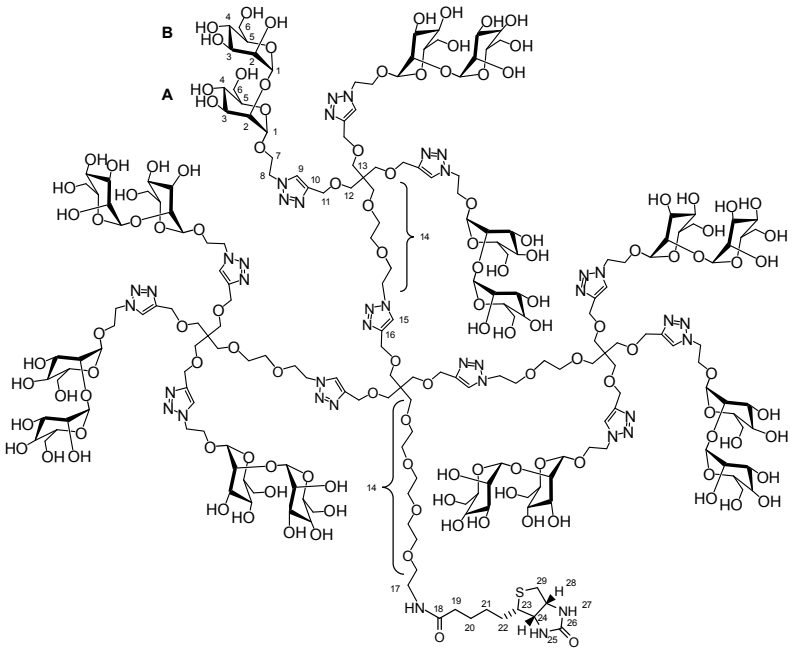
30min at the same temperature. Then, the solvent was evaporated, MeOH (10 mL) was added and the catalyst was removed by adding the metal scavenger resin QuadraSil® Mercaptopropyl (a spatula tip). The mixture was stirred for 30 min, filtered with MeOH, and the solvent was removed under vacuum and the resulting crude was purified by size exclusion chromatography, Sephadex LH20 (100% MeOH). The compound shown the presence of TBTA, by NMR. For this reason, the solid was dissolved in water millipore and centrifugated for 30 min at 3500 rpm. The supernatant was removed and Liophilized yo afford the desired glycodendron **127** as a white foam. (15.5 mg, 85 %.) ^{1}H NMR (400 MHz, MeOH-d_4): δ 8.03 (s, 3H, H9), 5.14 (d, *J* = 1.7 Hz, 3H, H1ManB), 4.97 (d, *J* = 1.7 Hz, 3H, H1ManA), 4.74 – 4.47 (m, 13H, H8, H11 and H26), 4.34 (m, 1H, H22), 4.12 (ddd, *J* = 11.0, 6.9, 3.8 Hz, 3H, H7), 4.03 – 3.85 (m, 6H, H2ManB, H2ManA, H7'), 3.85 – 3.42 (m, 60H, H3ManA, H3ManB, H4ManA, H4ManB, H5ManA, H5ManB, H6ManA, H6ManB, H12, H14, H15), 3.42 – 3.18 (m, 1H, H21), 2.95 (dd, *J* = 12.8, 4.9 Hz, 1H, H27), 2.73 (d, *J* = 12.7 Hz, 1H, H27'), 2.25 (t, *J* = 7.3 Hz, 2H, H17), 1.84 – 1.54 (m, 2H, H20), 1.46 (m, 2H, H19), 1.31 (s, 2H, H18). ^{13}C NMR (101 MHz, MeOH – d_4): δ 174.7 (C16), 164.7 (C24), 144.8 (C10), 124.4 (C9), 102.8 (C1ManB), 98.3 (C1ManA), 79.0 (C2ManA), 73.7 (C14), 73.5 (C5ManB), 71.0 (C5ManA), 70.5 (C3ManA), 70.4 (C14), 70.1 (C2ManB), 69.8 (C4ManB), 69.1 (C12), 68.7, 67.3 (C4ManA), 65.3 (C7), 64.0 (C11), 61.8 (C21), 61.5 (C6ManA), 60.6 (C6ManB), 60.2 (C26), 55.6 (C22), 49.7 (C8), 45.2 (C13), 39.0 (C15), 38.9 (C27), 35.3 (C17), 28.3 (C19), 28.1 (C20), 25.4 (C18). ESI-MS m/z calcd. for $C_{74}H_{124}N_{12}O_{42}S$: 868.4 $[M]^{+}$; found 965.4 $[M + 2Na]^{+}$, 1907.6 $[M + Na]^{2+}$.

