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SYNTHESIS AND CHARACTERIZATION OF GLYCOCONJUGATED PRODUCTS AS POTENTIAL VACCINES AGAINST TUBERCULOSIS

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CHAPTER 1: INTRODUCTION

1.1 BIOLOGICAL AND PHARMACOLOGICAL ROLE OF CARBOHYDRATES

1.1.1 CARBOHYDRATES ROLES

Carbohydrates are the major class of biomolecules. Usually they are covalently linked with other macromolecules as lipids or proteins. The last ones can be O-glycosylated (carbohydrates are attached with serine or threonine) or N-glycosylated (attached to asparagine residues)[1,2]. They have important biological roles in receptor functions, cell adhesion, cell-cell signalling, in inflammation and immune response. In particular, glycoconjugates often mediate the first step of infection process by viruses, bacteria and fungi[3].

Many scaffolds, based, for example, on proteins, polymers, gold nanoparticles or fullerenes, conjugated with carbohydrates form nanomaterials with anti-adhesive and cell-targeting properties. This kind of structures interfere with host cells-pathogen recognition process and have the potential to block this and avoid the infections.

Another way to fight against pathogens is based on the possibility to use glycoconjugates as vaccines and immunomodulators[3]. Carbohydrates have been thoroughly studied as potential molecular target in the development of glycoconjugate vaccines against many diseases, (such as cancer). However, they have demonstrated to be poorly immunogenic; indeed, they are incapable of stimulating T cell-dependent immune responses, required for developing immunity. Nevertheless, the chemical conjugation to immunogenic molecules such as a protein capable of inducing T-cell dependent responses can enhance their immunogenicity[4,5].

1.1.2 BIOLOGICAL ROLE OF LECTINES: MANNOSE RECEPTOR

Lectins are a class of proteins that interact with carbohydrates. They are a heterogeneous group of several proteins having different size, shape, structure and molecular organization. Each lectin contains specific sites for one or two carbohydrates and this bond is reversible and highly selective.

The concern on them depends on their role in cell recognition of biological process: it means they

are recognition determinants of clearance of glycoproteins in the blood, they have role in the control of adhesion of infectious agents and recruitment of leucocytes to inflammation sites. So this protein-carbohydrate interaction has an important task in immune response[6].

One important type of lectin involved in immunity is C-type lectin to which the mannose receptor (MR) belongs. This class of lectin is called C-Type because they need Ca^{2+} for their activity and they are divided in three groups : endocytic, collections and selectins. MR belongs to endocytic lectins.

It is a type I transmembrane protein, its extracellular domains consist in a unique N-terminal segment rich in cysteine, a fibronectin type II repeat (the most conserved one) and a region near to membrane consisting in eight CRDs (Carbohydrate recognition domain)[7]. (Figure 1.1)

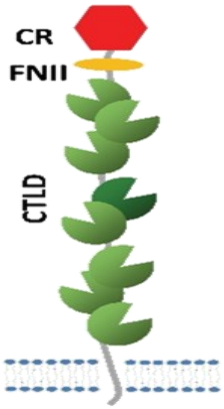


FIGURE 1.1: Mannose receptor and its three domains[8].

The C-type lectin-like domains (CTLDs) bind and internalise endogenous and exogenous molecules (for example allergens and microbial products and in particular glycoconjugates having mannose, fucose or N-acetylglucosamine as terminal part).

Some studies have demonstrated that the principal portion fundamental for the binding with mannose polysaccharides are CRD_4 and CRD_5 (above all it was proved that CRD_4 was the only region able to have affinity with sugars in absence of other CRD_5). However also CRD_{6-8} help the link, increasing the affinity with mannose molecules. Instead CRD_{1-3} are not essential for an high affinity binding[9].

The bond is similar to what MBPs (maltose binding proteins) do, the two main interactions are the coordination of $Ca^{2+}$and the formation of hydrogen bonds with amino acids by equatorial hydroxyl groups (3-OH and 4-OH): one lone pair of them is used in the coordination with $Ca^{2+}$and the other

one acts as hydrogen bond acceptor from an amine group of asparagine residue; instead the protons of these OH are the donors to acid oxygen atoms[10].

1.1.2.1 CONCANAVALIN A AS MR MODEL

A legume lectin, Concanavalin A, is the putative model in biological assay to evaluate the affinity and so the recognition between carbohydrates and mannose receptors. MR can't be used because of its difficult extraction, then Con A is a valid substitute.

Contrary, these proteins are really easy to extract and purify and also they present a variety of sugars specificities even if they have a strong sequence conservation [11]. As mentioned above, the most suitable lectin for this aim is the concanavalin A, a lectin isolated from jack beans (*Canavalia ensiformis*).

Usually it is a tetramer (considered as "dimer of dimer") and each subunit contains two metal sites, one for transition metal ions and the other mainly for calcium ions[12]. The subunits have a dome shape composed by two antiparallel β-sheets (six and seven strands). (Figure 1.2)

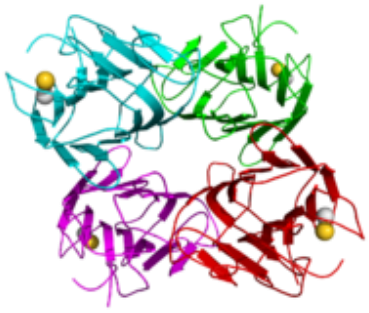


FIGURE 1.2: Concanavalin A.

The ions Mn^{2+} and Ca^{2+} are situated 4.25 Å apart and they contribute to position in a correct way the amino acids responsible of the interaction with carbohydrates. These metal ions are not bound directly with sugars, but linked to four amino acids side chains (aspartic acid residues). Therefore, Concanavalin A binds glucose and mannose thanks to the aspartate oxygen that accepts hydrogen bonds from carbohydrate hydroxyl groups (6-OH and 4-OH), instead the amine group of asparagine donates this bond to 4-OH group[7].

Both MR and Concanavalin A use Ca^{2+} ions for the interaction, but in two different ways: an indirect role for Con A where the ion ensure the right position of peptide chain for the linking with

the sugar; and a direct involvement of this ion in MR interaction where Ca^{2+} actively participates by coordination[13].

1.1.2.2 MANNOSE RECEPTOR EPITOPE

In order to develop new biological applications of oligosaccharides targeting MR (for example new drugs or vaccines) it's necessary to understand which is the carbohydrate epitope that binds these receptors. In the literature, there are some studies reporting the main natural polymannans portions identified as the most recognized by mannose receptor (precisely, in these studies MR is substituted by Con A as explained above).

In 1994, Mandal et al.[14] discovered, by microcalorimetry analysis, that manα1,2man disaccharide was recognized by Con A better than the monosaccharide and other different disaccharides (Figure 1.3).

According to this article and confirmed by other studies [15], α-(1,2) mannose oligosaccharides seem to have greater affinity with concanavalin A, probably due to their ability to increase the probability of binding, thanks to the presence of multiple internal and nonreducing terminal residues with free 3-, 4-, and 6-hydroxyl groups (binding residues).

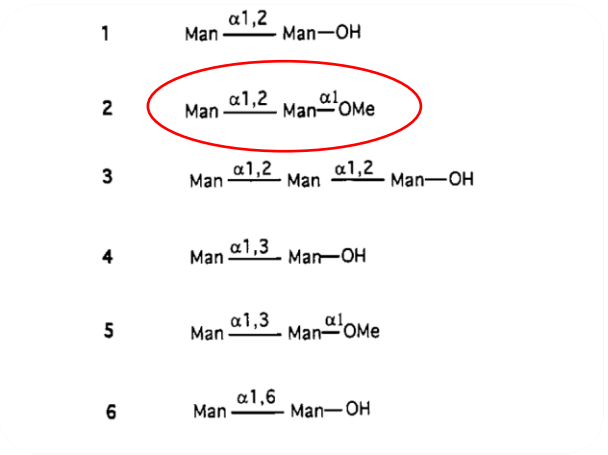


Table 1: Thermodynamic Parameters Derived from the Titration of Con A with Various Saccharides at 25 °C

carbohydrate	carbohydrate conc. (mM)	lectin conc. (mM)	K_a[a] (M^{-1})
	Native Con A at pH 7.2[b]		
MeαGlc	70.0	0.895	1.96 (±0.05) × 10^3
MeαGlcNAc	72.0	0.918	1.08 (±0.04) × 10^3
maltose	68.8	0.918	1.31 (±0.07) × 10^3
maltotriose	80.6	0.904	1.34 (±0.03) × 10^3
MeαMan	46.0	0.483	0.82 (±0.02) × 10^4
Meα2-dGlc	61.7	0.730	2.75 (±0.07) × 10^3
1	26.7	0.390	4.17 (±0.08) × 10^4
2	26.5	0.380	1.41 (±0.04) × 10^5
3	5.15	0.125	3.79 (±0.26) × 10^5
4	20.8	0.256	1.41 (±0.02) × 10^4
5	14.8	0.247	3.35 (±0.12) × 10^4
6	42.9	0.483	1.34 (±0.04) × 10^4

FIGURE 1.3: Microcalorimetry results.

Moreover, the main part that interact in a major way with Concanavalin A was discovered to be the branched trimannoside Manα1,6(Manα1,3)Man (Figure 1.4, blue).

In 2004, the Gutierrez Gallego et al.[16] showed that the optimum epitope for the interaction with Con A, confirmed by surface plasmon resonance (SPR) , consists of a tetramannoside unit Manα1,2Manα1,6(Manα1,3)Man that combine the presence of the manα1,2man moiety with the presence of branched α-1,3 moiety (whose importance in the interaction was demonstrated previously[17,18]) (Figure 1.4, red).

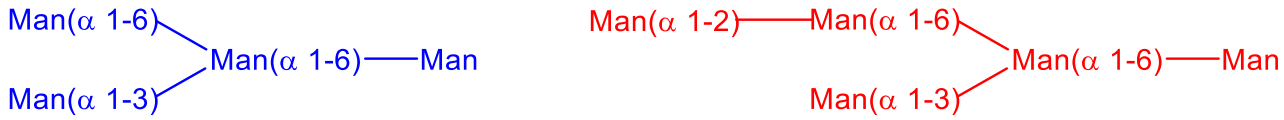


FIGURE 1.4: Epitope recognized by Con A according to Mandel *et al. (blue)* and Gutierrez Gallego *et al. (red).*

Further evidence to confirm the good affinity of these epitopes was presented by Orwenyo et al.[19] who demonstrated the importance of C-6 hydroxyl group of the α-1,6-branched trimannoside for the Con A recognition. This hydroxyl group forms a hydrogen bond with an aspartic acid residue in the binding site. This hydrogen bond seems essential, since its lack due to deoxygenation or fluorination of C-6 hydroxyl group led to a dramatic decrease of affinity.
Eventually, in 2017, Leelayuwapan et al.[20] presented an interesting study about the relation between α-(1,6) mannans chain length and their affinity binding. It seems that longer chain of α-(1,6) mannans leads to a higher affinity with Concanavalin A, as measured by SPR.

1.1.2.3 MANNOSE RECEPTOR IN IMMUNE RESPONSE

The fact that MRs are expressed on antigen presenting cells (APCs), led to the awareness MR is involved in immune process. Indeed, it seems MR could have a scavenger function, acting to clear potentially dangerous molecules in bloodstream; among them pathogens such as *Mycobacterium tuberculosis* (MTB) and HIV virus, which expose high density of mannose on their surface.
MR mediate the recognition of viral and microbial glycans inducing the consequent internalization of the pathogens (up-take) by phagocytosis. Once inside, it is digested and transformed into shorter oligopeptides appropriate for recognition by class II MHC molecules.
Therefore MR, able to distinguish from self and not-self structures, has a double function in homeostasis processes as well as in pathogen recognition and antigen exposition. Indeed, it has an

important homeostatic task in the clearance of endogenous molecules (lysosomal hydrolases and hormones) but in addition it plays an important role in the uptake of foreign antigens, which during pathological conditions could be used to activate the acquired immune system[8].

This process is the first step for the stimulation of an immune response toward specific antigens and, consequently, an efficient uptake induces a strong immune response[21].

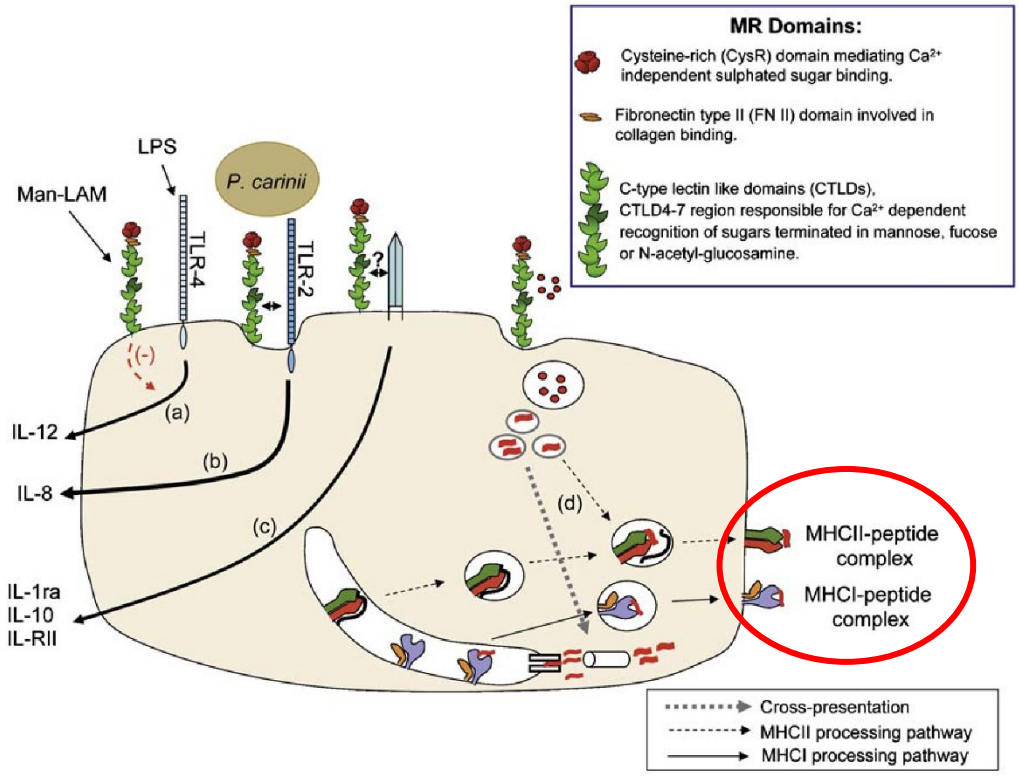


FIGURE 1.5: Involvement of Mannose receptor in immune response[21].

1.1.3 FROM HIV VACCINE TO MANNOSYLATED ANTIGENS

The discovery of the role of MR has stimulated the research towards the development of mannosylated antigens in order to increase immune response[22]. In the literature, there are several studies regarding natural high-mannosylated GP120 fragments (Figure 1.6) as potential HIV vaccine[23,24], for example in the publication by *Geng et.al*.[25]**.** In HIV virus high mannose glycans are included in the important envelope glycoprotein GP120, that is essential for the entrance of the virus in the host cell. Indeed, the infection process of HIV virus is mediated by the interaction of GP120 with MR present on APC cells surface. This glycoprotein is composed of a natural polymannan that is also able to stimulate antibody response.

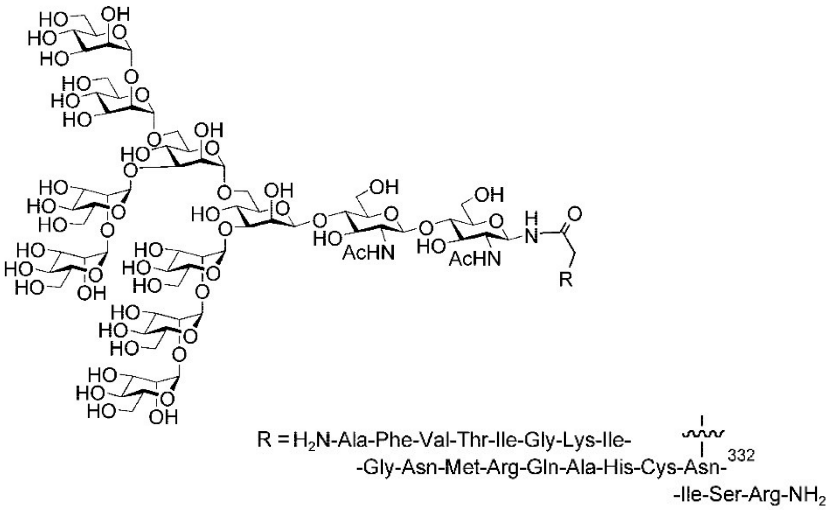


FIGURE 1.6: Vaccine GP120-like[25].

1.1.3.1 CARRIERS FOR POTENTIAL GLYCOCONJUGATE VACCINE

There are several examples of mannans conjugated to different carriers that have been investigated as potential HIV vaccine. The main strategies are the following:

1) The design of HIV vaccine composed by a cyclic decapeptide template (Figure 1.7) that has been used as scaffold for binding high-mannose glycans on one face and two T-helper epitope peptides on the other face[26]

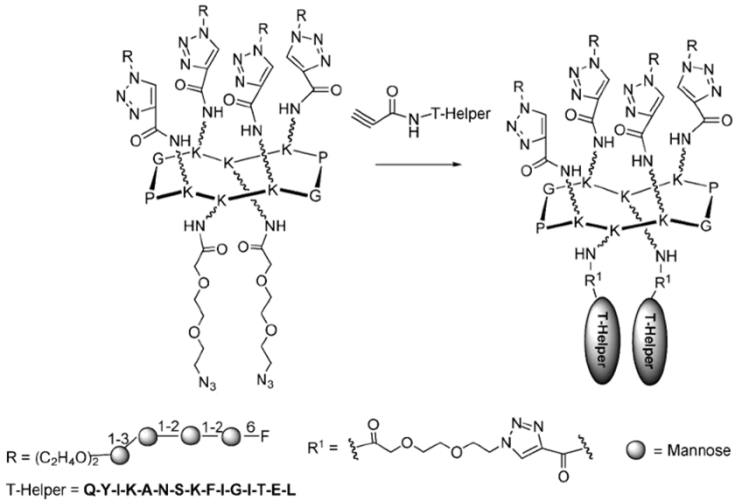


FIGURE 1.7: Potential HIV vaccine by using cyclic decapeptide template[26].

2) The development of HIV vaccine by using the icosahedral capsids of bacteriophage Qb and cowpea mosaic virus (CPMV) as scaffold for glycoconjugates binding (Figure 1.8)[27].

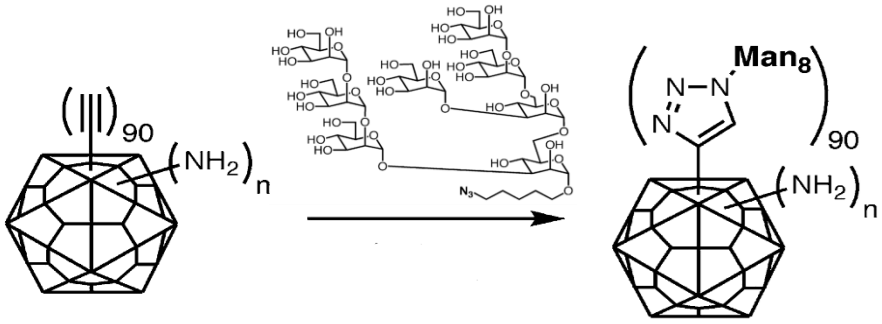


FIGURE 1.8: Potential HIV vaccine by using a bacterial capsid[27].

1.1.3.2 NEW STRATEGY: MANNOSYLATED ANTIGENS

Unfortunately, this approach resulted poorly efficient and not useful for the development of an efficient HIV vaccine candidate, but it has suggested the idea that the natural polymannan or its analogues, being well-recognized by mannose receptor of APC, could be used in order to improve the antigenic activity of peptide or proteins by mannosylation.

Accordingly, it was investigated the use of the natural polymannan of HIV-GP120, conjugated with different antigenic peptide, in order to produce an efficient vaccine for a specific disease. *Fairbanks et. al.*[22] developed a chemo-enzymatic synthesis of mannosylated glycopeptides obtained by conjugation of the synthetic natural-like polymannan with one antigenic peptide sequence of human cytomegalovirus (CMV). Biological tests have proved the real stronger immune response of the mannosylated antigen compared to the non-glycosylated antigenic peptide.

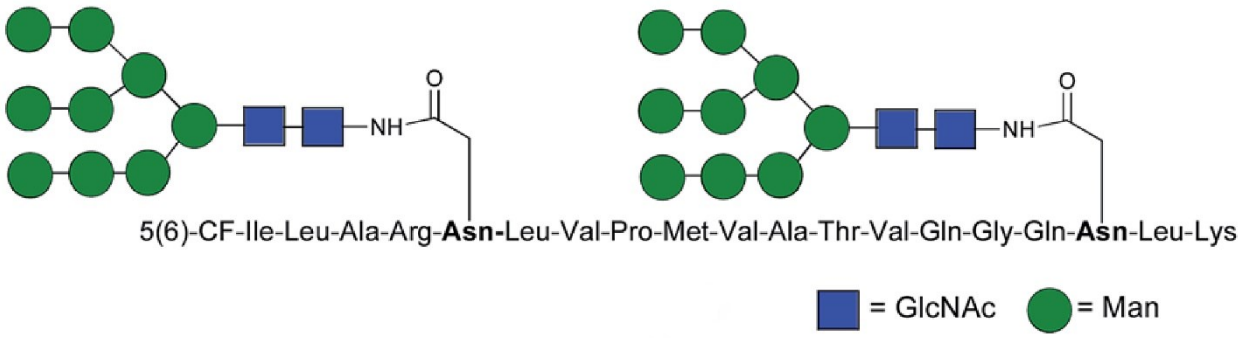


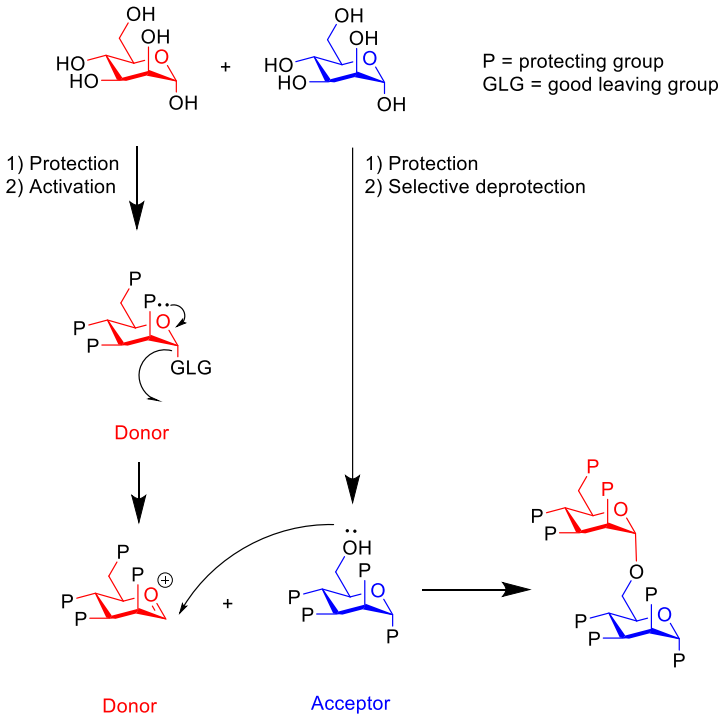
FIGURE 1.9: Mannosylated antigen[22].

1.1.4 SYNTHESIS OF OLIGOSACCHARIDES

As just described, oligosaccharides are widely present in nature and expressed in bacteria and viruses and they could have an important role in future vaccine applications. However, these applications are still limited because the synthesis of oligosaccharides is complicated as consequence of their structural complexities while the extraction from natural sources has low yields and allows mixtures of product with not well defined structures[28].

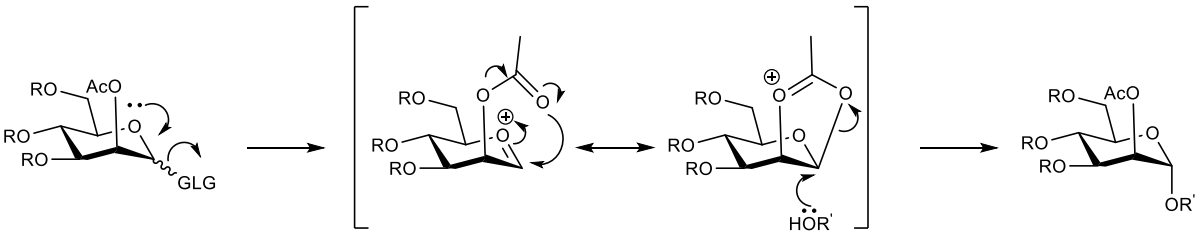
The synthesis of oligosaccharides is normally achieved by linking different monosaccharides building blocks. Indeed, the hydroxyl groups of monosaccharides are characterized by the similar reactivity, except for the one linked to anomeric carbon.

Thus, the synthesis involves a monosaccharide "donor", presenting a good leaving group, and a monosaccharide "acceptor", presenting only one free hydroxyl group (Scheme 1.1). For the glycosylation reaction, sugar acceptors with only one free hydroxyl group prepared using various protecting groups are required in order to avoid the formation of different isomers [29]. The glycosidic linkage is formed through the activation of the glycosyl donor to create a reactive electrophilic species that could react with the hydroxyl group of glycosyl acceptors allowing the synthesis of the desired disaccharide.



SCHEME 1.1: Mechanism of glycosylation.

The second difficulty is to obtain selectively α or β glycosylation, depending if the nucleophilic substitution takes place over(β) or under(α) the plane. The neighbouring group participation (also known as anchimeric assistance) helps in this process. Indeed, in the case of mannose, the neighbouring group induce an α glycosylation. When an acyl group (e.g. acetyl or benzoyl) is present at the C-2 position, it can form a stable cycle by oxocarbenium ion and consequently nucleophilic attack can happen only under the plane. The mechanism is shown in scheme 1.2.



GLG= Good leaving group
R= Protective group
R'= acceptor monosaccharide

SCHEME 1.2: Mechanism of neighbouring group participation.

However, the preparation of a monosaccharide building block specifically deprotected is difficult. Classical chemical methods employ many protection reactions, with different protecting groups. This is a complicated way that often affords low yields. Indeed, protection reactions have low regioselectivity because of similar reactivity of hydroxyl groups[30].

With the aim to improve yields of the synthetic processes for the preparation of oligosaccharides, new strategies have been developed:

- One-pot computer aided synthesis: the one-pot approach uses a computer program to automate glycan synthesis. The strategy exploits difference in the anomeric reactivity of a large set of diverse building blocks. These intermediates are monosaccharides that bear in anomeric position different leaving group with different reactivity and present only one free hydrolxyl group. Mixing these building blocks in sequence (from high reactivity to low reactivity), will be generated the desired product in a protected form. The key advantage to use a one-pot approach is the rapid planning and ordering of the synthetic steps. In addition, a programmable one-pot synthesis eliminates intermediate purification steps utilized in traditional synthetic schemes[31].

- Solid phase synthesis: glycosyl acceptor is covalently linked to a solid matrix. The glycosyl donor and an activator are added and a the new glycosidic linkage is obtained. Side products, reagents and unreacted starting material are washed away while the desired glycan remains covalently attached to solid matrix[32].
- Chemoenzymatic synthesis: enzymes are used as catalysts as they have demonstrated to be regio- and stereoselective[33]. In this context, enzymatic hydrolysis of acetylated monosaccharides has proved to be a valid alternative to classical chemical synthesis of building blocks: deprotection is regioselective and allows one-pot preparation of the sugar acceptor only using acetyl group for protection [34].
- Enzymatic glycosylation could avoid the use of protecting groups[35].

1.2. BIOCATALYSIS

1.2.1 ROLE OF BIOCATALYSIS

Enzymes are proteins that act as catalists of biochemical reactions in living organisms. They are characterized by high catalytic acitivity with high specificity for a particular reaction or a particular type of chemical bond or functional group. These proteins, generally, operate in an acqueous environment (buffer added with low % of organic solvents), in mild pH conditions (pH value between 5.00 and 8.00) and at room temperatures (from 20 °C to maximum 40 °C).
Biotransformations are, compared to classical chemistry, faster, cheaper and safer, in agreement with key principles of green chemistry[36]. Moreover, enzymes are reusable, overall if immobilized on a solid matrix[37,38].
The green chemistry aim is to reduce or eliminate the use or production of hazardous substances in manufacture, application and design of chemical products. The biocatalysis satisfies exactly this, and the huge increase of its use in organisc synthesis, above all in API (Active Pharmaceutical Ingredients) production, demonstrates it.
In fact, the chemical processes to obtain API are the most inquinant because of the complex structure on most drugs that require many synthetic steps for its preparation, leading to a huge amount of waste and scrap that have an impact on the environment. It was estimated that between 25 to 100 kg of waste result from every kilogram of pharmaceutical synthesized.

In this context, the use of enzymes and biocatalysts can be an important tool to pursue the green chemistry path.

Therefore, summarizing, the biocatalysis advantages are the following[39]:

- Enzymes are often chemo-specific (enzyme acts on a single type of functional group), regio-specific (enzymes can distinguish between functional groups which are situated in different regions of the substrate) and stereospecific.
- Biocatalysis is a Green chemistry technology
- Less steps compared to organic synthesis
- Biocatalysts can be reused and reycled.
- Mild reaction condictions (pH and temperature).

However, it is important to remark some limitations of biocatalysis:

- pH, temperatures and buffer concentration that allow an optimum enzyme activity are not always favorable for the rate of the desired reaction;
- enzymes mainly operate in aqueous medium; however, not all the organic molecules are water soluble;
- some enzymes require co-factors that can be expensive and not reusable;
- elevated substrate or product concentrations, as required at preparative scale, could inhibit the enzymes[36].

There are almost 3000 enzymes classified by International *Union of Biochemistry* (only about 10% is commercialy avaible). In this classification, each enzyme is characterized by a number and a name that identified a specific enzymatic reaction. The main classes of enzymes are six[40]:

Oxidoreductases	EC1	Oxidation and reduction reactions
Transferases	EC2	Transfer a functional group
Hydrolases	EC3	Hydrolysis reaction
Lyases	EC4	Addition or removal of functional group
isomerases	EC5	Transfer functional group in order to obtain isomer
Ligases	EC6	Synthesis of new C-C, C-S, C-O e C-N bindings

1.2.2 HYDROLASES

The hydrolases are a class of enzyme able to catalyse hydrolysis reactions. They can divided in different groups: esterase and lipase (for esters and lipids), phosphatase (detachment of phosphate group), glycosidase (for polysaccharides) and peptidase (for peptide bonds).

Lipases (triacylglycerol acyl-hydrolases EC 3.1.1.3) are a subclass of esterases, and catalyse hydrolysis of ester bonds of triglycerides, releasing glycerol and fatty acids[41]. They are the most widely used enzymes; indeed, they are stable (in same case also in organic solvent), easy to handle and they have a broad substrate specificity and a high enantio- and regioselectivity.

They are presents in many animals, plants and microorganisms where they can be isolated and purified from. In this way there are a huge variety of lipase sources with different functional and structural characteristics.

They are commercially available in free or immobilized forms. The reactions catalysed by lipases are reversible in non-aqueous systems, so these enzymes can also catalyse the formation of esters from acyl donors (as anhydrides) and alcohols[42].

Their reaction mechanism is enough complicated. Their activity is enhanced near lipid-water interface (phenomena known as " lipase interfacial activation")[40]. In absence of this, the lipase are very low reactive and they have a closed structure, inaccessible for substrates. Otherwise the presence of the interface stabilize the open conformation of the enzyme, and in this way the substrates can reach easily the catalytic site[43,44]. This mechanism is convenient since natural substrate are lipids, and also because they can use in organic chemistry on apolar substrates (as protected sugars).

The lipase third structure is characterized of a hydrophobic pocket containing the catalytic site, a protein scaffold and a lid[45]. During closed conformation, the lipase presents the catalytic site surrounded by hydrophobic region covered by the lid (with an hydrophobic portion that is oriented towards the hydrophobic region of active site, meanwhile the hydrophilic part is in exposed in the aqueous environment)[46].

In the open conformation, in presence of hydrophobic interface, the lid doesn't cover the active site (because the hydrophobic face is exposed towards the interface) that is consequently ready to react with substrates. This conformation is instable in aqueous solvents where the closed and inactive conformation is more frequent[47].

The active site of lipases is a catalytic triad of amino acids: the first amino acid has a hydroxylated lateral chain and acts as neutrophil, the second is involved in hydrogen bonds, the third acts as an acid compound. Generally, the amino acids involved in the reactions are Ser/Thr-His-Asp, but several exceptions have been observed[48].

Lipases are different from other esterases because of their kinetic model, it is not the michaelis-menten one (figure 1.10): lipases are inactive under a specific CMC (critical concentration) and then their activity increases with substrate concentration rising a *plateau* (saturation)[36].

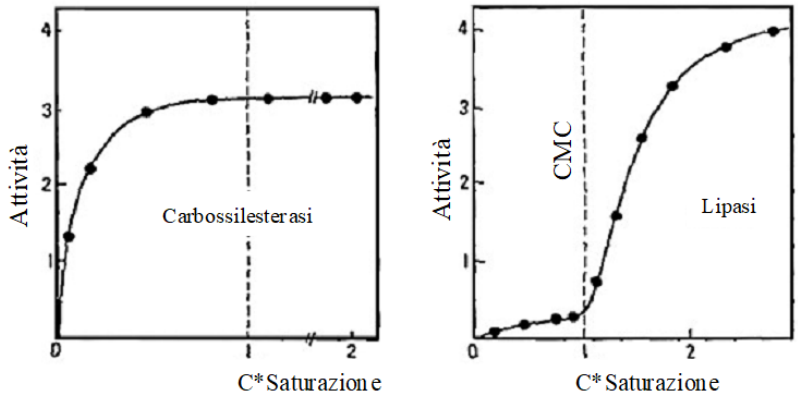


FIGURE 1.10: Comparation between an esterase and a lipase.

In the case of esterases, the absence of the lid makes not required an interfacial activation for stabilising the active form that only depends from the interaction with the substrate in solution.

Lipases and esterases represent an alternative to the chemical methods for the regioselective deprotection of carbohydrates in order to obtain building blocks with free hydroxyl groups in the desired position.

1.2.2.1 CANDIDA RUGOSAE LIPASE

Candida rugosa is one of the most popular enzymes used in the biotransformations. It is a fungus that secretes lipases: 5 isoenzymes have been cloned and sequenced. The presence of five different isoenzymes can influence the reproducibility of reactions where this catalyst is employed.

Lipases from *Candida rugosa* (CRL) are formed by a polypeptide chain of 543 amino acids and its activity is mediated by the classical catalytic triad. There are many applications and studies of this

lipase, also using immobilized CRL, and they are used in both synthetic (in organic solvents) or hydrolysis reactions (in aqueous solvents)[34,49].

1.2.2.2 ACETYL XYLAN ESTERASE FROM BACILLUS PUMILUS

Acetyl xylan esterases (AXE, EC 3.1.1.72) are a subclass of esterases. They catalyse the deacetylation of xylans and xylo-oligosaccharides. Their active site is composed of a catalytic triad of aminoacids common to all the hydrolases. The xylan is a typical component of plant cell wall and it is formed by β-1,4-linked xylose residues with side branches of α-arabinofuranose and α-glucuronic acids[50]. Biodegradation of hemicellulose, composed principally by xylan, is a process that require several enzymes. Among these, *Bacillus pumilus* is particularly interesting as it has many enzymes able to split xylan in smaller molecules[51]. One of these enzymes is acetyl xylan esterase. AXE from *Bacillus pumilus* represents an alternative to the chemical methods for the regioselective deprotection of carbohydrates[52].

Even if this enzyme was poorly considered as a catalyst in organic chemistry, there are some examples of its use in literature.

Moen et al.[53] screened the deacetylation capacity of this enzyme on some glycosides with two different anomeric groups (methyl and benzyl). Comparing AXE with other enzymes (as lipases and porcine liver esterase) its reactions resulted simpler and more specific, giving less products mixtures. Moreover, glycosides having benzyl group in anomeric position could be affected by this bulky group because they took ten time to reach complete hydrolysis respect to ones with methyl group.

Bavaro et al.[54] obtained a selective deacetylation in C-6 position of glycoside moiety of “lacto” carbohydrates. The behaviour of enzyme depended on substrate, the regioselectivity was observed depending on the absence of acetoxyl group in C1 position. In this paper were prepared intermediates useful for the complex synthesis of oligosaccharides with biological and pharmaceutical interest.

1.2.2.3. PREPARATION OF SUGAR BUILDING BLOCKS BY REGIOSELECTIVE HYDROLYSIS

Esterases and lipases have been applied in regioselective hydrolysis of mono- and disaccharides protected by acetyl groups and with different groups in anomeric position, in order to obtain the

specific intermediates for the synthesis of oligosaccharides. This last strategy is very simple. Once the sugar is protected only by acetyl groups, the reaction of selective deprotection catalysed by immobilized hydrolases can lead to obtain intermediates with a specific deprotected position.

This chemoenzymatic synthesis allows to use only one type of protecting group (normally the acetyl one) and allows to obtain the desired mono-deprotected sugar in one step. There are some studies about deprotection of anomers α and β of monosaccharides as glucose, galactose and theirs derivatives. It seems that α-anomers are more reactive and it is hydrolysed faster and with better yields than the β anomers.

Moreover depending on the enzyme used and the conditions (pH and temperature), it is possible to obtain different products and yields. In addition, the same enzyme can lead to different regioselectivity on the basis of the structural characteristic of the substrate and the type of enzyme immobilization[55].

1.3. TUBERCULOSIS

1.3.1 BACKROUND

1.3.1.1 SPREAD OF TUBERCULOSIS

Tuberculosis (TB) is an infective and contagious disease caused by MTB and is still very common, mainly in less developed countries as India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (6%), Nigeria (4%), Bangladesh (4%) and South Africa (3%). Only 6% of cases were in Europe and America (Figure 1.11)[56].

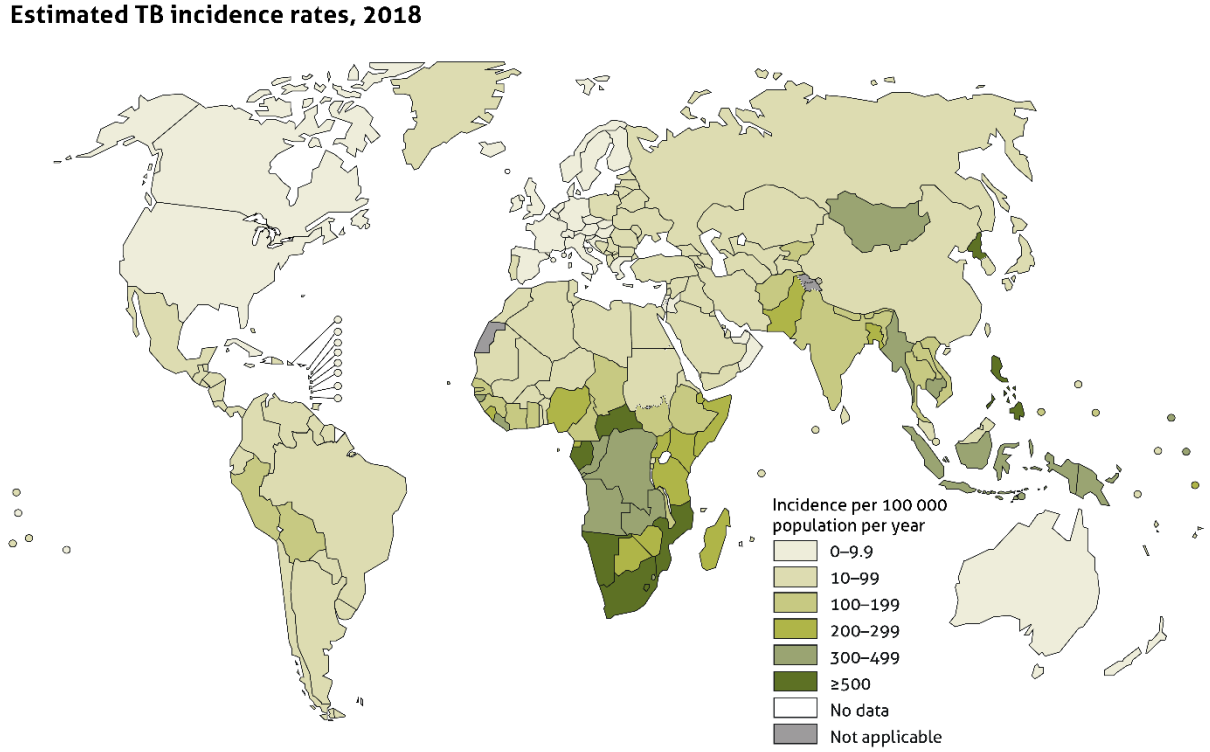


FIGURE 1.11: Estimated TB incidence rates 2018[56].

Tuberculosis (TB), also known as Koch's bacillus, is a chronic and slowly progressive infection. The most common form of TB is a chronic pulmonary disease with extensive focal inflammation: the classic symptoms are persistent inflammation[57], a chronic cough with blood-containing sputum, fever, night sweats and weight loss. The lungs damage, after the development of granulomas, ultimately causes death by suffocation, due to anoxia. Extrapulmonary TB, when the infection affects other organs, can cause a wide range of symptoms. Other forms of TB are, for example, tuberculomas or tubercular meningitis[58]. TB is spread through the air when people expel bacteria by coughing, spitting, speaking, or sneezing. Only people with developed TB spread the disease. Those whose immune system is damaged by diseases like AIDS, malnutrition or diabetes and long-term smokers are more likely to get TB.

TB is the ninth leading cause of death in the world because of late diagnosis, lack of access to treatment and associated infections such as HIV, although great efforts have been made to prevent and treat this disease. In 2016, TB caused 1.3 million deaths among HIV-negative people and an additional 374000 deaths among HIV-positive people. Moreover, 10.4 million is the number estimated for people that have been fallen with tuberculosis: 90% adults, 65% male, 10% HIV-positive[59].

According to WHO, this disease killed 1.6 million people in 2017[59]. In addition, it is reported that nearly 2 billion people have been exposed to the tuberculosis bacillus and are at risk of developing active disease. In 2018 the data showed a slight decrease, about 10 million people sick and 1.2 million deaths.

Unfortunately, these data show that the disease is still widespread in the world, also because of the increasingly drug resistance and the lack of efficient vaccines.

1.3.1.2 MYCOBACTERIUM TUBERCULOSIS

MTB is a member of the complex *M. tuberculosis* (MTBC) including other six related species : *M. bovis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae* e *M. canettii*[60].

It is an aerobic bacillus, acid-resistant (AFB, Acid-Fast Bacillus), without a capsule and able to infect the host, growing in oxygenated tissue where can stay a long time unidentified[61]. Their unusual cell wall has resulted to be essential for growth and survival in the infected host. Indeed, most of effective antimycobacterial drugs act at inhibiting the biosynthesis of the cell wall components.

The small size of mycobacteria in comparison to other bacteria, their hydrophobicity, cellular permeability, antigenic response inhibition and acid-fast staining are peculiar aspects of the bacillus connected to the uncommon cell wall[62].