Dimannose monovalent compound 128



To a solution of disaccharide **23** (0.03 g, 0.073 mmol) and **131** (0.015 g, 0.033 mmol) in a mixture of DMSO/H_2O (0.2 mL/0.25 mL), $CuSO_4 \cdot 5H_2O$ (0.0082 g, 0.033 mmol), sodium ascorbate (0.016 g, 0.08 mmol) and TBTA (0.035 g, 0.066 mmol), all three dissolved in DMSO (0.3 mL) were added. The reaction was treated under microwave irradiation at 60°C for 30 min. Then, the solvent was evaporated, MeOH (10 mL) was added and the catalyst was removed by adding the metal scavenger resin QuadraSil® Mercaptopropyl (a spatula tip). The mixture was stirred for 30 min, filtered with MeOH, and the solvent was removed under vacuum. The resulting crude was purified by size exclusion chromatography, Sephadex LH20 (100% MeOH). The compound by NMR showed the presence of TBTA. For this reason, the solid was dissolved in water millipore and centrifugated for 30 min at 3500 rpm. The supernatant was removed and lyophilized to afford the desired compound **128** as a white foam. (27 mg, quant.) ^{1}H NMR (400 MHz, D_2O): δ 8.05 (s, 1H, H9), 4.98 (s, 1H, CH, H1ManB), 4.90 (s, 1H, CH, H1ManA), 4.66 – 4.50 (m, 5H, CH_2, H8, H11 and H24), 4.42 – 4.31 (m, 1H, H20), 4.00 (m, 2H, H2ManB, H2ManA), 3.92 – 3.42 (m, 28H, H3ManA, H3ManB, H4ManA, H4ManB, H6ManA, H6ManB, H7, H12 and H13), 3,25 (m, 1H, H19), 2.95 (m, 3H, H5ManA, H5ManB, H25), 2.78 – 2.59 (m, 2H, H25'), 2.18 (m, 4H, 2H, H15-18), 1.55 (m, 2H, H17), 1.33 (s, 2H, H16). ^{13}C NMR (101 MHz, D_2O): δ 176.8 (C13), 165.3 (C22), 143.9 (Ctriazol, C10), 125.6 (Ctriazol C9), 102.2 (C1ManB), 98.0 (C1ManA), 78.5 (C2ManA), 73.2 (C5ManB), 72.8 (C5ManA), 70.2 (C3ManA), 70.0 (C2ManB), 69.9 (C4ManB), 69.6, 69.6, 69.5, 69.4, 68.9, 68.8 (C12), 66.8 (C4ManA), 66.5, 65.6 (C7), 63.1 (C11), 62.0 (C19), 61.1 (C6ManA), 60.6 (C6ManB), 60.2 (C24), 55.3 (C20), 50.0 (C8), 39.6 (C14), 35.4 (C15), 27.8 (C17), 27.6 (C18), 25.1 (C16). ESI-MS m/z calcd. for $C_{35}H_{60}N_6O_{17}S$: 868.4 $[M]^+$; found: 691.3 $[M + Na]^+$.