Mycobacterial cell wall is composed by a plasma membrane, similar to other bacteria membrane [63]. Above this, there is a layer of peptidoglycan (PG) that confers a rigidity to the cell wall, allowing it to maintain osmotic pressure, cell integrity and shape. This PG forms a covalent bond with the mycolyl-arabinogalactan (mAG) by phosphodiester bond , leading to the formation of the basal layer for the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex.

Another important component of mycobacterial cell wall is lipoarabinomannan (LAM). It extends from plasma membrane across the entire envelope. It is demonstrated that it is involved in virulence and immuno-pathogenesis of mycobacterial diseases[58].

1.3.2 MECHANISM OF INFECTION AND IMMUNE RESPONSE

The therapies available today for TB are not enough efficient for eradicating this disease.

MTB is a gram-positive acid causing damages to respiratory system by a persistent inflammation and it can evolve in other serious manifestations. Because this is essential to have a suitable

knowledge of the pathogenesis, the mechanism of infection and how the mycobacterium cause the disease.

1.3.2.1 HOST'S IMMUNE SYSTEM

There are two different type of immune reactions that protect the organism and they depend on the timing of action, specifically on the speed of response (Figure 1.12): one precocious, known as **innate immune system** and one tardive, **adaptive immune system** that guarantees specificity and memory[64].

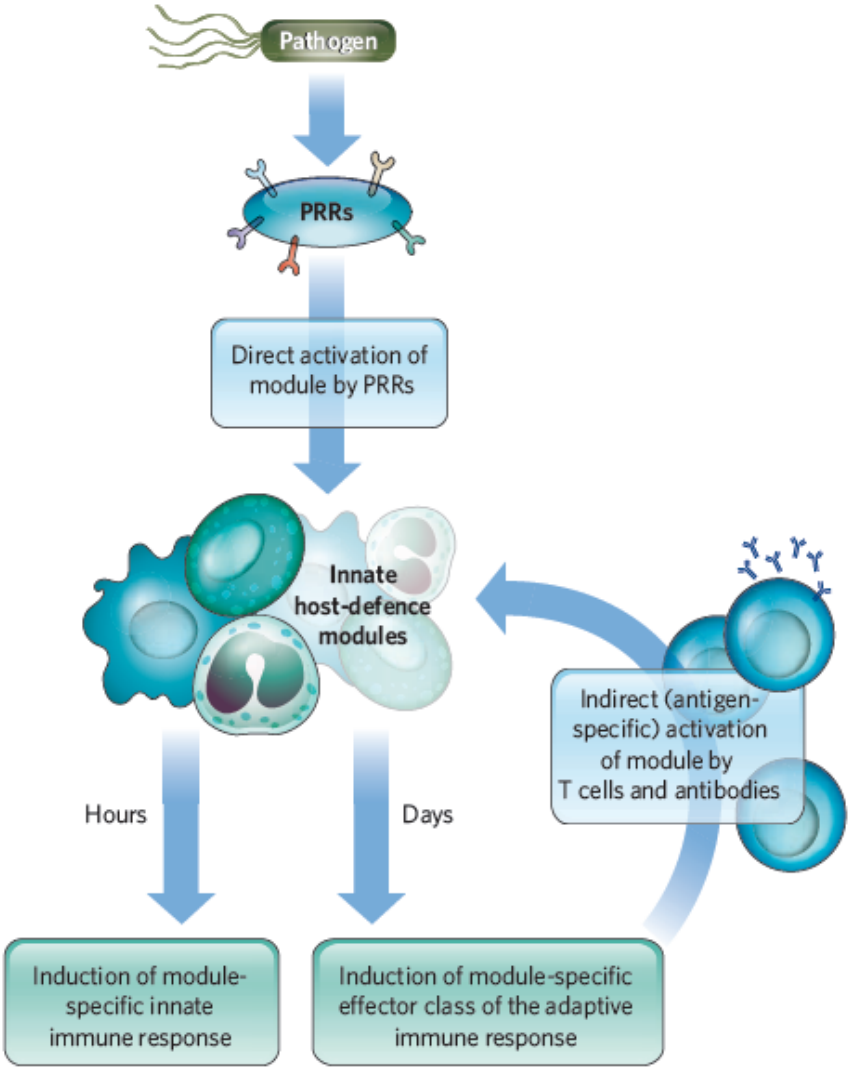


FIGURE 1.12: Type of immune reactions[64].

The innate immune system consists in protective mechanisms that are ready and act very quickly when the infection starts. It's always active, regular and aspecific. Its team is composed by chemical and physical barriers (e.g. secretions, epithelium), phagocytes (macrophages, granulocytes), dendritic cells (DCs) and natural killer cells (NKs).Generally, these agents, the first line of defence, recognise infectious agents by pattern recognition receptors (PRRs).

The role of innate immune system is fundamental for the immune defence, but is not always enough and so an adaptive immune system was evolved to protect the organism against more dangerous infections. In fact, pathogens that overcome the innate immune system are recognized by the components of the adaptive immune system, which has a specificity for different molecules, the ability to memorize and provide a fast response in case of repeated exposure of the same microbe. The cells of the innate immune system, unlike the adaptive immune system, do not give long lasting immunity[57].

There are cooperative mechanisms between innate immunity and adaptative immunity, indeed the innate response actives and stimulates adaptive response which eliminates pathogens by means of mediators and cells belonging to innate immunity.

Antigens are the substances that induce a specific immune response. Adaptive or specific immunity is mainly mediated by lymphocytes B and T. It is divided into two different immune responses depending on the type of lymphocytes: humoral immunity (B cells) and cell-mediated immunity (T-cells).

Lymphocytes B originate and maturate in the bone marrow, they are unique cells able to produce antibodies or immunoglobulins; these glycoproteins are useful for the activation of the B cells and are the molecules assigned for the antigen elimination.

Instead, in the case of cell-mediated immunity, the protagonists are lymphocytes T (originate from the bone marrow but maturate in thymus). They express on their surface proteins that act as a marker called Clusters of Differentiation (CD). In base of these CDs, T cells are subdivided into: T-helper lymphocytes (Th1 and Th2), expressing CD4 protein and able to produce cytokines and useful to coordinate the several phases of immune response and cytotoxic lymphocytes (CTL), expressing CD8 protein and directly employed to eliminate the infective and modified cells.

Three are the classes able to recognition the antigens and gets start to the specific immune response: the antibodies, B and T cells receptors (BCR and TCR) and the major histocompatibility complex (MHC, divided in class I and class II).

This last class has a very important role, the task to bind antigens fragments and present them to lymphocytes T. For T $CD8^+$ cells is the MHC class I to present the antigen, contrary, T $CD4^+$ cells recognise it only if presented by MCH class II.

Therefore, T-cells, differently to B-cells, are not able to recognise antigen unless it is presented to them in fragments by specific cells, called APCs. DCs are the most representative APCs. DCs, belonging to innate immunity, are resident in peripheral tissue and are able to internalize small or infectious antigens. Once captured these antigens, DCs become active and migrate in secondary lymphoid tissue (e.g. lymph nodes) to present the antigen to lymphocytes T. Also other cells, as macrophages and B cells, could accomplish this task in order to get start the immune response[65,66].

1.3.2.2 PATHOGENESIS OF TB

MTB is an extremely dangerous facultative intracellular pathogen that causes TB in humans and animals. It principally affects lungs. The bacteria are internalized by alveolar macrophages and create outbreaks of infection in the lung tissue. These foci expand through bacterial growth and the recruitment of macrophages and lymphocytes that build the granuloma determines the infection. The granuloma appears to support limited bacterial growth and prevents infection metastasis; however, it also protects the bacterium from the immune response and is probably responsible for the persistent or latent nature of the infection[67].

MTB has evolved developing several mechanisms that can subvert many of the host immune defences and it can escape innate immune effector cells, such as macrophages and airway epithelial cells[68]. For this reason, it is a human pathogen hard to eradicate.

Indeed, if MTB is not killed by the innate immune response, and consequently it can replicate and disseminate, so efficient anti-microbial substances are needed[69].

As described above, TB typically affects lungs: MTB is inhaled through nose and mouth and passes along the respiratory apparatus (trachea, bronchus, bronchioles and alveoli). Thus, the respiratory mucosa is the first line of defence against MTB. Indeed, lung microenvironment consists of a complex setting of pattern recognition molecules and alveolar epithelial cells are the first cell types to encounter MTB. These cells act in a coordinate way whit “professional” immune cells to secrete chemokines and recruit leukocytes[69].

Most infected people show no progression of the disease. Only 3-5% develop tuberculosis in the first year after infection. A further 3-5% develop tuberculosis later in their lives. HIV-positive people infected with *M. tuberculosis* have a 50% chance of developing tuberculosis reactivation (post-primary) at some time in their lives.

It is generally recognised that cell-mediated immunity (CMI) plays a critical role in protection against *Mycobacterium tuberculosis* because it is an intracellular pathogen (Figure 1.13). In fact, MTB has both intracellular and extracellular phases in its infectious cycle. The interdependence and synergy between cellular and humoral immunity has been demonstrated[57].

In particular, the activities of T-helper cells (Th1 and Th2) have been shown to be involved in responses against MTB. Th1 cells are involved in cellular immunity, which fights against viruses and intracellular pathogens (the most important effectors are IFN-γ, IL-12 and TNF). Th2 cells are involved in humoral immunity and in the up-regulation of antibody production to fight extracellular pathogens.

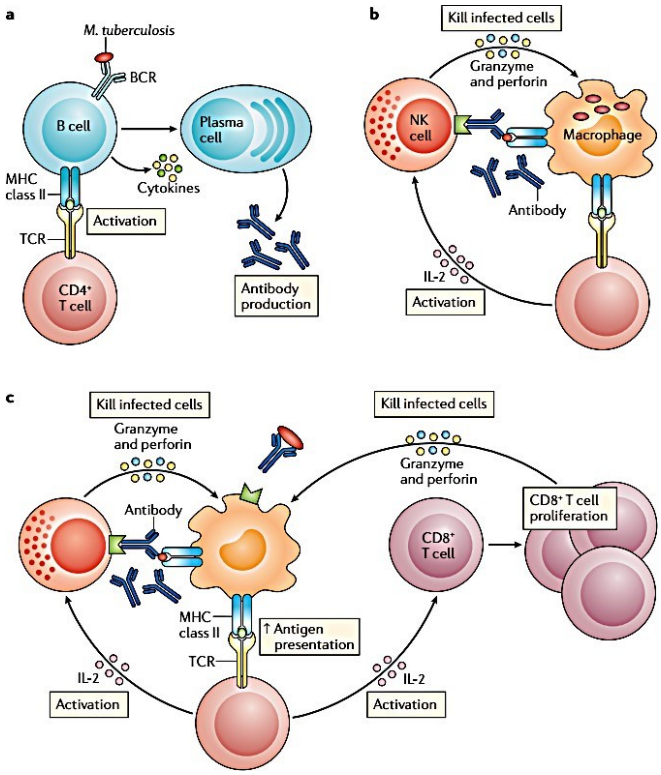


FIGURE 1.13: Synergy between cellular and humoral immunity.

Pathogen-associated molecular patterns (PAMPs) on MTB surfaces can be recognized by PRRs. All these receptors mediate the production of cytokines and help to build an efficient immune response, binding the pathogen to epithelial cells.

Moreover, recruitment and activation of DCs, T cells, and B cells is induced by the activated innate immune response in order to increase antigen recognition, antibody production and other different mechanisms of adaptive immune response. These components are useful to stimulate a synergy between epithelial cells and B and T cells, DCs and macrophages[69]. The latter have multiple roles in protection against TB, including antigen processing and presentation, production of lysosomal enzymes and of reactive nitrogen intermediates, generation of reactive oxygen intermediates and apoptosis[70] .

Other cells involved in the antimicrobial defence to MTB are natural killer (NK). These cells can recognise infected macrophages and lyse them. Moreover, they generate IFN-γ to activate new macrophages and they deliver cytokines to increase $CD8^+$ T cells and NK T cell populations.

Not all the MTB are eradicated in the upper airways. Some of them reach the alveoli. The alveoli are protected by alveolar macrophages (AMs), dendritic cells, neutrophilis and by a line of type I epithelial cells (useful also for gas exchange) and type II epithelial cells (involved in the production of antimicrobial molecules). MTB can be phagocyted by alveolar macrophages. The process initiates after bacterial contact with macrophage mannose receptors and/or complement receptors. MTB enters the host and it is taken up into the phagosomes of resident alveolar macrophages.

When normal phagosomal maturation cycle takes place and phagosome-lysosome fusion occurs, MTB can be defeated. It encounters a hostile environment: acid pH, reactive oxygen intermediates, lysosomal enzymes and toxic peptides. However, MTB is capable of blocking phagosome acidification and inhibiting the acquisition of hydrolytic enzymes and anti-microbial peptides. Thus, maturation of phagosomes is mainly blocked by LAM and an the MTB protein TACO[71,72].

The exposition to a toxic intracellular environment is another way that immune system uses to fight MTB. For example, oxidative killing is induced by phagocytes generating reactive oxygen species (ROS). Bacterial elimination is also achieved with heavy metal poisoning, such as zinc[73].

1.3.3 OBSTACLES IN TREATMENT AND PREVENTION OF TB

As previously reported, the incidence of tuberculosis still causes high levels of deaths due to a failure of global tuberculosis control strategies. The spread of this disease depends on several factors such as overcrowding and low levels of sanitisation, particularly in developing countries.

More than 60 million people have been documented as treated and cured since 2000 and the rate of cases and deaths has steadily decreased. The incidence rate has probably stabilised, but the challenge is to reverse the trend and reduce global mortality and, in the long run, eliminate the disease.

The risk of infection is related to the lack of timely diagnosis and failure of current pharmacological treatments consisting of very protracted multi-drug therapies. Moreover, the only vaccine authorised and used since 1921 is the Bacillus Calmette-Guérin (BCG) vaccine from *Mycobacterium bovis*. Both strategies are not enough to ensure health security, in particular because of the drug-resistant cases and the variable efficacy of BCG[74,75].

1.3.3.1. DRUG TREATMENT

Almost 5000 people die daily from TB worldwide, the majority of them in developing countries. However, TB is becoming more and more important in Europe where MTB strains resistant to two or more drugs (Multidrug Resistant – MDR - or Extensively Drug Resistant – XDR - strains) have been identified with increasing frequency and showed a particularly high mortality rates due to the lack of effective drug regimens.

Indeed, in the last 45 years, new drugs have not been discovered and introduced; the last novel first line anti-TB drug introduced was rifampicin in 1963. The only improvement to the therapy has been the personalization of the treatment, focused on the patient. Moreover, even if the duration of treatment has been reduced to 6 months thanks to the combination of different drugs, patient compliance, strictly connected to resistance insurgence, is still a serious problem. The first phase of the treatment lasts 2 months. Isoniazid, rifampicin, pyrazinamide and ethambutol are used in combination. The second phase lasts 4 months. Isoniazid and rifampicin are used.

The development of MDR and XDR-TB has required the setting up of new treatment protocols. According to this protocol, 5 or 6 drugs are used simultaneously (Table 1.1).

TABLE 1.1: main anti-TB drugs.

I. Oral first line drugs	• Isoniazid • Rifampicin • Pyrazinamide • Ethambutol
II. Fluoroquinolones	• Ofloxacin • Levofloxacin • Ciprofloxacin
III. Injectable drugs	• Streptomycin • Kanamycin • Amikacin • Capreomycin
IV. Second line drugs	• Ethionamide • *p*-Aminosalicylic acid • Cycloserine

Recently, MTB genome has been sequenced and different genetic technologies have been developed to manipulate and study MTB. Indeed, essential genes have been mapped and the biochemical function of several enzyme targets has been characterized. However, these findings have resulted inadequate to easy develop new drugs for the treatment of TB, due to all the complexities and the peculiarities of the mycobacterium and of the resultant infection. For example, granulomas, created by immune system, are dense structures poorly vascularized and are formed by acellular cores. These structures are a barrier to drug penetration, also hampered by the resistant MTB cell envelope[61].

Thus, finding new drugs against MTB is a challenge. There are two main needs: discover drugs against drug-resistant TB and find drugs which may allow shorter treatments compared with the actually used therapies. Many novel drugs are in clinical development, for example, PNU-100480, TMC-207, OPC-67683, PA-824 and rifapentine. Other drugs are in pre-clinical development and it is hoped that at least some of these will reach clinical development and patient testing. Bedaquiline and delamanid have received accelerated or conditional regulatory approval based on Phase IIb trial results (Figure 1.14), despite their cardiac toxicity and prolongation of the QT interval remain a problem[76].

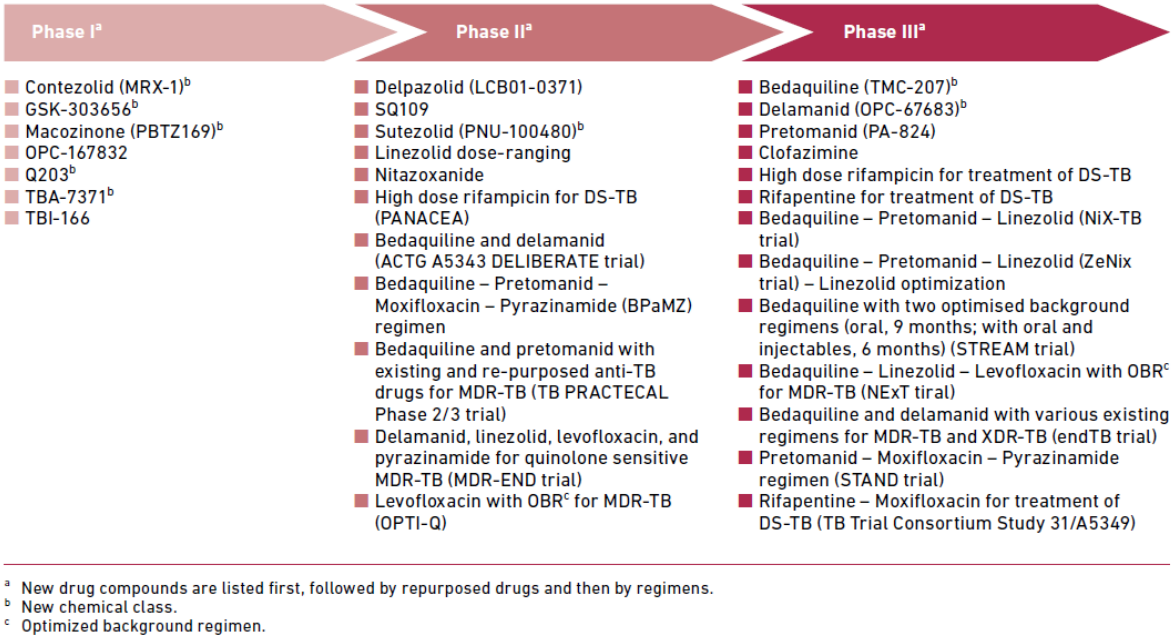


FIGURE 1.14: Development pipeline for new anti-TB drugs[77].

However, MTB has the strong ability of generate frequent and spontaneous chromosomal mutations and thus creating new karyotypes. Indeed, 6% of new cases of TB and 20% of retreatment cases are MDR cases, they are resistant to isoniazid and rifampicin. In 2016, 600000 new patients developed a resistance to rifampicin, the first-line drug, and 490000 cases of MDR-TB.

Three categories are recognized as drug resistant: 1) RR-TB is TB resistant to rifampicin and requires treatment with second-line drugs, 2) MDR-TB is TB resistant to both rifampicin and isoniazid, the two most potent TB drugs and therefore requires treatment with a second-line regimen and 3) XDR-TB is defined as MDR-TB plus resistance to at least one fluoroquinolone and a second-line injectable agent (amykacin, goatomycin or kanamycin).

Generally, the combination of three specific drugs is needed in order to avoid drug resistance[78,79]. It is thought that resistance to different antibiotics and chemotherapeutic agents could be related to the unusual cell wall of MTB.

1.3.3.2 BCG VACCINE

Vaccination is a more successful prophylactic tool to reduce the morbidity and mortality of a disease caused by infection and, since tuberculosis is one of the top ten causes of death worldwide, the prevention regime suggests the use of vaccine[80].

Currently, *mycobacterium bovis* BCG is the only licensed vaccine against TB, which is received by more than 3 billion people worldwide. Albert Calmette and Camille Guérin developed the vaccine at the Institute Pasteur, Lille, France from 1908 to 1921. It is an attenuated strain of *M. bovis* derived from a virulent strain after more than 13 years of continuous in vitro passage[81].

Now the BCG vaccine is prepared by genetic engineering and maintains its position as the world's most widely used vaccine, however, it is not effective and safe to satisfy today's needs. Many studies showed that BCG vaccine has a decent effect on children, where efficacy in the prevention of tuberculous meningitis and miliary tuberculosis is 73% and 77%, respectively[82]. However, when the vaccinated becomes teenagers and adults, the protective efficacy is controversial due to the fact that efficacy can vary from 0 to 80% in different countries and areas[83]. Generally, countries in North America can be well protected by BCG, while BCG's efficacy is particularly poor in tropical and subtropical regions[84]. Lalor MK argued that the environmental factors, maturity of the body's immune system, T-cell response, mother-to-child transmission related diseases, and other vaccination effects may lead to the difference[85]. Besides, the effect of BCG vaccine for HIV-infected individuals is problematic. Mansoor N. [86] suggested that HIV infection may seriously affect BCG-induced specific cellular responses and increase the risk of BCG-osis (infection due to the vaccine) in HIV-infected infants. Currently, TB is the leading cause of death in HIV-infected people because of the high incidence of dual infections and accompanying inhibition of the immune system by the two diseases. It was reported that co-infection with HIV and MTB increases the risk of developing TB 30-fold[87]. Lastly, an adverse reaction is another affair that needs to be well considered.

In general, BCG vaccine is relatively safe, and incidence of inflammation is less than 1‰, and the incidence of life-threatening BCG-related diseases is less than 2 per 1 million. However, with the increase in the diagnosis of immune-deficient diseases, the increase in disseminated pulmonary tuberculosis and severe disease-deficient disease, BCG has received much attention in the vaccination of immunodeficient infants[88].

Human antibodies induced by BCG vaccination protect human body against mycobacterial infection in different ways: 1) first, LAM-specific IgG production is increased; 2) phagocytic cells enhance internalization of BCG; 3) BCG-induced antibodies significantly increase the inhibitory effects of neutrophils and macrophages on mycobacterial growth; 4) BCG-induced antibodies augment IFN-γ production in mycobacterium-specific $CD4^+$ T cells and $CD8^+$ T cells, as well as the proportion of proliferating and degranulating $CD8^+$ T cells[70,89].

The biggest issue of BCG is that immunological memory after one vaccination wanes over time. A huge amount of data from meta-analysis of clinical trials supports this hypothesis[80]. Its protective efficacy wanes significantly over a period of 10–15 years[70]. BCG could not be used for a secondary booster immunization later in life; indeed, deep studies have demonstrated a very limited efficacy of revaccination with BCG in adolescents and adults[90].

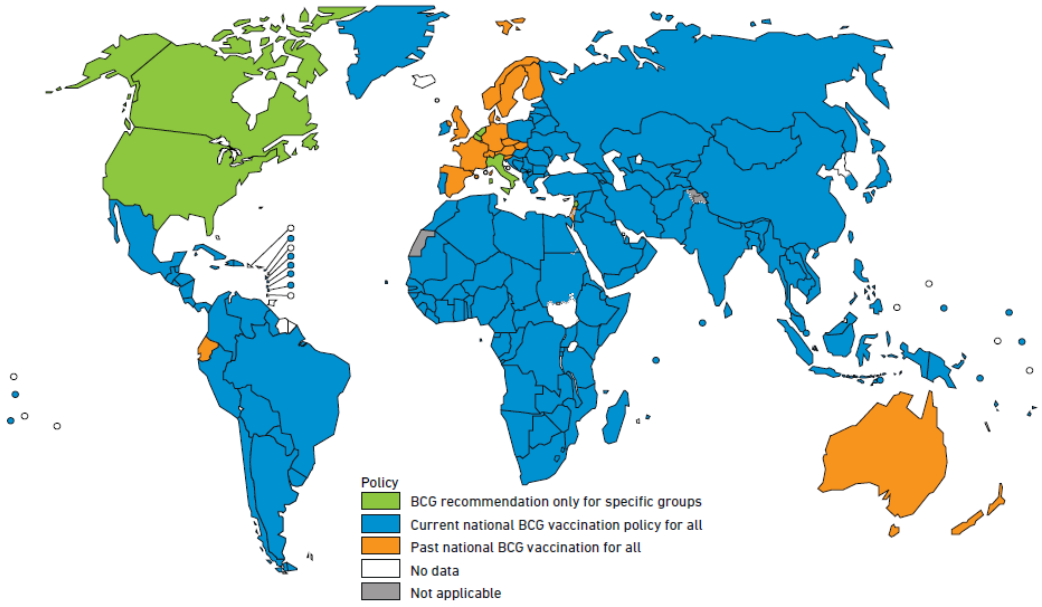


FIGURE 1.15: BCG vaccination which depends on the geographical areas.

1.3.4 NEW VACCINE CANDIDATES

Despite the high coverage of BCG vaccination in childhood immunisation programmes, the slow decline in TB incidence globally highlights the need for a much more effective vaccine that provides protection against all forms of tuberculosis in all age groups. There is currently no vaccine that is effective in preventing tuberculosis disease in adults before or after exposure to tuberculosis infection. However, in the last years scientists are studying this topic in order to find a solution and new type of TB vaccines[91].

1.3.4.1 CURRENT PROGRESS

Depending on the time of taking, there are two potential types of vaccines against TB nowadays, those given before and after exposure to pathogen. Vaccines given before exposure are called pre-exposure or prophylactic vaccines, while those given after exposure are called post-exposure or therapeutic vaccine[92]. Two strategies are commonly considered to develop the pre-exposure

vaccine. The first strategy is designing a longer-lasting and more protective vaccine to replace BCG using novel recombinant BCG or attenuated *M. tuberculosis* vaccine. The other strategy is hammering at boosting and prolonging BCG's immunity in individuals who have already been BCG vaccinated[93]. So using BCG for priming and a subunit vaccine as a booster may provide a direction for pre-exposure vaccine's development. Post-exposure vaccination is targeting to latently infected individuals accounting for over one third of the world's population. Since BCG was once used as a therapeutic vaccine against active disease and caused many deaths from treatment, antigens need to be well considered before using.

However, now it is commonly thought that carefully selected antigens will not cause safety problems as a therapeutic vaccination in latently infected individuals[94]. The reverse vaccinology approach allowed the development of protein-based sub-unit vaccines and new BCG vaccines over expressing selected antigens[95]. Furthermore, the characterization of the epitopes of these proteins inspired the rational design of new vaccine products including chimeric proteins obtained by genetic fusion of different antigens, as well as DNA or RNA based vaccines. The subunit vaccines are commonly composed by one or more antigens selected among the pool of protein secreted by MTB. In fact, this pathogen secrete more than 30 different proteins[96]. The most abundant proteins-antigen secreted by MTB is the protein-complex Antigen (Ag) 85, but other predominant antigens are proteins belonging in the ESAT6 family and Mpt64. The Ag85 complex is a 30-32 kDa family of three mycolyl transferases (Ag85 A, B and C) involved in the coupling of mycolic acids with the arabinogalactan in the cell wall[97]. Ag85B is the most powerful *M. tuberculosis* antigen and induces both humoral and cell-mediated immune response. For this reason, it has been considered for the development of most of the new vaccines products under clinical investigation.

Various studies were performed for the characterisation of Ag85B epitopes and in some case homologous such as Ag85B from *M. Bovis* or *M. Smegnatis* were considered[97-101]. As reported in table 1, for T-cell activity human subjects and different animal models have been investigated, while for B-cell activity studies were carried out in mouse. Different sequences have been indicated as putative T-cell epitope (Table 1.2) in the region included between positions Leu11-Ala35 and Ala90-Arg113 while two sequences (Leu101-Arg113 and Ser126-Pro140) have been indicated to be able to stimulate both the T and B cell antigenic activity against Ag85B (Ser126-Pro140 and Thr261-lys275).

The ESAT6 family includes some low-molecular-mass proteins such as ESAT-6, TB10.4 and CFP10 strongly recognized by T cell[102]. About ESAT-6, nine synthetic 20AA peptides were prepared and

tested[103], allowing the identification of two sequences with T-cell activity: ESAT-6_{51-70} (YQGVQQKWDATATELNNALQN), and ESAT-6_{1-20} (MTEQQWNFAGIEAAASAIQ). It was also discovered that the ESAT-6_{1-20} sequence also include an epitope for B-cells[104]. Recently, in the case of TB10.4 two in silico methods indicate the sequences Met2-Met5, His14-Tyr21 and Gly13-Thr24 as putative epitopes in the N-terminal region, Gln37-Gl41 in the central part of this protein and one epitope near the C-terminal region (Val64-Thr88)[105].

Finally, in the ESAT6 family, CFP10 is an important protein because it is not present in BCG and consequently can be associated to BCG or used as boost for subject already vaccinated with BCG. This protein induces both CD4 and CD8 T-cell immunity and Li et al synthesized and tested 26 peptides[106] allowing the identification of four epitopes: CFP10 $_{35–43}$ (TAGSLQGQW), CFP10 $_{75-83}$ (NIRQAGVQY), CFP10 $_{3-11}$ (EMKTDAATL) and CFP10 $_{13-21}$ (QEAGNFERI).

There are several studies also for the characterization of T-cell[107] and B-cell epitopes[108] of Mpt64, including in *silico* predictions[109], while the research of other MTB protein-antigens has been reported[110] and is object of continuous investigation.

Many antigenic proteins have been considered for the development of new vaccine products against MTB. Nowadays, although no vaccine can totally replace BCG's role in vaccination, several vaccines candidates are evaluated in phase I, II or III trials and some of them are likely to be substitutions in the future.

TABLE 1.2: . Epitopes T and B of Ag85B already reported in literature.

Epitope	**Sequence in Ag85B of MTB**	***Mycobacterium***	***Host***
T-Cell (CD4)	LQVPSPSMGRDIKVQFQ (11-27) LQVPSPSMGRDIKVQFQSGG (11-30) GRDIKVQFQSGGNNSPA (19-35) AGCQTYKWETFLTSE (90-104) CQTYKWETFLTSELPQW (92-108)	*M. Tuberculosis*	Human
	LTSELPQWLSANR (101-113) SMAGSSAMILAAYHP (126-140) THSWEYWGAQLNAMK (261-275)	*M. Tuberculosis*	Mouse
	YYQSGLSIVM (61-70)	*M. Bovis*	Cattle
	MPVGGQSSFY (70-79)	*M. Bovis*	Human
B-Cell	LTSELPQWLSANR (101-113) SMAGSSAMILAAYHP (126-140) THSWEYWGAQLNAMK (261-275)	*M. Smegnatis*	Mouse

1.3.4.2 NEW VACCINES

The vaccines currently studied (Figure 1.16) can be divided in three categories depending on the stage of infection they are intended to be used.

1) Those administered before infection, preexposure or priming vaccines are studied essentially as replacements for BCG. Infants are generally the target population for this category. As BCG belongs to this category, it is required that priming vaccines have an efficacy at least equal to BCG and generate longer-lasting immunity than BCG. The lead candidates for this approach are genetically modified attenuated MTB or recombinant BCG. The supplement of antigens absent or under-expressed in the current BCG vaccine strains or the addition of proinflammatory genes such as that for IFN-γ are studied with the purpose of generating long lasting memory response. VPM1002 and rBCG30 are two examples of recombinant BCG candidates. rBCG30 was developed by recombining the MTB secretory antigen Ag85B; however, as it contained an antibiotic resistance marker, its development was stopped[111]. VPM1002, studied with the purpose of enhancing major histocompatibility complex class (MHC)-I-related immune responses, it has demonstrated to be safe, well-tolerated and immunogenic in new born infants[112]. It successfully passed Phase I trials and completed the Phase II randomized clinical trial in healthy, HIV and non-HIV infants in South Africa. Now in India a phase II/III trial is ongoing for the prevention of tuberculosis in adults.

   The first live attenuated vaccine entered in clinical trials in January 2013, it is a derivative of genetic modification of MTB: it is called MTBVAC[113]. It is the only vaccine able to induce specific immune responses for CFP10 and ESAT6 and these responses are effective in protecting against pulmonary tuberculosis[114]. It seems to be safe, well-tolerated and highly immunogenic in adults. It is proposed as a preventive vaccine in children[115].

2) Vaccines intended to be given to infected or vaccinated individuals, frequently adolescents and adults with latent TB infection (LTBI), are called booster or postexposure vaccines.

   They can supplement BCG or another priming vaccine, increasing immunity generated by these prior vaccinations. Different possibilities have been proposed, such as recombinant proteins or DNA, antigens expressed in nonmycobacterial vectors such as viruses, protein–adjuvant combinations, composed of fusion proteins in appropriate adjuvant[61,90,116].

   Subunit protein vaccines is another approach, they are composed principally by antigens. They can be divided into recombinant adjuvant proteins, such as M72/AS01E and H56/IC31 and viral vector systems. M72/AS01E vaccine combines two MTB antigens (32A and 39A) with an adjuvant (AS01E), with monophosphoric lipids A and QS-21 in a liposomal

formulation. Although some adverse events have been reported due to the adjuvant AS01E, they were not so severe and can be resolved within one week, and may reduce the development of active disease[117]. H56/IC31 is composed by the fusion protein H56, that contains Ag85B, ESAT6 and Rv2660c antigens, and adjuvant IC31. It has an acceptable safety and tolerability profile, no serious adverse events have been reported[118].

ChAdOx185A – MVA85A, Ad5 Ag85A and TB/FLU-04L are viral vectors systems, they are like their viruses, highly immunogenic, and are able to stimulate both CD4 and CD8 T-cell responses. ChAdOx185A-MVA85A is an adenovirus; it is the first new post-exposure tuberculosis vaccine to be subjected to clinical trials in 2002. MVA85A is a recombinant modified vaccinia virus Ankara expressing the immunodominant MTB Ag85A[119].

Ad5 Ag85A is a recombinant non-replicating adenovirus expressing the Ag85A protein. Its safety and immunogenicity have been tested on healthy people with and without BCG immunization, and it has been confirmed that it is safe, well tolerated and immunogenic.

TB/FLU-04L is a mucosal vectored vaccine containing influenza virus with an attenuated replication deficiency and expresses the Ag85A and ESAT-6 antigens[120].

Its safety and immunogenicity have been explored in healthy adults with BCG vaccination and no serious adverse effects have been reported, and a study in Latent TB Infected (LTBI) peoples is ongoing[77], [91], [120].

3) Therapeutic vaccines are designed to treat patients with active TB in adjunct to chemotherapy or patients suffering from XDR and MDR TB[80]. Two vaccines are in clinical trial:
   a) vaccae, a whole heat-inactivated mycobacterium vaccae art. 112; it is proposed that M. vaccae promotes a Th1 response, overcoming the pathogenic Th2 response of individuals with tuberculosis by providing epitopes to stimulate a protective immune response[121]. The vaccine induces strong Th1 immune responses, and is safe and immunogenic in adults with HIV infection and vaccinated with BCG.
   b) RUTI®, an inactivated TB vaccine formed by detoxified and fragmented MTB, is designed to be used in conjunction with a short intensive antibiotic treatment [122]. It has a good safety profile[123] and it is promising for further development for use in LTBI. A study is underway in patients with MDR TB.

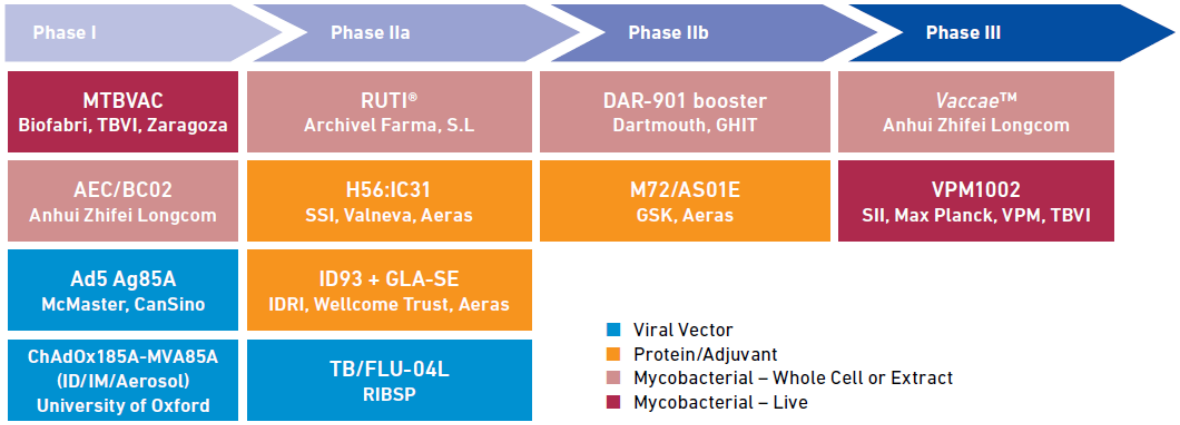


FIGURE 1.16: Global development of new tuberculosis vaccines[77].

1.3.4.3 GLYCOCONJUGATE VACCINES

A further improvement in the development of efficient subunit vaccine could be the design and the study of *neo*-glycoproteins obtained by the chemical conjugation of protein antigens with antigenic oligosaccharides.

The first example of semisynthetic glycoprotein (*neo*-glycoprotein) vaccine against TB was the carbohydrate-protein conjugate vaccine obtained using the arabinomannan (AM) portion of LAM. After removing the lipid portion from the natural MTB lipopolysaccharide the AM portion obtained was covalently linked to Tetanus Toxoid (TT) similarly to that performed for vaccine against other pathogens. In addition, AM was conjugated also with different MTB antigenic proteins, including Ag85B[83]. In this case, the conjugation with proteins was performed via a thioether linkage method previously described by Pawlowski et al[124] (Scheme 1.3) that can be efficiently applied to saccharides with free reducing terminal groups.

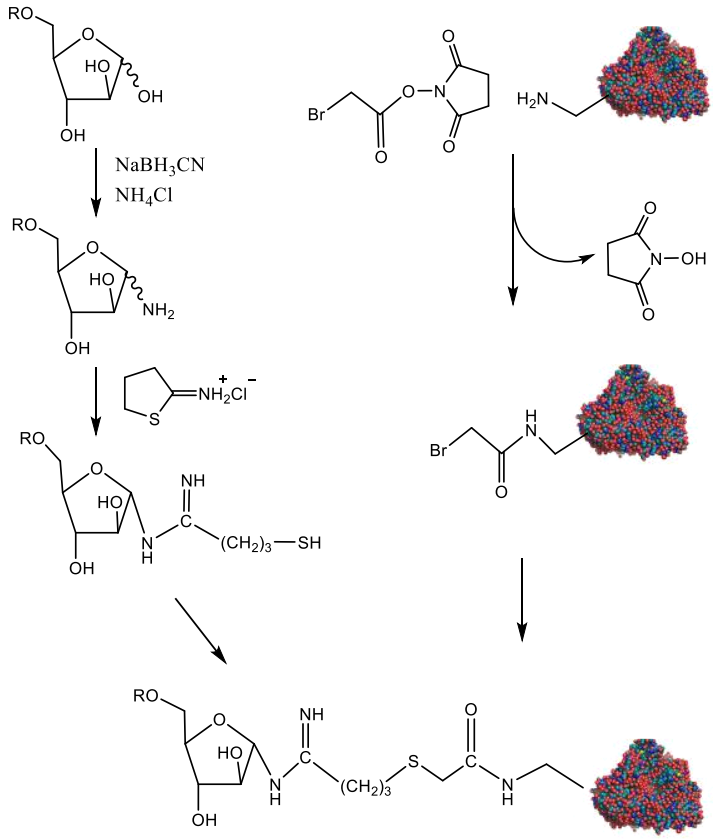
The studies performed in different animal models showed that the AM–protein conjugate all induced vigorous IgG-Ab responses specific for AM improving the survival compared with control groups[83]. The experimental AM-protein conjugate vaccines induced both cellular and Ab responses against MTB, but the antibody response was reported to be the main protective component. In fact, the immunization effect observed seems related to a T-cell-dependent B-cell activation that induce the production of AM-specific antibodies, which efficiently reduce the depressive effects exerted by LAM towards the immunological system during the infection.

In this context, the conjugation of AM with recombinant MTB antigenic proteins could be considered an innovative strategy for the development of highly efficient vaccines, by combining

the activity of different antigens. However, this approach could be affected by the glycosylation of the epitopes that reduce the antigenic activity of the protein[22].

For this reason, Temporini et al[4]developed an analytical strategy, combining enzymatic digestions and LC–MS, for a complete characterization of *neo*-glycoconjugate products. This study was first performed using recombinant TB10.4 (rTB10.4) having only a lysine residue and a seven aminoacid N-tag the seven amino acids pre-sequence (AMAISDP) originating from the cloning procedure and expression[105].

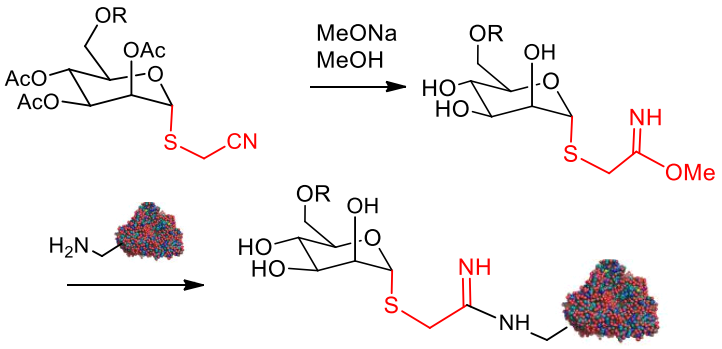
The analytical approach proposed allowed to define the efficiency and selectivity of the glycosylation process (two glycosylation method have been compared). In particular, the HPLC-MS analysis of the peptides and glycopeptides obtained after enzymatic digestion of each neo-glycoconjugate provided information concerning the glycosylation sites and their relative abundance.



SCHEME 1.3: Conjugation method via thioether linkage. R=Arabinomannane (**AM**).

TB10.4 has only one lysine (K103 in the rTB10.4). When glycosylation was performed via iminomethoxyethyl thioglycoside (IME) (Scheme 1.4) the reaction was highly specific towards this Lysine. Consequently, based on the previous optimization of the glycosylation condition, it was possible to achieve almost complete glycosylation of rTB10.4 allowing the production of only one glycoform, obtained by reaction of the sole lysine (K103) of this MTB protein. This aminoacid (corresponding to lysine 96 in the sequence of the WT TB10.4) is not included in the putative T-cell epitopes proposed for this protein. Accordingly, the T-cell activity of rTB10.4 was not affected after glycosylation[125].

The effect of the glycosylation on the antigenic activity of rAg85B has recently been also investigated using a recombinant form of this protein (Figure 1.17) expressed in *E. coli*. Similarly to the rTB10.4, compared with the Wild Type protein, the recombinant form of Ag85B (rAg85B) present the seven amino acids pre-sequence (AMAISDP) originating from the same cloning procedure and expression above described for TB10.4[105].



SCHEME 1.4: Glycosylation *via* iminomethoxyethyl thioglycoside (IME).
R= H or α-D-Mannose; Protein= TB10.4 or Ag85B.

In Figure 1.17 the primary sequence of rAg85B is reported with all the T or B epitopes already identified (Compared with the WT protein the amino acids numeration of the epitope sequences is shifted of 7 positions according to the presence of the 7AA pre-sequence). This protein includes 8 lysine residues and, according to Table 2, many of these are included in T and/or B epitopes.

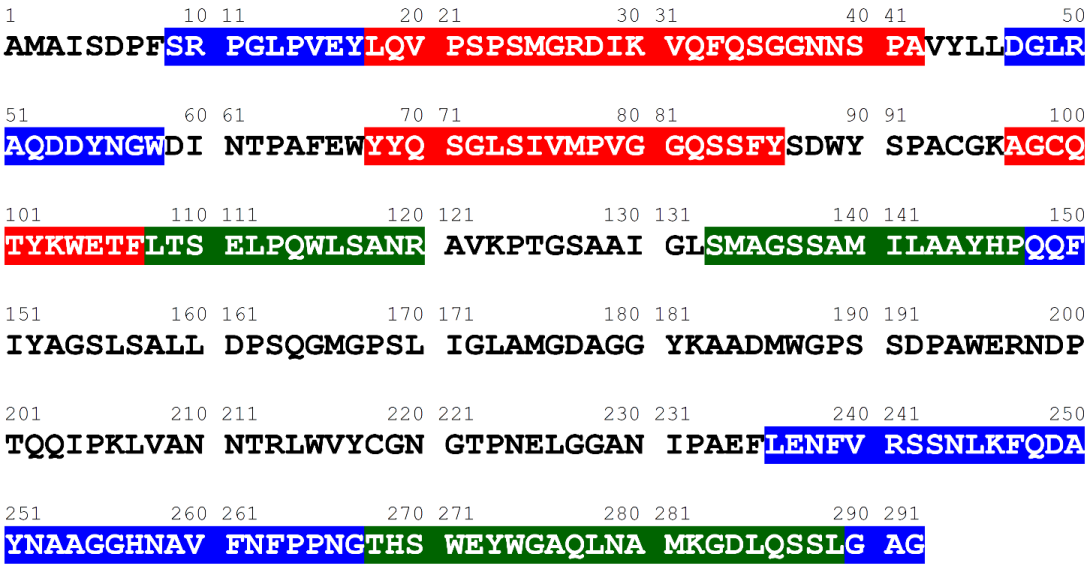


FIGURE 1.17: B-cell epitopes in Blue. T-cell epitopes in Red. Both B- and T- cell in green.

Unlike TB10.4, in this case, the immunogenic activity strongly decreased after glycosylation with mono and disaccharides activated with IME group (Scheme 1.4). A strong reduction of the T-cell activity was in fact observed because the two mayor glycosylation sites, K_{30} and K_{282} (corresponding to K_{23} and K_{275} in the sequence of the wt protein) are included in two important T-cell epitopes[125].

Also, B-cell activity decreased after glycosylation as observed in another study[126] performed by SPR analysis of the affinity of the *neo*-glycoproteins obtained by glycosylation of rAg85B for specific antibodies. This effect of the glycosylation can be explained considering that one of the two main glycosylation sites, K_{282} of rAg85B (K_{275} in the wt protein), is included also in a B-cell epitope (Figure 1.17).

Rinaldi et al. [127]also observed that substituting Lysine K_{30} and K_{282} of rAg85B with arginine, the antigenic activity of the protein was preserved. In fact, single (K30R and K282R) and double (K30R/K282R) point variants of rAg85B were cloned, produced and tested by ELISPOT and the K/R mutation resulted conservative for the antigenic activity of the protein. In addition, arginine is not reactive with IME-glycosides and, consequently, glycosylation of the mutated position 30 and 282 was avoided[127] Accordingly, by ELISPOT test was observed that the glycoderivative of the mutated proteins, unlike the rAg85B, maintained their T-cell activity after glycosylation with IME-glycosides.

This result confirms that K_{23} and K_{275} of wt Ag85B are important residues involved in the T-epitopes of this protein and that the mutant forms of rA85B (with arginine in position 30 and 282) could be considered as optimal antigenic carriers for the development of new glycoconjugate vaccine against TB obtained by conjugation of MTB antigenic proteins with LAM or LAM-mimetic synthetic oligosaccharides.

1.3.4.4 NANO-VACCINES: ELASTING-LIKE RECOMBINAMERS

A new emerging field, the engineering of materials that can modulate the immune system, was developed over the last years. Biomaterials, now being engineered to delivery antigens, have become a key target of research towards vaccine delivery and above all as encouraging candidate adjuvants to improve vaccine efficacy [128,129]. Their particle sizes, particle shape, hydrophobicity and others properties can influence the nature of the immune response and its magnitude.

Among these new materials, elastin-like recombinamers (ELRs) stood out thanks its advantageous characteristics helpful in vaccine delivery. Elastin and its precursor tropoelastin explicate their duty in providing elasticity and resilience to the tissues, thanks to their polymeric peptide sequences. Moreover, they are able to self-assemble in determinate conditions. These features can be exploited for different biological applications. Thanks to Dan W Urry's work[130], demonstrating the versatility of these motif, several "elastin-mimetic" and "elastin-like" polypeptides started to be studied and produced.

The use of recombinant bio-synthesis makes these new materials even more interesting and advantageous (no organic solvents traces, reproducibility and high yields, no problems in introducing even complex variations, etc.). The new term "recombinamers" was adopted in place of polymers, to clarify the origin and the main characteristics of this new class of materials.

ELRs are composed of a pentapeptide (VPGXG) repeated several times, where X is any amino acid but not proline[131]. The ELRs have allowed the formation of a wide range of biomaterial-based structures, in a wide range of sizes, morphologies, and functional possibilities, thanks to elastin's elasticity and ability to self-assemble. Some of the important features that allow the ELRs to be an exceptional candidate as a carrier in the field of vaccine developments is its lack of immunogenicity as well as their biodegradability and biocompatibility for humans.

Moreover, the nature of ELRs allows the formation of different amphiphilic elastin-like block core combinamers (ELbcRs), which can self-assemble with high reproducibility and homogeneity, into highly biocompatible, multivalent, monodisperse, and stable nanoparticles with a hydrodynamic diameter of (≈ 50- 60) nm. The self-assembly is a thermally driven process in an aqueous medium (when T> transition temperature), as illustrated in (figure 1.18) and this transition temperature depends on the composition of the material (it can be controlled by adjusting their hydrophobicity and molecular weight). Thus, when it is in aqueous solution above this critical temperature, the hydrophobic moiety aggregates forming a nano-structure that exposes the hydrophilic part in contact with water[132-134].

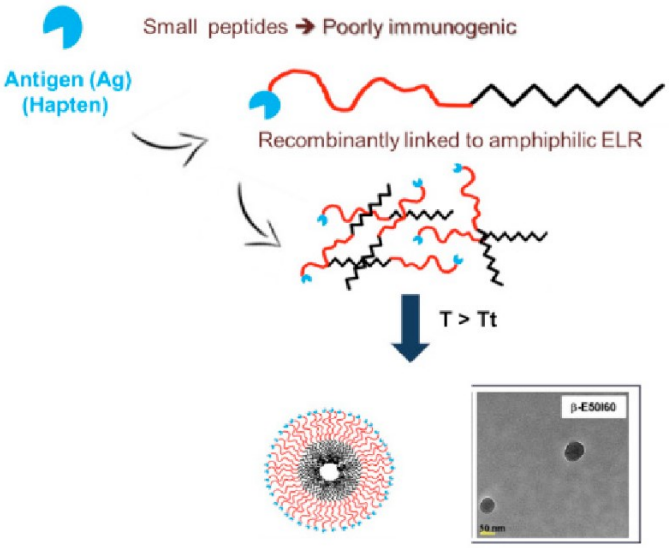


FIGURE 1.18: Self-assembly of the ELR into spherical nanoparticles displaying a corona of antigens from a M. tuberculosis major membrane protein[134].

By genetically encoded synthesis it is possible to incorporate an antigenic molecule at the hydrophilic terminus of an ELbcR. Theoretically in this way, the antigenic portions is well-exposed on the surface, while the elastin support (just a vehicle) remains hidden from the immunity system. Moreover, decorating this nanoparticle with the antigenic sequences, just using recombinant synthesis, avoids the use of bioconjugate chemistry to link proteins to the nanomaterial.

Another quality of ELRs that can be exploited include the stability during secondary processes (sterilization, lyophilization, packaging and reconstitution of the dried powder), so no cryoprotectants and cold chain are necessary for their stockpiling.

A first application of these new biomaterials for Tuberculosis was shown by Flos D.M.' et al.[135], describing the expression and immunogenicity of a fusion protein composed by a elastin-like polymer with two important antigens, previously seen (ESAT-6 and Ag85B).

The protein was produced in transgenic tobacco plants (treated by inverse transition cycle) and was tested in mice. The rate of antigen expression increased, but the polymers tested did not allow efficient antigen release so adjuvants were added in the formulation. However, poor results were obtained even increasing the dose.

Conversely, García-Arevalo et al. [134],working with ELbcR, demonstrated its positive contribute as potential TB vaccine vehicle. In this work was tested an immunodominant antigenic sequence (belongs to 16 Kda, one of the major membrane constituent of MTB) recombinant synthesized with an ELbcR, composed of a hydrophilic block based on glutamic acid (E) as guest residue at the amino terminus (E50) and a hydrophobic block based on isoleucine pentamers (I) at the carboxy terminus (I60). The antigenic sequence included in this recombinant molecule (β-E50I60) is "$_{92}$EFAYGSFVRTVSLPVGADE$_{110}$", the main epitope of 16Kda previously identified by Bosze[136]. In this study was developed a new promising potantial ELBcR-based vaccine for Tuberculosis. It favors a long presented antigen half-life in circulation, increasing time available for the uptake and processing. The assay in mice showed a biphasic response : an innate immune phase due to an early secretion of chemotactic cytokines followed by and adaptive immune phase due to induction of a T helper 2 responses (IL-5 production and up-regolation of IgM and IgG). Therefore, it was confirmed that the optimum characteristics, just described, makes ELBcR highly suitable for further studies as vaccine vehicle.

1.4. FULLERENE

1.4.1. MAIN CHARACTERISTICS

1.4.1.1 DISCOVERY

Only three allotropes of carbon were known until 1985: diamond, graphite and amorphous carbon. In 1985 was discovered another type, C_{60} Fullerene, a soccer ball shape pure carbon structure and later carbon nanotubes as spin-off product of C_{60}.

Kroto et al. discovered it during some experiments about carbon involvement in space and stars processes[137]: “Buckminsterfullerene” (Fullerene in short), called in this way with reference to architect Buckminster Fuller, who designed geodesic domes with a structure comparable to the structure of this carbon compound.

Some scientists before him hypothesized existence of another carbon structure[138] (maybe a cage of carbon), it was also produced a carbon cluster beams but without isolating and identifying any compounds[139]. Only Kroto’s team was able to synthetize and above all isolate it. They suggested also a hypothesis of fullerene structure (without having any analysis tool yet to verify it[140]. They won a Nobel prize in 1996 for this discovery.

This new material due to its size and composition has attracted attention of scientist belonging to different fields. The discovery opened also a new study field; some scientists are still working for finding an efficient method for fullerene synthesis.

As written before, Kroto and his team were the first scientists able to produce C_{60} by vaporisation of carbon species.

In 1990 Kratschmer devised the “Kratschmer–Huffman method” for gram-size fullerene production: it consisted in evaporating graphite electrodes via resistive heating in an atmosphere of about 100 torr of helium. After few years, other applications based on this method was patented (for example Smalley’s[141]).

Other strategies are “combustion processes” and “chemical approaches”, by using high energy laser and surface-catalysed cyclodehydrogenation process[142] or the pyrolysis of naphthalene[143].

Even if fullerene increasingly is studied and used in many research fields and for many applications, the amount of produced fullerene remains low and this affects the price of the material that is still expensive.

1.4.1.2 STRUCTURE

Fullerenes are generally represented by a formula Cn, where n is the number of carbon that makes up the cage, and it can be classified into “classical” or “non classical” fullerene. The first one is a closed carbon cage containing hexagons and 12 pentagons, while a nonclassical fullerene can have heptagons, octagons, and an additional number of pentagons or squares.

Theoretical calculations predicted that fullerenes larger than C_{76} should have at least two isomeric forms. Fullerenes with n> 84 are difficult to isolate.

C_{60} and C_{70} are the most representative of the fullerene family: the first one is perfectly round (as football ball), whereas C_{70} is oval (as rugby ball) (Figure 1.19).

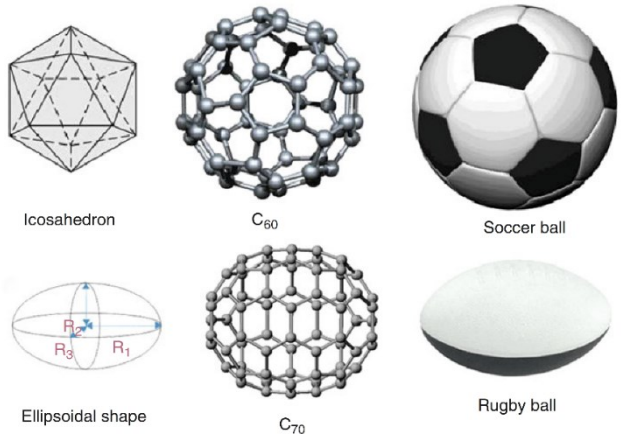


FIGURE 1.19: C_{60} and C_{70} representation[144].

Since 1985 more and more studies and analysis have been done to verify and understand this new material.

Until 1990 Fullerene remained a matter of theory because the spectroscopy analysis was the only method to detect it: they hypothesized a truncated icosahedron, a polygon with 60 vertices and 32 faces, 12 pentagons located around the vertices and 20 hexagon rings placed in the middle of icosahedral faces.

Many studies focused on stability and on the fact the truncated icosahedron is the most probable geometrical figure to guarantee this stability[145]: empirical rules that link the stability to the disposition of pentagonal rings[146] prove the C_{60} structure.

After some years also experimental studies had confirmed this structure theory (by using analytical instruments as DCC, mass spectrometry and NMR)[147].

C_{60}, the smallest stable fullerene, has also high symmetry, confirmed by ^{13}C-NMR analysis and studies on crystalline C_{60}[148].

The IUPAC name is (C_{60}-I_h)[5,6] Fullerene that describes exactly the number of carbon 60, the symmetry and the ring sizes that make it up (pentagon and hexagon explained by number 5 and 6)[149].

The diameter is 10.34 Å. There are two different type of bonding: [5,6] bond has a single bond character, of 1,45 Å between pentagon and hexagon; the second is [6,6] bond of 1,38 Å between two hexagons, has a double bond character. The reactivity of fullerene depends on this last bond (Figure 1.20).

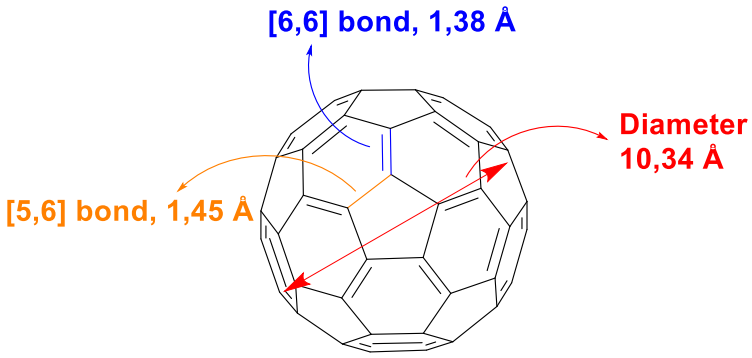


FIGURE 1.20: Fullerene size.

1.4.1.3 SOLUBILITY AND BIOLOGICAL PROPERTIES

Solubility is an important parameter to evaluate fullerene for possible chemical transformations and reactions. Ruoff R.S. et al. [150] checked the solubility of C_{60} in different solvents:

- completely insoluble in polar solvents and H-bonding solvents (THF, Acetone, MeOH etc..);
- sparingly soluble in alkanes (Pentane and hexane and also cyclic ones) with the solubility increasing in Chloroalkanes (DCM, Chloroform).
- appreciably soluble in aromatic solvents (benzene, toluene etc..), the introduction of bromine or chlorine on the ring increases the solubility (o-dichlorobenzene is the best solvent with the highest solubility).

These data are also confirmed by computational studies[151].

Pure C_{60} is highly hydrophobic and soluble only in organic solvent, even just in aromatic ones. This is incompatible with a possible human assumption. Moreover, the lack of solubility in aqueous solvent leads to the consequent formation of aggregates.

This problem can be solved with chemical or supramolecular approach: by using cyclodextrins, by co-solving with PVP in chloroform or by using artificial lipids membrane[152].

The most common method regards modifications of surface by addition of chain and chemical groups that change the physical and chemical property of the compound.

Depending on the choose of attached group, the derivatization increases the solubility in organic or aqueous solvent. The increase of solubility in water leads to a possible application in human body. In addition, it seems that the toxicity decreases with increasing of functionalization on fullerene[153].

Even if fullerene was discovered more than thirty years ago, it is considered a "new material" because chemical modification and the introduction of fullerene derivatives in biomedical application is recent. Consequently, the toxicity is one of these characteristics that are still studied.

The pure C_{60} potential toxicity is due to three factors[154]:

- Its dominant apolar character and its ability to penetrate and merge with biological membrane.
- The propensity to form aggregates that can have an impact mainly on the environment. [155].
- The capacity of reacting with a lot biochemical compounds.

The fullerene ability of reacting allows the change of chemical and physical properties. The addition of some polar groups gives fullerene the possibility to interact with aqueous environment and avoids the aggregations. Colvin et al. hypothesized that toxicity decreases as much as fullerene is soluble in water and it is derivatized[153], confirmed and stated also by other studies and works[156-158]. Indeed, fullerene has been investigated, for example against cancer[153].

Actually the toxicity depends on several features as dose, concentrations, time-dependent but also the type of fullerene (C_{60}, C_{70} etc..), the functional groups used for derivatizing it, and the method of administration[159].

So the crucial aspect is conducting a specific toxicity assay on the final product of interest[160-161].

1.4.2 CHEMICAL MODIFICATION

The unique geometry, the high symmetry and shape of fullerene is at the basis of its reactivity.

This particular shape forces carbons to have no planarity that change the hybridation of sp2 σ and π orbitals [162]; this leads to a sort of deactivation of the aromatic nature and so a different

reactivity too respect a normal aromatic compounds. It's better to use the term "pseudo-aromaticity".

Fullerene is considered an electronegative molecule because of its low LUMO level and the electronical effects just described. This electronic acceptor character makes it appropriate for addition and redox reactions.

The [6,6] bonds are the main reactive part that can be easily reacted with nucleophilic groups. The addition can be verified in different positions: 1,2 positions (for no bulky reagents) and 1,4 position (principally for bulky reagents because of steric effects)[163]. (Figure 1.21)

It could be considered a very good dienophile ready to form a cycloaddition.

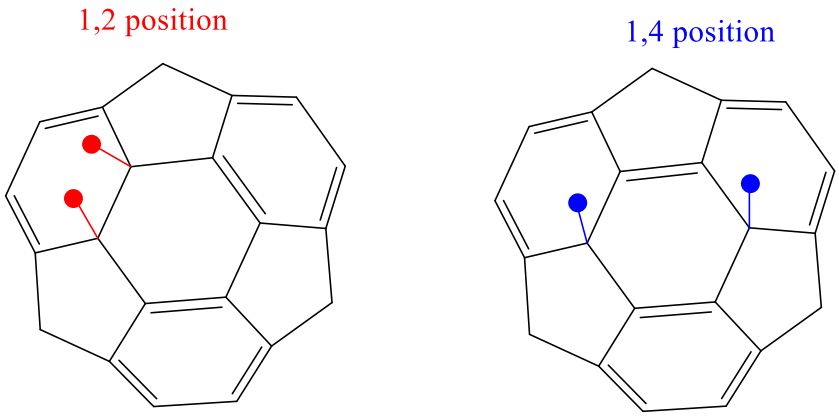
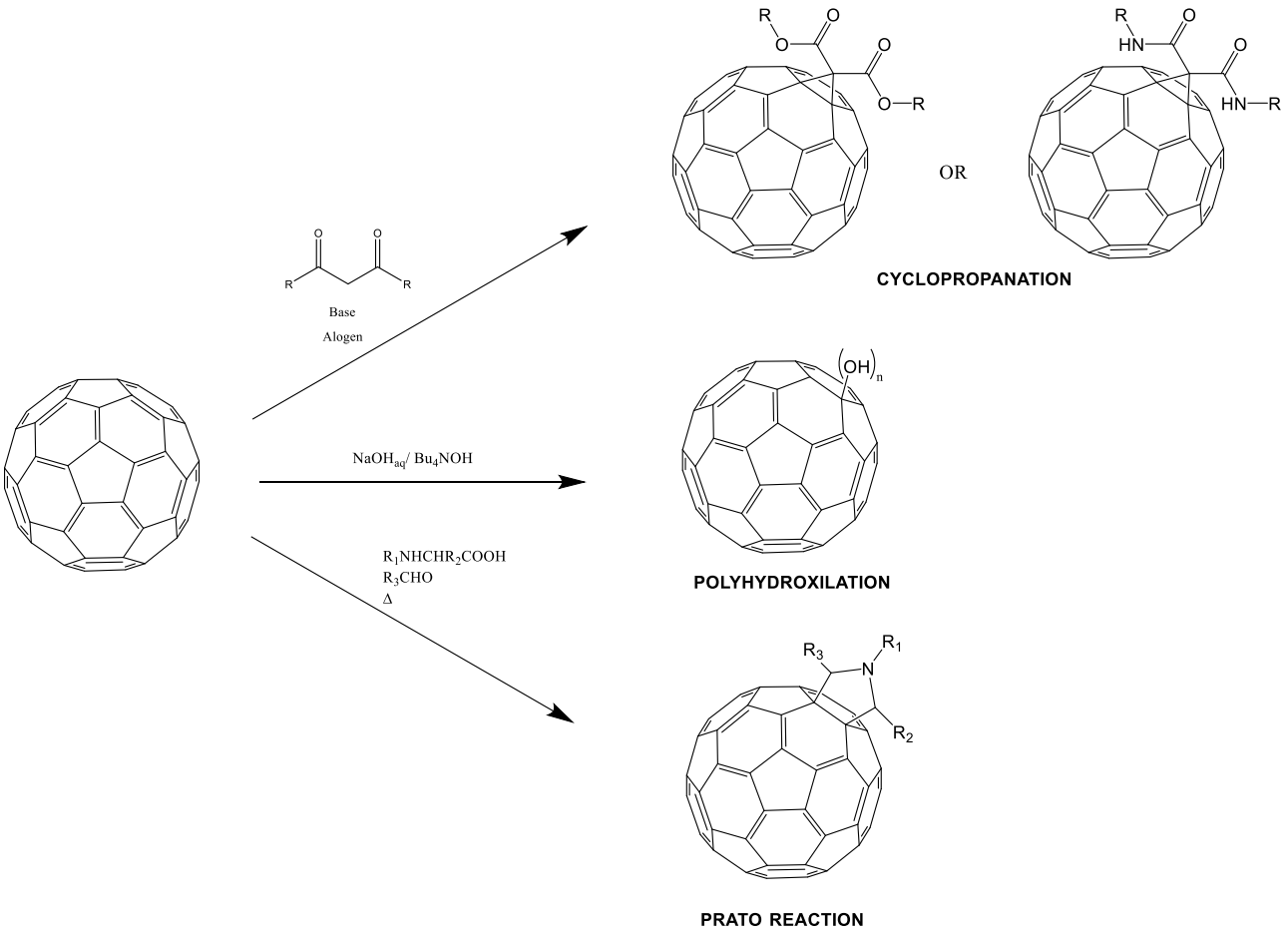


FIGURE 1.21: Position for nucleophilic addition.

1.4.2.1 MAIN STRATEGIES OF CONJUGATION

As written above, different strategies to make fullerene compatible with a drug delivery applications are necessary for using it for human and biological approaches.

The most versatile methodology for doing this seems to be the chemical modification of the surface. In order to make fullerene soluble in water and aqueous solvents, a lot of different functional group have been used to increase the hydrophilicity (-OH,-NH_2, -COOR). There are three main synthetic approaches to obtain these derivatives (scheme 1.5): the cyclopropanation, polyhydroxylation and cycloaddition azomethine ylides (known also as Prato reaction)[164].



SCHEME 1.5: Main synthetic approach.

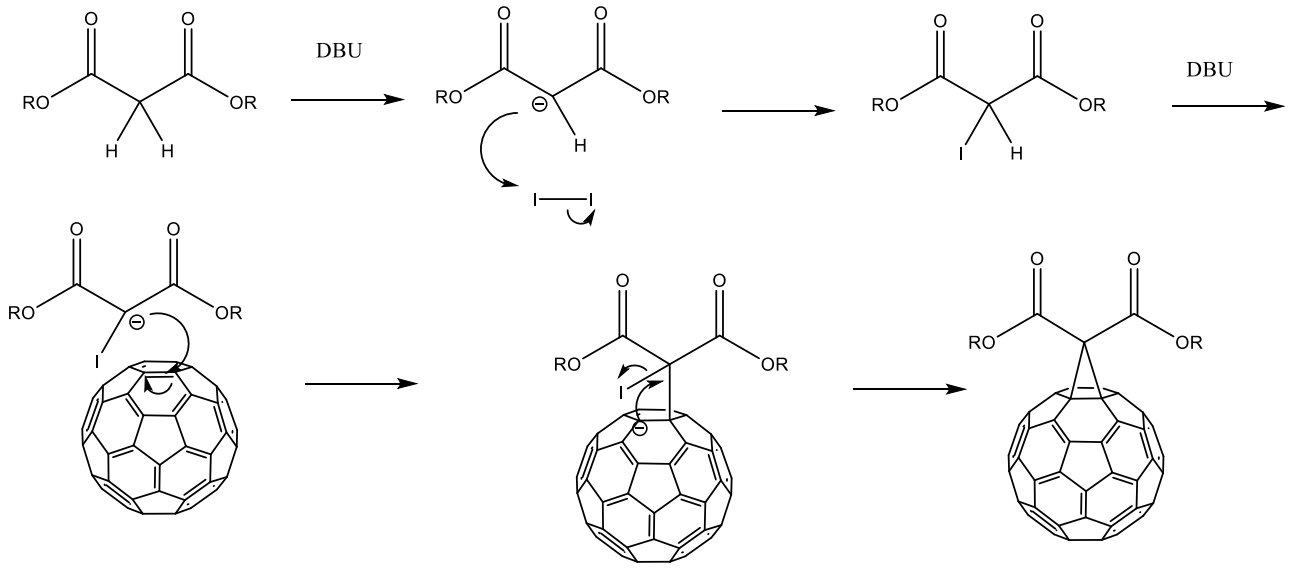
The cyclopropanation strategy is the most used. It can be obtained by different methods as the addition of frees carbenes, the terminal addition of diazo compounds and the nucleophilic cyclopropanation with addition-elimination mechanism. The last one is the most common strategy (gives the best yields) and it's also known as Bingel reaction[165].

It was discovered by C. Bingel in 1993 , he described a kind of Michael reaction between an α-halo carbanion with fullerene followed by intramolecular substitution of halogen in order to form a methanofullerene[166].

Initially NaH was used as base for the reaction, afterwards also other bases were employed like pyridine, triethylamine and sometimes also a stronger base as LDA (lithium diisopropylamide)[167].

In order to improve the yields, some other conditions was tested and the next idea was a one-step reaction, using α-halomalonates generated *in situ* (in presence of I_2 or CBr_4) and DBU (1,8-diazabicyclo[5.4.0]undec- 7-ene) as base. The use of I_2 is preferable in mono-addition (maximum three conjugations), otherwise tetrabromomethane for higher adducts[168].

The mechanism is always the same: the base removes an acid proton from malonate forming a carbanion which reacts with the halogen building an α-halomalonate. Again the base removes the other malonate acid proton and the new carbanion attacks the electron deficient double bond of fullerene. The carbanion just formed on fullerene removes the halogen by intramolecular nucleophilic substitution (Scheme 1.6).



SCHEME 1.6: Bingel reaction mechanism.

These strategies are a starting point for further methods and applications.

For example by using Prato reaction you can introduce significant functional group and be able to link also peptide on fullerene: Aroua S. et al., starting by Bis-*t*-butyl ester, prepared a scaffold for obtaining soluble-water fullerene as peptidofullerene or C_{60}-PEG conjugates[169].

Otherwise by using cyclopropanation it is possible to functionalize fullerene with two different groups in order to derivatize it with two different molecules but with a same chain: one group is a propargyl, so ready to react to azido group by click chemistry; the other one is a thiol maleimide with which is possible link also aminoacids and peptides[170].

There are also other functionalizing methods based on some particular characteristics of fullerene. They include radical additions, metal complex formations, oxidations and reactions with electrophiles[171].

1.4.2.2 CARBOHYDRATE LINKING STRATEGIES

Conjugation of fullerene with carbohydrates for biological application has been already investigated[172].

The main way to link sugars with C_{60} is through a multiple cyclopropanation: six ester malonate chains having azido terminal groups are bond in one step on Fullerene by Bingel reaction. Now it is easy to link carbohydrates (mainly monosaccharides) by click chemistry, since sugar has a terminal propargyl group. It works also vice versa, with propargyl on ester chain and azide on carbohydrate[173] (Figure 1.22).

However the CuuAc (Cu(I) alkyne–azide cycloaddition reaction) (**A,** Figure 1.22) is not the only way to link sugars once malonate chain is linked to C_{60}. Ramos-Soriano et al.[174] designed a Cyclooctyne fullerene hexakis adducts by cupper free click chemistry (SPAAC)[175]. (**B,** Figure 1.22)

Otherwise, always through cyclopropanation is possible to link just one chain already presenting the carbohydrates [176] without any click reaction (**C,** Figure 1.22).

According to literature there are also other ways to conjugate sugars.

The first is the cyclopropanation mediated by carbene addition (**D**, Figure 1.22) the reaction between C_{60} and glycosylidene carbenes, derived from diazirine , lead to spiro-linked C-glycosides with good yields. The reaction is enantioselective[177-179].

There is also a method suitable for linking in one-step reaction five carbohydrates, by using a thiolate/alkyl halide coupling reaction (**E,** Figure 1.22) :in the first step is prepared the pentathiol fullerene followed by nucleophilic substitution reaction of thiolate with bromide-sugars. The aqueous solvent, in which these reactions take place, eliminate the protecting groups and accelerate the reaction[180].

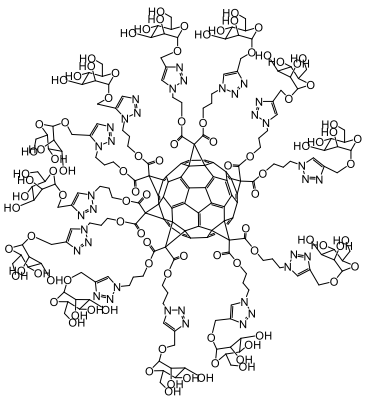
A particular method use iron salt and oxidant to link directly the sugar through two hydroxyl groups (**F**, Figure 1.22) [181].

The link can also occur between azide and the double bond of fullerene *via* 1,3-dipolar cycloaddition[182]: For the monoadducts theoretically there are four isomers, but the most common are 5,6-open[183] (**G1**, Figure 1.22) (adduct preferred by alkyl azide) and 6,6-close (**G2**,

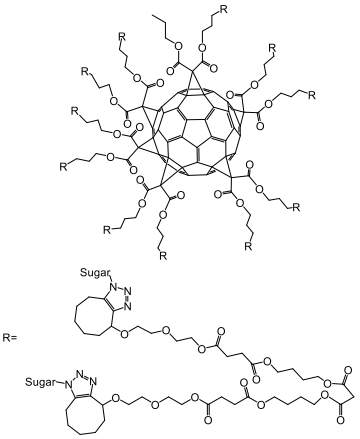
Figure 1.22) (preferred by acyl azide). It is also possible obtain bisadduct involving the all-carbon framework of fullerene[184] (**G3**, Figure 1.22).

Another synthetic way involves a mixture of sarcosine, sugar aldheydes and C_{60} in reflux[185]. 1,3-dipolar sugar azomethine ylide intermediate is formed as firs step and after it is linked to fullerene (attacks 6,6-ring junction) forming a fulleropyrrolidine glycoconjugate (**H,** Figure 1.22).

In all these way several molecules, even of biological interest, can be linked to Fullerene, for example cyclodextrins[186] or carbohydrates (as mannose)[187].



A



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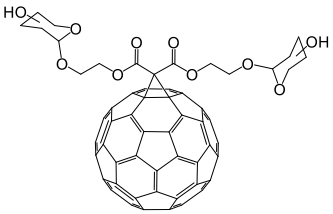

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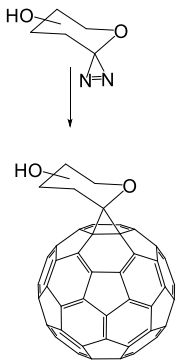

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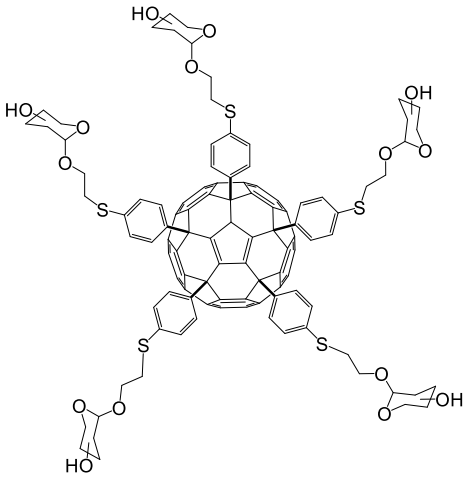

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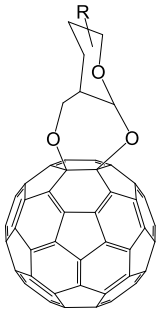

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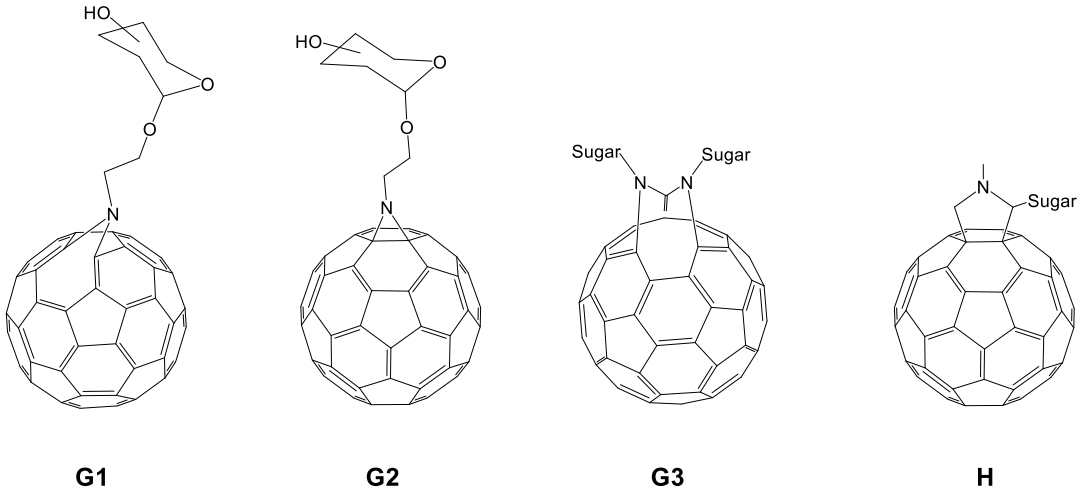


FIGURE 1.22: Sugar conjugation: A,B) Multiple Bingel cyclopropanation ; C)Single cyclopropanation ; D) Carbene addition ; E) thiolate/alkyl halide coupling reaction F) Iron salt and oxidant reaction; G) via Azide; H)sarcosine, sugar aldheydes and C_{60} reaction.

1.4.3 TERAPEUTIC APPLICATION OF C_{60}

Fullerene initially was not evaluated for biological application because its insolubility in water and aqueous solvents, it was later considered for therapeutical application once there were discovered a lot of strategies to functionalize it.

This molecule and its derivatives can be considered for this purpose because of its implicit therapeutic characteristics and its physical and chemical properties that make fullerene a perfect candidate as nanomaterial for drug delivery, as active molecule for specific disease or both of them.

Depending on the kind of derivatization the applications can be more specific for one purpose or another, it could be used as a vector to link drug or directly for its curative properties.

First of all it's interesting to evaluate the fullerene characteristic that makes useful C_{60} in biological and therapeutically field.

The main important properties for drug delivery application are the following: size (it is considered a nanomaterial since its dimension is around 0.7-1 nm), high surface area that is also regular and reproducible, solubility in water when it is derivatized, reversable aggregation and potential covalent conjugation (very stable).

Moreover we can't forget also its important chemical properties that makes fullerene a potential therapeutic molecule: fullerene has a dual ability to be either a ROS generator or a radical

scavenger depending on the presence or absence of light[188]. Under light exposition C_{60} is able to generate ROS, photoexcited C_{60} form ROS by two pathways, *via* energy transfer reaction (from the triplet excited state of C_{60}) and via electron transfer reaction (from the C_{60} radical anion)[169]. Conversely in the dark, because of its intrinsic electronic properties it has an exceptional capacity for radical scavenging, in fact it's also called "radical sponge"[189].
Such features and advantages make fullerene a very good candidate for drug delivery, for photodynamic therapy (PTD)[190] , for antioxidant therapy or in diagnosis technics[191].

1.4.3.1 C_{60} AS POTENTIAL VACCINE CARRIER

In the last few years, preliminary studies were made also in a possible application of fullerene as vaccine delivery system [192,193]: they have vast surfaces and this makes them more detectable and easy-recognized by APC cells, their size is so small that they can pass easily trough cells walls and endothelium and so they can reach zonas in which there are more macrophages, for these features it is possible to administer them in a few amount respect to no nanomaterial vaccines.
Moreover Fullerene has other advantages than other nanomaterials (as ceramics, metals, polymers): their surfaces is regular and reproducible, and the size is smaller than the others, even if derivatized fullerene remains very small. In addition, the size and shape of possible aggregates in water are reversible and fluxional[194].
Another advantage is the potential covalent conjugation, that covalently attach the nanomaterial with antigen.
According to Masalova et al.[195] amino acids (Sodium salt of aminocaproic acid and GMDP) linked on fullerene could be valid adjuvant and increase IgG production.
It was confirmed also by Xu et al.[196] work, where fullerene-tutfsin conjugates, compared with the natural tutfsin, showed a significant enhancement of immunostimulant activity (increased presence of phagocytes, major chemotaxis activity in macrophages stimulated with this conjugates). Moreover the presence of fullerene led to two important and favorable consequences: 1) fullerene protects the amino acids from enzyme degradation, and this could prolong the blood circulating time of antigen; 2) due to the nano size of fullerene, the conjugates preferentially enter in the mononuclear phagocyte system improving the bioavailability of antigen. These two considerations are a further proof that underlines a possible favorable use of fullerene in the vaccine delivery system.

CHAPTER 2: SYNTHESIS OF CARBOHYDRATES

The aim of this first part of the project is the chemical synthesis and characterization of analogues of natural polymannan that will be then tested as specific ligands for APC receptors. To this purpose it was designed and synthesized di- and tri-mannan oligosaccharides with α1,6man and/or α1,2man motifs, simpler than natural polymannans that are composed by 10 or more mannose units (Figure 1.6), bearing in anomeric position a reactive linker suitable for conjugation with protein and fullerene.

The synthesis of the different mannose-based glycans has been designed by means of a chemoenzymatic approach, based on the use of engineered enzymes, and the classical chemical synthesis pathways. The enzymatic approach was an essential tool to obtain these desired oligosaccharides, a resource to exploit for the production of increasingly complex analogues. The structural diversity and complexity of natural oligosaccharides, including polymannan, make their synthesis or the production from natural sources and characterization extremely complicated.

In the synthesis of oligosaccharides, the preparation of sugar acceptor building blocks bearing only one free hydroxyl group in the desired position represents the main bottleneck. Usually, the chemical synthesis of mono-deprotected monosaccharides and disaccharides is performed through orthogonal multistep processes, and frequently result in low yields[197,198].

The use of enzyme-catalyzed reactions provides, instead, a more straightforward route: hydrolases have been successfully employed as catalysts in the regioselective deprotection of peracetylated mono[34,55,199,200] and disaccharides[54,201,202] under mild reaction conditions.

Thus, two enzymatic screenings were carried out during this part of the work, precisely with the aim to gain intermediates helpful for speed-up the synthesis of mannose-based sugars. By using immobilized enzyme, a library of acetylated mannose-based monosaccharide and disaccharide building blocks, was prepared.

This biocatalytic approach is versatile and simple since the building blocks can be obtained directly by enzymatic regioselective hydrolysis, starting from sugars with different reactive group/linker in anomeric position and using acetyl as the only protecting group in the other positions. Among these outcoming products we chose the suitable acceptors for the synthesis of the disaccharides and linear tri-saccharides considered in this project (Figure 2.1): mannose disaccharides with α-1,6 and α-1,2 glycosidic bond (compounds **1**-**4**) and trisaccharides with α1,6-α1,6 and α1,2-α1,6

glycosyl bonds (compounds **5-8**). According to literature these compounds could be recognized by mannose receptor (see section 1.1.2.2)

The products were prepared with two different anomeric group according to their distinct targets. The carbohydrates with thiocyano methyl group have been designed for the conjugation with TB proteins (to obtain antigenic *neo*glycoprotein active against tuberculosis), instead propargyl derivatives are needed for conjugation with Fullerene by click chemistry reaction.

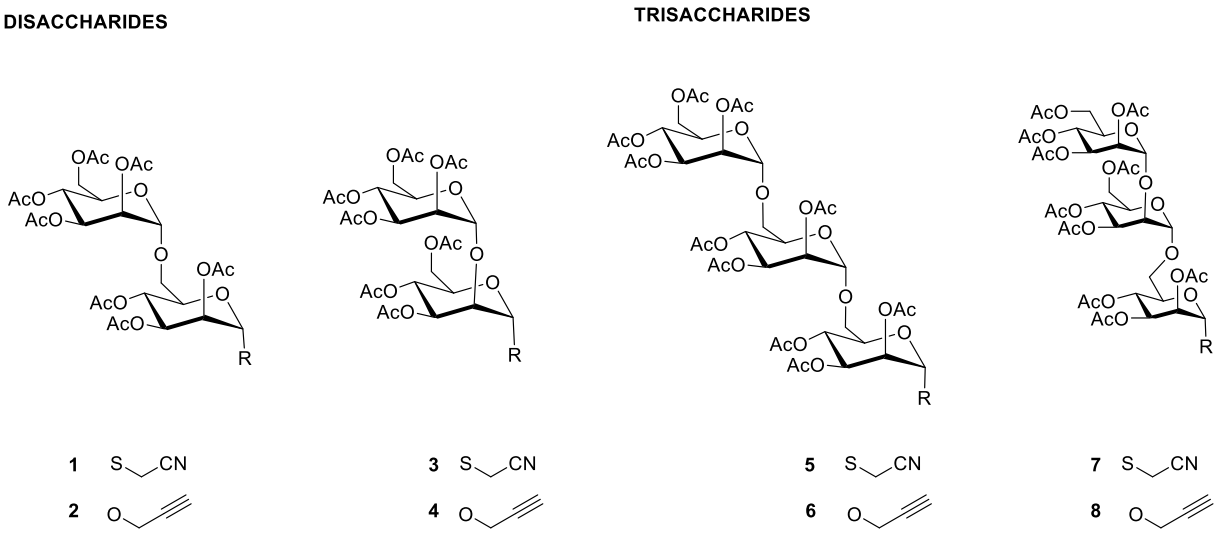


FIGURE 2.1: di- and tri-saccharides chosen.