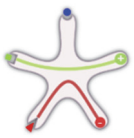
Dimannose nonavalent dendron 129



To a solution of diMannose trivalent dendron short chain with azide at focal point **107** (0.05 g, 0.0313 mmol) and scaffold **130** (0.005 g, 0.0075 mmol) in a mixture of DMSO/H_2O (0.5 mL/1.0 mL), $CuSO_4{\cdot}5H_2O$ (0.002 g, 0.0045 mmol), sodium ascorbate (0.002 g, 0.009 mmol) and TBTA (0.005 g, 0.0054 mmol), all three dissolved in DMSO (0.5 mL) were added. The reaction was treated under microwave irradiation at 60°C for 30 min; Then, one portion more of $CuSO_4{\cdot}5H_2O$ (0.3 eq) was added and the reaction was irradiated again under MW for 30 min at the same temperature. Then, seeing by TLC presence of the starting product, one portion more of $CuSO_4{\cdot}5H_2O$ (0.3 eq) was added and the reaction was irradiated again under MW for other 30min at the same temperature (a total of 1.2 eq $CuSO_4{\cdot}5H_2O$ have been added in three times) with the reaction finished, the solvent was evaporated, MeOH (10 mL) was added and the catalyst was removed by adding the metal scavenger resin QuadraSil® Mercaptopropyl (a spatula tip). The mixture was stirred for 30 min, filtered with MeOH and the solvent was removed under vacuum. The resulting crude was purified by size exclusion chromatography, Sephadex LH20 (100 % MeOH). The compound

showed presence of TBTA by NMR. For this reason, the solid was dissolved in water millipore and centrifugated for 30 min at 3500 rpm. The supernatant was removed and lyophilized. By NMR, it was posible to see the presence of the starting trivalent reactant. The solution was put in an Amicon Ultra centrifugal filter (Ultra-15, MWCO 30 kDa) and after 6 cycles of washing with distilled water in a centrigufe at 4000rpm, the aqueous phase collected was lyophilized, affording the diMannose nonavalent dendron **129** as a white foam (0.0235 g, 59 %). ^{1}H NMR (400 MHz, D_2O): δ 7.95 (s, 9H, H9), 7.89 (s, 3H, H15), 5.01 – 4.94 (m, 9H, H1ManB), 4.93 – 4.84 (m, 9H, H1ManA), 4.65 – 4.33 (m, 46H, H7, H8, H11, H28), 4.29 (m, 9H, H7'), 4.08 – 3.90 (m, 18H, H2ManB, H2ManA), 3.91 – 3.19 (m, 151H, H3ManA, H3ManB, H4ManA, H4ManB, H6ManA, H6ManB, H12, H14, H15, H23), 2.96 (m, 18H, H5ManA, H5Manb), 2.84 (m, 1H, H29), 2.73 (m, 1H, H29'), 2.70 – 2.59 (m, 2H, H19), 2.26 – 2.08 (m, 2H, H22), 1.67 – 1.35 (m, 2H, H21), 1.24 (d, *J* = 6.9 Hz, 2H, H20). ^{13}C NMR (101 MHz, D_2O): δ 174.7 (C18), 165.8 (C26), 144.3 (C10), 144.2 (Ctriazol, C16), 125.3 (C9), 125.1 (C15), 102.3 (C1ManB), 98.0 (C1ManA), 78.6 (C2ManA), 73.2 (C5ManB), 72.8 (C5ManA), 70.4 (C3ManB), 70.3 (C3ManA), 70.1 (C2ManB), 69.9 (C4ManB), 69.7, 69.4 (C14), 69.0, (C12), 68.7, 68.3 (C14), 66.9 (C4ManA), 66.6 (C14), 65.6 (C7), 63.6 (C11), 62.0 (C23), 61.1 (C6ManA), 60.6 (C6ManB), 60.2 (C28), 55.3 (C24), 50.0 (C8), 44.8 (C13), 39.7 (C29), 38.9 (C19), 28.0 (C21), 27.7 (C22), 25.2 (C20). ESI-MS m/z calcd. for $C_{212}H_{348}N_{38}O_{124}S$: 5442.4 $[M]^{+}$; found: 1383.7 $[M + 4Na]^{4+}$, 1837.4 $[M + 3Na]^{3+}$.