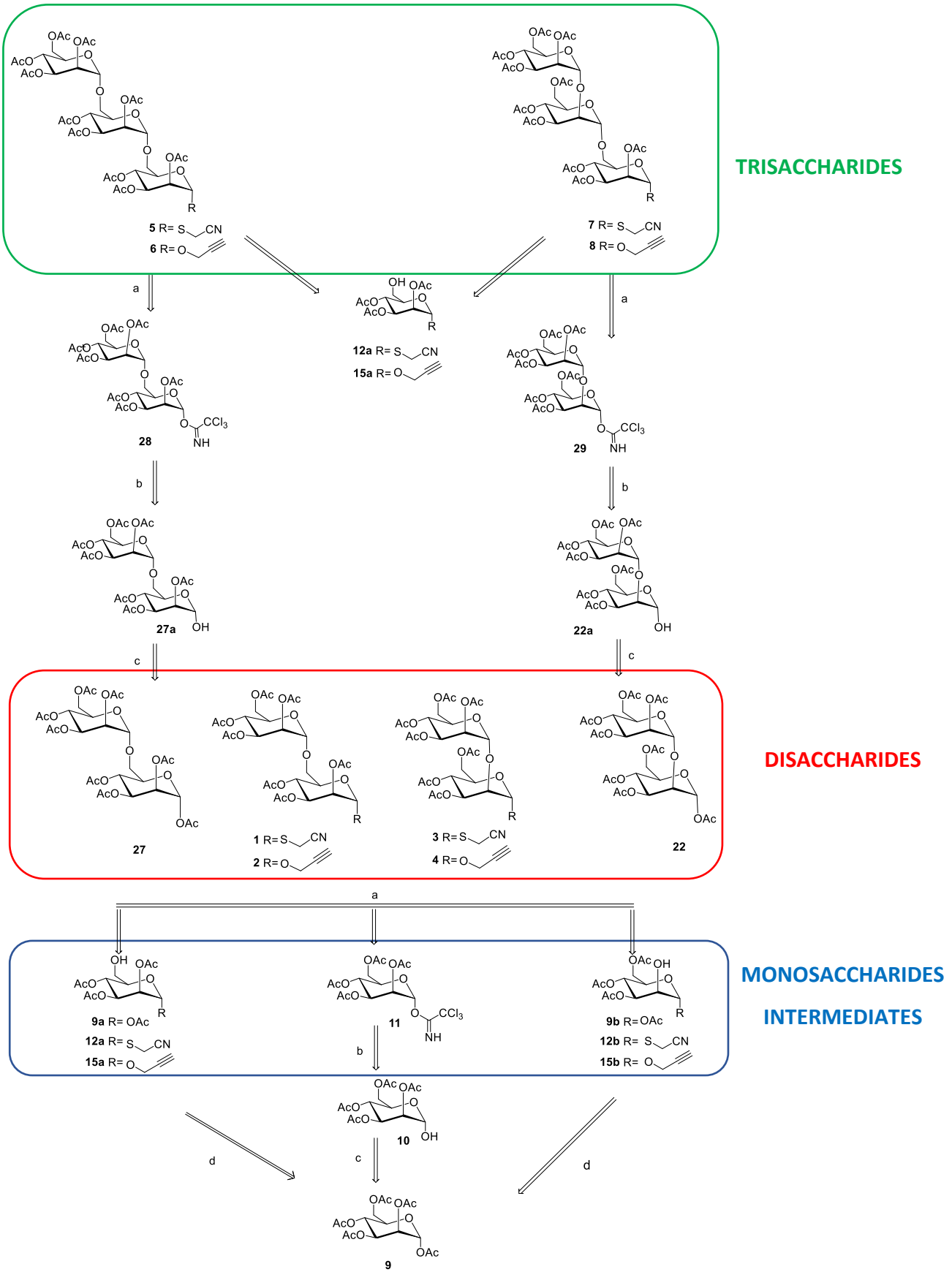
As explained in the next paragraphs, the enzymatic screening was very useful to obtain the desired oligosaccharides. The first one was carried out by hydrolysis of different mannose derivatives to obtain monosaccharides suitable to be used as building blocks for the synthesis of disaccharides and linear oligosaccharides. Indeed, a second study was performed using, as substrates, different disaccharides, in order to obtain advanced building blocks potentially convenient for the synthesis of complex analogues, such as branched mannose-based oligosaccharides.

2.1. SYNTHETIC STRATEGY

As previously described in the introduction, the glycosidic linkage is formed through the activation of a glycosyl donor to create a reactive electrophilic species that could react with a glycosyl

acceptor with a free hydroxyl group. Protecting groups are required in order to avoid side-reactions, and in this thesis we have designed the synthesis of the desired compounds in order to use exclusively the acetyl group for the protection of the sugar building blocks. The acetyl group was selected for its various advantages. First, the protection by acetylation is relatively simple and allows high yields. Second, the acetyl group can be easily and regioselectively removed by biocatalytic reactions. Finally, the presence of and acetyl group in C-2 allows α-mannoside formation, *via* anchimeric assistance (see paragraph 1.1.4).

The retro-synthetic pathways required for the preparation of the target molecules are reported in the Scheme 2.1. First of all, starting from acetylated mannose **9** (obtained by acetylation of the commercially mannose) were prepared monosaccharide intermediates by using enzymatic or chemical approach to obtain all the sugar acceptors with a free hydroxyl group in C6 or C2 (**9a**,**9b**,**12a**,**12b**,**15a**,**15b**) and the donor **11**. These building blocks were used for the synthesis of the disaccharides of interest (**1**-**4**) as potential ligands for MR, as well as other disaccharides having the acetyl group in anomeric position (**22**,**27**) that are useful to continue the chain elongation for synthesis of trisaccharides. In the last case, once the anomeric acetyl group was removed and after activation as trichloacetimidate (**28**, **29**), the disaccharides reacted with the monosaccharides **12a** and **15a** to obtain the trisaccharides **5**-**8**.



SCHEME 2.1: Retro-synthetic strategy: monosaccharide intermediates, disaccharides and trisaccharides

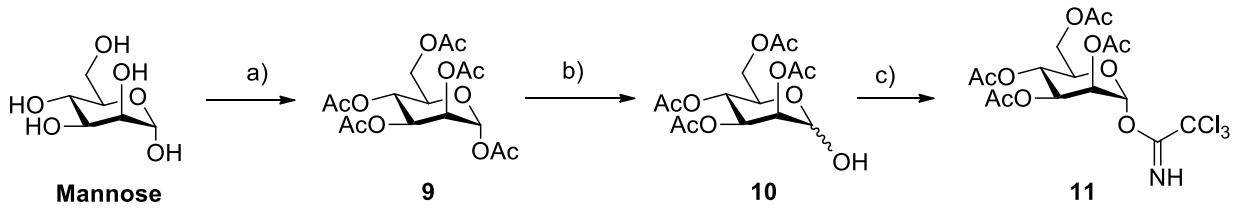
a) Glycosylation reaction, b) Imidate synthesis, c) Anomeric deprotection, d) Enzymatic and chemical deprotection.

2.2 PREPARTION OF THE MONOSACCHARIDE INTERMEDIATES

2.2.1 DONOR

In a glycosyl donor an efficient leaving group is required at the anomeric position. The typical leaving groups are halide, thioether or imidate. Trichloroacetimidate group, (TCA) was used as leaving group for the synthesis of the oligosaccharides prepared in this thesis. Thus, peracetylated mannose activated with TCA (compound **11**) was prepared according to the literature (scheme 2.2).

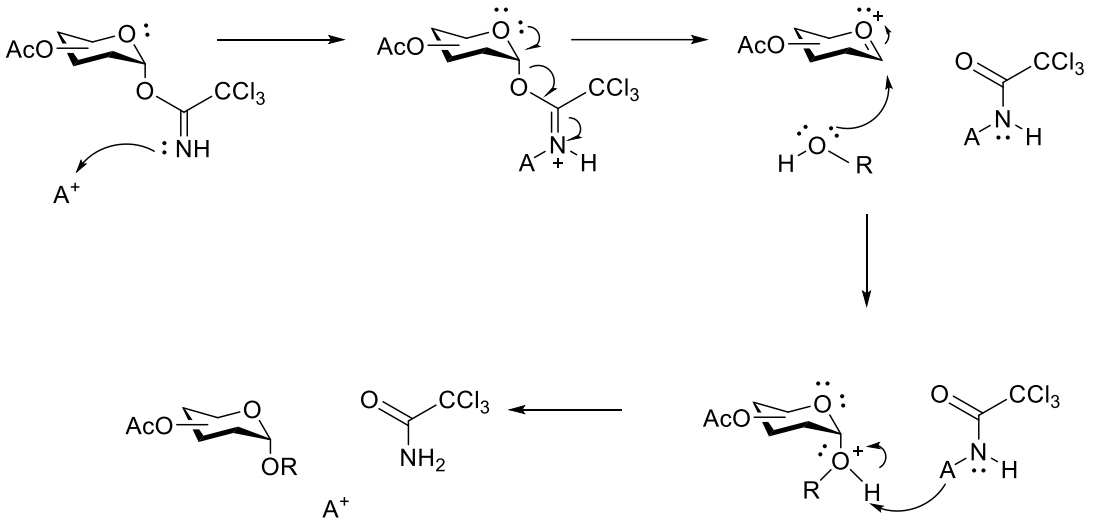
First 1,2,3,4,6-*Penta*-*O*-acetyl-α-D-mannopyranoside (**9**) was obtained by performing first the acetylation of the commercial D-mannopyranose[203]. The synthesis of the TCA-donor from **9** needs two steps: deprotection of anomeric hydroxyl group and subsequent addition of trichloroacetonitrile in that same position. We know that it is easy to remove chemically acetyl group in anomeric position without affecting the other positions. Initially, it was used hydrazine in DMF, but given the high toxicity of this compound it was replaced with benzylamine. Therefore according to literature[204] compound **9** was dissolved in THF with benzylamine and was obtained 2,3,4,6-*Tetra*-*O*-acetyl-α/β-D-mannopyranose (**10**). Once acetyl was removed by C1 position, the glucosyl donor was prepared: compound **10** was dissolved in DCM and reacted with trichloroacetonitrile and DBU to obtain 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) as reported in scheme 2.2.



SCHEME 2.2: Synthesis of mannose-TCA. Experimental conditions: a) Acetic anhydride, pyridine,DMAP,r.t.,1h (80%); b) Benzylamine,THF,r.t,20h(66%); c) trichloroacetonitrile, DBU, dry DCM, 0°C, 4h (80%).

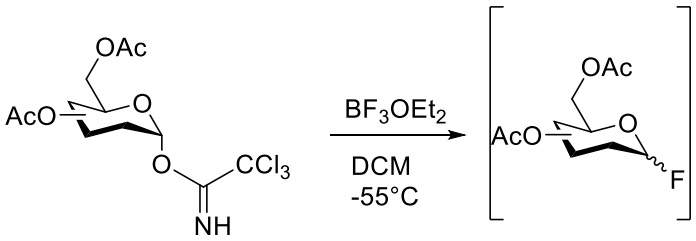
The most widely accredited mechanism of activation of TCA is described in Scheme 2.3. The Lewis acid (BF_3) reacts with nitrogen belonging to TCA, its activation to a very good leaving groups leads

to a oxocarbenium ion intermediate followed by the attack (generally a SN1 reaction), by a nucleophile, the free hydroxyl group of glycosyl acceptor.



SCHEME 2.3: Mechanism of glycosylation reaction with TCA leaving group.

However, Qiao et al.[205] investigated the glycosylation reaction at low temperature, analysing it with ^{1}H- and ^{19}F-DOSY and discovered a new intermediate of acid catalysed glycosylation reaction by using BF_3OEt_2, the glucosyl-fluoride. So the nucleophilic substitution would take place thanks to this intermediate (Scheme 2.4).



SCHEME 2.4: Possible intermediate assigned by Qiao et al.

2.2.2 ACCEPTORS

a) SYNTHESIS OF BUILDING BLOCKS BY ENZYMATIC REACTION

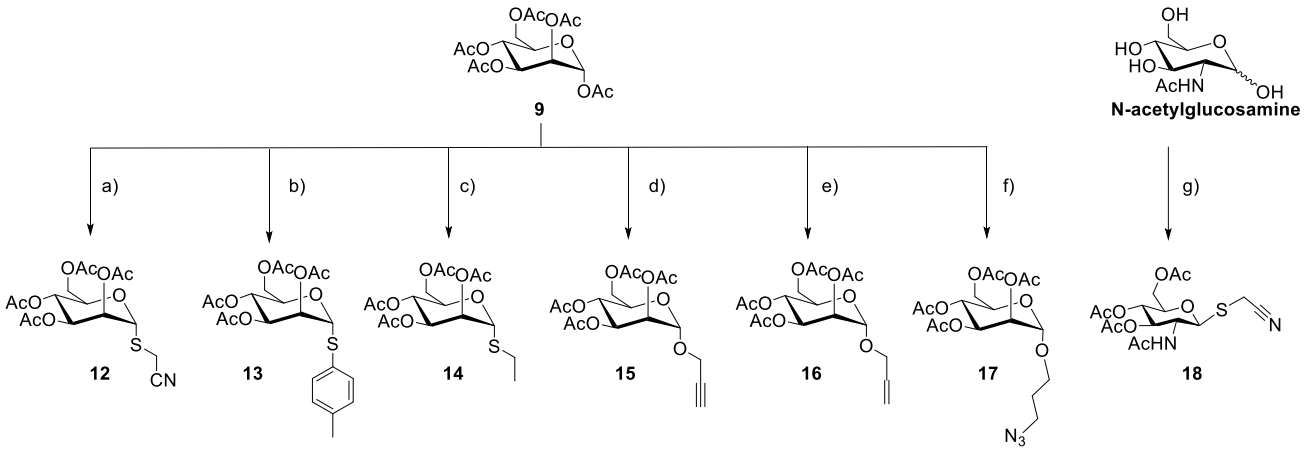
As previously reported, the chemical synthesis of oligosaccharides is impeded by the complexity of the chemical strategies required for their preparation. In particular, one of the critical steps is the synthesis of building blocks with only one free hydroxyl group in the desired position, due to the difficult control of the regioselectivity. The use of enzymatic reactions for regioselective deprotection of acetylated sugars, can overcomes these problems and it is a good green alternative approach that use only acetyl groups for the protection of the glycans[49,55]. Accordingly, most of the acceptors used in this thesis have been synthesized by means of immobilized lipase or esterase.

The use of immobilized hydrolases is highly sought since peracetylated sugars are scarcely soluble in aqueous medium; thus, the use of organic co-solvents is required in the reaction medium. Stabilization of enzymes via immobilization techniques is a valuable strategy to enhance the stability of biocatalysts in the presence of organic co-solvents and their easy recovery from reaction mixture and re-use[206]. The immobilization of lipases on hydrophobic carriers, through adsorption (e.g., on octyl-Sepharose or octadecyl-Sepabeads), is a well-established methodology for obtaining highly active biocatalysts[207] with good stability in the presence of organic co-solvents[208]. Conversely, this simple methodology is not suited for esterases due to their different 3D-architecture and kinetics[209,210]. However acetyl xylan esterase from *Bacillus pumilus* was successfully immobilized by covalent interaction on glyoxyl-agarose (GLX-AG)[211].

Thus, by using Candida rugosa lipase (CRL) immobilized on octyl-agarose (OC-AG) and acetyl xylan esterase from Bacillus pumilus (AXE) immobilized on glyoxyl-agarose (GLX-AG), in aqueous solvent (Phosphate buffer) with a low percentage of organic solvent (acetonitrile), we have investigated the synthesis of different mannosides building blocks with specific free hydroxyl group.

Various useful intermediates were obtained with a simple procedure and good or high yields, starting from mannose derivatives presenting different groups in anomeric position. Specifically, some intermediates with good leaving groups in anomeric position are useful in glycosylation reactions, instead others bearing propargyl or azide group could be used in click chemistry reactions, while the anomeric thiocyano methy group has been reported to be a suitable reactive linker for protein glycosylation[55].

First, starting from peracetylated mannose **9** and using procedures already reported in the literature, we first prepared the substrates that have been used for enzymatic reactions. Thus, several mannose derivatives with different anomeric function were synthesised: with thiocyano methy [212], S-tol [213], S-ethyl [214], propargyl [215], allyl [216,217] and azide [218]. In addiditon, a glucosamine derivate bearing a thiocyano methyl group[199] was considered too.

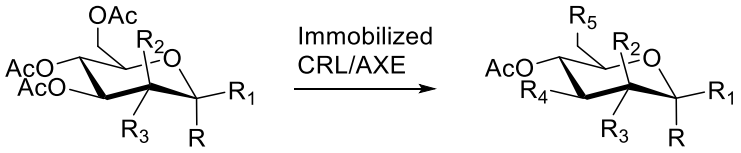


SCHEME 2.5: Substrates for the enzymatic screening. Experimental conditions: a) (i) Titanium tetrachloride, dry DCM, r.t,24h(85%), (ii) Thiourea,dry acetonitrile, reflux 80°C,10h(80%), (iii) Chloroacetonitrile,sodium meta bisulphite, potassium carbonate,H_2O/acetone,r.t.,2h (45%); b) *p*-thiocresol, BF_3OEt_2, dry DCM,0°C to r.t.,48h(85%); c) Ethanethiol, BF_3OEt_2,dry DCM, 0°C to r.t, 7h(70%); d) Propargyl alcohol, BF_3OEt_2, dry DCM,0°C to r.t.,5 days(84%); e) Allyl alcohol, BF_3OEt_2, dry DCM,0°C to r.t.,7 days(61%); f) 3-azido-1-propanol, BF_3OEt_2, dry DCM,0°C to r.t., overnight(50%); g) (i) Pyr,Ac_2O,DMAP, r.t ; (ii) Titanium tetrachloride, dry DCM, r.t,24h, (iii) Thiourea,dry acetonitrile, reflux 80°C,10h (iv) Chloroacetonitrile,sodium meta bisulphite, potassium carbonate,H_2O/acetone,r.t.,2h (98%).

Recent studies [55] about enzymatic hydrolysis of acetylated mannoses show that optimal pH value is 4, because the enzyme is highly selective. Thus, at this pH value we performed the enzymatic hydrolysis (scheme 2.6) of the different substrates (**9** and **12-18)** at 10 mM of concentration (except for compound **18**, that was hydrolysed at pH=5) and using 20% of acetonitrile in order to have a complete substrate solubilization in the aqueous solvent.

The results obtained are shown in Table 2.1. CRL was highly selective hydrolyzing preferably the primary position (C6) for compounds **9** and **13**-**17**, forming, respectively, **9a**[55], and **13a-17a**[219] in good yields (65–81%). Compound **12** was deacetylated by CRL simultaneously in position C6 and

C2, giving access to **12a** (50%) and **12b** (40%), both useful building blocks for further oligosaccharides synthesis[55]. Compared to CRL, AXE seemed less specific for the same compounds, showing the same regioselectivity as CRL but lower yields (24–50%) due to the formation of undesired by-products (di- or multiple deacetylation of starting material).



9 R= OAc, R_1=H, R_2= OAc, R_3=H

12 R= SCH_2CN, R_1=H, R_2= OAc, R_3=H

13 R= STol, R_1=H, R_2= OAc, R_3=H
14 R= SCH_2CH_3, R_1=H, R_2= OAc, R_3=H
15 R= OCH_2CCH, R_1=H, R_2= OAc, R_3=H

16 R= $OCH_2CH{=}CH_2$, R_1=H, R_2= OAc, R_3=H
17 R= $O(CH_2)_3N_3$, R_1=H, R_2= OAc, R_3=H
18 R= H, R_1=SCH_2CN, R_2= H, R_3=NHAc

9a R= OAc, R_1=H, R_2=OAc, R_3=H, R_4=OAc, R_5=OH
10 R/R_1= H/OH, R_2=OAc, R_3=H, R_4=OAc, R_5=OAc
12a R= SCH_2CN, R_1=H, R_2=OAc, R_3=H, R_4=OAc, R_5=OH
12b R= SCH_2CN, R_1=H, R_2=OH, R_3=H, R_4=OAc, R_5=OAc
13a R= STol, R_1=H, R_2=OAc, R_3=H, R_4=OAc, R_5=OH
14a R= SCH_2CH_3, R_1=H, R_2=OAc, R_3=H, R_4=OAc, R_5=OH
15a R= OCH_2CCH, R_1=H, R_2=OAc, R_3=H, R_4=OAc, R_5=OH
15b R= OCH_2CCH, R_1=H, R_2=OH, R_3=H, R_4=OAc, R_5=OAc
16a R= $OCH_2CH{=}CH_2$, R_1=H, R_2=OAc, R_3=H, R_4=OAc, R_5=OH
17a R= $(CH_2)_3N_3$, R_1=H, R_2=OAc, R_3=H, R_4=OAc, R_5=OH
18a R= H, R_1=SCH_2CN, R_2=H, R_3=NHAc, R_4=OAc, R_5=OH

SCHEME 2.6: Regioselective enzymatic hydrolysis of peracetylated mannopyranosydes **9,12-18.** Experimental condition: 25 mM KH_2PO_4 pH 4.0 (except for compound 8, pH=5.0, 20-30% v/v acetonitrile, room temperature, [substrate]= 10 mM, CRL immobilized on octyl-AG or AXE immobilized on GLX-AG.

Interestingly, for compound **15**, CRL and AXE showed a different regioselectivity profile: CRL selectively deprotected the primary position (C6) giving **15a** (80%), while AXE preferred the C2 position, giving access to **15b** in acceptable yields (40%). These results highlighted a different and complementary selectivity behaviour of the two biocatalysts towards the same substrate. However, the formation of by-products (di-deacetylation) don't allow to reach higher yield for compound **15b**.

Compound **18** was selectively hydrolyzed by using AXE immobilized on GLX-AG, and the results were in agreement with those previously reported using the enzyme immobilized on acrylic epoxy carrier[199].

These monodeprotected products are useful building blocks in the synthesis of oligosaccharides and glycoconjugates. For example, compound **12a** and **18a** can be used to conjugate antigenic proteins through the thiocyanomethyl group in anomeric position. Compound **13a** can be used

both as acceptor or donor in further glycosylation reactions because, once used as acceptor, the product obtained can become a donor due to the good properties of S-Tol group as leaving group. Furthermore, compounds obtained by hydrolysis of **15** and **17** can be used as intermediates to prepare glycoproducts by click chemistry, thanks to their propargyl and azido groups in the anomeric position.

TABLE 2.1: Regioselective enzymatic mono-deprotection of monosaccharides.

Substrate	Enzyme	Time	Position	Product (Yield)	By-products
9	CRL	21h	6OH 1OH	**9a** (77%) **10** (13%)	(10%)
	AXE	24h	1OH	**10** (50%)	-
12	CRL	20h	6OH 2OH	**12a** (50%) **12b** (40%)	(10%)
	AXE	72h	6OH	**12a** (26%)	(74%)
13	CRL	48h	6OH	**13a** (65%)	(35%)
	AXE	72h		(27%)	(73%)
14	CRL	24h	6OH	**14a** (69%)	(31%)
	AXE	48h		(24%)	(76%)
15	CRL	20h	6OH 2OH	**15a** (80%) **15b** (8%)	(12%)
	AXE	24h	6OH 2OH	**15a** (5%) **15b** (40%)	(55%)
16	CRL	6h	6OH	**16a** (81%)	(19%)
	AXE	6h		(24%)	(48%)
17	CRL	24h	6OH	**17a** (70%)	(30%)
	AXE	24h	-	- -	-
18	CRL	48h	6OH	**18a** (68%)	(32%)
	AXE	24h		(90%)	(10%)

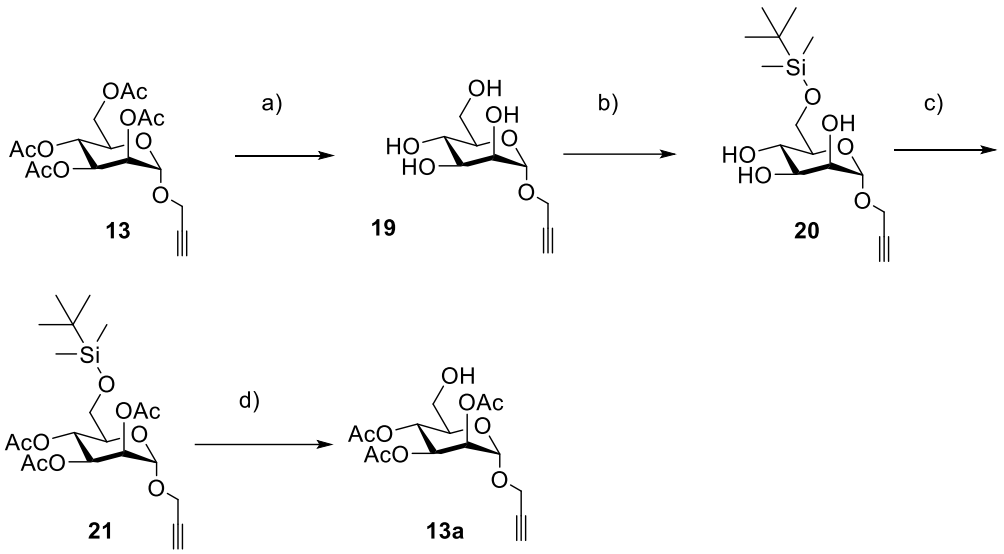
b) SYNTHESIS OF BUILDING BLOCKS BY CHEMICAL REACTION

*CHEMICAL SYNTHESIS OF COMPOUND **15a***

In order to compare the results obtained in the preparation of the monosaccharides building blocks by the enzymatic regioselective hydrolysis with the performance obtained by chemical synthesis, compound **15a** was also achieved via chemical preparation. In the first step, acetylated

propargyl mannose **15** was deacetylated by sodium in methanol. At this point, *tert*-Butyldimethylsilyl chloride (TBDMSCl) selectively protected the position C6 [220]. Once the sugar was protected again with acetyl groups, it was possible to remove TBDMSCl by using acid solution due to $KHSO_4$ salt[221].

As supposed, chemical approach resulted less performant respect to enzymatic one. In fact, starting from compound **15** the chemical synthesis needed four steps allowing **15a** in 43% overall yield, while the enzymatic approach provided the same product in one step and 80% of yield.



SCHEME 2.7: Chemical synthesis of compound 13a. Experimental conditions: a) Sodium, dry MeOH,r.t.,3h(96%); b) TBDMSCl, TEA, MeCN/DMF, r.t., 30min(83%); c) Acetic anhydride, pyridine, DMAP, r.t., 40min(94%); d) $KHSO_4$, H_2O/acetone, r.t., 5h(54%).

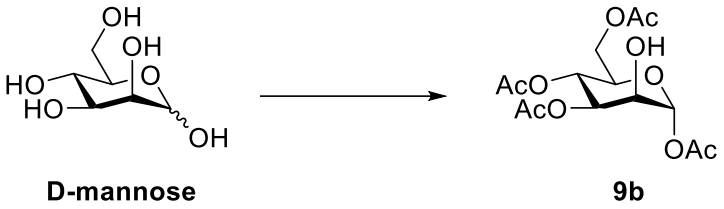
*DEPROTECTION IN C2 POSITION: synthesis of building block **9b***

Unfortunately, not all the desired monosaccharide intermediates can be prepared by enzymatic approach. In fact, the synthetic approach used in this work require building blocks with a free hydroxyl group in C2 position. This can be obtained by enzymatic reaction only when in anomeric position a thiocyanomethyl or propargyl groups are present.

For the synthesis of the desired trisaccharide **8** (containing 1,2 and 1,6 bonds), the intermediate 1,3,4,6- Tetra- O-acetyl-α-D-mannopyranose (**9b**) (with a free hydroxyl group in C2 and the acetoxy group in anomeric position) is required.

The synthetic chemical pathways already reported in literature to obtain mannosides building blocks selectively deprotected in C2, are very long and poorly efficient [222]. The first strategy already reported in literature consisted in using two different protective groups, the acetyl in C2

and benzyl for the others positions, except the anomeric that is protects as p-tolouyl tioglycoside. According to this method, starting from compound **9**, six steps are required to obtain the product with a free hydroxyl group in anomeric position with a global yield of 9%. In addition, the presence of benzyl protecting groups (that could be removed by reduction), make this approach not suitable for the synthesis of trisaccharides with propargyl or thiocyanomethyl group in anomeric position.
So we investigated a faster route of synthesis for achieving directly intermediate **9b** that uses only acetyl as protecting group based on an old article (1967) reporting a one-step reaction (Scheme 2.8)[223]. Starting from commercial mannose by carrying after acetylation, adding tribromide of phosphorus, water and sodium acetate, 1,3,4,6- Tetra- O-acetyl-D-mannopyranose (**9b**) was obtained in a *one-pot* process in a yield of 16% (practically identical as reported in the literature).



D-mannose **9b**

SCHEMA 2.8: One-pot chemical synthesis of compound **9b**. Experimental conditions: (i) Acetic anhydride, $HClO_4$, 40-45°C, 20min, (ii) PBr_3, 30-25°C, 90min, (iii) CH3COONa $3H_2O$, 35-40°C, 25min(16%).

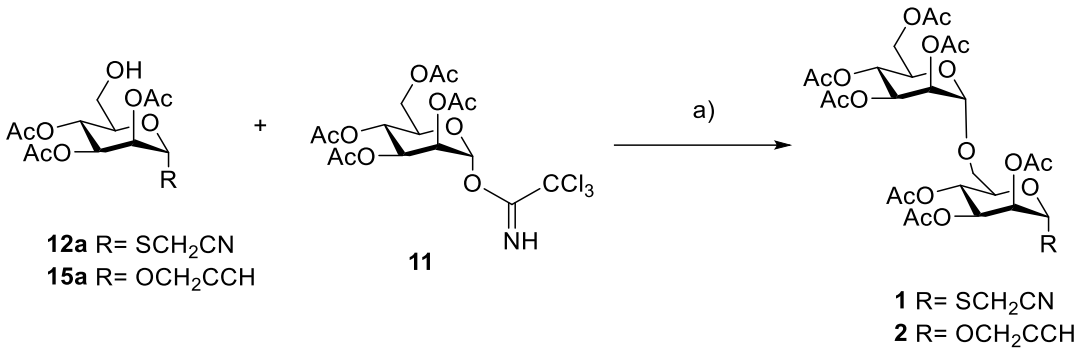
2.3 SYNTHESIS OF DISACCHARIDES

Once all monosaccharides intermediate were prepared, we synthesized various acetylated disaccharides with different groups in anomeric position, including the compounds (**1**-**4**) that we have considered as glycans for the preparation of the glycoconjugate products.
For these synthesis, the mono-deprotected building blocks, previously obtained by regioselective enzymatic hydrolysis have been considered as sugar acceptors for man-TCA **11** (the sugar donor). All of the glycosylation performed are acid catalysed reactions[224], but they were carried out under different conditions (time and temperature) depending on the glycosylation position.

*2.3.1 SYNTHESIS OF DISACCHARIDE **1** AND **2** BY GLYCOSYLATION IN POSITION C6*

For the synthesis of Thio-cyanomethyl 2',3',4',6'-*Tetra-O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri-O*-acetyl-α-D-mannopyranoside (**1**) and Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-

(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**2**), mono-deprotected building blocks **12a** and **15a**, were reacted with **11** as shown in scheme 2.9. The two building blocks were dissolved in dichloromethane at 0 °C and boron trifluoride-diethyl ether (BF_3OEt_2) was added. After six hours disaccharide **1** was obtained in 79% yield after purification. Instead disaccharide **2** needs four hours to be obtained in 90% yield after purification.



12a R= SCH_2CN
15a R= OCH_2CCH

11

1 R= SCH_2CN
2 R= OCH_2CCH

SCHEMA 2.9: Synthesis of disaccharide 1 and 2. Experimental conditions: synthesis of **1** a) BF_3OEt_2, dry DCM, 0°C to r.t., 6h (79%); synthesis of **2** a) BF_3OEt_2, dry DCM, 0°C to r.t., 4h(90%).

*2.3.2 SYNTHESIS OF DISACCHARIDES **3** AND **4** BY GLYCOSYLATION IN POSITION C2*

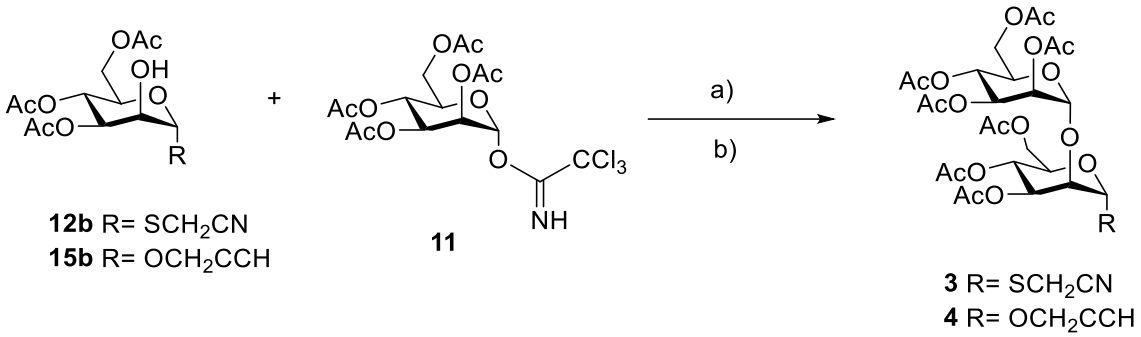
For the synthesis of Thio-cyanomethyl 2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (**3**), mono-deprotected building block **12b**, was reacted with glycosyl donor **11** at -70 °C (Scheme 2.10). After four hours disaccharide **3** was obtained in yield 52.6 % after purification.

The synthesis of Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (**4**) was investigated by means of both chemoenzymatic and chemical approach, in order to compare the two methods starting acetylated mannose **9**.

For the chemoenzymatic strategy, mono-deprotected building block **15b** (obtained by enzymatic regioselective hydrolysis of **15**) was reacted with the donor **11** and two Lewis acid were investigated. The reaction was tested with TMSOTf [225] (Trimethylsilyl trifluoromethanesulfonate) and BF_3OEt_2[226](scheme 2.11), following two different published procedures.

In the first case the two building blocks reacted in dichloromethane, at -30 °C, and TMSOTf was added. In 15 min disaccharide **4** was obtained in 53% yield after purification. Instead, the second Lewis acid (BF_3OEt_2) was added at -50 °C. The mixture was allowed to warm up to r.t. and after 18 hours **4** was obtained in 80% yield after purification (Scheme 2.10). Therefore, considering the

yields obtained in the preparation of building block **15a** from **9** (by two steps as reported in the paragraph 2.2.2.1) the chemoenzymatic synthesis of **4** can be obtained in 27% overall yield in 3 steps using BF_3OEt_2 as Lewis acid in the final glycosylation step.



12b R= SCH_2CN
15b R= OCH_2CCH

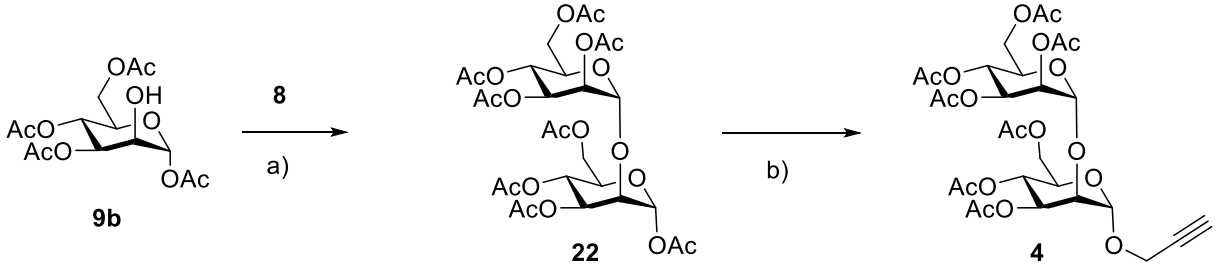
11

3 R= SCH_2CN
4 R= OCH_2CCH

SCHEMA 2.10: Synthesis of disaccharide **3**. Experimental conditions: a) BF_3OEt_2, dry DCM, -70°C,4h(79%). Synthesis of disaccharide **4:** Experimental conditions a) TMSOTf, dry DCM,-30°C,15min(53%); Experimental conditions b) BF_3OEt_2, dry DCM, -50°C to r.t.,18h(80%).

Alternatively, the disaccharide **4** was prepared by chemical synthesis. As describe in paragraph 2.2.2.b, the synthesis of intermediates **9b** can be obtained in a one-pot procedure starting from **9** in 16% yield. According with Scheme 2.11, 2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-1,3,4,6-tetra-O-acetyl-α-D-mannopyranose (**22**) was prepared by reacting mono-deprotected building block **9b** with **11** in dichloromethane at -70°C. Once BF_3OEt_2 was added and in only 10 minutes, disaccharide **22** was obtained in 81% yield.

At this point the propargyl group was reacted directly with this intermediate disaccharide, forming the desired disaccharide **4** after 7 days at room temperature in 58% of yield, that means the 7% overall yield starting from compound 9. Thus the chemoenzymatic synthesis resulted the most efficient.



9b

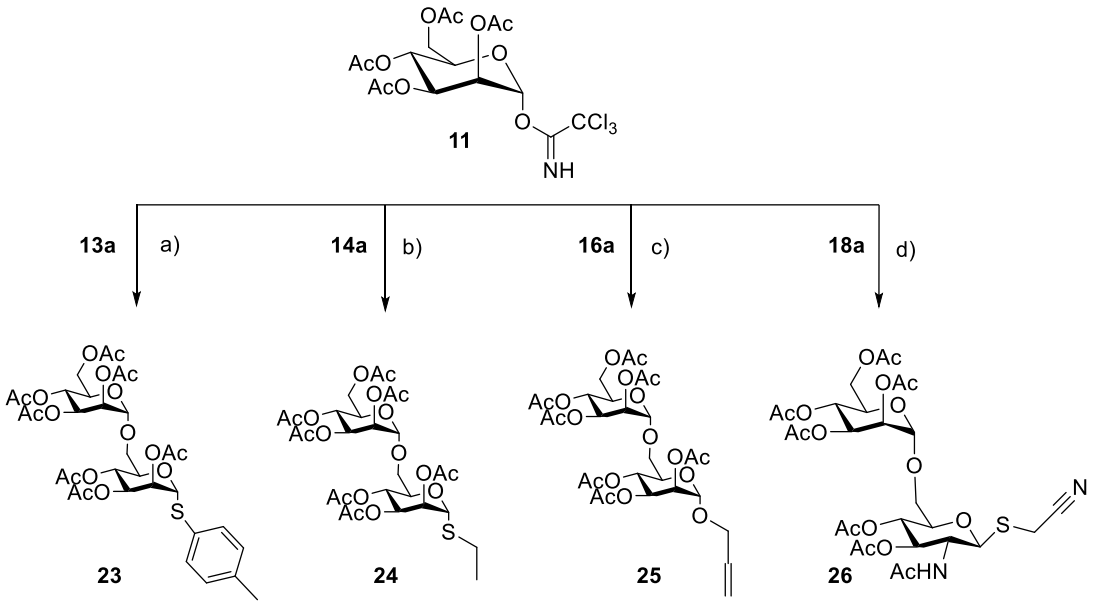
22

4

SCHEME 2.11: Chemical synthesis of Disaccharide **4** from building block **9b**. Experimental conditions: a) BF_3OEt_2, dry DCM, -70°C, 10min(81%); b) Propargyl alcohol, BF_3OEt_2, dry DCM, 0°C to r.t., 7days (58%).

2.3.3 SYNTHESIS OF OTHER DISACCHARIDES

Other disaccharide **23**-**26** were prepared starting from the mono-deprotected monosaccharides **13a**, **14a**, **16a** and **18a** by Lewis acid-catalyzed glycosylation with the trichloroacetimidate donor **11** (Scheme 2.12). The Man(1→6)Man disaccharides **23-25** were obtained in a range of 37% and 92% yield working at 0 °C after 2–4 h. The same conditions were used also for compound **26** obtained in very good yield (74%).



SCHEME 2.12: Chemical synthesis of Disaccharide 21-24. Experimental conditions: a) BF_3OEt_2, dry DCM, 0°C, 3.5h (92%); b) BF_3OEt_2, dry DCM, 0°C, 4h(37%); C) BF_3OEt_2, dry DCM, 0°C, 2h(50%); d) BF_3OEt_2, dry DCM, 0°C, 2.5h(74%).

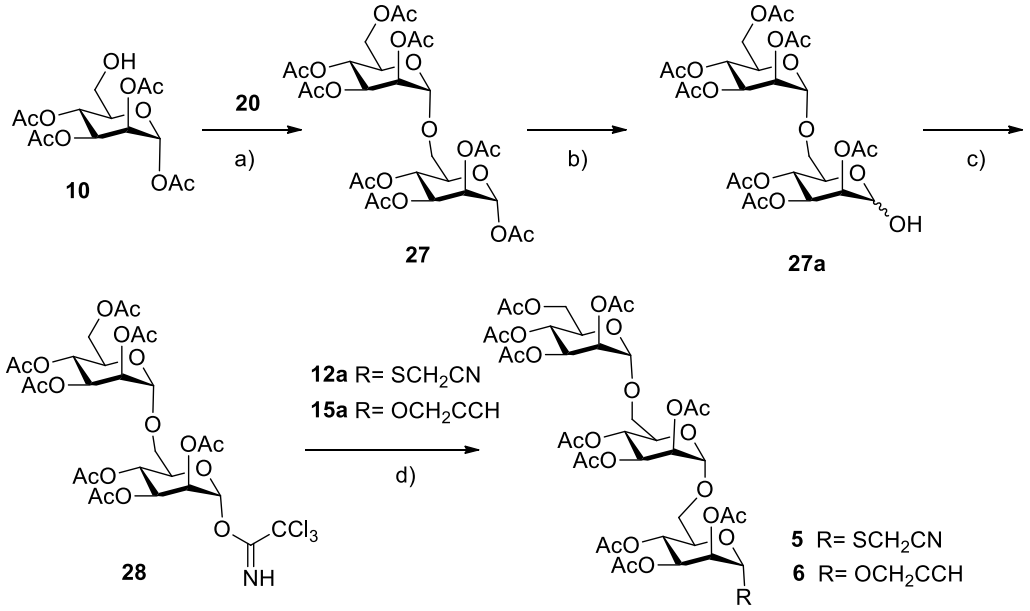
2.4 SYNTHESIS OF TRISACCHARIDES

*2.4.1 SYNTHESIS OF TRISACCHARIDES **5** AND **6***

The preparation of TCA-donors was the first step to synthesize trisaccharides **5** and **6** (Scheme 2.13) As reported in scheme 2.12, mono-deprotected building block **10** was reacted with glycosyl donor **11** to form 2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-1,2,3,4-tetra-*O*-acetyl-α-D-mannopyranose (**27**) in dichloromethane at 0 °C, using BF_3OEt_2. After two hours and half the disaccharide **27** was obtained in 86% yield. The activation of **27** as TCA was then performed first by anomeric deacetylation, performed with hydrazine and leading to the formation of 2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α/β-D-mannopyranose (**27a**) in 92%

of yield, and subsequent by reaction with trichloroacetonitrile to obtain the TCA activated disaccharide 2',3',4',6'- Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**28**), in a yield of 57 % .

Following, for the synthesis of Thio-cyanomethyl 2",3",4",6"-*Tetra*-*O*-acetyl- α-D-mannopyranosyl-(1→6)-2',3',4'-*tri*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranoside (**5**) and Propargyl 2",3",4",6"-*Tetra*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2',3',4'-*tri*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranoside (**6**), mono-deprotected building block **12a** and **15a** were reacted with **28** in dichloromethane, at 0 °C and using boron trifluoride-diethyl ether complex. After three hours trisaccharide **5** was obtained in 31% yield, while four hours were required for preparing trisaccharide **6** in 56% yield.



SCHEME 2.13 : Synthesis of trisaccharides **22** and **23**. Experimental conditions: a) BF_3OEt_2, dry DCM, 0°C ,2.5h(86%); b) Hydrazine, DMF, 50°C,5h(92%); c) trichloroacetonitrile, DBU, dry DCM,0°C,4h(57%); synthesis of **5** d) BF_3OEt_2, dry DCM, 0°C, 3h(31%); synthesis of **6** d) BF_3OEt_2, dry DCM, 0°C, 4h(56%).

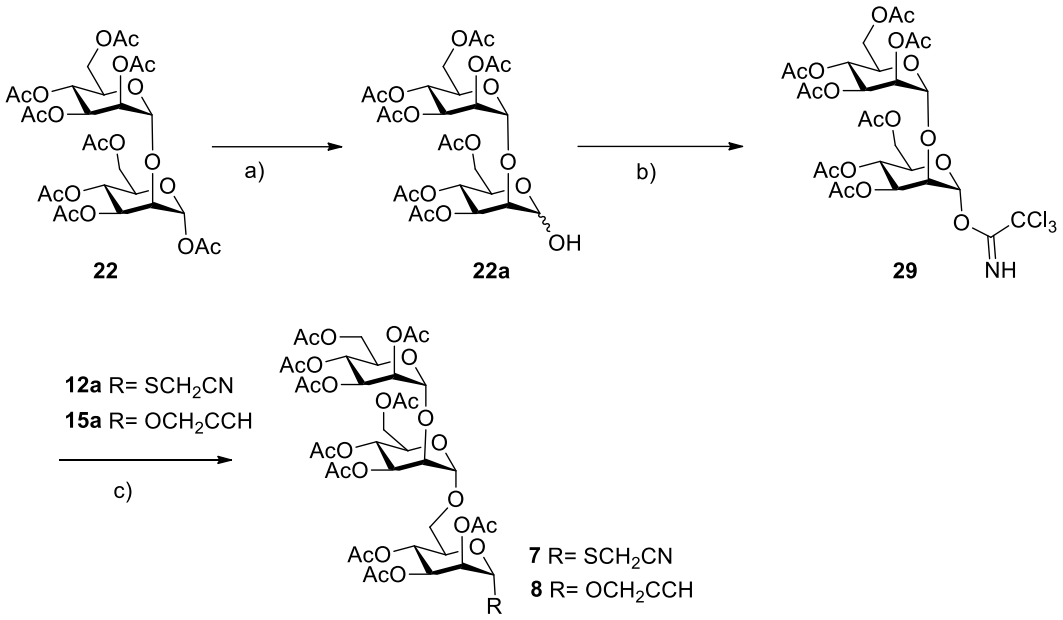
*2.4.2 SYNTHESIS OF TRISACCHARIDES **7** AND **8***

The same steps above described were followed for the preparation of trisaccharides **7** and **8**. Thus, 2',3',4',6'-tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-1,3,4,6-tetra-O-acetyl-α-D-mannopyranose (**22**), was prepared as described in paragraph 2.3.2.

This disaccharide was then activated to TCA. Thus, in the first step compound **22** was deacetylated in C1 position, with benzylamine, to obtain 2',3',4',6'-Tetra-O-acetyl-α-D-

mannopyranosyl-(1→2)-3,4,6-tri-O-acetyl -α/β-D-mannopyranose (**22a**) in 92% yield. Following, 2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**29**) was prepared by addition of trichloroacetonitrile in 63% yield as reported in scheme 2.14.

Once prepared the donor, for the synthesis of Thio-cyanomethyl 2",3",4",6"-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3',4',6'-tri-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-acetyl-α-D-mannopyranoside (**7**) and Propargyl 2",3",4",6"-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3',4',6'-tri-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-acetyl-α-D-mannopyranoside (**8**), mono-deprotected building block **12a** and **15a** were reacted with glycosyl donor **29** in dry dichloromethane, at -20 °C in presence of BF_3OEt_2. After twenty min trisaccharide **7** was obtained in 87% yield. Conversely, trisaccharide **8** was obtained after 1h in 53% yield.



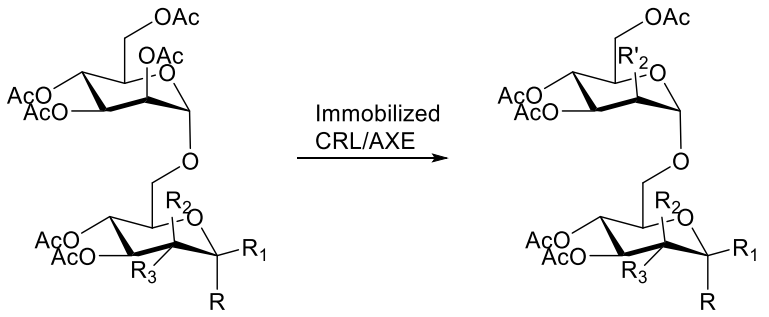
SCHEMA 2.14.: Synthesis of trisaccharides 7 and 8. Experimental conditions: a) Benzylamine, dry THF, r.t.,21h(92%); b) trichloroacetonitrile,dry DCM,0°C,4h(63%); synthesis of **7** d) BF_3OEt_2, dry DCM, -20°C, 20min(87%); synthesis of **8** d) BF_3OEt_2, dry DCM,-20°C, 1h(53%).

2.5 ENZYMATIC HYDROLYSIS OF THE DISACCHARIDES

In order to prepare advanced building blocks that could be useful for the preparation of polymannan analogues, we have investigated the regioselective hydrolysis of the acetylated disaccharides with different reactive groups in anomeric position previously prepared.

The disaccharides considered in this study are compounds **1-4** and **22-27** that have been prepared by chemoenzymatic synthesis as reported in the previous paragraph 2.3.

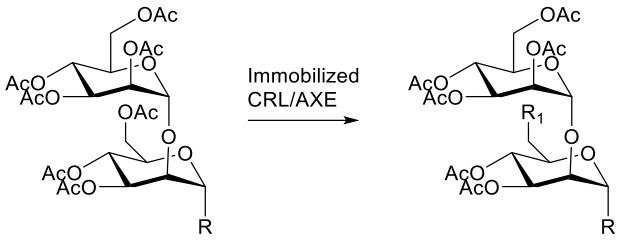
Thus, man-α–1,6man (Scheme 2.15) and man-α–1,2man (scheme 2.16) disaccharides were tested as substrates for a further enzymatic hydrolysis with the aim to obtain selectively deprotected disaccharides that can be used as advanced building blocks for the synthesis of complex linear and branched mannose based oligosaccharides. These compounds are new substrates submitted to a known enzymatic procedure. Both immobilized CRL and AXE were tested as catalysts but only AXE showed a relevant activity towards the different disaccharides (CRL was almost completely inactive towards all these substrates). The hydrolysis catalyzed by AXE derivative provided different results depending from the substrate as shown in Table 2.2.



1 R= SCH_2CN, R_1=H, R_2=OAc, R_3=H
2 R= OCH_2CCH, R_1=H, R_2=OAc, R_3=H
23 R= STol, R_1=H, R_2=OAc, R_3=H
24 R= SCH_2CH_3, R_1=H, R_2=OAc, R_3=H
25 R= $OCH_2CH{=}CH_2$, R_1=H, R_2=OAc, R_3=H
26 R= H, R_1=SCH_2CN, R_2=H, R_3=NHAc, R4=OAc
27 R= OAc, R_1 = H, R_2 = OAc, R_3 =H

1a R= SCH_2CN, R_1=H, R_2=OH, R_3=H, R'_2=OAc
2a R= OCH_2CCH, R_1=H, R_2=OH, R_3=H, R'_2=OAc
23a R= STol, R_1=H, R_2=OH, R_3=H, R'_2=OAc
24a R= SCH_2CH_3, R_1=H, R_2=OH, R_3=H, R'_2=OAc
25a R= $OCH_2CH{=}CH_2$, R_1=H, R_2=OH, R_3=H, R'_2=OAc
26a R= H, R_1=SCH_2CN, R_2=H, R_3=NHAc, R'_2=OH
27a R/R_1= OH/OAc, R_2=OAc, R_3=H, R'_2=OAc

SCHEME 2.15: Regioselective enzymatic hydrolysis of peracetylated 1→6 disaccharides.



3 R= SCH_2CN
4 R= OCH2CCH
22 R= OAc

3a R =SCH_2CN, R_1 = OH
4a R= OCH2CCH, R_1 = OH
22a R/R_1= OH/OAc

SCHEME 2.16: Regioselective enzymatic hydrolysis of peracetylated 1→2 disaccharides.

The fully acetylated man(1→6)man **27** and man(1→2)man **22** were deprotected at the anomeric position yielding product **27a** in 50% of yield (α/β mixture) after 48 h (63% of substrate consumption) and **22a** in trace (because of slow conversion). These results agree with those previously reported in the hydrolysis of fully acetylated lactose [227]. By introducing an alkyl or aryl glycoside or thioglycoside group (substrates **1**,**2**,**23**,**24**,**25** in Scheme 2.15) in anomeric position, the selectivity of AXE moved towards the C2 position for man(1→6)man disaccharides allowing the production of compounds **1a**,**2a**,**23a**,**24a**,**25a** in yields ranging from 23% to 52%.

When the man(1→2)man disaccharides **3** and **4** were tested as substrates of AXE (Scheme 2.16), the hydrolysis was observed for the position C6 (being position C2 involved in the glycosidic bond), yielding the products **3a** and **4a** with the thiocyanomethyl and the propargyl group in anomeric position. In these cases, the yields obtained were lower (16% and 20%, respectively) compared to the corresponding man(1→6)man disaccharides (compounds **1a** and **2a**). Considering this results, we wondered what selectivity AXE would have if positions 2 and 6 were not available for hydrolysis (compound **26** is the suitable candidate because presents a glycosidic bond in position 6 and an acetamido group in position 2). When man(1→6)GluNHAc **26** was submitted to hydrolysis, AXE was not able to hydrolyze the second position of the anomeric sugar (where an acetamido group is present instead of an ester) ;thus, the selectivity of the enzyme moved further towards C2'position of the mannose unit at the no*n*-reducing end. However, the reaction proceeded very slowly (168 h for achieving 40% of substrate consumption) and low yields (about 20% of product **26a**).

However, yields of these reactions are not so high, because some by-products were started to form when unconverted substrates was still present.

TABLE 2.2: Regioselective enzymatic deprotection of mannose-based disaccharides using AXE-GLX-AG.

Substrate	Glycosidic bond	Time (h)	Position	Product	Yield (%) [a]	Conversion (%) [b]
1	1,6	5	2OH	**1a**	23	53
2	1,6	26	2OH	**2a**	24	85
3	1,2	8.5	6OH	**3a**	16	46
4	1,2	24	6OH	**4a**	20	32
22	1,2	71	1OH	**22a** (α/β)	Trace	-
23	1,6	57	2OH	**23a**	24	60
24	1,6	7	2OH	**24a**	38	41
25	1,6	15	2OH	**25a**	52	84
26	1,6	168	2'OH	**26a**	21	40
27	1,6	48	1OH	**27a** (α/β)	50	63

Experimental conditions: 25 mM KH_2PO_4 pH 4.0–5.8, 30–35% *v/v* acetonitrile, room temperature, [substrate] = 5–10 mM, AXE immobilized on GLX-AG. [a] Yield was determined by isolation and purification by flash chromatography. [b] Conversion was calculated based on the isolated amount of unconverted substrate.

Therefore, AXE favourite hydrolysis positions were evaluated (depending on whether the positions are free or covered): 1OH>2OH>6OH>2'OH.

Unfortunately, this screening did not lead to a useful result for the synthesis of desired trisaccharides for this thesis project. However it led to the formation of interesting potential intermediates applied for the synthesis of branched oligosaccharides as analogues of natural polymannan. Further studies will be carried on.

2.6 MATERIALS AND METHODS

Reagents, chemicals, hydrophobic carrier Octyl Sepharose® CL-4B, and lipase from *Candida rugosa* (CRL) were purchased from Sigma-Aldrich (Milano, Italy). Acetyl xylan esterase (AXE) from *Bacillus pumilus* was from Dobfar (Tribiano, Italy). SepharoseTM CL-6B (agarose) was from GE Healthcare (Milan, Italy).

Compounds purification was performed by flash chromatography using Silica Gel high-purity grade, pore size 60 Å 70–230 mesh, 63–200 µm (Sigma-Aldrich). Analytical thin layer chromatography (TLC) was performed on silica gel F254 precoated aluminium sheets (0.2 mm layer, Merck, Darmstadt, Germany). Products were detected by spraying with 5% H_2SO_4 in ethanol, followed by heating to ca. 150 °C. Enzymatic reactions and activity assays were monitored by Titrator 718 stat (pH-Stat) Tritino from Metrohm (Herisau, Switzerland). Characterization of purified compounds was performed by NMR spectroscopy. NMR spectra were recorded in $CDCl_3$ on a Bruker Advance III 400 MHz spectrometer (Bruker Corporation, Billerica, MA, USA), available at the CGS of the University of Pavia. All 1D and 2D NMR spectra were acquired using the standard pulse sequences available with Bruker Topspin 3.6 software package. Chemical shifts (δ) are given in ppm and were referenced to the solvent signals (δ_H 7.28, δ_C 77.00). Signal multiplicities are abbreviated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Structures assignment was performed by means of 2D-COSY and HSQC and, in some cases, 2D-NOESY. Spectra analyses were carried out using Mestrenova reader software. For compounds **2**,**4**,**6**,**8**,**15a** and **15b** high resolution mass spectra (HRMS) were recorded with a Bruker Micro-TOF spectrometer in electrospray ionization (ESI) mode, using Tuning-Mix as reference. For all other compounds, mass spectra were recorded on an LCQ-DECA Thermo Finnigan Spectrometer by the ESI (Electron Spray Ionization) ionization method with an ionic source and with use of Xcalibur 2.2 software (Thermo-Finnigan, San Jose, CA, USA). Analyses were run under positive modality, and the experimental conditions were: voltage of the source 5.0 kV, voltage of the capillary 14 V, flow of the gas 35 (arbitrary units), and temperature 200 °C.

2.6.1 DETERMINATION OF ENZYMATIC ACTIVITY AND IMMOBILIZATION

The activity of the enzymes was determined following a standard protocol by using an automatic titrator pH-Stat. The hydrolytic activity was calculated based on NaOH consumption (mL of NaOH/min).

STANDARD ACTIVITY ASSAY

The activity of AXE was determined using 1-naphtyl acetate as standard substrate[54]. The activity of CRL was determined using tripropionin as standard substrate[228].

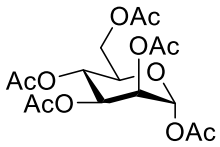
CRL IMMOBILIZATION ON OCTYL-SEPHAROSE® (OC-AG)

The crude extract of CRL (1.5 g; loading 2500 UI per gram of carrier) was suspended in KH_2PO_4 buffer (25 mM, pH 7.0). The mixture was allowed to stir on the rolling shaker for 30 min. Then, octyl-Sepharose® (3 g), previously conditioned with the same buffer, was added and the suspension was stirred at room temperature overnight. The enzyme derivative was filtered under reduced pressure on a Büchner funnel, rinsed thoroughly with distilled water, and stored at 4 °C till use.

2.6.2 SYNTHESIS OF MONOSACCHARIDE

A) SYNTHESIS OF THE SUBSTRATES SUBMITTED TO ENZYMATIC HDROLYSIS

1,2,3,4,6-*Penta*-*O*-acetyl-α-D-mannopyranose (9)

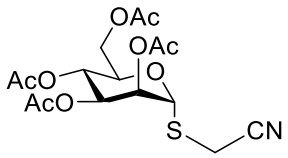


Acetic anhydride (31.67 ml, 33.3 mmol, 6 eq.) and 4-dimethylaminopyridine (catalytic quantity) were added to a solution of D-mannose (10 g, 55.5 mmol, 1 eq.) in pyridine (25 ml) and was stirred 1h at room temperature. The reaction was monitored by TLC (ethyl acetate/*n*-hexane 6:4. Rf:0.67).The mixture was transferred in an ice bath and pH was adjusted to 5.0 with HCl 6 N. The aqueous phase was extracted with 150 ml of ethyl acetate and the organic phase was washed with

$NaHCO_3$ 5% in water (100ml) and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The desired product (17 g, 80%) was obtained as a white solid after crystallization from diethyl ether.

^{1}H-NMR was in agreement with previously reported data[203].

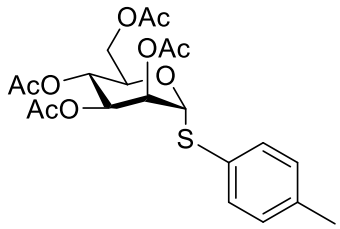
Thio-cyanomethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (12)



Thio-cyanomethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**12**) was synthesized as previously reported[204]

2,3,4,6-*Tetra*-*O*-acetyl-α-D-mannopyranosyl-1-thiouronium chloride (7.3 g, 0.017 mmol, 1 eq.), sodium meta bisulphite (6.28 g, 0.034 mmol, 2 eq.), and potassium carbonate (2.81 g, 0.0204 mmol, 1.2 eq) were dissolved in acetone/water (50:50, 80 mL). Subsequently, chloroacetonitrile (21.73 mL, 0.34 mmol, 20 eq.) was added, and the reaction was incubated for 2 h at room temperature. The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 5:5, Rf = 0.60). Upon completion, 60 mL of ice water were added to the solution, and the mixture was stirred for 45 min. The reaction was extracted with dichloromethane, and the combined organics extracts were washed with brine, filtered, dried over anhydrous Na2SO4, and concentrated in *vacuo*. The mixture was then crystallized from hot methanol. The desired product (3 g, 45%) was obtained as a white crystalline solid. ^{1}H-NMR was in agreement with previously reported data[204].

(4-Methylphenyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside (13)

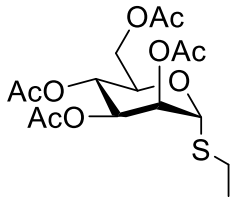


4-Methylphenyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside (**13**) was synthesized, slightly modifying the protocol reported by Janssens J. et al.[213].

To a mixture of 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranoside (**9**) (3.372 g, 8.6 mmol, 1 eq.) and *p*-thiocresol (0.955 g, 7.6 mmol, 1.2 eq.) in dichloromethane (35 mL), boron trifluoride diethyl etherate (700 µL, 5.6 mmol, 1.5 eq.) was added dropwise. The mixture was stirred at room temperature for 48 h, under nitrogen atmosphere. The reaction mixture was monitored by TLC (ethyl acetate/toluene 3:7, Rf = 0.57). The reaction mixture was then diluted with dichloromethane (28 mL) and washed with saturated $NaHCO_3$ twice and water. The organic layer was dried over $MgSO_4$. The solvent was removed, and the residue was purified by column chromatography (SiO_2, ethyl acetate/toluene 3:7). The desired product (3.21 g, 85%) was obtained as a white solid.

^{1}H-NMR was in agreement with that previously reported[229].

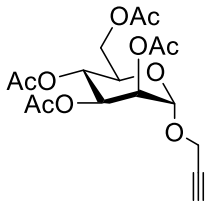
Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside (14)



Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside (**14**) was synthesized following a slightly modified protocol reported by Calosso M. et al[214].

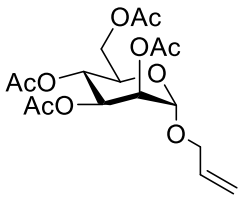
To a solution of 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**9**) (3.062 g, 7.85 mmol, 1 eq.) in anhydrous dichloromethane, ethanethiol (0.79 mL, 11 mmol, 1.4 eq.) in the presence of 4 Å molecular sieves was added. The reaction was cooled to 0 °C, and BF_3OEt_2 (1.65 mL, 13.345 mmol, 1.7 eq.) was added dropwise. The reaction was monitored by TLC (ethyl acetate/*n*-hexane 6:4, Rf = 0.64). After 7 h, the reaction was washed with 40 mL of a saturated solution of $NaHCO_3$, and the aqueous phase was washed with dichloromethane. The organic phase was dried with anhydrous Na_2SO_4, filtered and concentrated in *vacuo*. The reaction crude was purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 6:4). The desired product (2.15 g, 70%) was obtained as a white solid. ^{1}H-NMR was in agreement with that previously reported[230].

Propargyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (15)



1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranoside (**9**) (1.043 g, 2.68 mmol, 1 eq.) was dissolved in anhydrous dichloromethane (8 mL) under nitrogen atmosphere in presence of activated molecular sieves. Propargyl alcohol (0.156 mL, 2.68 mmol, 1 eq.) was added. The mixture was cooled to 0 °C, and BF_3OEt_2 (0.661 mL, 5.36 mmol, 2 eq.) was added dropwise. The reaction mixture was allowed to warm up to room temperature and allowed to stir for 5 days. The solution was diluted with dichloromethane, washed with saturated $NaHCO_3$ then water, dried over anhydrous Na_2SO_4, and concentrated in *vacuo*. The reaction was monitored by TLC (ethyl acetate/*n*-hexane 5:5, Rf = 0.30). Column chromatography (SiO_2, ethyl acetate/*n*-hexane 5:5) gave the desired compound (2.64 g, 84%) as white solid. ^{1}H-NMR was in agreement with that previously reported[215].

Allyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (16)

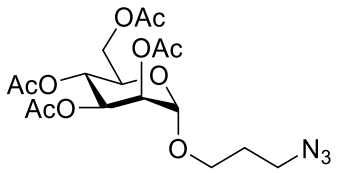


Allyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**16**) was synthesized modifying slightly the protocol reported by Balcerzak A. K. et al.[216].

1,2,3,4,6-Penta-O-acetyl-α-D-mannopyranoside (**9**) (1 g, 25.6 mmol, 1 eq.) was dissolved in anhydrous dichloromethane (8 mL) under nitrogen atmosphere in presence of activated molecular sieves. Allyl alcohol (0.175 mL, 25.6 mmol, 1 eq.) was added. The mixture was cooled to 0 °C, and BF_3OEt_2 (0.633 mL, 51.2 mmol, 2 eq.) was added dropwise. The reaction mixture was allowed to warm up to room temperature and allowed to stir for 7 days. The solution was diluted with dichloromethane, washed with saturated $NaHCO_3$ and then water, dried over anhydrous Na_2SO_4,

and concentrated in *vacuo*. The reaction was monitored by TLC (ethyl acetate/*n*-hexane 5:5, Rf = 0.65). Column chromatography (SiO_2, ethyl acetate/*n*-hexane 5:5) gave the desired product (610 mg, 61%) as a colorless oil[216]. ^{1}H-NMR was in agreement with that previously reported[217].

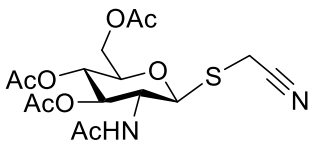
(3-Azidopropyl) 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (17)



(3-Azidopropyl) 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**17**) was synthesized as previously reported[219].

3-azido-1-propanol (0.31 mL, 3.08 mmol, 1.2 eq.) and $BF_3{\cdot}Et_2O$ (0.49 mL, 3.87 mmol, 1.5 eq.) were added to a solution of 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**9**) (1.0 g, 2.54 mmol, 1 eq.) in dichloromethane (20 mL) at 0 °C, and the mixture was stirred at room temperature overnight. The reaction was monitored by TLC (ethyl acetate/*n*-hexane 5:5, Rf = 0.25). The reaction mixture was quenched by adding dichloromethane (10 mL) and saturated $NaHCO_3$ to neutralize the remaining BF_3Et_2O. The aqueous layer was extracted by dichloromethane (30 mL), and the combined organic layers were washed with brine and then dried over anhydrous $MgSO_4$, affording the crude product. The crude product was purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 1:1) to obtain the desired product (0.58 g, 50%) as colorless oil.
^{1}H-NMR was in agreement with that previously reported[218].

Thio-cyanomethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (18)



Cyanomethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-α-D-glucopyranoside (**18**) was synthesized as reported by Zheng C. et al.[199].

1-thiourea-2-acetamido-3,4,6,-tri-*O*-acetyl-2-deoxy-α-D-glucopyranoside (245 mg, 0.554 mmol, 1 eq.) was dissolved in 1:1 water:acetone mixture (2.6 mL), and sodium meta bisulphite (0.212 g,

1.115 mmol, 2 eq.), potassium carbonate (0.093 g, 0.672 mmol, 1.2 eq.) and chloroacetonitrile (0.712 mL,11.08 mmol, 20 eq.) were added. The mixture was stirred at room temperature, and reaction was monitored by TLC (dichloromethane/methanol 9:1, Rf = 0.64). Upon completion, 8 mL of ice water were added to the solution that was stirred for 45 min. The reaction was extracted with dichloromethane, and the combined organics extracts were washed with brine and dried over anhydrous anhydrous Na_2SO_4 and concentrated in *vacuo*. The reaction crude was purified by flash chromatography (SiO_2, dichloromethane/methanol 95:5). The desired product solid (230 mg, 98%) was obtained as a white. ^{1}H-NMR was in agreement with that previously reported[199].

B) ENZYMATIC SYNTHESI OF MONOSACCHARIDES BUILDING BLOCKS

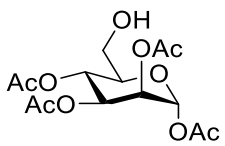
***General procedure of enzymatic monosaccharides deprotection*.**

The deacetylated monosaccharides were produced following a general procedure of hydrolysis.

The substrates (10 mM final concentration) were dissolved in acetonitrile (20–30% *v*/*v* depending on substrate solubility) under magnetic stirring, and then phosphate buffer (50 mM, pH 4.0–5.0) was added slowly. The reaction was started through the addition of immobilized CRL and/or AXE, previously conditioned with reaction buffer. The reactions were performed at 25 °C under mechanical stirring; the pH of the solution was maintained constant by automatic titration. Reaction course was monitored by TLC.

After complete consumption of the starting substrate or before an excessive formation of undesired products, the reactions were stopped by enzymatic derivative filtration on Büchner funnel. Acetonitrile was evaporated under reduced pressure, and the solution was brined and extracted with ethyl acetate. The organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated in *vacuo*. The mixture obtained was purified by flash chromatography.

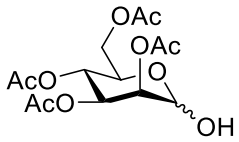
1,2,3,4-*Tetra*- *O*-acetyl-α-D-mannopyranose (9a)



1,2,3,4,6-Penta-O-acetyl-α-D-mannopyranoside (**9**) was hydrolyzed to the corresponding 1,2,3,4-tetra-O-acetyl-α/β-D-mannopyranose (**10**) following the general procedure for enzymatic hydrolysis, by using CRL-OC-AG as reported by Bavaro T. et al.[55]. The reaction mixture was

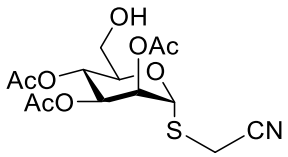
monitored by TLC (ethyl acetate/*n*-hexane 6:4) and purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 6:4, Rf=0.45). The desired product (87%) was obtained as a white solid.
^{1}H-NMR was in agreement with that previously reported data[231].

2,3,4,6-*Tetra*-*O*-acetyl-α/β-D-mannopyranose (10)



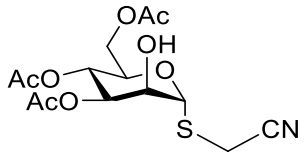
1,2,3,4,6-Penta-*O*-acetyl-α-D-mannopyranoside (**9**) (30 mg, 5mM) was hydrolyzed to the corresponding 2,3,4,6-tetra-*O*-acetyl-α/β-D-mannopyranose (**10**) following the general procedure for enzymatic hydrolysis, by using AXE-GLX-AG. Column chromatography (SiO_2, ethyl acetate/petroleum ether 6:4. Rf=0.5) gave the desired product (2.9 g, 66%) as yellow oil. ^{1}H-NMR was in agreement with previously reported data[203].

Thio-cyanomethyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (12a)



Thio-cyanomethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**12**) was hydrolysed to the corresponding Thio-cyanomethyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**12a**) following the general procedure for enzymatic hydrolysis, by using CRL-OC-AG, as reported by Bavaro T. et al.[55].
The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 6:4. Rf=0.45) and purified by flash chromatography (ethyl acetate/*n*-hexane 5:5). The desired product (70%) was obtained as a white solid. ^{1}H-NMR was in agreement with previously reported data[55].

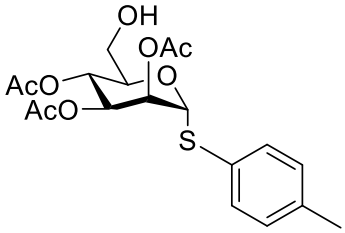
Thio-cyanomethyl 3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (12b)



Thio-cyanomethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**12**) was hydrolysed to the corresponding Thio-cyanomethyl 3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (**10b**) following the general procedure for enzymatic hydrolysis , by using CRL-OC-AG, as reported by Bavaro T. et al.[55].

The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 6:4. Rf=0.52) and purified by flash chromatography (ethyl acetate/*n*-hexane 5:5). The desired product (30%) was obtained as colourless sticky solid. ^{1}H-NMR was in agreement with previously reported data[55].

(4-Methylphenyl) 2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranoside (13a)



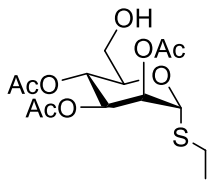
(4-Methylphenyl) 2,3,4,6-tetra-O-acetyl-1-thio-α-D-mannopyranoside (**13**) was hydrolyzed to the corresponding (4-methylphenyl) 2,3,4-tri-O-acetyl-1-thio-α-D-mannopyranoside (**13a**) following the general procedure for enzymatic hydrolysis: substrate (1g, 10 mM) was solubilized in 255 mL of phosphate buffer 50 mM pH 4.0 and 30% v/v of acetonitrile. The reaction was started through addition of CRL-OC-AG (7000 UI). The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 6:4) and purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 6:4, Rf = 0,56). The desired product (590 mg, 65%) was obtained as a white solid.

^{1}H-NMR (400 MHz, CDCl3): δ 7.38 (d, 2H, J = 8.0 Hz, Ar), 7.12 (d, 2H, J = 8.0 Hz, Ar), 5.50 (s, 1H), 5.42 (s, 1H), 5.30-5.35 (m, 2H), 4.30 (m, 1H), 3.7 (m, 2H), 2.32 (s, 3H, Ph-CH3), 2.13, 2.09, 2.02 (3s, 9H, OAc).

^{13}C-NMR (100 MHz, CDCl3): 170.74, 169.97, 169.82 (COOCH3), 138.49, 132.67, 130.04, 128.83 (Ar), 86.12, 71.74, 70.98, 69.21, 66.62, 61.30, 21.12 (Ph-CH3), 20.87, 20.75, 20.66 (COOCH3).

MS: m/z = 435.13 [M + Na+] (calcd 435.45).

Ethyl 2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranoside (14a)



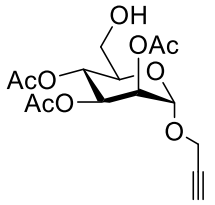
Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside (**14**) was hydrolyzed to the corresponding ethyl 2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranoside (**14a**) following the general procedure for enzymatic hydrolysis: substrate (120 mg, 10 mM) was solubilized in 30.6 mL of phosphate buffer 50 mM pH 4.0 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of CRL-OC-AG (500 UI). The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 6:4) and purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 6:4, Rf = 0.46). The desired product (56.8 mg, 53%) was obtained as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.36-5.28 (m, 4H, H-1, H-2, H-3, H-4), 4.16-4.14 (m, 1H, H-5), 3.70-3.67 (m, 2H, H-6_{ab}), 2.67-2.62 (m, 2H, SC*H*$_2$CH$_3$), 2.09, 2.02, 1.93 (3s, 9H, OAc), 1.30 (t, 3H, SCH_2CH_3).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.93, 170.07, 169.79 (*C*OOCH$_3$), 82.15 (C-1), 71.27, 71.06, 69.27, 66.69, 61.23 (5C, ring carbon), 25.39 (S*C*H$_2$CH$_3$), 20.91, 20.74, 20.65 (COO*C*H$_3$), 14.68 (SCH$_2$*C*H$_3$).

MS: *m*/*z* = 373.10 [M + Na^+] (calcd 373.38).

Propargyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (15a)



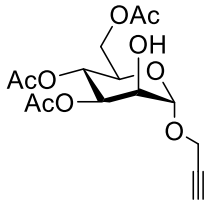
Propargyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**15**) was hydrolyzed to the corresponding propargyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**15a**) following the general procedure for enzymatic hydrolysis: substrate (500g, 10 mM) was solubilized in 255 mL of phosphate buffer 50 mM pH 4.0 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of CRL-OC-AG (7000 UI). The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 6:4, Rf = 0.37) and purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 5:5). The desired product (311 mg, 80%) was obtained as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.33 (dd, 1H, *J* = 3.4, 10.3 Hz, H-3), 5.27-5.16 (m, 2H, H-2, H-4), 4.98 (s, 1H, H-1), 4.21 (d, 2H, *J* = 2.4 Hz, OC*H*$_2$C≡CH), 3.78-3.72 (m, 1H, H-5), 3.65 (dd, 1H, *J* = 2.4, 12.7 Hz, H-6$_a$), 3.56 (dd, 1H, *J* = 4.23, 12.7 Hz, H-6$_b$), 2.41 (t, 1H, *J* = 2.4 Hz, C≡C*H*), 2.17-2.09-2.02 (3s, 9H, CH_3COO).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.76, 169,97, 169.80 (3C, CH_3*C*OO), 96.34 (C-1), 78.06 (CH≡*C*-), 75.50 (*C*H≡C-), 71.14, 69.42, 68.72, 66.34, 61.16, 54.96(CH≡C*C*H$_2$), 20.84, 20.72, 20.66 (3C, *C*H_3COO).

HRMS: *m*/*z* = 367.0996 [M + Na$^+$] (calcd 367.100).

Propargyl 3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (15b)



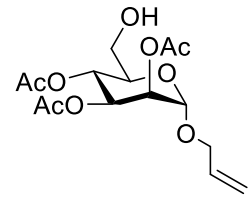
Propargyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (**15**) was hydrolyzed to the corresponding propargyl 3,4,6-tri-O-acetyl-α-D-mannopyranoside (**15b**) following the general procedure for enzymatic hydrolysis: substrate (500 mg, 10 mM) was solubilized in 234 mL of phosphate buffer 50 mM pH 4.0 and 30% v/v of acetonitrile. The reaction was started through addition of immobilized AXE (787.5 UI). The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 6:4, Rf = 0.41) and purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 6:4). The desired product was obtained as a white sticky solid (178 mg, 40%).

^{1}H-NMR (300 MHz, CDCl3): δ 5.29 (t, 1H, J = 9.9 Hz, H-4), 5.17 (dd, 1H, J = 9.9, 3.2 Hz, H-3), 5.01 (d, 1H, J = 1.8 Hz, H-1), 4-26-4.15 (m, 3H, H-6a, OCH2C≡CH), 4.06 (d, 1H, J = 2.5 Hz H-6), 4.02 (m, 1H, H-2), 3.90 (m, 1H, H-5), 2.43 (t, 1H, J = 2.5 Hz, C≡CH), 2.02, 2.01, 1.97 (3s, 9H, COCH3).

^{13}C-NMR (100 MHz, CDCl3): δ 170.85, 169.99, 169.89 (3C, CH3COO), 98.16 (C-1), 78.30 (CH≡C-), 75.33 (CH≡C-), 71.53 (C-3), 69.02, 68.83 (C-2 C-5), 66.12 (C-4), 62.35 (C-6), 54.77 (CH≡C-CH2-O), 20.83, 20.73, 20.67 (3C, CH3COO).

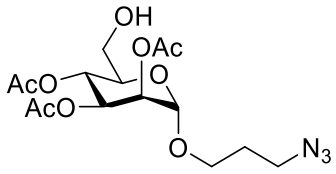
HRMS: m/z = 367.100 [M + Na+] (calcd 367.100).

Allyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (16a)



Allyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**16**) was hydrolyzed to the corresponding allyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**16a**) following the general procedure for enzymatic hydrolysis: substrate (300 mg, 10 mM) was solubilized in 77.3 mL of phosphate buffer 50 mM pH 4.0 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of CRL-OC-AG (4372.5 UI). The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 5:5, Rf = 0.4) and purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 5:5). The desired product (214 mg, 80%) was obtained as a colorless oil. ^{1}H-NMR was in agreement with that previously reported[232].

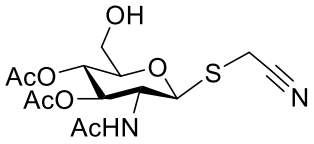
(3-Azidopropyl) 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (17a)



(3-Azidopropyl) 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**17**) was hydrolyzed to the corresponding (3-Azidopropyl) 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**17a**) by using CRL-OC-AG as reported by Li Z. et al[219]. The substrate (10 mM), immobilized CRL (1400 UI/g, 3 g in 100 ml), 30% v/v of acetonitrile, phosphate buffer 50 mM pH 4.0 for 24 hours.

The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 6:4 ,Rf=0.36) and purified by flash chromatography (ethyl acetate/*n*-hexane 6:4). The desired product (70%) was obtained as a sticky solid. ^{1}H-NMR was in agreement with previously reported data[219].

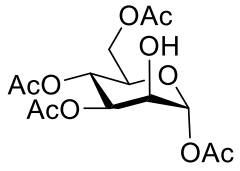
Thio-cyanomethyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (18a)



Cyanomethyl 2-acetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside (**18**) was hydrolyzed to the corresponding Thio-cyanomethyl-2-acetamido-3,4-di-*O*-acetyl-β-D-glucopyranoside (**18a**) following the general procedure for enzymatic hydrolysis, by using by CRL-OC-AG as reported by Zheng C. et al [199]. The reaction mixture was monitored by TLC (dichloromethane: MeOH 95:5. Rf=0.21) and purified by flash chromatography (dichloromethane: MeOH 95:5). ^{1}H-NMR was in agreement with that previously reported[199]**.**

C) CHEMICAL SYNTHESIS MONOSACCHARIDES BUILDING BLOCKS

1,3,4,6-Tetra-O-acetyl-α-D-mannopyranose (9b)



1,3,4,6- Tetra- O-acetyl-α-D-mannopyranose (**9b**) was synthesized as previously reported[223].

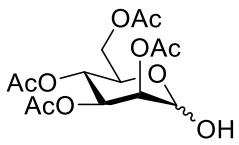
Acetic anhydride (25 ml, 0.265 mol, 7.3 eq.) and few mg (about 20-30 mg) of D-mannose were put in a flask. Two drops of 70% perchloric acid was add ($HClO_4$,70%). Other D-mannose (6.6 g, 0.036 mol, 1 eq) was added portion wise to this yellow solution during 20 min and the internal temperature was kept at 40-45 °C. The mixture was kept at R.T. for 1h and after cooled to 15°C and phosphorus tribromide (PBr3, 4.3 ml, 0.045 mol, 1.25 eq.) was added dropwise and the internal temperature kept at 20-25°C. Then water (2.3 ml) was added, and the mixture was stirred at R.T. for 90 min. A solution of sodium acetate trihydrate (20 g, 0.147 mol, 4 eq.) in water (25 ml) at 5°C was added dropwise to the mixture (internal temperature kept at 35-40°C) and was stirred for 25 min at R.T.

The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 1:1, Rf=0.13).

The reaction was moved into a beaker containing ice and was extracted with chloroform (3x30ml). the organic phase was washed with cold water, cold NaHCO3 solution then cold water again and dried over anhydrous $NaSO_4$, filtered and concentrated *in vacuo*.

The residue was crystallized from dry diethyl ether to give the desired product (2.38g, 19%) as a white solid. ^{1}H-NMR was in agreement with that previously reported data[223].

2,3,4,6-*Tetra-O*-acetyl-α/β-D-mannopyranose (10)

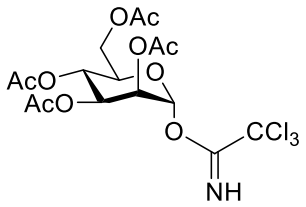


1,2,3,4,6-Penta-O-acetyl-α-D-mannopyranoside (**9**) (5 g, 12.82 mmol, 1 eq.) and benzylamine (2ml 19.23 mmol, 1.5 eq.) were solubilized in 50 ml of THF. The reaction mixture was stirred at R.T. for

20 hours. The reaction was monitored by TLC (ethyl acetate/petroleum ether 6:4). The solvent was evaporated, and the residue dissolved in dichloromethane and washed progressively with HCl 1M, saturated NaHCO3 then water. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.*

Column chromatography (SiO_2, ethyl acetate/petroleum ether 6:4. Rf=0.5) gave the desired product (2.9 g, 66%) as yellow oil.

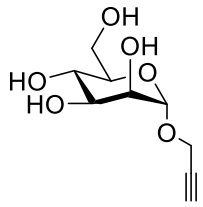
2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (11)



2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**9**) was synthesized following the procedure reported by Ekholm F.S. et al[233].

Trichloroacetonitrile (4.97 mL, 49.55 mmol, 5 eq.) and 1,8-diazabicyclo-[5,4,0]-7-undecene (DBU, 0.74 mL, 4.95 mmol, 0.5 eq.) were added to a solution of 2,3,4,6-tetra-*O*-acetyl-α/β-D-mannopyranose (**10**) (3.45 g, 9.91 mmol, 1 eq.) in dry dichloromethane (30 mL) at 0 °C under nitrogen atmosphere. The mixture was stirred for 3 h at 0 °C and then concentrated in *vacuo*. The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 5:5, Rf = 0.8) and purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 5:5). The desired product (3.89 g, 80%) was obtained as a yellow sticky solid.c^{1}H-NMR was in agreement with that previously reported[203].