CHAPTER 8
References



8. References

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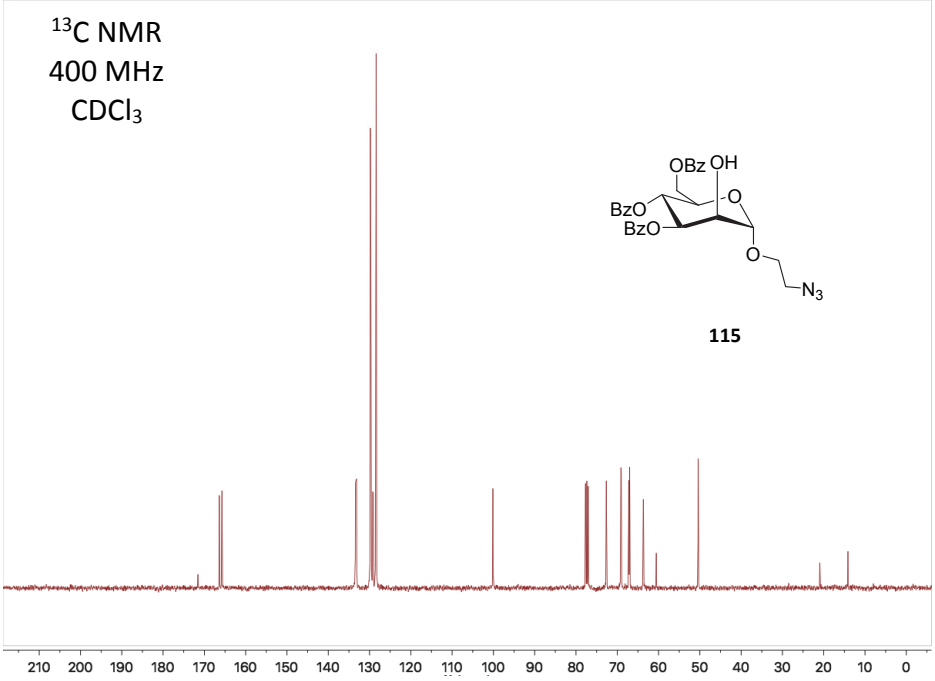
Appendix

Esta parte de la tesis se publicará con un retraso de 12 meses desde su aprobación y defensa.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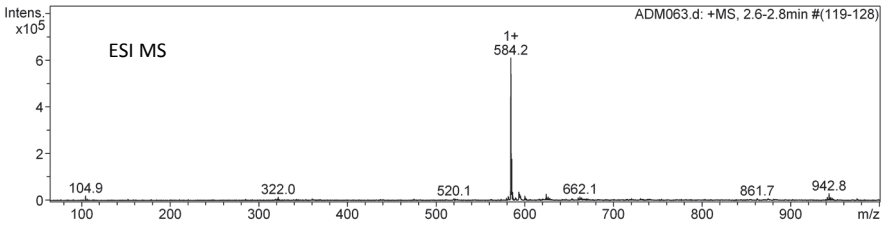
BzO
BzO
O
O
N_3
115
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0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662.1
861.7
942.8
m/z

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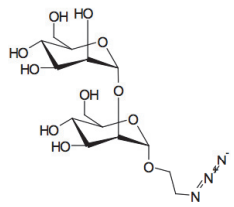
D_2O



23

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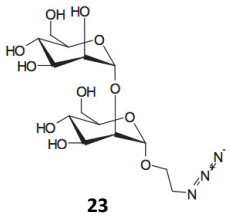
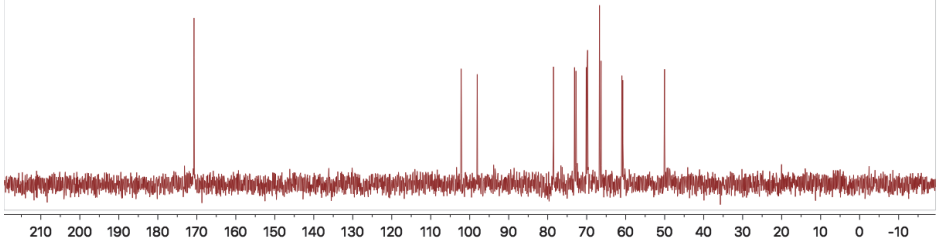
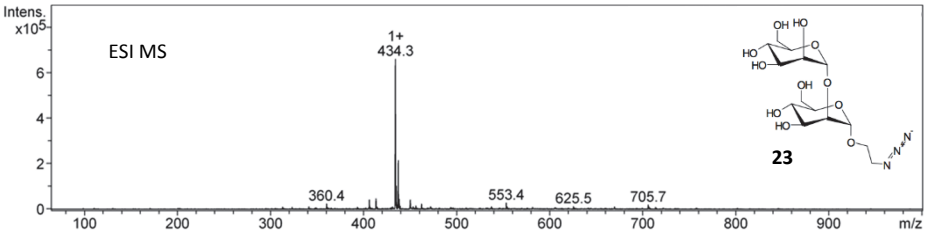
400 MHz
D_2O