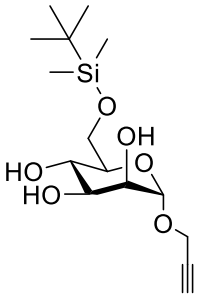
Propargyl α-D-mannopyranoside (19)



Propargyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**15**) (80mg, 0.207 mmol) was stirred 20min in 1ml solution of Na/dry MeOH (2mg/ml) under argon atmosphere. The reaction mixture was monitored by TLC (dichloromethane/MeOH 5:1 ,Rf=0.05). The solution was filtered over a short

column of Dowex® 50WX8-200 (H^+ resin form). The resin was washed with MeOH (15 mL) and H2O (15 mL) and the fractions containing the product were concentrated under reduced pressure to afford the desired product (44mg, 97%) as a white solid. ^{1}H-NMR was in agreement with that previously reported[234].

Propargyl 6-*tert*-butyldimethylsilyl-α-D-mannopyranoside (20)



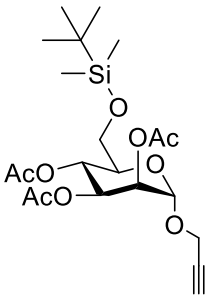
Propargyl α-D-mannopyranoside (**19**) (49 mg, 0.224 mmol, 1 eq.) was dissolved in 1.1 mL of solvent (CH_3CN/DMF, 10:1). Triethylamine (56 µL, 0.403 mmol, 1.8 eq.) and *tert*-butyldimethylsilyl chloride (TBSCl)(50.8 mg, 0.336 mmol, 1.5 eq.) were added and the mixture was stirred at r.t. for 30 min and after concentrated in *vacuo*. The reaction was monitored by TLC (ethyl acetate/cyclohexane 5:1, Rf = 0.43). Column chromatography (SiO_2, ethyl acetate/cyclohexane 5:1) gave the desired compound (61.5 mg, 83%) as white solid.

^{1}H-NMR (400 MHz, CDCl3): δ 5.00 (s,1H), 4.24 (d, 2H, J= 2.5 Hz, OC*H*2C≡CH), 4.00-3.79 (m, 7H), 3.74 (t, 1H, J= 9.3 Hz), 3.62-3.55 (m, 1H), 2.46 (t, 1H, J=2.4 Hz, OCH2C≡C*H*), 0.92 (s, 9H, -$(CH_3)_3$), 0.11 (s, 6H, Si-$(CH_3)_2$).

^{13}C-NMR (100 MHz, CDCl3): δ 98.17 (C-1),78.82, 74.89, 72.02, 71.63, 70.31, 69.44, 64.20, 54.05, 25.89, 18.27, 5.34, 5.37.

MS: *m*/*z* = 355.2167 [M + Na^+] (calcd 355.46).

Propargyl 2,3,4-tri-*O*-acetyl-6- *tert*-butyldimethylsilyl-α-D-mannopyranoside (21)



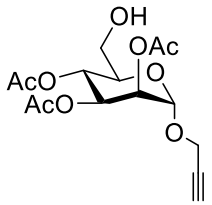
Acetic anhydride (77.8 µL, 0.823 mmol, 4.5 eq.) and 4-dimethylaminopyridine (catalytic quantity) were added to a solution of Propargyl 6-*tert*-butyldimethylsilyl-α-D-mannopyranoside (**20**) (61 mg, 0.183 mmol, 1 eq.) in pyridine (1 ml) and was stirred 1h at room temperature. The reaction was monitored by TLC (ethyl acetate/*n*-hexane 6:4. Rf:0.67).The mixture was transferred in an ice bath and pH was adjusted to 5.0 with HCl 6 N. The aqueous phase was extracted with dichloromethane and the organic phase was washed with saturated $NaHCO_3$ solution and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.* The desired product (79 mg, 94%) was obtained as a colorless oil.

^{1}H-NMR (400 MHz, CDCl3): δ 7.35-7.18 (m,3H), 4.99 (s, 1H), 4.24 (d, 2H, J= 2.5 Hz, OC*H*2C≡CH), 3.78 (dd, 1H, J= 9.31 Hz, J= 4.2 Hz), 3.70-3.66 (m, 2H), 2.46 (t, 1H, J=2.4 Hz, OCH2C≡C*H*), 2.12, 2.01, 2.97 (3s, 9H, CH_3COO), 0.89 (s, 9H, -$(CH_3)_3$), 0.08 (s, 6H, Si-$(CH_3)_2$).

^{13}C-NMR (100 MHz, CDCl3): δ 169.94, 169.90, 169.53 (3C, CH3*C*OO), 95.58 (C-1),78.09, 75.32, 71.76, 69.54, 69.29, 66.39, 62.15, 54.22,25.77, 20.77, 20.71, 20.65 (3C, *C*H3COO), 18.20, 5.42, 5.46.

MS: *m*/*z* = 481.2362 [M + Na^+] (calcd 481.57).

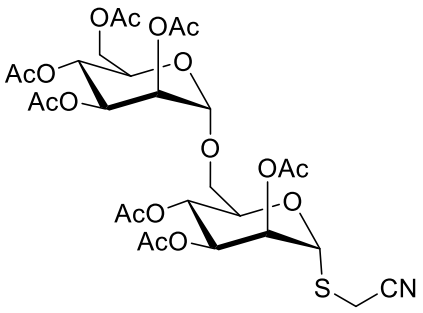
Propargyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (15a)



Propargyl 2,3,4-tri-*O*-acetyl-6- *tert*-butyldimethylsilyl-α-D-mannopyranoside (**21**) (79 mg, 0.172 mmol, 1 eq.) was dissolved in 5ml acetone/water (3:7). $KHSO_4$ (23 mg, 0.172 mmol, 1 eq.) was added and the mixture was stirred 5h at 35°C. The acetone was removed under reduced pressure and the aqueous solution was extracted with ethyl acetate. The organic phase was dried over anhydrous Na_2SO_4, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (ethyl acetate/cyclohexane 6:4, Rf = 0.33) and purified by flash chromatography (SiO_2, ethyl acetate/cyclohexane 6:4). The desired product (32 mg, 54%) was obtained as a white solid. Analysis are shown above.

2.6.3 SYNTHESIS OF DISACCHARIDES

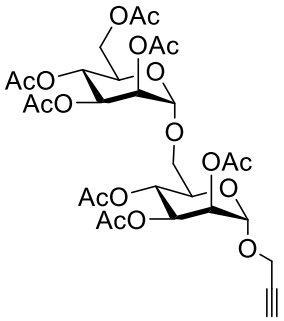
Thio-cyanomethyl 2',3',4',6'-*Tetra*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranoside (1)



Cyanomethyl (2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranose (**11**) was synthesized as reported Bavaro T. et al.[55].

2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (226 mg, 0.460 mmol, 2 eq.) and Thio-cyanomethyl 3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (**12a**) (83 mg, 0.230 mmol, 1 eq.) were dissolved in dry dichloromethane (5 mL) in presence of activated molecular sieves and cooled to 0 °C with dry ice under nitrogen atmosphere. BF_3OEt_2 (56.6 µL, 0.460 mmol, 2 eq.) was added, and the mixture was stirred at room temperature for 4 h. The reaction was quenched with triethylamine (64.2 µL, 0.460 mmol, 2 eq.), stirred for 5 min, filtered, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 5:5, Rf = 0.32) and purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 5:5). The desired product (125 mg, 79%) was obtained as a white solid. ^{1}H-NMR was in agreement with that previously reported[55].

Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (2)



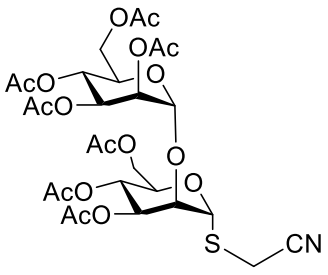
2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (1.1 g, 2.233 mmol, 2 eq.) and propargyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**15a**) (0.384 g, 1.115 mmol, 1 eq.) were dissolved in dry dichloromethane (35 mL) in presence of 4 Å molecular sieves and cooled to 0 °C under argon atmosphere. BF_3OEt_2 (137.6 µl, 1.115 mmol, 1 eq.) was added, and the mixture was stirred at 0 °C for 4 h. The reaction was quenched with triethylamine (155 µL, 1.115 mmol, 1 eq.), stirred for 5 min, filtered over Celite, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (dichloromethane/acetone 9:1, Rf = 0.61). Column chromatography (SiO_2, dichloromethane/acetone 9:1) gave the desired product (0.676 g, 90%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.38-5.25 (m, 6H, H-4', H-3', H-2', H-4, H-3, H-2), 5.03 (d, 1H, *J* = 1.8 Hz, H-1), 4.87 (d, 1H, *J* = 1.7 Hz, H-1'), 4.32 (d, 2H, *J* = 2.4 Hz, OC*H*$_2$C≡CH), 4.28 (dd, 1H, *J* = 5.3 Hz, 12.4 Hz, H-6'$_a$), 4.15 (dd, 1H, *J* = 2.4 Hz, 12.2 Hz, H-6'$_b$), 4.12-3.98 (m, 2H, H-5', H-5), 3.80 (dd, 1H, *J* = 5.7 Hz, 11 Hz, H-6$_b$), 3.60 (dd, 1H, *J* = 2.6 Hz, 11 Hz, H-6$_a$), 2.53 (t, 1H, *J* = 2.4 Hz, C≡C*H*), 2.18, 2.17, 2.12, 2.07, 2.06, 2.01, 2.00 (7s, 21H, $COCH_3$).

^{13}C-NMR (150 MHz, $CDCl_3$): δ 170.59, 170.04, 169.94, 169.83, 169.79, 169.74, 169.70 (7C, CH_3*C*OO), 97.51 (C-1'), 96.03 (C-1), 78.05 (OCH$_2$*C*≡CH), 75.60 (OCH$_2$C≡*C*H), 69.85(C-5), 69.35, 69.29, 69.01, 68.98, 68.65 (C-5'), 66.64 (C-6), 66.44, 65.97, 62.42 (C-6'), 54.95 (O*C*H$_2$C≡CH), 20.84, 20.75, 20.72, 20.70, 20.70, 20.64, 20.62 (7C, *C*H$_3$COO).

HRMS: *m*/*z* = 697.1943 [M + Na$^+$] (calcd 697.1950).

Thio-cyanomethyl 2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (3)



2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (202 mg, 0.41 mmol, 1 eq.) and Thio-cyanomethyl 3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (**12b**) (148 mg, 0.41 mmol, 1 eq.) were dissolved in dry dichloromethane (5 mL) in presence of activated molecular sieves and cooled to -63 °C under argon atmosphere. BF_3OEt_2 (50.6 µL, 0.41 mmol, 1 eq.) was added, and the

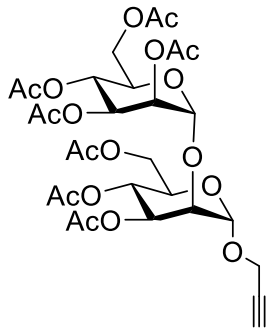
mixture was stirred for 30 min. After, the solution was allowed to warm to room temperature and stirred for 1h. The reaction was quenched with triethylamine (57 µL, 0.41 mmol, 1 eq.), stirred for 5 min, filtered over Celite, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (dichloromethane/acetone 9:1, Rf = 0.42). Column chromatography (SiO_2, dichloromethane/acetone 9:1) gave the desired product (149 mg, 52.6%) as white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.63 (d, 1H, *J* = 1.9 Hz, H-1), 5.45-5.39 (m, 2H, H-4, H-3'), 5.31 (t, 1H, *J* = 9.9 Hz, H-4'), 5.27 (dd, 1H, *J* = 3.4, 1.9, H-2'), 5.16 (dd, 1H, *J* = 9.6, 3.3, H-3), 4.96 (d, 1H, *J* = 1.9 Hz, H-1'), 4.37-4.25 (m, 3H, H-6_a, H-6_b, H-5), 4.23-4.12 (m, 4H, H-2, H-5', H-$6'_a$, H-$6'_b$), 3.51 (d, 1H, *J* =17.1 Hz, SC*H*$_2$CN), 3.40 (d, 1H, *J* = 17.1 Hz, SC*H*$_2$CN), 2.17, 2.17, 2.13, 2.12, 2.08, 2.07, 2.04 (7s, 21H, C*H*$_3$COO).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.74, 170.65, 170.32, 169.87, 169.74, 169.51, 169.25 (7C, CH_3*C*OO), 115.75 (SCH_2*C*N), 99.15 (C-1'), 83.33 (C-1), 76.70 (C-2), 70.35 (C-3), 70.04 (C-5), 69.60, 69.60 (C-2', C-5'), 68.30, 66.48, 65.95 (C-4, C-4', C-3'), 62.60 (C-6'), 61.77 (C-6), 20.84, 20.76, 20.68, 20.64, 20.64, 20.62, 20.62 (7C, *C*H_3COO), 15.91 (S*C*H_2CN).

MS: *m*/*z* = 714.21 [M + Na^+] (calcd 714.65).

Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (4)



CHEMOENZYMATIC SYNTHESIS

2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (212 mg, 0.43 mmol, 1 eq.) and propargyl 3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (**15b**) (150 mg, 0.43 mmol, 1 eq.) were dissolved in dry dichloromethane (8 mL) in presence of activated molecular sieves. The solution was cooled to -50 °C under argon atmosphere. BF_3OEt_2 (49.7 µL, 0.43 mmol, 1 eq.) was added to the flask, and the solution was allowed to warm to room temperature and stirred for 18 h. The reaction was quenched with triethylamine (155 µL, 1.115 mmol, 1 eq.), stirred for 5 min, filtered

over Celite, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (dichloromethane/acetone 9:1, Rf = 0.70). Column chromatography (SiO_2, dichloromethane/acetone 9:1) gave the desired product (232 mg, 80%) as a white solid.

CHEMICAL SYNTHESIS

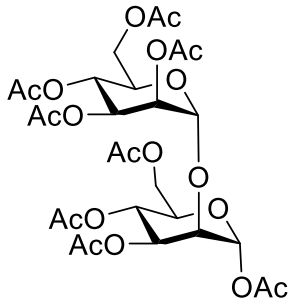
2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-1,3,4,6-tetra-O-acetyl-α-D-mannopyranose (**22**) prepared as following reported (161 mg, 0.237 mmol, 1 eq.) was dissolved in anhydrous dichloromethane (3 mL) under argon atmosphere. Propargyl alcohol (13.8 µL, 0.237 mmol, 1 eq.) was added. The mixture was cooled to 0 °C, and BF_3OEt_2 (58 µL, 0.474 mmol, 2 eq.) was added dropwise. The reaction mixture was allowed to warm up to room temperature and allowed to stir for 7 days. The solution was diluted with dichloromethane, washed with saturated $NaHCO_3$ then brine, dried over anhydrous Na_2SO_4, and concentrated in *vacuo*. The reaction was monitored by TLC (ethyl acetate/cyclohexane 5:5, Rf = 0.27). Column chromatography (SiO_2, ethyl acetate/cyclohexane 5:5) gave the desired compound (92 mg, 58%) as white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.45-5.27 (m, 5H, H-3', H-4, H-4', H-3, H-2'), 5.18 (d, 1H, *J* =2.0 Hz, H-1), 4.95 (d, 1H, *J* = 2.0 Hz, H-1'), 4.29 (d, 2H, *J* = 2.4 Hz, OC*H*$_2$C≡CH), 4.28-4.22 (m, 2H, H-6$_a$, H-6$_b$,), 4.20-4.12 (m, 3H, H-5', H-6'$_a$, H-6'$_b$), 4.07(dd, 1H, *J* =1.2, 2.0 Hz H-2), 4.00-3.95 (m, 1H, H-5), 2.51 (t, 1H, *J* = 2.4 Hz, C≡C*H*), 2.17, 2.16, 2.11, 2.10, 2.06, 2.05, 2.03 (7s, 21H, C*H*$_3$COO).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.86, 170.54, 170.33, 169.83, 169.70, 169.44, 169.33 (7C, CH_3*C*OO), 99.20 (C-1'), 96.92 (C-1), 78.11 (OCH$_2$*C*≡CH), 76.75 (C-2), 75.54 (OCH$_2$C≡*C*H), 70.06 (C-2'), 69.75 (C-3), 69.24 (C-5'), 69.09 (C-5), 68.41 (C-3'), 66.31(C-4'), 66.09 (C-4), 62.35(C-6$_a$), 62.03(C-6'), 55.00 (O*C*H$_2$C≡CH), 20.90, 20.87, 20.74, 20.71, 20.67, 20.67, 20.65 (7C, *C*H$_3$COO).

HRMS: *m*/*z* = 697.1950 [M + Na$^+$] (calcd 697.1950).

2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-1,3,4,6-tetra-O-acetyl-α-D-mannopyranose (22)

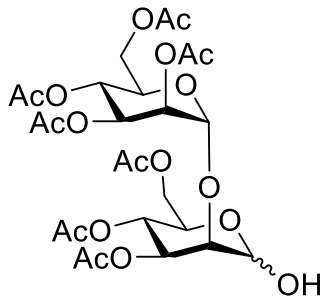


2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (496 mg, 1.009 mmol, 1.3 eq.) and 1,3,4,6- *Tetra*- *O*-acetyl-α-D-mannopyranose (**9b**) (270 mg, 0.776 mmol, 1 eq.) were dissolved in 15 ml of dry dichloromethane in presence of 4 Å molecular sieves and cooled to -70°C under argon atmosphere. BF_3OEt_2(95.8 µL, 0.776 mmol, 1 eq.) was added and the mixture to warm to room temperature and stirred for 10 min.

The reaction was quenched with triethylamine (108 µl, 0.776 mmol, 1 eq.), stirred for 5 minutes, filtered and concentrated *in vacuo*. The reaction mixture was monitored by TLC (ethyl acetate: cyclohexane 6:4. Rf=0.36).

Column chromatography (SiO_2, ethyl acetate: cyclohexane 6:4) gave the desired product (426 mg, 81%) as white solid. ^{1}H-NMR was in agreement with that previously reported[225].

2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-O-acetyl-α/β-D-mannopyranose (22a)



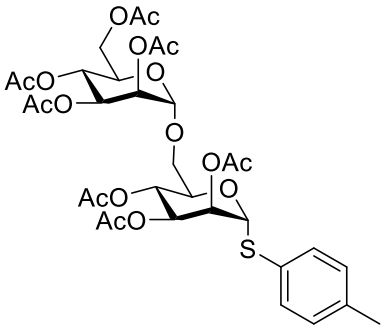
2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-1,3,4,6-tetra-*O*-acetyl-α-D-mannopyranose (**22**) (50 mg, 0.074 mmol, 1 eq.) and benzylamine (12.1 µL, 0.111 mol, 1.5 eq.) were dissolved in 1 ml of THF. The reaction mixture was stirred at R.T. for 21 hours. The solution was evaporated,

taken into chloroform and washed progressively with HCl 1M, saturated $NaHCO_3$ then brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo.*

The reaction mixture was monitored by TLC (ethyl acetate: cyclohexane 6:4. Rf=0.50).

Column chromatography (SiO_2, ethyl acetate/*n*-hexane 6:4) gave the desire product (43.3mg, 92%) as pale-yellow sticky solid. ^{1}H-NMR was in agreement with that previously reported[225].

(4-Methylphenyl)(2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranoside (23)



2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (366 mg, 0.743 mmol, 2.5 eq.) and (4-methylphenyl) 2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranoside (**13a**) (140 mg, 0.298 mmol, 1 eq.) were dissolved in dry dichloromethane (7 mL) in presence of 4 Å molecular sieves and cooled to 0 °C under nitrogen atmosphere. BF_3OEt_2 (73.5 µL, 0.596 mmol, 2 eq.) was added, and the mixture was stirred at 0 °C for 3.5 h. The reaction was quenched with triethylamine (83.2 µL, 0.596 mmol, 2 eq.), stirred for 5 min, filtered, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 5:5, Rf = 0.28) and purified by flash chromatography (SiO_2, ethyl acetate/*n*-hexane 5:5). The desired product (204 mg, 92%) was obtained as a white solid.

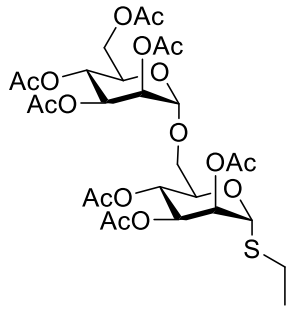
^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.30 (d, 2H *J* = 8.0 Hz, Ar), 7.08 (d, 2H, *J* = 8.0 Hz, Ar), 5.50 (dd, *J* = 1.7, 3.2 Hz, 1H, H-3), 5.42–5.20 (m, 6H, H-4, H-2', H-3', H-2, H-1, H-4'), 4.84 (d, 1H, *J* = 1.5 Hz, H-1'), 4.43–4.38 (m, 1H, H-5), 4.21 (dd, 1H, *J* = 5.0, 12.2 Hz, H-6'$_a$), 3.99 (dd, 1H, *J* = 12.3, 2.4 Hz, H-6'$_b$), 3.95–3.88 (m, 1H, H-5'), 3.74 (dd, 1H, *J* = 11.3, 5.0 Hz, H-6$_a$), 3.59 (dd, 1H, *J* = 11.3, 2.7 Hz, H-6$_b$), 2.25 (s, 3H, C*H*$_3$-Ar), 2.08, 2.09, 2.02, 2.02, 1.99, 1.95, 1.92 (7s, 21H, COC*H*$_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.60, 170.14, 169.90, 169.87, 169.74, 169.72, 169.56 (7C, CH_3COO), 138.34, 132.58, 130.08, 129.08 (Ar), 97.98 (C-1'), 86.17 (C-1), 70.91 (C-3), 70.22 (C-5),

69.44, 69.31, 69.07, 68.62 (C-5'), 66.85 (C-6), 66.65, 66.00, 62.31 (C-6'), 21.13(CH_3-Ar), 20.85, 20.78, 20.75, 20.72, 20.72, 20.66, 20.62 (7C, *C*H_3COO).

MS: *m*/*z* = 765.18 [M + Na^+] (calcd 765.20).

Ethyl (2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranoside (24)



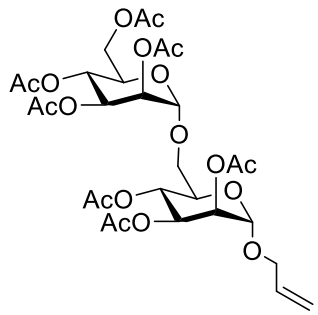
2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (58.7 mg, 0.119 mmol, 1 eq.) and ethyl 2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranoside (**14a**) (41.8 mg, 0.119 mmol, 1 eq.) were dissolved in dry dichloromethane (1 mL) in presence of 4 Å molecular sieves and cooled to 0 °C under nitrogen atmosphere. BF_3OEt_2 (14.7 µL, 0.119 mmol, 1 eq.) was added, and the mixture was stirred at room temperature for 4 h. The reaction was quenched with triethylamine (17 µL, 0.119 mmol, 1 eq.), stirred for 5 min, filtered, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (dichloromethane/acetone 9:1, Rf = 0.71) and purified by flash chromatography (SiO_2, dichloromethane/acetone 9:1). The desired product was obtained as a white solid (28 mg, 37%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.38–5.22 (m, 7H, H-2', H-3', H-4', H-1, H-2, H-3, H-4), 4.85 (d, 1H, *J* = 1.7 Hz, H-1'), 4.39 (ddd, 1H, *J* = 9.1, 6.4, 2.3 Hz, H-5), 4.25 (dd, 1H, *J* = 12.2, 5.4 Hz, H-6'$_a$), 4.16 (dd, 1H, *J* = 12.2, 2.4 Hz, H-6'$_b$), 4.06 (ddd, 1H, *J* = 9.5, 5.4, 2.4 Hz, 1H, H-5'), 3.82 (dd, 1H, *J* = 10.9, 6.4 Hz, H-6$_a$), 3.56 (dd, 1H, *J* = 10.8, 2.4 Hz, H-6$_b$), 2.74–2.63 (m, 2H, SC*H*$_2$CH$_3$), 2.18, 2.17, 2.13, 2.08, 2.05, 2.00, 1.99 (7s, 21H, COC*H*$_3$), 1.34 (t, 3H, *J* = 7.4, SCH$_2$C*H*$_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.61, 170.05, 169.99, 169.88, 169.77, 169.74, 169.66 (7C, CH$_3$*C*OO), 97.33 (C-1'), 81.47 (C-1), 71.04, 69.56, 69.54 (C-5), 69.41, 68.88, 68.59 (C-5'), 66.81 (C-6), 66.53, 66.05, 62.39 (C-6'), 25.08 (S*C*H$_2$CH$_3$), 20.86, 20.84, 20.74, 20.74, 20.69, 20.63, 20.63 (7C, *C*H$_3$COO), 14.57 (SCH$_2$*C*H$_3$).

MS: *m*/*z* = 703.12 [M + Na^+] (calcd 703.19).

Allyl (2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (25)



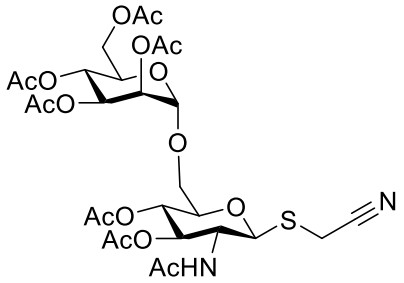
2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (226.4 mg, 0.460 mmol, 1 eq.) and allyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**16a**) (159 mg, 0.460 mmol, 1 eq.) were dissolved in dry dichloromethane (5 mL) in presence of activated molecular sieves and cooled to 0 °C under nitrogen atmosphere. BF_3OEt_2 (56 µL, 0.460 mmol, 1 eq.) was added, and the mixture was stirred at 0 °C for 2 h. The reaction was quenched with triethylamine (64.2 µL, 0.460 mmol, 1 eq.), stirred for 5 min, filtered, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (dichloromethane/acetone 9:1, Rf = 0.74) and purified by flash chromatography (SiO_2, toluene/methanol 9:1). The desired product (156 mg, 50%) was obtained as white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.98-5.88 (m, 1H, CH_2CH=CH_2), 5.41-5.23 (m, 8H, H-2, H-3, H-4 H-2', H-3', H-4', CH_2CH=CH_2), 4.88 (d, 1H, *J* = 1.8 Hz, H-1), 4.86 (d, 1H, *J* = 1.8 Hz, H-1'), 4.30-4.21 (m, 2H, H-6'$_a$, CH_2CH=CH_2), 4.16 (dd, 1H, *J* = 12.2, 2.4 Hz, H-5), 4.14-3.97 (m, 3H, H-5', H-6'$_b$, CH_2CH=CH_2), 3.80 (dd, 1H, *J* = 10.9, 6.0 Hz, H-6$_a$), 3.58 (dd, 1H, *J* = 10.9, 2.5 Hz, H-6$_b$), 2.17, 2.17, 2.13, 2.07, 2.06, 2.01, 2.00 (7s, 21H, CH_3COO).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.63, 170.18, 169.99, 169.89, 169.89, 169.77, 169.69 (7C, COO), 133.04 (CH_2CH=CH_2), 118.42 (CH_2CH=CH_2), 97.43 (C-1), 96.26 (C-1'), 69.61, 69.42, 69.36, 69.17, 68.95, 68.64, 68.55 (CH_2CH=CH_2), 66.63, 66.63, 66.04 (C-6), 62.42 (C-6'), 20.87, 20.82, 20.74, 20.74, 20.70, 20.70, 20.64 (7C, CH_3COO).

MS: *m*/*z* = 699.25 [M + Na^+] (calcd 699.21).

Thio-cyanomethyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (26)



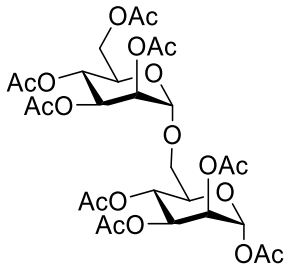
Thio-cyanomethyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**26**) was synthesized as reported by Zheng C. et al.[199]. 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (235 mg, 0.478 mmol, 2 eq.) and Thio-cyanomethyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**18a**) (86 mg, 0.239 mmol, 1 eq.) were dissolved in dry dichloromethane (25 mL) in presence of activated molecular sieves and cooled to 0 °C under nitrogen atmosphere. BF_3OEt_2 (59 µL, 0.478 mmol, 2 eq.) was added, and the mixture was stirred at room temperature for 2.5 h. The reaction was quenched with triethylamine (67 µL, 0.478 mmol, 2 eq.), stirred for 5 min, filtered, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (ethyl acetate/diethyl ether 3:2, Rf = 0.39) and purified by flash chromatography (SiO_2, ethyl acetate/diethyl ether 3:2). The desired product (122mg, 74%) was obtained as a as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.95 (d, 1H, *J* = 9.2 Hz, NH), 5.39–5.17 (m, 4H, H-4', H-3', H-2', H-3), 5.05 (t, 1H, *J* = 9.3 Hz, H-4), 4.86 (s, 1H, H-1'), 4.80 (d, 1H, *J* = 10.3 Hz, H-1), 4.29 (dd, 1H, *J* = 12.4, 5.1 Hz, H-6'$_a$), 4.25-4.09 (m, 2H, H-2, H-6'$_b$), 4.02 (m, broad, 1H, H-5'), 3.83-3.76 (m, 2H, H-6$_a$, H-5), 3.73 (d, 1H, *J* = 17.1 Hz, C*H*CN) 3.63-3.54 (m, 1H, H-6$_b$), 3.38 (d, 1H, *J* = 17.1 Hz, C*H*CN), 2.17, 2.13, 2.08, 2.07, 2.06, 2.01, 1.99 (21H, C*H*$_3$COO).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.22, 170.68, 170.53, 170.09, 169.90, 169.72, 169.42 (7C, CH_3*C*OO), 116.40 (SCH_2*C*N), 97.36 (C-1'), 83.30 (C-1), 76.85 (C-5), 73.23 (C-3), 69.38 (C-3'), 68.96 (C-5'), 68.86 (C-2'), 68.83 (C-4), 66.70 (C-6), 66.03 (C-4'), 62.41 (C-6'), 52.76 (C-2), 23.14 (CH_3, NHAc), 20.87, 20.79, 20.72, 20.67, 20.65, 20.61 (6C, *C*H_3COO), 14.62 (S*C*H_2CN).

MS: *m*/*z* = 713.10 [M + Na^+] (calcd 713.18).

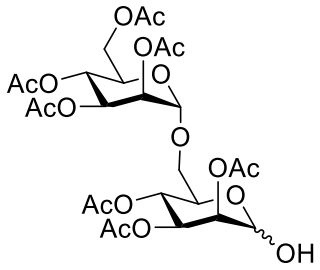
2′,3′,4′,6′-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-1,2,3,4-tetra-*O*-acetyl-α-D-mannopyranose (27)



2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**11**) (3.77 g, 7.662 mmol, 1.6 eq.) and 1,2,3,4-tetra-*O*-acetyl-α-D-mannopyranose (**9a**) (1.67 g, 4.798 mmol, 1 eq.) were dissolved in dry dichloromethane (50 mL) in presence of activated molecular sieves and cooled to 0 °C under nitrogen atmosphere. BF_3OEt_2 (591 µL, 4.798 mmol, 1 eq.) was added, and the mixture was stirred at room temperature for 2.5 h. The reaction was quenched with triethylamine (668 µL, 4.798 mmol, 2 eq.), stirred for 5 min, filtered, and concentrated in *vacuo*. The reaction mixture was monitored by TLC (dichloromethane/methanol 9:1, Rf = 0.89) and purified by flash chromatography (SiO_2, dichloromethane/methanol 9:1). The desired product (2.83 g, 87%) was obtained as a white solid.

^{1}H-NMR was in agreement with that previously reported[235].

2′,3′,4′,6′-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α/β-D-mannopyranose (27a)



2′,3′,4′,6′-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-1,2,3,4-tetra-*O*-acetyl-α-D-mannopyranose (**27**) (3.37 g, 4.96 mmol, 1 eq.) and hydrazine acetate (0.45 g, 4.96 mmol, 1 eq.) were solubilized in 70 ml of dimethylformamide. The reaction mixture was stirred at 50 °C for 5 hours. The solution

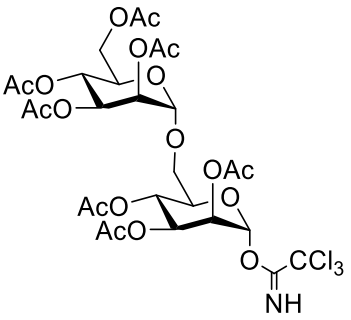
was diluted with ethyl acetate (100 ml) and extracted three times with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*.

The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 6:4. Rf=0.45).

Column chromatography (SiO_2, ethyl acetate: cyclohexane 6:4) gave the desired product (2.9 g, 92%) as a white sticky solid.

The product was identified by comparing the reaction mixture with standard material[236].

2',3',4',6'- Tetra-*O*-acetyl-α-D-mannopyranosyl -(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (28)

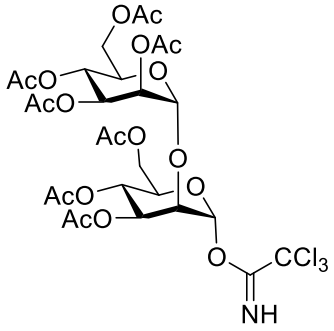


Trichloroacetonitrile (1.061 ml, 10.61 mmol, 15 eq.) and 1,8-diazabicyclo-[5,4,0]-7-undecene (DBU, 0.42 ml, 2.83 mmol, 4 eq.) were added to a solution of 2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α/β-D-mannopyranose (**27a**) (450 mg, 0.708 mmol, 1 eq.) in dry dichloromethane (10 ml) at 0°C under N_2 atmosphere. The mixture was stirred for 4 hours at 0°C, then concentrated *in vacuo*. The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 7:3 Rf=0.75). Column chromatography (SiO_2, ethyl acetate/*n*-hexane 6:4) gave the desired product (315 mg, 57%) as a pale-yellow solid.

MS: m/z = 804.17[M + Na+] (calcd 803.92).

^{1}H-NMR was in agreement with that previously reported[237].

2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (29)



Trichloroacetonitrile (94.6 µL, 0.94 mmol, 15 eq.) and 1,8-diazabicyclo-[5,4,0]-7-undecene (DBU37.5 µL, 0,25 mmol, 4 eq.) were added to a solution of 2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α/β-D-mannopyranose (**22a**) (40 mg, 0.063 mmol, 1 eq.) in dry dichloromethane (1ml) in presence of 4 Å molecular sieves at 0°C under N_2 atmosphere. The mixture was stirred for 4 hours at 0°C, then concentrated *in vacuo*.

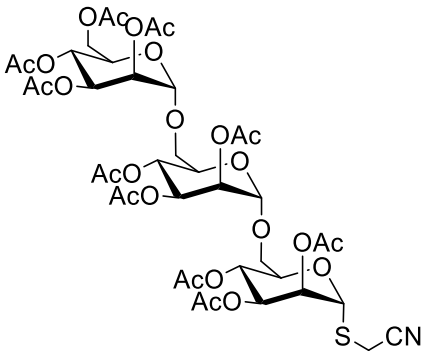
The reaction mixture was monitored by TLC (ethyl acetate/*n*-hexane 5:5. Rf=0.40). Column chromatography (SiO_2, ethyl acetate/*n*-hexane 5:5) gave 2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri- acetyl-α-D-mannopyranoside trichloracetimidate (31mg, 63%) as a pale-yellow solid.

MS: m/z = 802.08 [M+ Na^+] (calcolata 779,09)

^{1}H-NMR was in agreement with previously reported data[225].

2.6.4 SYNTHESIS OF TRISACCHARIDES

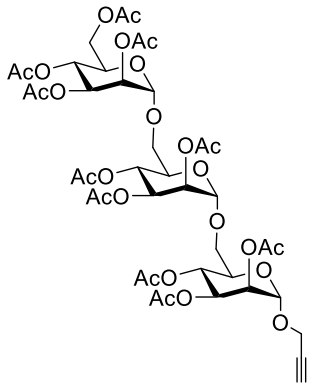
Thio-cyanomethyl 2",3",4",6"-*Tetra*-*O*-acetyl- α-D-mannopyranosyl-(1→6)-2',3',4'-*tri*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranoside (5)



2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**28**) (1,29 g, 1,662 mmol, 2 eq) and Thio-cyanomethyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**12a**) (0,300 g, 0,831 mmol, 1 eq) were dissolved in 25 mL of dry dichloromethane in presence of activated molecular sieves 4Å. The reaction was cooled to 0°C and BF_3OEt_2 (135.6 µl, 0.831 mmol, 1 eq) was added dropwise. The reaction was quenched with triethylamine after 3 hours (30.57 µl, 0.415 mmol, 0.5 eq.), stirred for 5 minutes, filtered and concentrated *in vacuo*.

The reaction mixture was monitored by TLC (dichloromethane /acetone 9:1.Rf=0.57). Column chromatography (SiO_2, dichloromethane /acetone 9:1) gave the desired product (252 mg, 31%) as a pale-yellow solid. ^{1}H-NMR was in agreement with that previously reported[236].

Propargyl 2",3",4",6"-*Tetra*-*O*-acetyl- α-D-mannopyranosyl-(1→6)-2',3',4'-*tri*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranoside (6)



2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**28**) (250 mg, 0.658 mmol, 2 eq.) and propargyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**15a**) (0.113 g, 0.329 mmol, 1 eq.) were dissolved in dry dichloromethane (10 ml) in presence of activated molecular sieves and cooled to 0°C under nitrogen atmosphere. BF_3OEt_2 (40.6 µl, 0.329 mmol, 1 eq.) was added and the mixture was stirred at 0°C for 4 hours. The reaction was quenched with triethylamine (45.644 µl, 0.329 mmol, 1 eq.), stirred for 5 minutes, filtered and concentrated *in vacuo*.

The reaction mixture was monitored by TLC (acetone/dichloromethane 1:9 Rf=0.52 or ethyl acetate/*n*-hexane 5:5. Rf=0.19) and purified by flash chromatography (acetone/dichloromethane 1:9 and ethyl acetate/*n*-hexane 5:5). The desired product (56%) was obtain as a white solid.

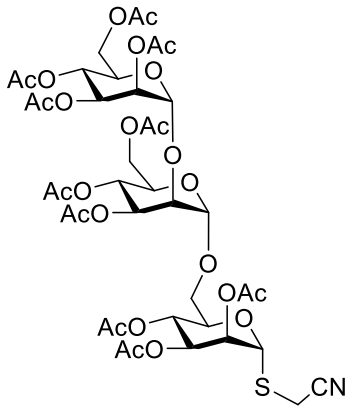
^{1}H NMR (400 MHz, $CDCl_3$): δ= 5.40-5.28 (m, 9H, H-2, H-3, H-4, H-2', H-3', H-4', H-2'', H-3'', H-4''), 5.04 (d, 1H, J=1.8Hz, H-1), 4.89 (d, 1H, J=1.8Hz, H-1') 4.87 (d, 1H, J=1.8Hz, H-1''), 4.32 (d, 2H, J= 2.4 Hz, OC*H*$_2$C≡CH), 4.30 (dd, 1H, J=5.6Hz, 12.3Hz), 4.15 (dd, 1H, J= 2.5Hz, 12.3Hz), 4.10-3.97(m,3H, H-5, H-5', H5'') 3.81 (td, 2H, J= 5Hz, 11.2Hz) 3.65-3.55 (m, 2H), 2.53 (t, 1H, J = 2.4 Hz, C≡CH), 2.20, 2.18 ,2.17, 2.13, 2.08, 2.08, 2.07, 2.02, 2.00, 2.00 (10s, 30H, $COCH_3$).

^{13}C NMR (150 MHz, $CDCl_3$): δ= 170.55, 170.17, 170.09, 169.90, 169.84, 169.75, 169.71, 169.69, 169.67, 169.57 (10 x CH_3*C*OO), 97.67, 97.60 (C-1', C-1''), 96.04 (C-1), 78.20 (OCH$_2$*C*≡CH), 75.60 (OCH$_2$C≡*C*H), 69.95, 69.41, 69.38, 69.29, 69.26, 69.09, 69.07, 68.99, 68.65, 66.52, 66.45, 66.32,

65.98, 62.39 (15 x ring carbons), 54.88 (O*C*H_2C≡CH), 20.83, 20.75, 20.72 (x3), 20.71, 20.68, 20.65, 20.64, 20.60 (10 x *C*H_3COO).

HRMS: m/z = 985.2796 [M + Na^+] (calcd 985.2796) err[ppm]=0.1.

Thio-cyanomethyl 2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3',4',6'-tri-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-acetyl-α-D-mannopyranoside (7)



2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl trichloracetimidate (**29**) (15 mg, 0.019 mmol, 1 eq.) and Thio-cyanomethyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**12a**) (9.01 mg, 0.025 mmol, 1.3 eq.) were dissolved in dry dichloromethane (1 ml) in presence of activated molecular sieves and cooled to -20°C under nitrogen atmosphere. BF_3OEt_2(4.27 µL, 0.035 mmol, 1.8 eq.) was added and the mixture was stirred for 10 minutes. The reaction was quenched with triethylamine (4.83 µl, 0.035 mmol, 1.8 eq.), stirred for 5 minutes, filtered and concentrated *in vacuo*.

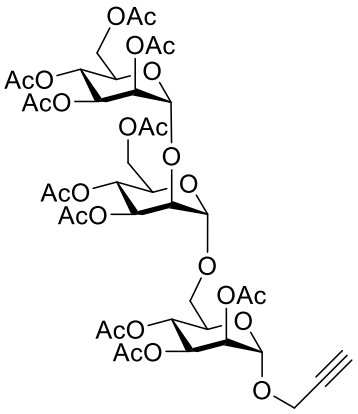
The reaction mixture was monitored by TLC (ethyl acetate/ciclohexane 6:4 Rf=0.10) and purified by flash chromatography (acetone/dichloromethane 1:9). The desired product (87%) was obtain as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.49 (m, 1H), 5.43-5.37 (m, 3H), 5.30-5.23 (m, 4H), 5.01 (d, 1H, J=1.8 Hz), 4.92 (d, 1H, J=1.8 Hz), 4.32 (m, 1H), 4.26-4.14 (m, 6H),4.05 (t, 1H, J=2.6), 3.99 (dt, 1H, J=2.8, 9.9 Hz), 3.89 (dd, 1H, J=6.8, 10.6 Hz), 3.58 (dd, 1H, J=2.3, 10.6 Hz), 3.54 (d, 1H, J=17.3 Hz), 3.40 (s, 1H, J=17.3 Hz), 2.21 (s, 3H, OAc), 2.18 (s, 3H, OAc), 2.17 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.02 (s, 3H, OAc).

^{13}C NMR (100 MHz, $CDCl_3$): δ= δ 171.33, 170.96, 170.88, 170.33, 170.22, 170.16, 170.13, 170.12, 169.96, 169.91 (10C, CH_3COO), 116.15 (SCH_2CN), 99.67, 98.20 (C-1', C-1"), 81.90 (C-1), 70.80, 70.36, 70.14, 69.79, 69.62, 69.51, 69.15, 69.70, 66.74, 66.69, 66.46, 65.91, 62.81, 62.37, 23.08 (CH_3, NHAc), 21.31, 21.17, 21.15, 21.11, 21.11, 21.09, 21.09, 21.07, 21.00 (10C, CH_3COO), 15.67 (SCH_2CN).

MS: m/z = 1002.83 [M + Na^+] (calcd 1002.90).