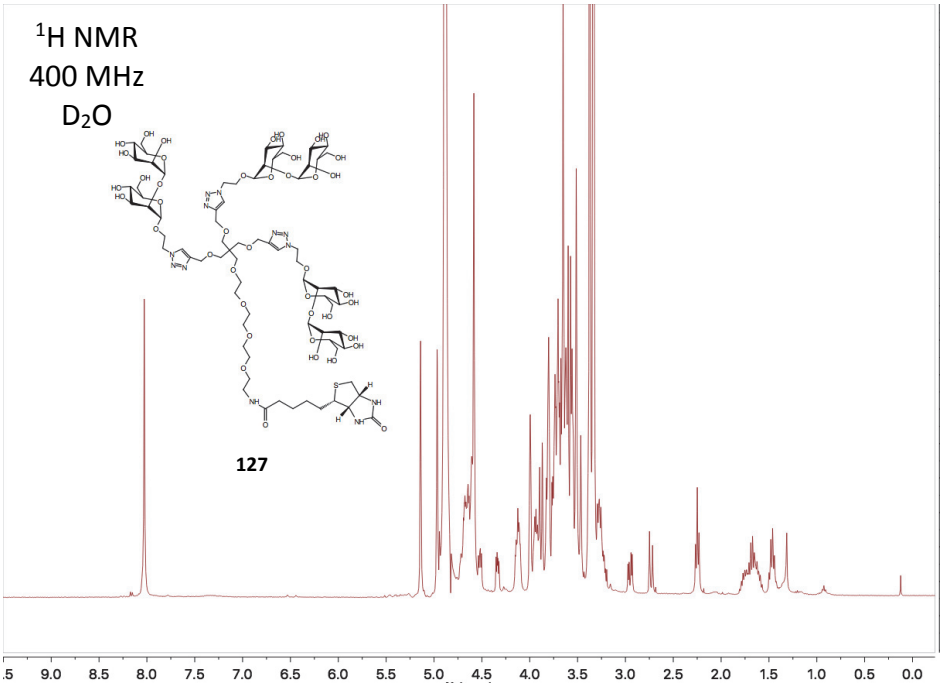


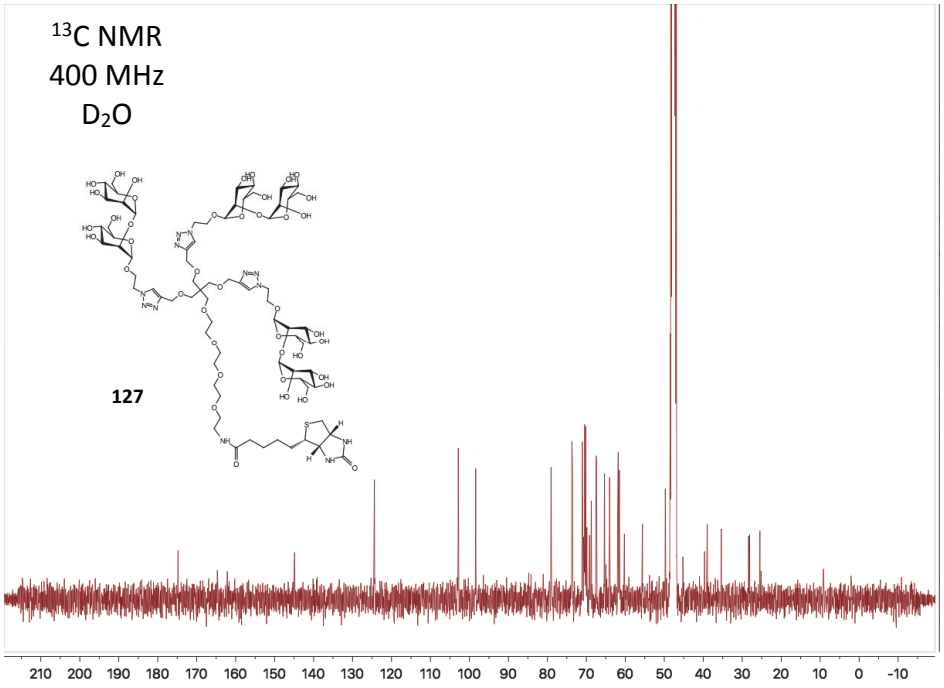
23





- NMR spectra of glycodendron with biotin

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127
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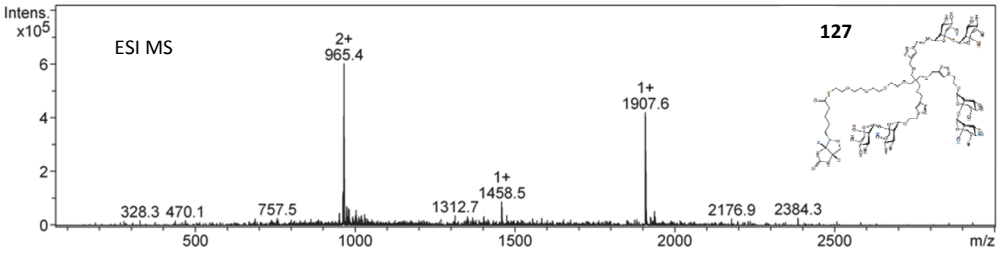
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x10^5
ESI MS