Propargyl 2",3",4",6"-*Tetra*-*O*-acetyl- α-D-mannopyranosyl-(1→2)-3',4',6'-*tri*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranoside (8)



2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranoside trichloracetimidate (**29**) (70 mg, 0.103 mmol, 1 eq.) and propargyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**15a**) (46.2 mg, 0.134 mmol, 1.3 eq.) were dissolved in dry dichloromethane (5 ml) in presence of activated molecular sieves and cooled to -20°C under argon atmosphere. BF_3OEt_2 (22.9 µL, 0.185 mmol, 1.8 eq.) was added and the mixture was stirred for 1 hour. The reaction was quenched with triethylamine (25.7 µl, 0.185 mmol, 1.8 eq.), stirred for 5 minutes, filtered over Celite and concentrated *in vacuo*. The reaction mixture was monitored by TLC (dichloromethane/acetone 9:1. Rf=0.61). Column chromatography (SiO_2, acetone/ dichloromethane 1:9) gave the desired product (52.5mg ,53%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 5.45-5.22 (m, 8H, H-3, H-4, H-2', H-3', H-4', H-2'', H-3'', H-4''), 5.02 (s, 1H, H-1), 4.98 (s, 1H, H-1'), 4.91 (s, 1H, H-1''), 4.30 (d, (d, 2H, J= 2.4 Hz, $OCH_2C{\equiv}CH$), 4.27-3.97 (m, 8H), 3.81 (dd, 1H, J=5.4, 10.6 Hz), 3.58 (dd, 1H, J=9.5), 2.56 (t, 1H, ≡CH), 2.18, 2.16 (x2), 2.10, 2.09, 2.06 (x2), 2.05, 2.02, 2.00 (10s, 30H, $COCH_3$).

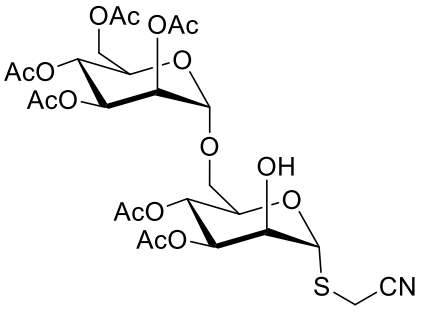
^{13}C-NMR (100 MHz, $CDCl_3$): δ = 170.88, 170.49, 170.28, 169.98, 169.85 (x2), 169.75 (x2), 169.45 (x2) (10 x CH_3COO) 99.28, 98.19, 96.03 (C-1, C-1', C-1''), 78.01 ($OCH_2C{\equiv}CH$), 76.99 (C-2'), 75.69 ($OCH_2C{\equiv}CH$), 70.25, 69.82 (x2), 69.31, 69.18, 68.97, 68.71, 68.39, 66.84, 66.52, 66.39, 65.86, 62.49, 62.14 (15 x ring carbons), 54.94 ($OCH_2C{\equiv}CH$), 20.88, 20.80, 20.73. 20.69 (10 x CH_3COO).
HRMS: m/z = 985.2796 [M + Na^+] (calcd 985.85).

2.6.5 ENZYMATIC HYDROLYSIS OF DISACCHARIDES

General procedure of enzymatic disaccharides deprotection: The deacetylated disaccharides were produced following a general procedure of hydrolysis reported for monosaccharides in paragraph 2.3.

The substrates (10 mM final concentration; 5 mM for **23**) were dissolved in acetonitrile (20–30% *v*/*v* depending on substrate solubility) under magnetic stirring, and then phosphate buffer (50 mM, pH 4.0-5.8) was added slowly. The reaction was started through the addition of immobilized CRL and/or AXE, previously conditioned with reaction buffer. The reactions were performed at 25 °C under mechanical stirring; the pH of the solution was maintained constant by automatic titration. Reaction course was monitored by TLC. After almost complete consumption of the substrate and before an excessive formation of undesired products (that can occur by a second hydrolysis), the reactions were stopped by enzyme derivative filtration on Büchner funnel. Acetonitrile was evaporated under reduced pressure, and the solution was extracted with ethyl acetate. The organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated in *vacuo*. The mixture obtained was purified by flash chromatography.

Thio-cyanomethyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-3,4-di-*O*-acetyl-α-D-mannopyranose (1a)



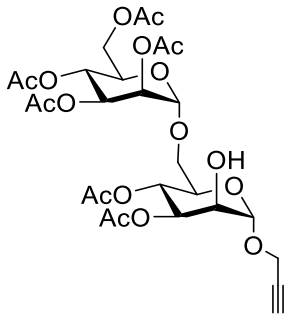
Thio-cyanomethyl 2',3',4',6'-*Tetra*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranoside (**1**) was hydrolyzed to the corresponding Thio-cyanomethyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-3,4-di-*O*-acetyl-α-D-mannopyranoside (**1a**) following the general procedure for enzymatic hydrolysis: substrate (69 mg, 10 mM) was solubilized in 8.5 mL of phosphate buffer 50 mM pH 5.3 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of AXE-GLX-AG (560 UI). The reaction mixture was monitored by TLC (dichloromethane/acetone 8:2, Rf = 0.53). Column chromatography (SiO_2, dichloromethane/acetone 8:2) gave the desired product (15 mg, 23%) as a white sticky solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.58 (d, 1H, *J* = 1.4 Hz, H-1), 5.36 (t, 1H, *J* = 9.9 Hz, H-4), 5.33-5.29 (m, 2H, H-4', H-3'), 5.24 (t, 1H, *J* = 2.2 Hz, H-2'), 5.18 (dd, 1H, *J* = 9.9, 3.2 Hz, H-3), 4.90 (d, 1H, *J* = 1.7 Hz, H-1'), 4.33-4.24 (m, 2H, H-6'$_a$, H-5), 4.22 (dd, 1H, *J* = 3.3, 1.4 Hz, H-2), 4.17 (dd, 1H, *J* = 12.2, 2.6 Hz, H-6'$_b$), 4.13-4.07 (m, 1H, H-5'), 3.87 (dd, 1H, *J* = 10.8, 7.0 Hz, H-6$_a$), 3.60 (dd, 1H, *J* = 10.7, 2.3 Hz, H-6$_b$), 3.56 (d, 1H, *J* = 17.3 Hz, SC*H*$_2$CN), 3.38 (d, 1H, *J* = 17.3 Hz, SC*H*$_2$CN), 2.18, 2.14, 2.12, 2.08, 2.08, 2.02 (5s, 18H, C*H*$_3$COO).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.83, 170.22, 170.08, 169.44, 169.81, 169.80 (6C, *C*OO), 115.88 (SCH$_2$*C*N), 97.00 (C-1'), 83.33 (C-1), 71.69 (C-3), 70.37 (C-5), 69.53 (C-2, C-2'), 68.92 (C-5'), 68.72, 66.49 (C-4), 65.96 (C-6), 65.88, 62.53 (C-6'), 20.88, 20.80, 20.77, 20.71, 20.67, 20.67 (6C, *C*H$_3$COO), 15.21 (S*C*H$_2$CN).

MS: *m*/*z* = 672.09 [M + Na$^+$] (calcd 672.16).

Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-3,4-di-*O*-acetyl-α-D-mannopyranoside (2a)



Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranose (**2**) was hydrolyzed to the corresponding propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-3,4-di-*O*-acetyl-α-D-mannopyranose (**2a**) following the general procedure

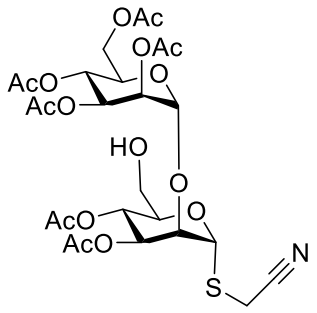
for enzymatic hydrolysis: substrate (81 mg, 10 mM) was solubilized in 12 mL of phosphate buffer 50 mM pH 4.0 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of AXE-GLX-AG (404 UI). The reaction mixture was monitored by TLC (dichloromethane/acetone 8:2, Rf = 0.41) and purified by flash chromatography (SiO_2, dichloromethane/acetone 8:2). The desired product (18.4 mg, 24%) was obtained as a as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.38-5.25 (m, 5H, H-4', H-3', H-2', H-4, H-3), 5.10 (d, 1H, *J* = 1.8 Hz, H-1), 4.92 (d, 1H, *J* = 1.8 Hz, H-1'), 4.31 (d, 2H, *J* = 2.4 Hz, OC*H*$_2$C≡CH), 4.26 (dd, 1H, *J* = 5.5 Hz, 12.4 Hz, H-6'$_a$), 4.19-4.10 (m, 2H, H-6'$_b$, H-5'), 4.09 (dd, 1H, *J* = 2.9, 1.9 Hz, H-2), 4.00 (ddd, 1H, *J* = 8.9, 6.0, 2.3 Hz, H-5), 3.81 (dd, 1H, *J* = 5.9 Hz, 11 Hz, H-6$_b$), 3.61 (dd, 1H, *J* = 2.4 Hz, 11 Hz, H-6$_a$), 2.52 (t, 1H, *J* = 2.4 Hz, C≡C*H*), 2.17, 2.13, 2.11, 2.07, 2.06, 2.01 (6s, 18H, COC*H*$_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.78, 170.16, 169.88, 169.88, 169.88, 169.81 (6C, CH_3*C*OO), 97.86 (C-1), 96.35 (C-1'), 78.32 (OCH$_2$*C*≡CH), 75.36 (OCH$_2$C≡*C*H), 71.48, 69.83 (H-5), 69.58, 69.18, 68.99 (C-2), 68.65 (C-5'), 66.67 (C-6), 66.60, 66.04, 62.46 (C-6'), 54.82 (O*C*H$_2$C≡CH), 20.88, 20.88, 20.77, 20.72, 20.72, 20.68 (6C, *C*H$_3$COO).

MS: *m*/*z* = 655.58 [M + Na$^+$] (calcd 655.19).

Thio-cyanomethyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4-di-*O*-acetyl-α-D-mannopyranoside (3a)



Thio-cyanomethyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (**3**) was hydrolyzed to the corresponding Thio-cyanomethyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4-di-*O*-acetyl-α-D-mannopyranoside (**3a**) following the general procedure for enzymatic hydrolysis: substrate (95 mg, 10 mM) was solubilized in 13.10 mL of phosphate buffer 50 mM pH 5.0 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of AXE-GLX-AG (477 UI). The reaction mixture was monitored by TLC

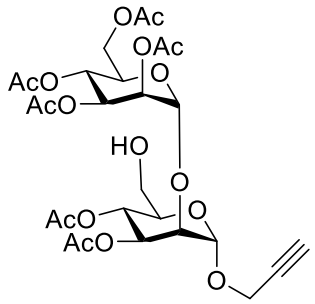
(dichloromethane/acetone 8:2, Rf= 0.33). Column chromatography (SiO_2, dichloromethane/acetone 8:2) gave the desired product (14.5 mg, 16%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.55 (d, 1H, *J* = 1.8 Hz, H-1), 5.30 (dd, 1H, *J* = 9.9, 3.4 Hz, H-3'), 5.23 (t, 2H, *J* = 10.3 Hz, H-4, H-4'), 5.18 (dd, 1H, *J* = 3.4, 1.9, H-2'), 5.11 (dd, 1H, *J* = 9.7, 3.3, H-3), 4.86 (d, 1H, *J* = 1.9 Hz, H-1'), 4.22 (dd, 1H, H-6'$_a$), 4.16-4.09 (m, 2H, H-2, H-5'), 4.06 (dd, 1H, *J* = 11.9, 2.9 Hz, H-6'$_b$), 4.00 (m, broad, 1H, *J* = 10.1, 4.5, 2.4 Hz, H-5), 3.75-3.58 (m, 2H, H-6$_a$, H-6$_b$), 3.41 (d, 1H, *J* = 17.2 Hz, SC*H*$_2$CN), 3.31 (d, 1H, *J* = 17.2 Hz, SC*H*$_2$CN), 2.08, 2.05, 2.04, 2.02, 2.01, 1.95 (6s, 18H, C*H*$_3$COO).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.88, 170.36, 170.34, 169.91, 169.91, 169.67 (6C, CH_3*C*OO), 116.06 (SCH_2*C*N), 99.06 (C-1'), 83.49 (C-1), 76.86 (C-2), 72.27 (C-5), 70.27 (C-3), 69.51, 69.41 (C-5', C-2'), 68.46 (C-3'), 66.46, 65.87 (C-4, C-4'), 62.83 (C-6'), 61.07 (C-6), 20.86, 20.84, 20.74, 20.71, 20.64, 20.61 (6C, *C*H_3COO), 15.02 (S*C*H_2CN).

MS: *m*/*z* = 672.09 [M + Na^+] (calcd 672.16).

Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4-di-*O*-acetyl-α-D-mannopyranoside (4a)



Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranose (**4**) was hydrolyzed to the corresponding propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4-di-*O*-acetyl-α-D-mannopyranose (**4a**) following the general procedure for enzymatic hydrolysis: substrate (38 mg, 10 mM) was solubilized in 5.6 mL of phosphate buffer 50 mM pH 5.8 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of AXE-GLX-AG (102 UI). The reaction mixture was monitored by TLC (dichloromethane/acetone 8:2). Column chromatography (SiO_2, dichloromethane/acetone 9:1, Rf = 0.17) gave the desired product (7 mg, 20%) as a white solid.

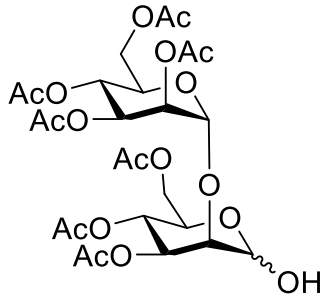
^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.40 (dd, 1H, *J* = 10.0, 3.3 Hz, H-3'), 5.37-5.26 (m, 4H, H-3, H-4, H-4', H-2'), 5.19 (d, 1H, *J* = 2.0 Hz, H-1), 4.95 (d, 1H, *J* = 1.9 Hz, H-1'), 4.29 (d, 2H, *J* = 2.4 Hz, OC*H*$_2$C≡CH),

4.27-4.15 (m, 3H, H-5', H-6'$_a$, H-6'$_b$,), 4.10 (dd, 1H, *J* = 3.2, 2.0 Hz, H-2), 3.78 (ddd, 1H, *J* = 9.6, 4.4, 2.3 Hz, H-5), 3-75-3.71 (m, 2H, H-6$_a$, H-6$_b$), 2.50 (t, 1H, *J* = 2.4 Hz, $OCH_2C{\equiv}C\mathit{H}$), 2.17, 2.13, 2.11, 2.09, 2.08, 2.03 (6s, 18H, $C\mathit{H_3}COO$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.91, 170.39, 170.35, 169.92, 169.89, 169.63 (6C, $CH_3\mathit{C}OO$), 99.09 (C-1'), 96.86 (C-1), 78.20 ($OCH_2\mathit{C}{\equiv}CH$), 76.75 (C-2), 75.53 ($OCH_2C{\equiv}\mathit{C}H$), 71.33 (C-5), 69.96, 69.64, 69.13 (C-5'), 68.60 (C-3'), 66.58, 66.29, 62.65 (C-6'), 61.28 (C-6), 54.89 ($O\mathit{C}H_2C{\equiv}CH$), 20.87, 20.83, 20.73, 20.73, 20.73, 20.67, (6C, $\mathit{C}H_3COO$).

MS: *m*/*z* = 655.20 [M + Na^+] (calcd 655.19).

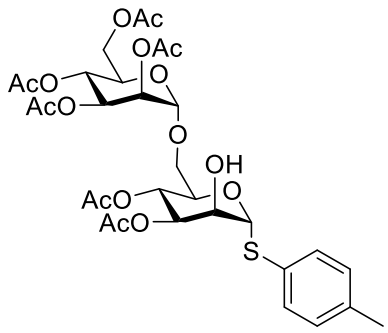
2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-O-acetyl-α/β-D-mannopyranose (22a)



2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-1,3,4,6-tetra-*O*-acetyl-α-D-mannopyranose (**22**) was hydrolyzed to the corresponding 2',3',4',6'-Tetra-O-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-O-acetyl-α/β-D-mannopyranose (**22a**) following the general procedure for enzymatic hydrolysis: substrate (10 mg, 10 mM) was solubilized in 1.7 mL of phosphate buffer 50 mM pH 4.0 and 30% v/v of acetonitrile. The reaction was started trough addition of AXE-GLX-AG (51 UI). The reaction mixture was monitored by TLC (dichloromethane/methanol 9:1, Rf=0.82).

The product was identified by comparing the reaction mixture with the reference standard prepared by chemical synthesis as reported in paragraph 2.6.2.

(4-Methylphenyl) (2′,3′,4′,6′-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-3,4-di-*O*-acetyl-1-thio-α-D-mannopyranoside (23a)



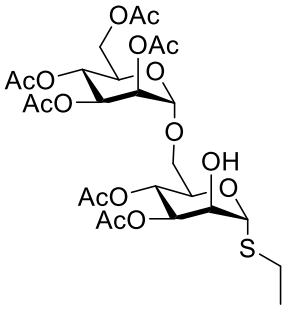
(4-Methylphenyl)(2′,3′,4′,6′-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranoside (**23**) was hydrolyzed to the corresponding (4-methylphenyl) (2′,3′,4′,6′-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-3,4-di-*O*-acetyl-1-thio-α-D-mannopyranoside (**23a**) following the general procedure for enzymatic hydrolysis: substrate (75 mg, 10 mM) was solubilized in 20.37 mL of phosphate buffer 50 mM pH 5.4 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of AXE-GLX-AG (420 UI). The reaction mixture was monitored by TLC (dichloromethane/acetone 9:1, Rf = 0.22). Column chromatography (SiO_2, dichloromethane/acetone 9:1) gave the desired product (17 mg, 24%) as a white sticky solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.39 (d, 2H *J* = 8.0 Hz, Ar), 7.17 (d, 2H, *J* = 8.0 Hz, Ar), 5.46 (d, *J* = 1.9 Hz, 1H, H-1), 5.42 (d, 1H, *J* = 9.9 Hz, H-4) 5.36-5.24 (m, 4H, H-2′, H-3′, H-4′, H-3), 4.92 (d, 1H, *J* = 1.6 Hz, H-1′), 4.50 (ddd, 1H, *J* = 10.0, 5.0, 2.4 Hz, H-5), 4.35-4.31 (m, 1H, H-2) 4.28 (dd, 1H, *J* = 12.3, 5.1 Hz, H-6′$_a$), 4.09 (dd, 1H, *J* = 12.3, 2.5 Hz, H-6′$_b$), 4.03 (ddt, 1H, *J* = 7.5, 5.1, 2.2 Hz, 1H, H-5′), 3.85 (dd, 1H, *J* = 11.4, 5.0 Hz, H-6$_a$), 3.62 (dd, 1H, *J* = 11.4, 2.5 Hz, H-6$_b$), 2.34 (s, 3H, C*H*$_3$-Ar), 2.16, 2.12, 2.11, 2.09, 2.07, 2.02 (6s, 18H, COC*H*$_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.72, 170.17, 169.98, 169.76, 169.76, 169.74 (6C, CH_3COO), 138.07, 132.21, 130.06, 129.39 (Ar), 97.81 (C-1′), 88.06 (C-1), 71.86 (C-3), 70.59 (C-2), 70.28 (C-5), 69.62, 69.00, 68.61 (C-5′), 66.84 (C-6), 66.69 (C-4), 66.05, 62.35 (C-6′), 21.13(CH_3-Ar), 20.89, 20.89, 20.75, 20.73, 20.73, 20.67 (6C, CH_3COO).

MS: *m*/*z* = 723.24 [M + Na^+] (calcd 723.19).

Ethyl (2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-3,4-di-*O*-acetyl-1-thio-α-D-mannopyranoside (24a)



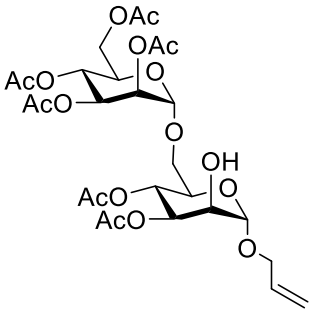
Ethyl(2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranose (**24**) was hydrolyzed to the corresponding ethyl (2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-3,4-di-*O*-acetyl-1-thio-α-D-mannopyranose (**24a**) following the general procedure for enzymatic hydrolysis: substrate (28 mg, 10 mM) was solubilized in 4.39 mL of phosphate buffer 50 mM pH 4.8 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of AXE-GLX-AG (160 UI). The reaction mixture was monitored by TLC (dichloromethane/acetone 8:2, Rf = 0.62). Column chromatography (SiO_2, dichloromethane/acetone 9:1) gave the desired product (10 mg, 38%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.35 (m, 1H, H-1) 5.33-5.22 (m, 5H, H-4, H-4', H-3', H-2',H-3,), 4.90 (d, 1H, *J* = 1.8 Hz, H-1'), 4.40 (ddd, 1H, *J* = 9.3, 6.5, 2.2 Hz, H-5), 4.26-4.14 (m, 3H, H-6'$_a$, H-6'$_b$, H-2) 4.11 (ddd, 1H, *J* = 9.7, 5.4, 2.4 Hz, 1H, H-5'), 3.83 (dd, 1H, *J* = 10.9, 6.6 Hz, H-6$_a$), 3.57 (dd, 1H, *J* = 10.9, 2.2 Hz, H-6$_b$), 2.75-2.59 (m, 2H, SC*H*$_2$CH$_3$), 2.17, 2.13, 2.10, 2.07, 2.06, 2.01 (6s, 18H, COC*H*$_3$), 1.35 (t, 3H, *J* = 7.4, SCH_2CH_3).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.82, 170.17, 169.94, 169.81, 169.81, 169.81 (6C, CH_3*C*OO), 97.39 (C-1'), 83.21 (C-1), 72.03, 70.64 (C-2), 69.61, 69.43 (C-5), 68.84, 68.53 (C-5'), 66.96, 66.47 (C-6), 66.15, 62.47 (C-6'), 24.79 (S*C*H$_2$CH$_3$), 20.90, 20.87, 20.78, 20.74, 20.70, 20.67 (6C, *C*H$_3$COO), 14.56 (SCH_2*C*H$_3$).

MS: *m*/*z* = 661.22 [M + Na^+] (calcd 661.18).

Allyl (2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-3,4-di-*O*-acetyl-α-D-mannopyranoside (25a)



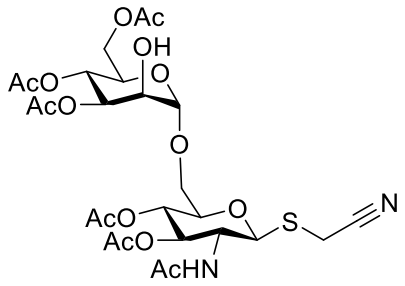
Allyl(2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranose (**25**) was hydrolyzed to the corresponding Allyl (2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-3,4-di-*O*-acetyl-α-D-mannopyranose (**25a**) following the general procedure for enzymatic hydrolysis: substrate (103 mg, 10 mM) was solubilized in 15.2 mL of phosphate buffer 50 mM pH 4.8 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of AXE-GLX-AG (504 UI). The reaction mixture was monitored by TLC (dichloromethane/acetone 8:2, Rf = 0.33). Column chromatography (SiO_2, dichloromethane/acetone 8:2,) gave the desired product (50 mg, 52%) as a white solid.

^{1}H-NMR (400 MHz, 45 °C, $CDCl_3$): δ 5.94 (dddd, 1H, *J* = 17.2, 10.4, 6.2, 5.3, $CH_2CH{=}CH_2$), 5.39-5.24 (m, 7H, H-3, H-4, H-2', H-3', H-4', $CH_2CH{=}CH_2$), 4.91 (d, 1H, *J* = 1.8 Hz, H-1) 4.90 (d, 1H, *J* = 1.8 Hz, H-1'), 4.28-4.21 (m, 2H, H-6'$_a$, $CH_2CH{=}CH_2$), 4.16 (dd, 1H, *J* = 7.9, 2.3 Hz, H-6'$_b$), 4.15-4.03 (m, 3H, H-2, H-5', $CH_2CH{=}CH_2$), 3.99 (ddd, 1H, *J* = 9.9, 6.1, 2.4 Hz, H-5), 3.80 (dd, 1H, *J* = 10.9, 6.2 Hz, H-6$_a$), 3.58 (dd, 1H, *J* = 10.9, 2.3 Hz, H-6$_b$), 2.17, 2.12, 2.10, 2.05, 2.06, 2.00 (6s, 18H, CH_3COO).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.79, 170.15, 169.94, 169.93, 169.83, 169.80 (6C, CH_3COO), 133.25 ($CH_2CH{=}CH_2$), 118.23 ($CH_2CH{=}CH_2$), 98.19 (C-1), 97.25 (C-1'), 71.73, 69.62, 69.35, 69.29, 68.91, 68.60, 68.40, 66.76, 66.63, 66.11, 62.46 (11C, carbon ring), 20.90, 20.89, 20.77, 20.74. 20.70, 20.67 (6C, CH_3COO).

MS: *m*/*z* = 657.25 [M + Na^+] (calcd 657.20).

Thio-cyanomethyl 3',4',6'-tri-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (26a)



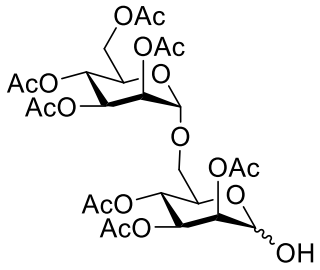
Thio-cyanomethyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**26**) was hydrolyzed to the corresponding Thio-cyanomethyl 3',4',6'-tri-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**26a**) following the general procedure for enzymatic hydrolysis: substrate (80.5 mg, 10 mM) was solubilized in 11.6 mL of phosphate buffer 50 mM pH 4.5 and 30% *v*/*v* of acetonitrile. The reaction was started through addition of AXE-GLX-AG (378 UI). The reaction mixture was monitored by TLC (dichloromethane/methanol 9:1, Rf = 0.42). Column chromatography (SiO_2, dichloromethane/acetone 8:2) gave the desired product (15.4 mg 20.5%) as a white sticky solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.02 (d, 1H, *J* = 9.3 Hz, NH), 5.39–5.07 (m, 4H, H-4', H-3', H-3, H-4), 4.95 (d, 1H, *J* = 1.9 Hz, H-1'), 4.75 (d, 1H, *J* = 10.4 Hz, H-1), 4.29 (dd, 1H, *J* = 12.3, 4.9 Hz, H-6'$_a$), 4.25-4.10 (m, 3H, H-2, H-2', H-6'$_b$), 3.98 (ddd, 1H, *J* = 9.9, 4.9, 2.4 Hz, H-5') 3.85-3.64 (m, 4H, C*H*CN, H-6$_a$, H-5, H-6$_b$), 3.34 (d, 1H, *J* = 17.3 Hz, C*H*CN), 2.12, 2.10, 2.08, 2.07, 2.05, 1.99 (6s, 18H, C*H*$_3$COO).

^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.33, 170.84, 170.60, 170.01, 169.84, 169.26 (6C, CH_3COO), 116.53 (SCH_2*C*N), 99.63 (C-1'), 83.36 (C-1), 77. 18 (C-5), 73.46 (C-3), 71.35 (C-3'), 68.92 (C-5'), 68.78 (C-2'), 68.67 (C-4), 66.23 (C-6), 65.95 (C-4'), 62.48 (C-6'), 52.57 (C-2), 23.11 (CH_3, NHAc), 20.90, 20.81, 20.4, 20.66, 20.64 (5C, CH_3COO), 14.50 (S*C*H_2CN).

MS: *m*/*z* = 671.21 [M + Na^+] (calcd 671.17).

2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α/β-D-mannopyranose (27a)



2',3',4',6'-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-1,2,3,4-tetra-*O*-acetyl-α-D-mannopyranose (**27**) was hydrolyzed to the corresponding 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α/β-D-mannopyranose (**27a**) following the general procedure for enzymatic hydrolysis: substrate (10 mg, 10 mM) was solubilized in 1.7 mL of phosphate buffer 50 mM pH 4.8 and 30% v/v of acetonitrile. The reaction was started trough addition of AXE-GLX-AG (52 UI). The reaction mixture was monitored by TLC (dichloromethane/methanol 9:1, Rf=0.76).
The product was identified by comparing the reaction mixture with standard material[236].

CHAPTER 3: NANO-GLYCOPROTEINS

The aim of this part of the research project regards the synthesis of potential nano-glycoproteins vaccines for Tuberculosis. They are composed by a nanomaterial, an elastin-like recombinamer (see paragraph 1.3.4.4) containing in its sequence an antigenic peptide to induce the immune response; linked to this immunogenic-carrier there is a sugar portion (di or tri- mannans synthesized as reported in chapter 2) with the aim to stimulate and induce a stronger response. These compounds will be evaluated by surface plasmon resonance (SPR) at Complutense university of Madrid in order to define the affinity of the sugar adjuvant linked on the ELBcR protein, to mannose receptors of dendritic cells (using concanavalin A as model).

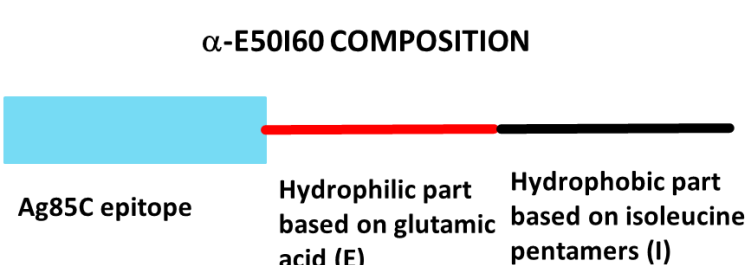
3.1 ELRS FOR THE DEVELOPMENT OF NEW GLYCOSYLATED NANO-VACCINES AGAINST TUBERCULOSIS

As described in the introduction, Elastin-like recombinamers, and more precisely ELbcRs (the self-assembled form) are promising particulate vaccine delivery systems with potential adjuvant activity. They showed their effectiveness in having higher and more efficient uptake by APC compared with soluble antigen and can be more proficiently modified to induce an effectual helper T cell response, so improved total immune response [131,238]. Thanks to this material, the incorporation of functional groups such as adjuvants without changing the bulk properties and the size of the formed nanoparticles is easily achievable [239].

The immunodominant antigenic peptide sequence from MTB membrane has been attached by recombinant encoding to the hydrophilic part of the amphiphilic ELbcR, to form α-E50I60 polymer (sequence in Figure 3.1A), which is used in this work to form the vaccine nanocarrier (in collaboration with Prof F. Javier Arias; university of Valladolid in Spain).

Figure 3.1B shows the mass spectrometry of α-E50I60.

A)



MESLLP- THSWPYWNEQLVAMKADIQH-[(VPGVG)2-(VPGEG)-(VPGVG)2]10[VGIPG]60-V

B)

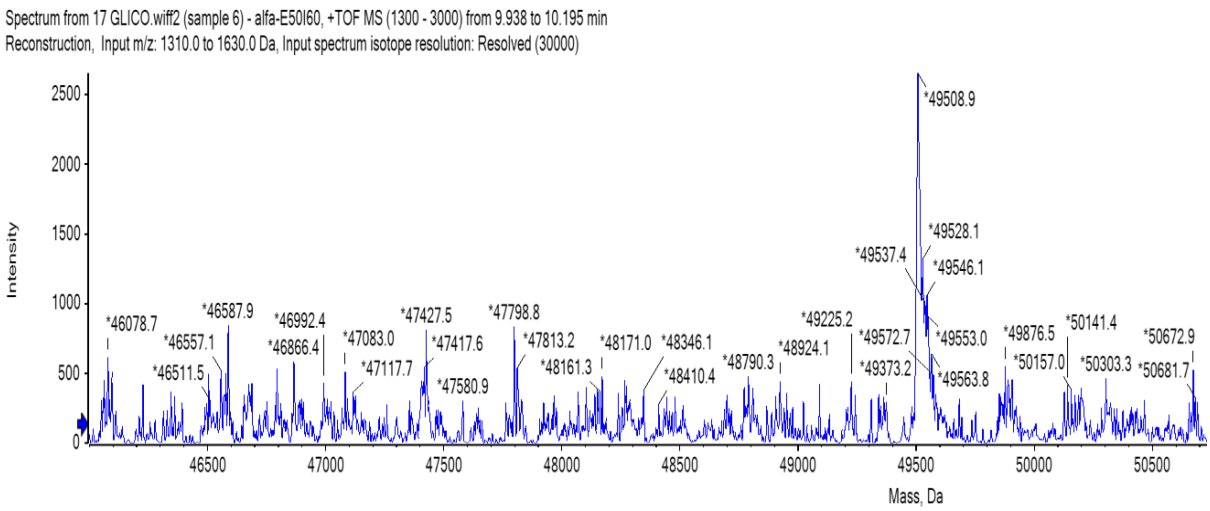


FIGURE 3.1: α-E50I60: It was synthesized by Bioforge Group, University of Valladolid, CIBER-BBN, Spain
A) nanoprotein sequence; B) Mass spectrometry analysis.

E50I60 polymer is composed of a hydrophilic part based on glutamic acid (E) at the amino terminus (E50) and a hydrophobic part based on isoleucine pentamers (I) at the carboxy terminus (I60), that will allow the presentation of the antigen on the surface of the self-assembled nanoparticles when contact with the physiological fluids [131]. Its composition attributes specific characteristics to the material, so the transition temperature that due to its hydrophobicity and size is 15°C.

The antigenic peptide of TB used in this study is referred to as alpha antigen, which corresponds to the peptide sequence "$_{261}$THSWPYWNEQLVAMKADIQH$_{280}$" from the Ag85C antigen[240]. This TB protein induces strong T-cell proliferation and gamma interferon (IFN-γ) production in most healthy individuals infected and also can stimulate strong humoral and cell-mediated immune responses [241].

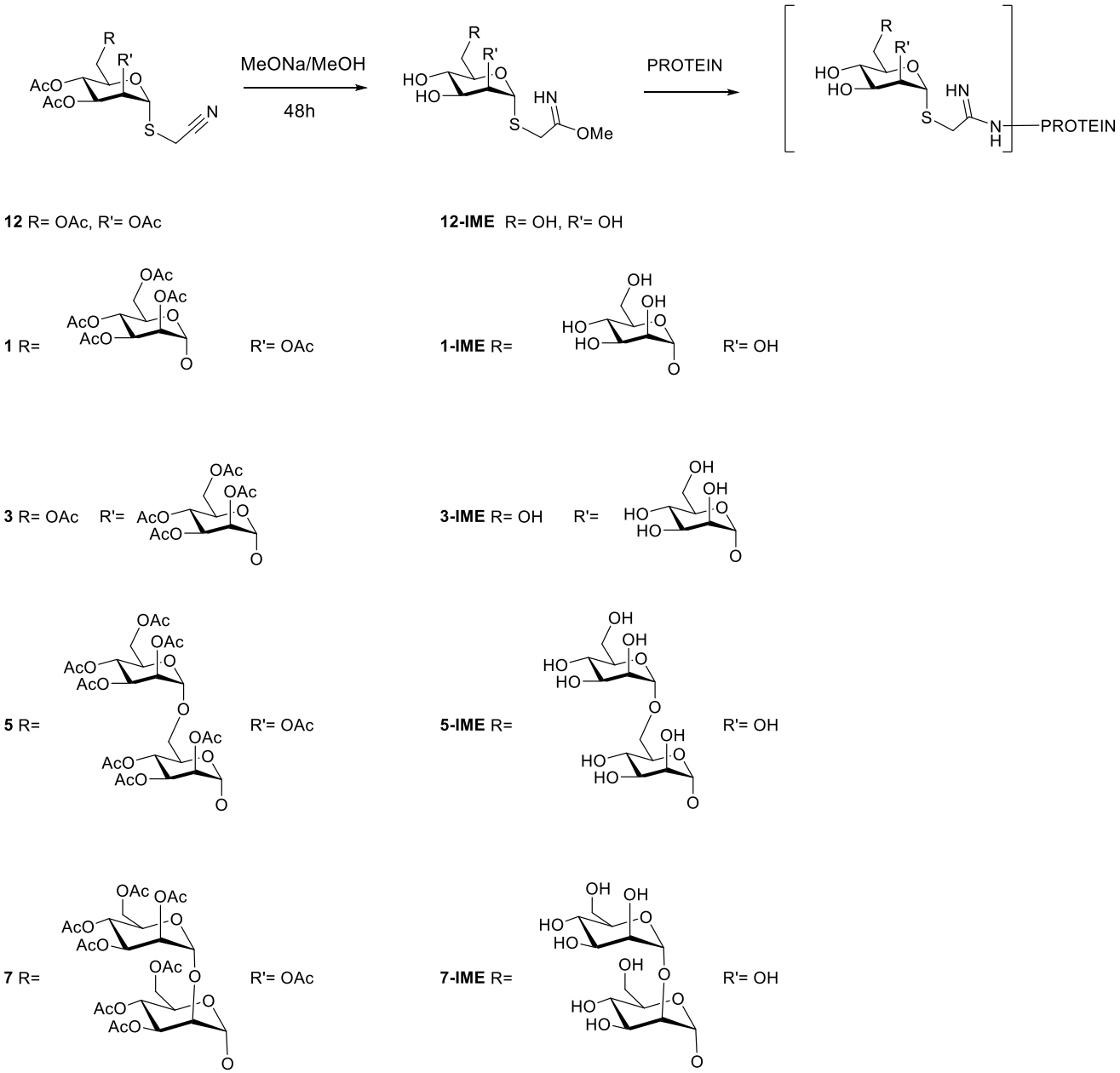
In this research, an attempt is made to find an optimal sugar adjuvant using analogues of natural polymannans, that could be used in the development of new family of nano glyco-conjugated TB vaccine.

3.2. METHOD OF PROTEIN GLYCOSYLATION MEDIATED BY IME GROUP

The glycosylation of mannose with the protein starts first with the activation of the linker in the anomeric position of the sugar moieties. A soluble carrier protein (ribonuclease A, RNase A, 124 amino acids, molecular mass 13681 Da) was used first for conjugation reaction, to test the glycosylation efficiency of the different activated mannose moieties and to optimize the reaction conditions (in term of temperature, sugar to protein molar/mass ratios, protein concentrations and buffer used).

The first activation relies on the use of 2-iminomethoxyethyl thioglycosides (IME) reactive linker. Taking the advantage of the high abundance of lysine residues on the model protein surface, an introduction of various mannose units for each molecule of protein is allowed.

The synthesis of peracetylated thiocyanomethyl mono-di-tri-mannose (**12**,**1**,**3**,**5** and **7**) moieties was done following B.G. Davis' procedure as described in the previous chapter[6-8]. The acetylated glycans (**12**,**1**,**3**,**5** and **7**) carry a thiocyanometyl group in anomeric position that is the precursor of the reactive IME and, consequently, can be simultaneously submitted to Zempl´en deacetylation reaction and C-1 activation to obtain the 2-iminomethoxyethyl (IME) group in a single synthetic step (scheme 3.1) . The purity of the activated mannose moieties (Compound **12-IME**, **1-IME**, **3-IME**, **5-IME** and **7-IME**) was checked using the electron spray ionization mass spectrometry (Finnigan LTQ MS), before the glycosylation with the protein. The IME glycans obtained can selectively react with the lysine residues on the protein surface to obtain the neo-glycoproteins.



SCHEME 3.1 : preparation of carbohydrates-IME

3.2.1 PREPARATION OF GLYCOPROTEINS WITH RNASE A

The commercially available Ribonuclease A, used in our experiments, contains 10 lysine residues needed for the binding with the IME group. Thanks to this high number of lysine a high glycosylation yield is expected (90-100%).

The glycosylation reaction is done according to B.G. Davis' protocol [242-244] with slight modification. The RNasi A and the IME-activated sugars (**12-IME**, **1-IME**, **3-IME**, **5-IME** and **7-IME**) were dissolved in sodium tetraborate buffer and stirred for 24h in different condition (37,24 and

10°C) and with different reagents ratio (100:1 and 50:1). The glycosylation reaction outcome are presented in (table 3.1) as resulting by HPLC-MS analysis.

TABLE 3.1: Optimization of experimental conditions for the conjugation reaction of **12-IME, 1-IME, 3-IME, 5-IME** and **7-IME** with RNase A.

ENTRY	MANNOSE	RATIO	T	MANNOSE BOUND /PROTEIN (mol/mol)	GLYCOSYLATION YIELD
1	**12-IME**	100:1	37	2.1	88%
2	**12-IME**	100:1	25	3.69	100%
3	**12-IME**	100:1	10	3.24	98%
4	**12-IME**	50:1	37	0.52	43%
5	**12-IME**	50:1	25	0.97	65%
6	**12-IME**	50:1	10	0.97	66%
7	**1-IME**	100:1	25	2	89%
8	**5-IME**	100:1	25	2.33	91%
9	**3-IME**	100:1	25	3.2	100%
10	**7-IME**	100:1	25	4.47	100%

Experimental conditions: 100 mM $Na_2B_4O_7$ buffer, pH 9.5, 24 hours.

For mono-mannose (**12-IME**), best glycosylation yields for the activated mono-mannose were 100% and 98% obtained when using a molar ratio of sugar to protein (100:1) at room temperature and 10^{o}C, respectively. Higher temperature (37^{o}C) affects sugar stability, which led to lower glycosylation yield. Using a molar ratio of sugar to protein (50:1) resulted in much lower glycoprotein yields, the amount of sugar is certainly not enough. 1,6 di-mannose (**1-IME**) glycosylation yielded 89% under room temperature, it can be optimized by adding more sugar. Generally, the increase in sugar size may affect its ability to conjugate with the protein. This can be overcome by increasing the amount of sugar ratio used per protein. Contrary, it seems that size didn't affect the glycosylation with compound **3-IME** and **7-IME** (100% yield).

3.2.2 PREPARATION OF GLYCOPROTEINS WITH ELR

Following, the activated mannose moieties with IME reactive linker (**12-IME**, **1-IME**, **3-IME**, **5-IME** and **7-IME**) were covalently glycosylated with the α-E50I60.

This protein only has one lysine included in the Ag85C epitope .Thus after self-assembly each protein expose this lone lysine residue on nanoparticle's. Of course, the glycosylation could strongly affect the antigenic activity of the epitopes. This means that the conjugation with the sugar should be controlled and must not be complete in order to have a suitable number of non-glycosylated epitopes. In this work we assumed to obtain a glycosylation degree of nanoparticle surface of 40-60% in order to combine the adjuvant effect of the mannose based oligosaccharide but maintaining a suitable number of free antigenic epitopes (Figure 3.2).

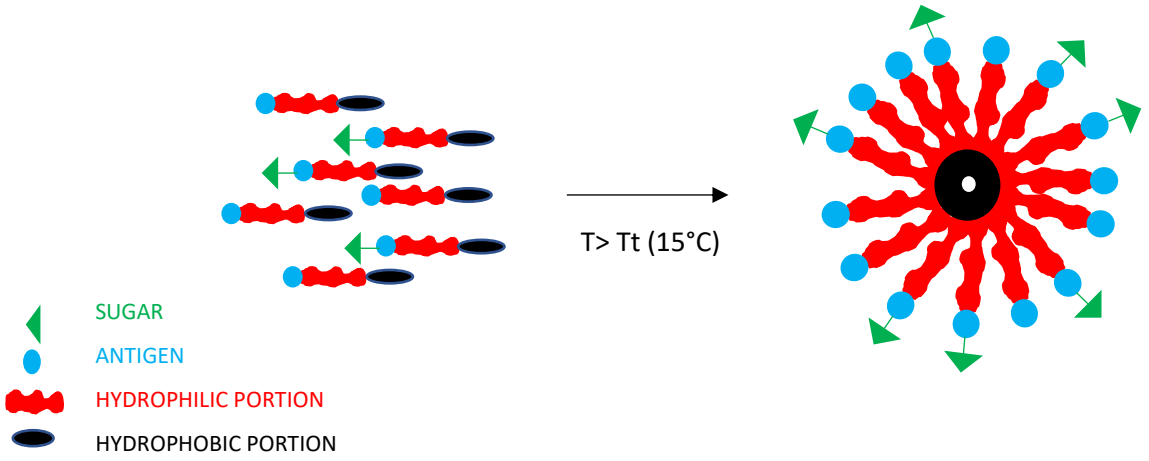


Figure 3.2: Nano-glycoprotein (40-60% glycosylated). Micella formation above its transition temperature.