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1+
1907.6
1+
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2384.3
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500
1000
1500
2000
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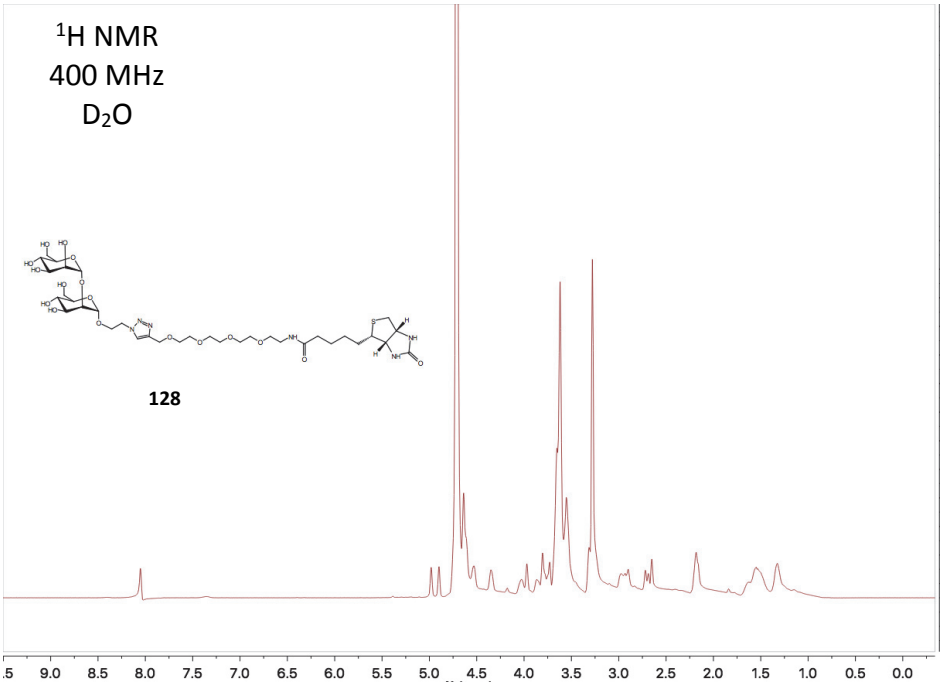
m/z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400 MHz
D_2O
128
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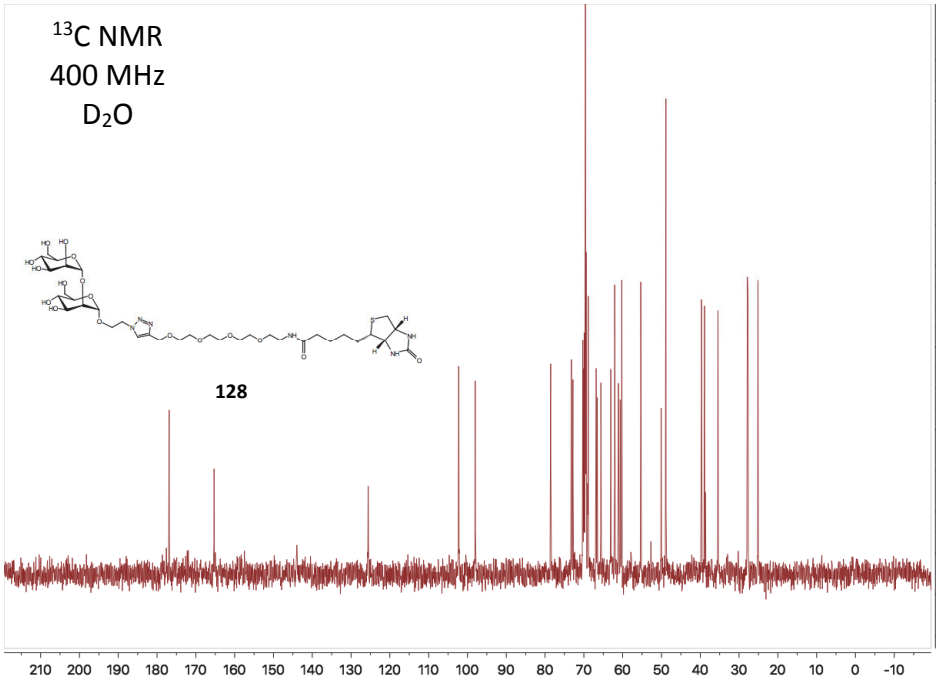
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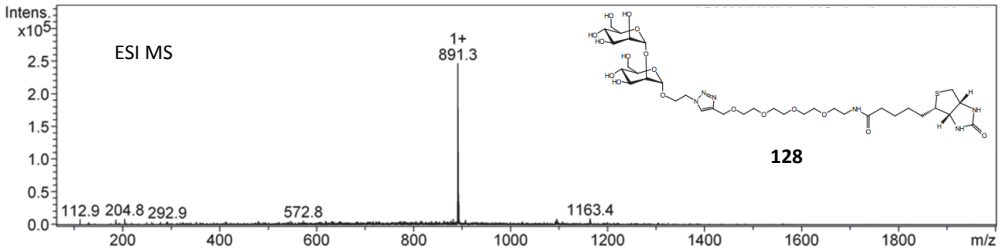
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572.8
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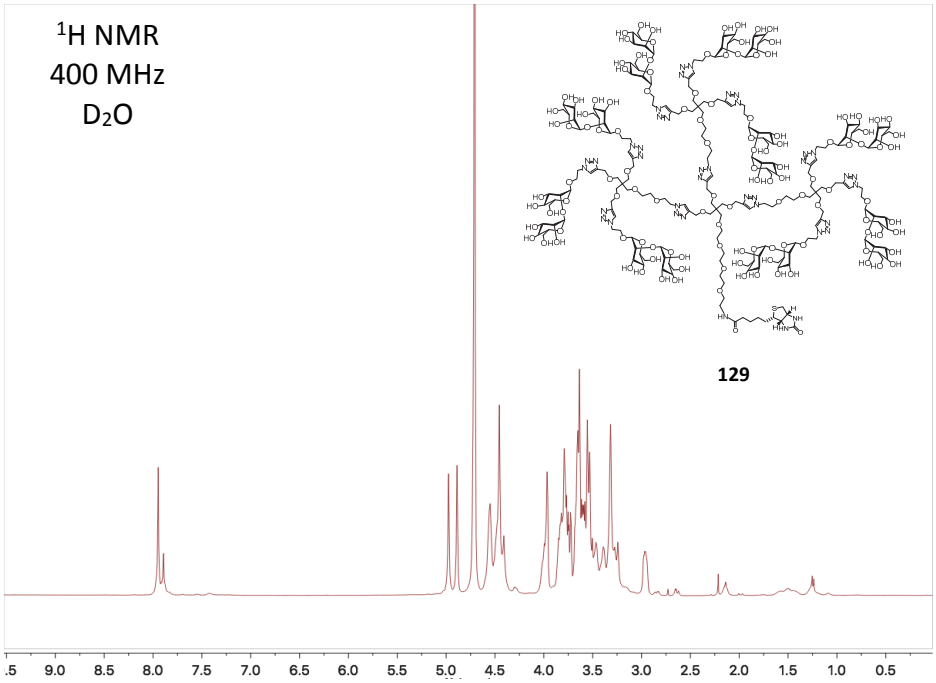
400
600
800
1000
1200
1400
1600
1800
m/z

128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2.0
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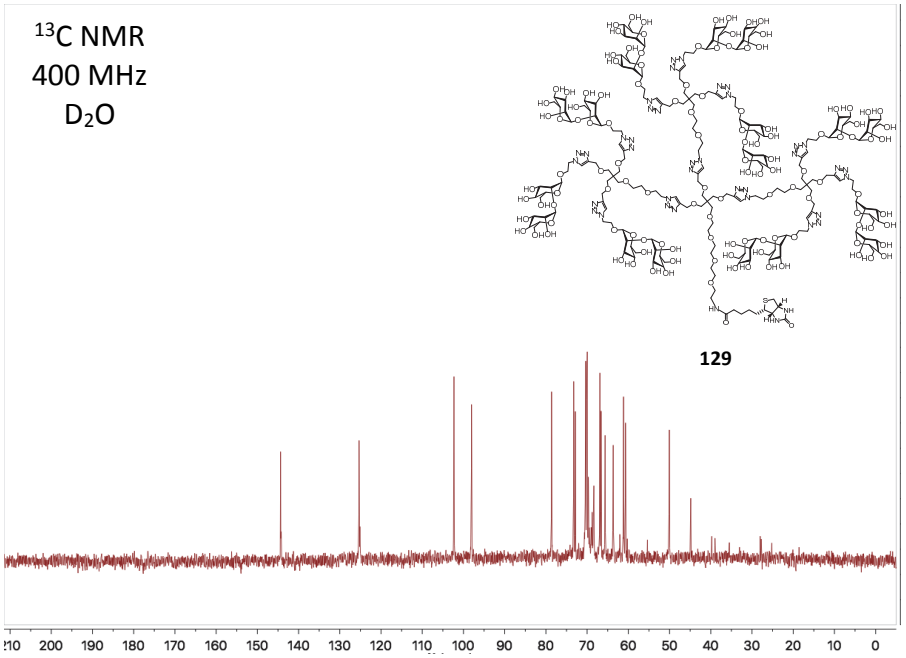
1.0
0.5

129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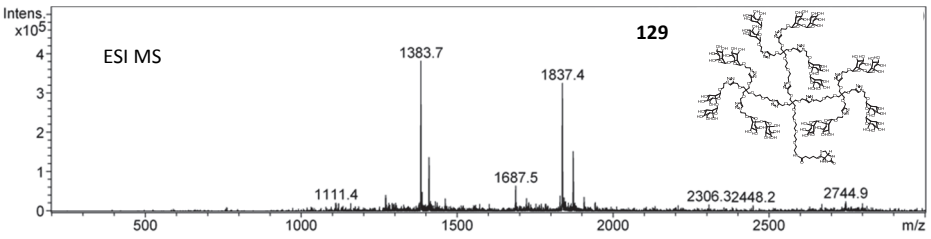
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2744.9
500
1000
1500
2000
2500
m/z

On the cover
The Tree of Life
Gustav Klimt, 1909
Austrian Museum of Applied Arts (MAK),
Vienna, Austria

In many mythologies, the image of the tree of life represents the connection among the earth and the heaven. Gustav Klimt's tree of life also creates another connection, with the underworld; it is signifying the final determinism governing over any living thing, that is born, grows, and then returns back into the earth. Tree's roots are deep into the earth, signifying that for life to be sustained, Mother Nature must be involved. The roots then come up to the above ground and meet into forming the tree's trunk that is on the earth. The swirling branches create mythical symbolism, suggesting the perpetuity of life. They extent to the heavens, and have various type of fruits and flowers that symbolize the life born from the tree. The black bird represent the Death. It is a reminder that everything that has a beginning, also has an end.

On the back cover, a detail of the painting, a woman in a pose reminiscent of ancient Egyptian art. It is called "The Expectation".

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