The experiments previously performed with the RNAse, demonstrate that the protein glycosylation can be performed at 10°C. Thus, in order to avoid the self-assembly of the α-E50I60 protein during the reaction, the glycosylation was performed at this temperature (10°C) and in a solution of sodium tetraborate buffer or in buffer and acetonitrile (75:25). The reactions were first performed using a sugar protein ratio of 200:1. However, as discovered in the glycosylation of RNAse, the size of the sugar can affect glycosylation and some attempts were performed with increased ratio (300:1 and 400:1). The analysis of the reactions was performed by HPLC-MS.

TABLE 3.2: Optimization of experimental conditions for the conjugation reaction of α-E50I60 with **12-IME**, **1-IME**, **3-IME**, **5-IME** and **7-IME**.

ENTRY	MANNOSE	RATIO	MANNOSE BOUND /PROTEIN (mol/mol)	GLYCOSYLATION YIELD
1	**12-IME**	200:1	0.41	41%
2	**1-IME**	200:1	0.51	51%
3	**3-IME**[a]	200:1	0.44	44%
4	**5-IME**	200:1	0.23	23%
5	**5-IME**	400:1	0.42	42%
6	**7-IME**[a]	300:1	0.45	45%

Experimental conditions: $Na_2B_4O_7$ buffer: CH_3CN (75:25), pH 9, temperature 10 ^oC, protein conc. 2mg/ml, 24 hours. a) without CH_3CN.

In all cases the reaction proceeded in the desired range (40-60% of protein glycosylation) but for trisaccharides (**5-IME** and **7-IME**) the desired yield was obtained by adding more sugar. Acetonitrile was added as an additional element to prevent micelle aggregation, but it was discovered that in some cases, for compounds **3-IME** and **7-IME**, the yields were good even without using this solvent.

An important aspect remains the conservation of the nanomaterial size (ELR is around 50-60nm). Once glycosylated it is important they don't change the size for maintaining the biological properties (for example being able to be uptaken by APC, or for cell-cell interaction). For this, the glycoproteins size was analysed at 37°C (physiological temperature) at 1 mg/ml of concentration by using Beckman coulter. The results confirmed the size was maintained after glycosylation (around 50 nm) and thus the conjugating of the sugar to the surface of nanoparticle is not affecting the size of the nanoparticle.

Moreover, sugar conjugation helped improving size homogeneity as clarified by reduced polydispersity index (PDI). Indeed this value was decreased from 0.424 to 0.372 for monosaccharide- conjugation to 0.051 after trisaccharide conjugation. This can be explained by the increase of the hydrophilic after sugar conjugation of the N-terminal part of the ELR polymer, which lead to the formation of more homogenous nanoparticle in term of size.

3.3 EXPERIMENTAL PARTS

The analysis was performed using High-performance liquid chromatography -mass spectrometry (HPLC-MS). The spectra were deconvoluted using BioworksBrowser and the abundance of the different species derived from their relative intensity in the deconvoluted spectrum. The ratio between the relative abundance of each glycoform and the total ion intensities of the pattern is used for the calculation of the average number of sugars linked to RNAse, while the glycosylation yield is derived from the abundance of unmodified protein [4,125].

3.3.1 PREPARATION OF ACTIVATED MANNOSE-IME[55]*:*

Mono, di, tri- mannose (**12**, **1**,**3**,**5** and **7**) were each dissolved in 5 ml of dry methanol. Sodium methoxide was added in a 1:3 (Sodium methoxide:sugar) molar ratio. The reactions were reacted for 2 days under nitrogen. After which the reactions were concentrated in vacuum, and solid form of the sugars were analysed by mass spectrometry.

Man-IME (**12-IME**), MS: m/z = 290.33 $[M + Na]^+$.

Man(1-6)Man-IME (**1-IME**), MS: m/z = 452.75 $[M + Na]^+$.

Man(1-2)Man-IME (**3-IME**), MS: m/z = 452.05 $[M+ Na]^+$.

Man(1-6)Man(1-6)Man-IME (**5-IME**), MS: m/z = 614.58 $[M + Na]^+$.

Man(1-2)Man(1-6)Man-IME (**7-IME**), MS: m/z = 614.08 $[M+ Na]^+$.

3.3.2 PREPARATION OF GLYCOPROTEINS WITH RNasi

The reactions were carried out in sodium tetraborate buffer 100mM and pH 9.5; the protein were dissolved in the buffer at 1mg/ml concentration and then sugar-IME (**12-IME**, **1-IME**, **3-IME**, **5-IME** and **7-IME**) was added to the solution with ratio 100:1 (glycoside:protein) and stirred for 24h at r.t. About monosaccharide (**12-IME**), once the solutions of sugar and protein were prepared with ratios 100:1 and 50:1, they were vortexed for 1 min and separated in three samples and stirred at 37, 25 and 10 °C for 24h.

3.3.3 PREPARATION OF GLYCOPROTEINS WITH α-E50I60

Conjugation of the activated Man-IME moieties (**12-IME**, **1-IME**, **3-IME**, **5-IME** and **7-IME**) was performed with the α-E50I60 polymer(molecular weight 49500 Da). The glycosylation of **12-IME**, **1-IME** and **5-IME** were carried out in sodium tetraborate buffer/acetonitrile solution (75:25), pH 9, meanwhile glycosylation reactions of **3-IME** and **7-IME** were performed in same condition but without acetonitrile.

α-E50I60 was dissolved in the water and kept overnight. The protein with a concentration of 2mg/ml was transferred into reaction solvent by dialysis.

Sugar: protein molar ratio of (200:1) was used for the all activated mannose moieties. As an optimization and to improve the glycoprotein yields, higher sugar molar ratio to protein of (300:1) and (400:1) was used respectively for **7-IME** and **5-IME** glycosylation reactions. The IME-sugars were added to the protein solution, samples to be incubated for 24 hours at 10 °C, under continuous gentle stirring.

CHAPTER 4: PREPARATION OF GLYCOCONJUGATE PRODUCTS WITH FULLERENE C_{60}

4.1. STRATEGIES AND AIMS

The third part of the project aims the investigation of the potential use of fullerene as carrier for both immunogenic sugars and the antigenic peptides in order to create a potential glycoconjugate vaccine for TB. In particular, the objective consisted in functionalizing the fullerene core with an appropriate linker suitable for reacting with the sugar by click chemistry in order to obtain a glycosylated product suitable to be further functionalized with an antigenic peptide.

Several methods for the functionalization of fullerene were developed over the past years (see paragraph 1.4.2.1). In this work we focused on the conversion of fullerene into methanofullerenes, taking advantage of Bingel reaction (cyclopropanation), which usually occurs upon treatment of fullerene with an halide-activated malonic acid derivative in the presence of a base[165]. Our idea was to exploit the malonic chain for designing derivatives bearing a terminal azide, which could undergo a copper-mediated Huisgen 1,3-dipolar cycloaddition with a terminal alkyne chain of the immunogenic mannoses and antigenic peptides in order to obtain C_{60} derivatives differently decorated (Figure 4.1).

In particular, in this work we report the investigation of two different approaches based on malonate or malondiamide bearing a terminal azide used as reactive linker in order to obtain functionalized Fullerene (C_{60}) with mannose based sugars.

In literature several example of cyclopropanation using malonic ester derivatives are reported [176,245-247]. However we have considered also the use of amide derivatives of malonic acid. Although the reactivity of malondiamide with C_{60} results not very high (few articles and low yields[184], [248])the amide bond is well-known to be much more stable than the ester function, allowing a major versatility in the reaction conditions. Importantly, the amide bond was considered in our work because it is stable to the conditions used for the deprotection of the hydroxy groups of the fully acetylated sugars obtained by our chemoenzymatic synthetic approach (see chapter 2). This feature allows the use of acetylated sugars in the click reaction with the malondiamide-azido linkers.

On the other side, esters of malonic acid are highly reactive in the conjugation conditions with C_{60}, allowing to link up to six malonate chains[249]. However, the ester group, in addition to its lower stability compared with the amide, is reactive in the conditions used for the deprotection of the acetylated sugars. Therefore, in this case only deprotected sugars can be used in the click reaction with the linkers.

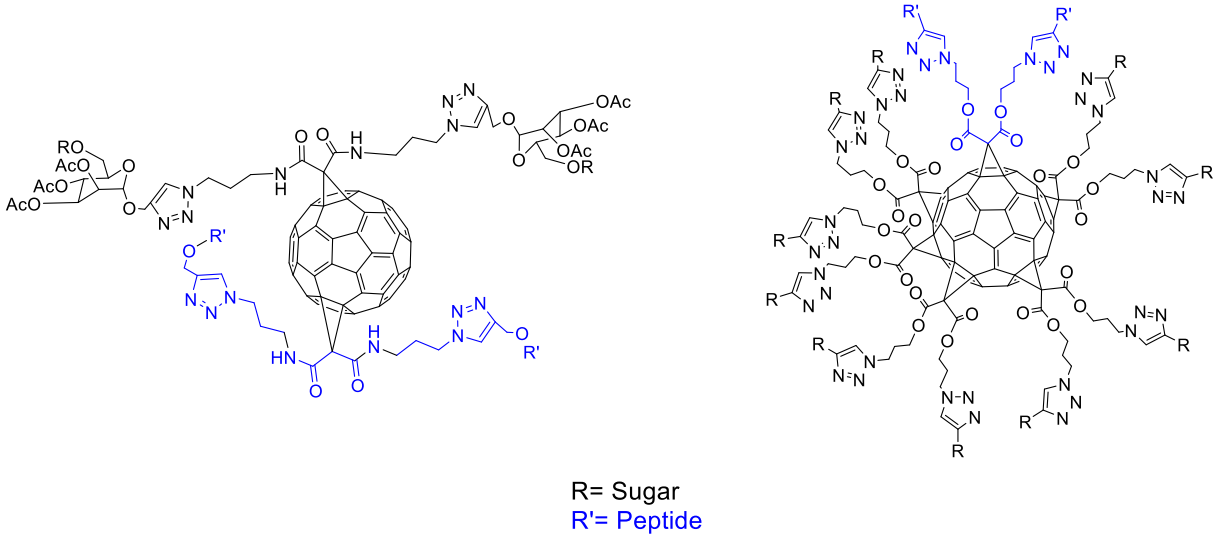
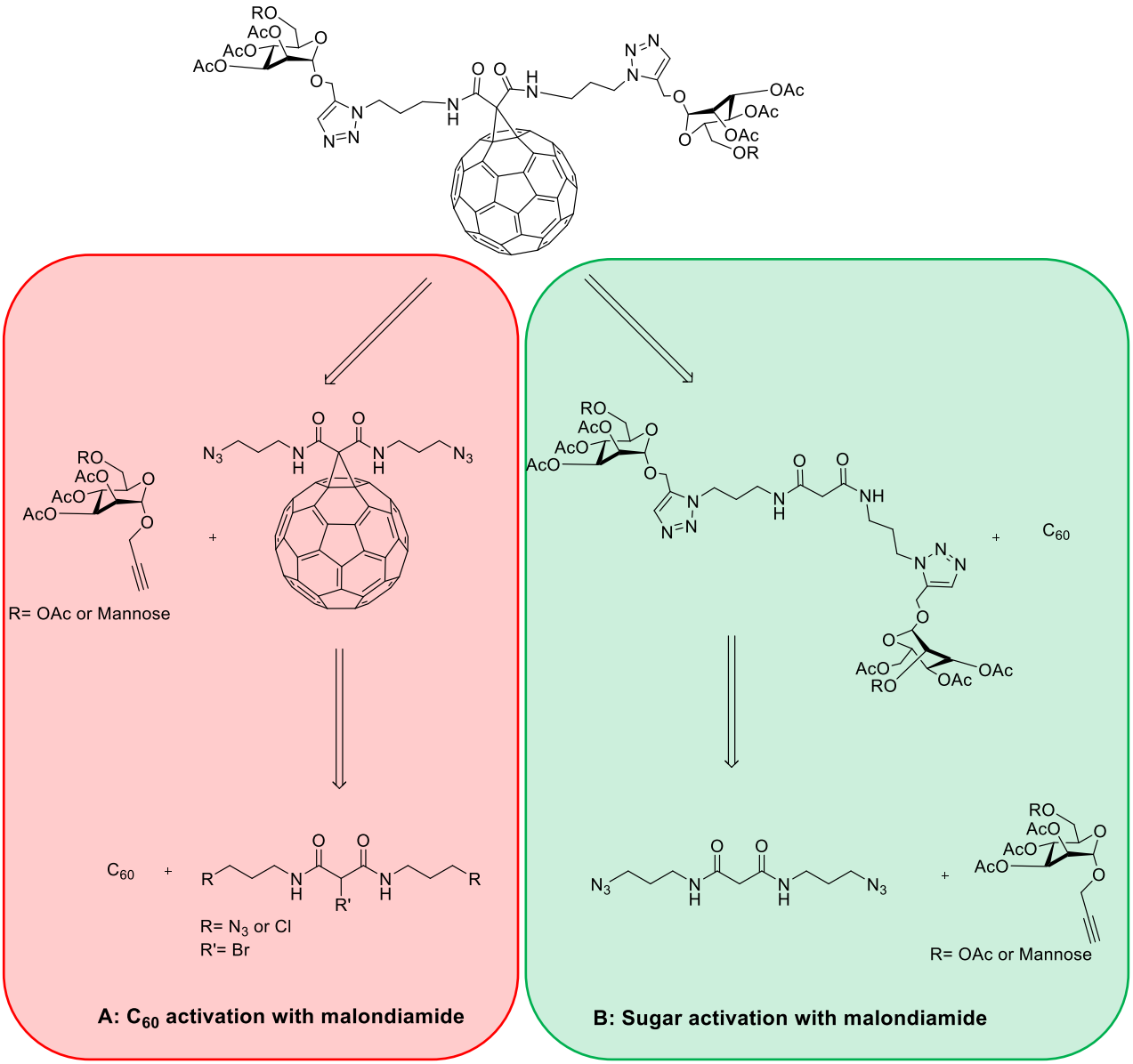


FIGURE 4.1: Example of-C-60 conjugated product using the malondiamide or malonate as linker.

4.2. PREPARATION OF GLYCOSYLATED C_{60} USING MALONDIAMIDE LINKERS

As previously described, the malondiamide can be used as linker in order to bind carbohydrates with C_{60} (Scheme 4.1). Two different approaches were envisioned. The first strategy consisted in the functionalization of C_{60} with malondiamide linkers to obtain an intermediate (functionalised with an azido group) suitable for binding the desired sugars (Scheme 4.1 A).

Alternatively, a second synthetic approach was designed in order to perform the click reaction between the sugar and the malondiamide before the conjugation with fullerene (Scheme 4.1B).

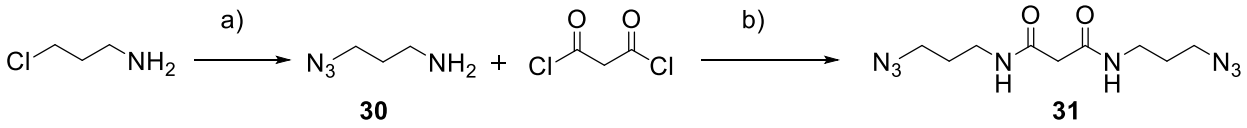


SCHEME 4.1: Retro-synthetic strategies with malondiamide (A and B).

4.2.1 FUNCTIONALISATION OF C_{60} WITH THE MALONDIAMIDE LINKER

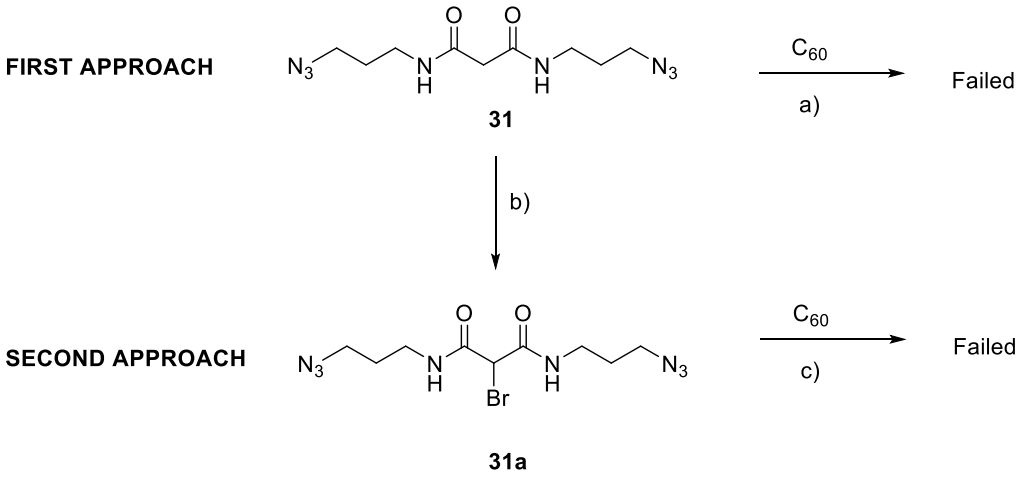
The first considered approach consisted in the activation of C_{60} with a malondiamide chain already bearing the azide functions required for click chemistry reaction with the sugars. This malondiamide chain was synthesized by a two-step reaction (Scheme 4.2). 1-Amino-3-chloropropane hydrochloride and sodium azide were reacted in water for six hours at 80°C to afford 3-azidopropan-1-amine (**30**) in 66% yield. This intermediate was then reacted with malonyl

chloride, in the presence of aqueous NaOH, at 0°C to give *N*[1],*N*[3]-bis(3-azidopropyl)malondiamide (**31**) in 44% yield.



SCHEME 4.2: Synthesis of N^1,N^3-bis(3-azidopropyl)malondiamide (4.2). Experimental conditions: a) 3-chloro propylamine, NaN_3, H_2O, 80°C, 6h(66%); b) malonyl chloride, aqueous NaOH,CH_3Cl, 0°C, 20 min(44%)

The azido malondiamide was reacted with C_{60} following two procedure (Scheme 4.3). The first approach (Scheme 4.3) was investigated in order to obtain the activated C_{60} by a one-pot reaction involving the *in situ* formation of the halide-activated malondiamide, the subsequent carbanion generation in a mixture of base and iodine or tetrabromomethane, followed by the final nucleophilic addition with the electron deficient fullerene double bond. However, the low reactivity of the malonate derivative highly affected the reaction outcome and the desired product was not obtained.



SCHEME 4.3: Procedure for synthesis of azido malondiamide C_{60}. Experimental conditions: a) DBU, I_2 or CBr_4, dry toluene, 0°C; b) Br_2, NEt_3, EtOAc(62%); c) DBU, dry toluene, 0°C.

Different reaction conditions were explored. Initially, N^1,N^3-bis(3-azidopropyl)malondiamide (**31**) , C_{60} and iodine were dissolved in dry toluene, and DBU was added at 0°C. After two hours, TLC analysis revealed only degradation products and by-products.

Unfortunately, not even the use of different reaction times (from hours to days) or solvents (o-DCB) or halogenating agent (CBr_4 instead of I_2) were enough to result in the formation of the desired product.

Assuming that the malondiamide chain could not be enough reactive in the conditions described above, an alternative approach was considered according to literature[250]. The malondiamide chain was previously activated as halide-derivative and then reacted with fullerene in the Bingel reaction conditions (Scheme 4.3).

The preparation of N^1,N^3-bis(3-azidopropyl)-2-bromomalondiamide (**31a**) was not trivial**.** Indeed, the reaction conditions lead to the formation of the *di*-brominated derivative as by-product. When, N^1,N^3-bis(3-azidopropyl)malondiamide (**31**) was reacted with TEA and Br_2 in dry DCM the bromomalondiamide **31a** was obtained in only 23% yield. The starting material was poorly reactive, and longer reaction times resulted only in increased amounts of the *di*-bromurated by-product (as shown in TLC analysis and confirmed by mass spectrometry analysis). However, using ethyl acetate in place of dichloromethane and adding Br_2 in four aliquots over 16 minutes, followed by the final addition of TEA, **31a** was afforded in 62% yield.

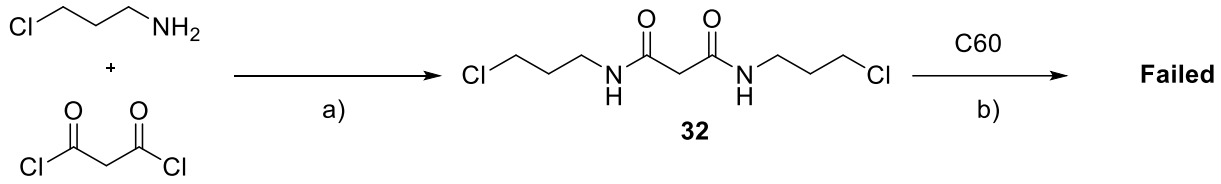
Compound **31a** was used in the cyclopropanation reaction that was performed in dry toluene, in the presence of DBU. Unfortunately, also in this case only degradation products and by-products were obtained.

The unsuccessful cyclopropanation could be due to the high reactivity of the terminal azide moiety that could react with the fullerene double bond. Notably, in the literature there are several studies reporting the direct binding of sugars or other chains to the fullerene core directly through an azide moiety[182], [184], [186], [187].

To avoid the side reactions mediated by the terminal azide with the C_{60}, a third strategy was designed (Scheme 4.4). Thus, a malondiamide linker bearing alkyl chloride chains in place of the alkyl azide chains, was synthesized. This intermediate was supposed to undergo Bingel reaction conditions and then converted into the azide derivative.

The synthesis of N^1,N^3-bis(3-chloropropyl)malondiamide **32** (Scheme 4.4), was performed starting from 3-chloropropylamine hydrochloride in chloroform with an aqueous NaOH solution, adding a solution of malonyl chloride in chloroform leading to the formation of the desired product in 18%

yield. The cyclopropanation step was performed in dry toluene in presence of DBU and I_2. Unfortunately, the reaction failed again. Even in the absence of the azide, only degradation products and by-products were obtained.



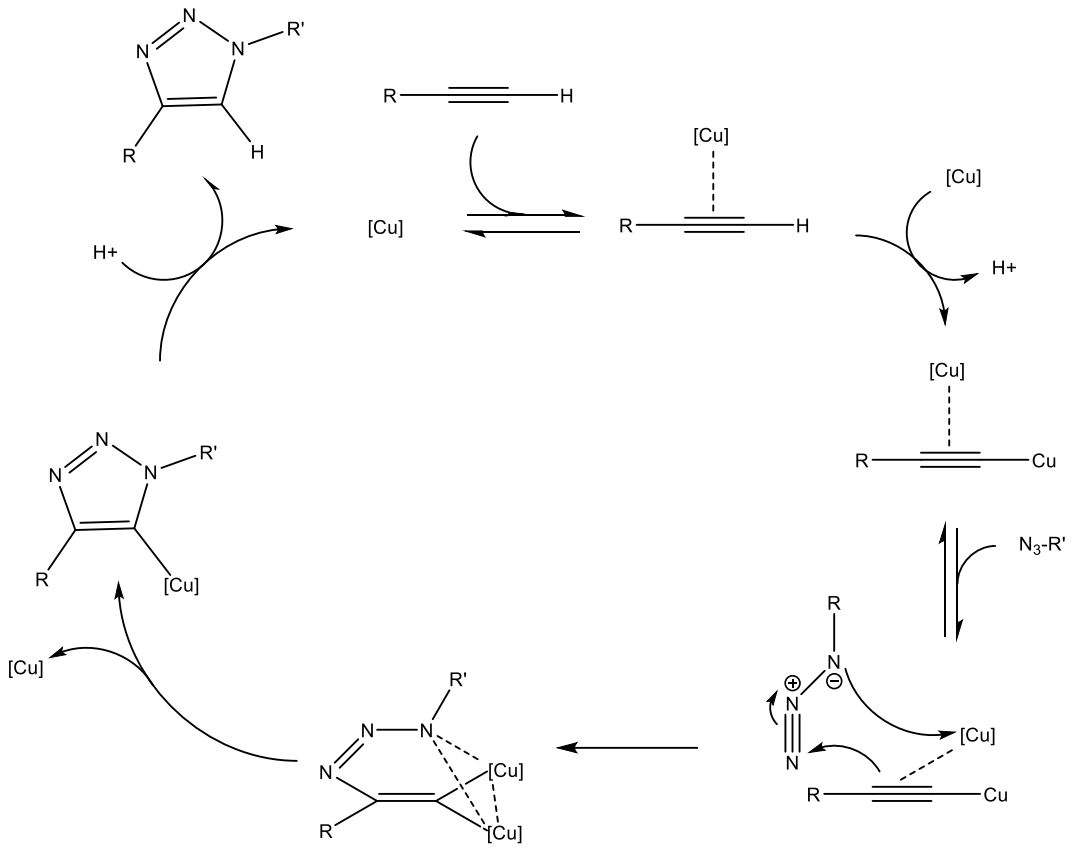
SCHEME 4.4: Third strategy to link malondiamide on C_{60}. Experimental conditions: a) aqueous NaOH, $CHCl_3$, 0°C, 20 min(18%), b) DBU, I_2, dry toluene.

4.2.2 SUGAR ACTIVATION WITH MALONDIAMIDE CHAIN

Since the first strategy did not work, we decided to invert the reaction order. Thus, the azido-malondiamide **31** chain was first submitted to click reaction with the propargyl-sugars to give the corresponding glycosylated 1,2,3-triazole malondiamide derivatives. Then, the reaction conditions to conjugate the glycosylated malondiamide chains with the fullerene C_{60} were studied.

a) CLICK CHEMISTRY REACTION

The Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) was employed for the 1,2,3- triazoles construction in this work. The reaction involves a 1,3-dipolar cycloaddition between an alkyne and an azide derivative[251] and is catalysed by copper(I), usually prepared by *in situ* reduction of copper sulphate (Cu^{II}) with a reducing agent, like sodium ascorbate. The Cu(I) species forms a π-complex with the triple bond. A second atom of Cu(I) results in the formation of the acetylide intermediate, after the deprotonation of the terminal hydrogen of the alkyne bond. The deprotonation step may be helped by the presence of a base. However, the copper-alkyne coordination complex can decrease the pKa of terminal alkyne C-H bond resulting in the deprotonation without a base. A copper atom is linked to the acetylide, whereas another atom of Cu(I) is needed to activate the azide through coordination of the nitrogen electrons. A copper-azide-acetylide complex is formed and the cyclization takes place, followed by final protonation (the proton derived from the deprotonation of the terminal alkyne). The product is formed and the copper can catalyse further reaction cycles. This mechanism is described in Scheme 4.5 [252].

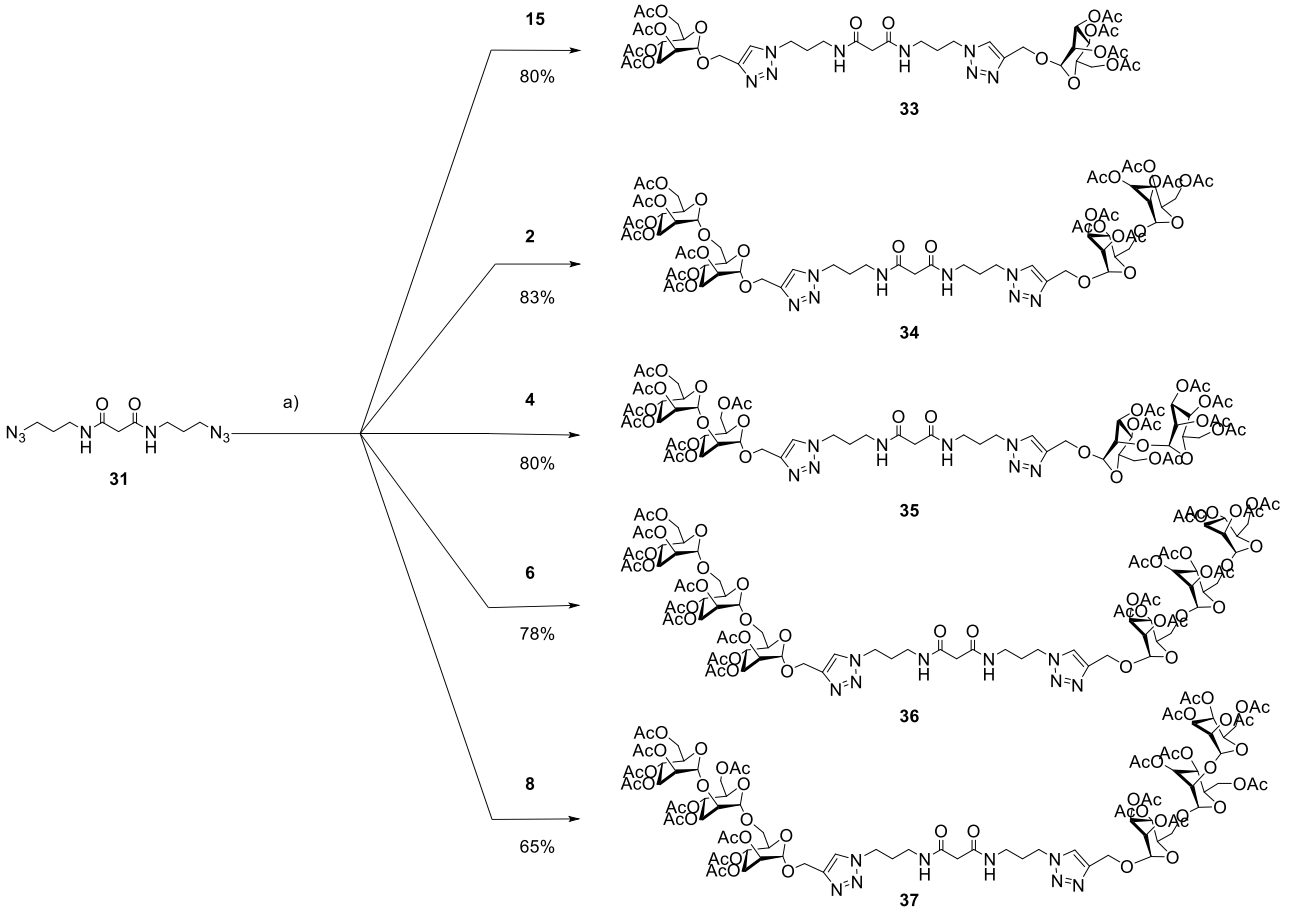


SCHEME 4.5: Click chemistry mechanism.

Copper is the main character of this reaction and it can derive from different sources, such as Cu(I) complex ($CuBrMe_2S$, CuCl, CuI)[253], metallic copper or from mixtures of copper (II) salts and reducing agents (the most convenient method).

Scheme 4.6 shows the click synthesis of the malondiamide chains prepared using the propargyl sugars previously synthesised (**2**,**4**,**6**,**8**,**15**, chapter 2). The click reaction was firstly performed between the malondiamide chain **31** and the acetylated monosaccharide (**15**) to obtain product (**33**). Then, the optimal conditions were applied for the conjugation of malondiamide **31** with di- and tri-saccharides.

The general procedure of the 1,3-dipolar cycloaddition consisted in dissolving the sugar in tetrahydrofuran, followed by the addition of the same amount of water. At this point copper sulphate and sodium ascorbate were added. The products were obtained in good yields (65-83%) after purification.

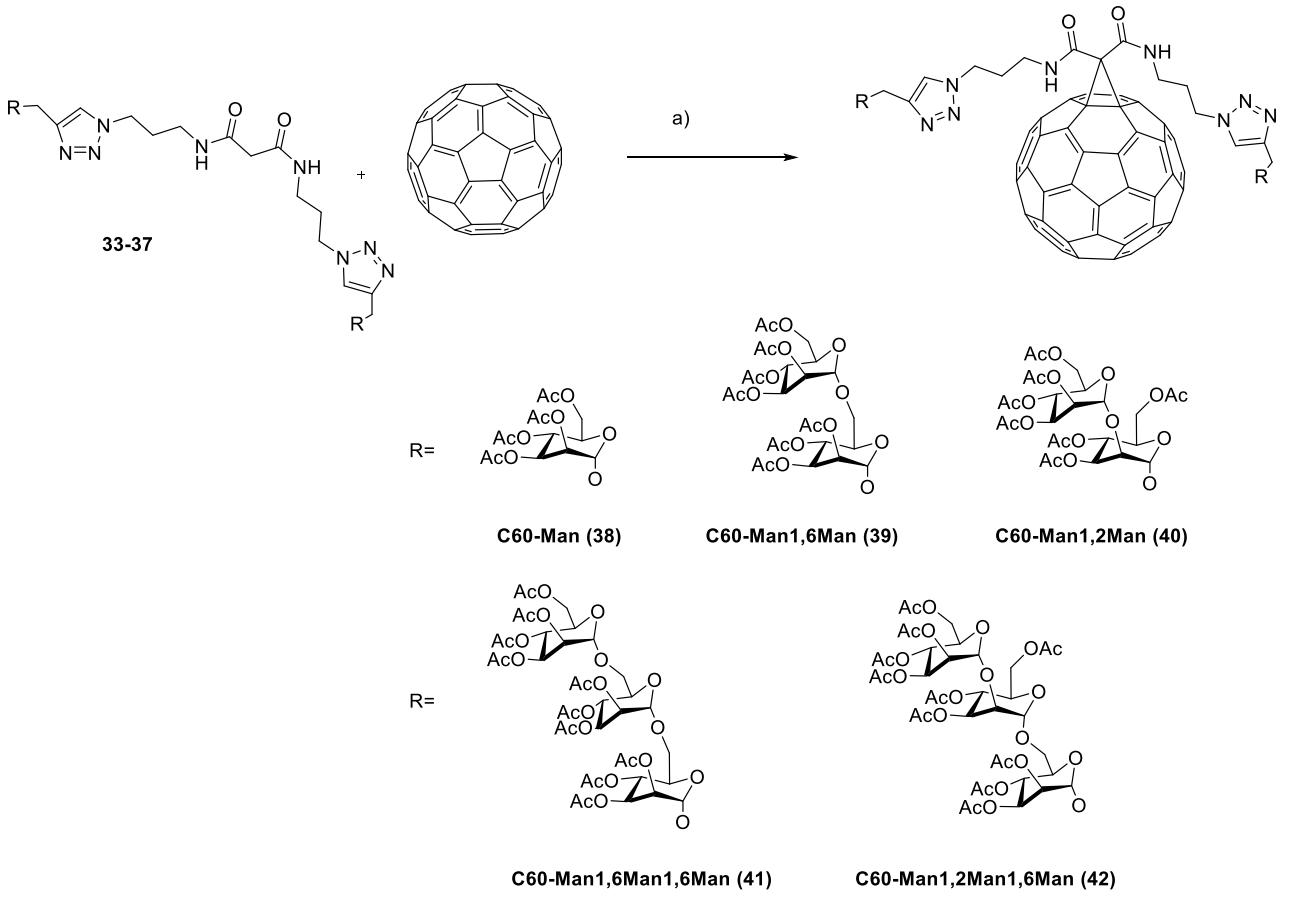


SCHEME 4.6: Sugars chains synthesis. Experimental conditions: a) $CuSO_4$, Na ascorbate, THF/H_2O.

Other conditions were attempted for the conjugation of the monosaccharide. Higher temperature (reflux at 100°C) or the use of a different catalyst and a ligand (Cu tetrakis and TBTA) afforded the desired product in comparable yields. For this reason, the simplest and fastest procedure described above was employed in the click reaction between the malondiamide azide and the other sugars.

b) BINGEL REACTION

Once prepared, the glycosylated malondiamide-sugar chains (**33-37**) were used in the cyclopropanation with fullerene by the Bingel reaction (Scheme 4.7) for the production of the glycosylated fullerenes. All the attempts and optimizations that have been made to obtain the desired products are described in detail below.



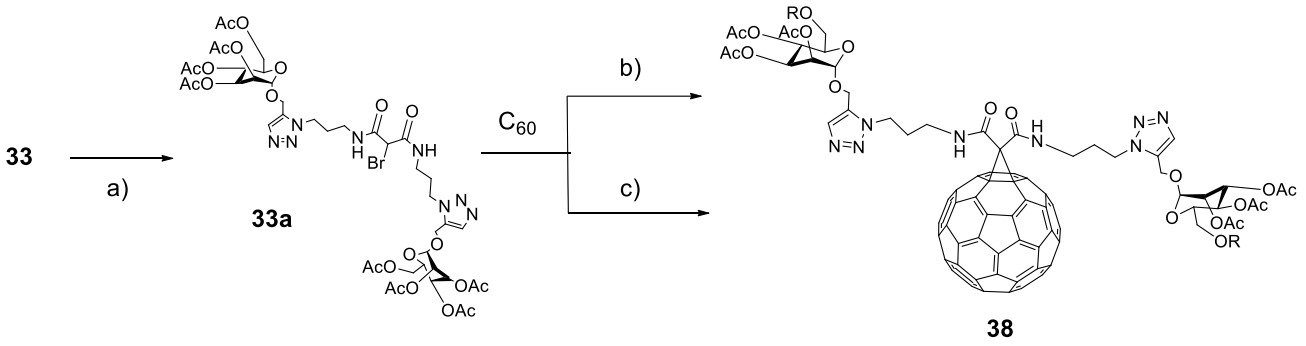
SCHEME 4.7: Bingel reaction for preparation of glycosylated fullerenes 38-42. Experimental conditions: a) DBU, I_2, dry toluene.

*Synthesis of Fullerene-Man (**38**)*

The chain **33** was used as model to optimize the reaction conditions (Table 4.1). In a first attempt, this malondiamide-Man chain **33** was dissolved in dry toluene with C_{60} and iodine. DBU was added at 0°C and the mixture was stirred at room temperature for 90 minutes. After purification, Fullerene-Man (**38**) was obtained in 17% yield in a one-pot reaction. This result was encouraging but further attempts were performed in order to improve the yield.

Following the approaches used with N^1,N^3-bis(3-azidopropyl)malondiamide (**31**), attempts were made to isolate in advance the activated halide-derivative to use it in the subsequent cyclopropanation step (Scheme 4.8). Hence, the bromination of **33** was carried out with bromine that was added in four aliquots, followed by triethylamine addition to afford the brominated malondiamide-Man chain (**33a**) in 35% yield. The moderate yield obtained was probably due to the formation of the *di*-brominated by-product.

Theoretically, the reaction should be improved by using the brominated chain, instead of the one-pot cyclopropanation. However the two step reaction via brominated malondiamide-Man chain (**33a**) afforded the desired product only in traces (<5%). Therefore, we tried to replace DBU with sodium hydride as base (that is used sometimes in this kind of reactions) but the product was obtained again in yield less than 5% (Table 4.1 entry 3).



SCHEME 4.8: Activated chain strategy. Experimental conditions: a) Br_2, NEt_3, DCM/EtOAc (1 :2), 16min (35%) ; b) DBU, dry toluene, r.t., 1.5h (<5%); c) NaH, dry toluene, r.t.,6.5h (<5%).

Consequently further efforts were made to optimize the one-pot conjugation of the mannosylated Chain (**33**) with fullerene. Assuming that the low yield could be due to the final product degradation, the reaction time was decreased and we were pleased to find out that this yield progressively increased. The product was obtained in 30% yield in 1 h and in 41% in 45 minutes (Table 4.1).

TABLE 4.1: Optimization of monosaccharide chain conjugation for the synthesis of **38**

ENTRY	MALONAMIDE	BASE	HALOGEN	TIME	YIELD
1	**33**	DBU	I_2	1.5h	17%
2	**33a**	DBU	-	1.5h	<5%
3	**33a**	NaH	-	6.5h	<5%
4	**33**	DBU	I_2	1h	30%
5	**33**	DBU	I_2	45 min	41%

The best conditions found for the conjugation of the monosaccharide chain **33** with fullerene were applied to the conjugation of C_{60} with the di- and trisaccharides malondiamide derivatives **34-37**. Surprising, each glycosylated chain required different reaction conditions (Table 4.2) and generally the reactivity decreased when the dimension of the sugar moiety increases. This could be due to the steric hindrance or the different glycosidic bonds, affecting the molecular spatial disposition.

*Synthesis of Fullerene-Man 1,6 Man (**39**)*

The Man1,6 man chain **34** was firstly reacted at room temperature for 90 min and 60 min to afford the desired product **39** in 10% and 12% yield, respectively. From these results, Man1,6 Man chain was supposed to be less reactive than the chain bearing the monosaccharide. However, similarly, decreasing reaction times the yields increased (16% and 24 % yield after 30 and 7 minutes, respectively). These data suggested that the product is less stable in the reaction condition and for this reason more easily degradable.

Indeed, by performing the reactions at 0°C, the yield increased up to 31 % in 2h (Table 4.2). When the reaction was performed during 1 hour, a similar yield was obtained (30%), suggesting that shorter reaction times would not lead to further improvements.

*Synthesis of Fullerene-Man 1,2 Man (**40**)*

The Man1,2man chain (**35**) showed different reactivity with respect to the other disaccharide. It is possible that the 1,2-glycosidic bond affects the spatial position of the two sugars and leads to higher steric hindrance, resulting in a strong decreased reactivity in the conjugation reaction.

Indeed, when the best conditions developed for the preparation of Fullerene-Man1,6Man **39** were applied to the Man1,2man malondiamide **35** , the desired product **40** was obtained in only 8% yield. Reducing the temperature to -20°C (to avoid an eventual chain or product degradation) the reaction outcome was not improved (8% yield in 3 h and <5% in 1.5 h). Conversely, when the reaction was performed at room temperature for three hours, the product was obtained in 11% yield, giving the best result. Longer reaction times resulted again in decreased yield (5%). Also changing the reagents ratio (0.7 eq of sugar and 1 eq of C_{60}) did not give any improvement.

*Synthesis of Fullerene-Man 1,6 Man1,6Man (**41**)*

The Man1,6man1,6Man chain (**36**) was supposed to have reactivity similar to Man1,6man chain (**34**).

Therefore, the optimized condition developed for the synthesis of C_{60}-Man1,6Man **39** was applied also for the synthesis of C_{60}-Man 1,6 Man1,6Man (**41**) by reaction with the Man1,6man1,6Man malondiamide **36**. The desired product was obtained but in lower yield (12%) compared to that obtained with the disaccharide Man1,6Man (24%), maybe because of the larger size of the trisaccharide. Temperature changes did not affect the reaction outcome. The reaction time was reduced to twenty minutes, causing a slight increase of the yield (15%).

*Synthesis of Fullerene-Man 1,2Man1,6Man (**42**)*

The reactivity of the man1,2man1,6Man chain (**37**) seems affected by both the large size of the substituent and the presence 1,2 glycosidic bond. In fact the synthesis of Fullerene-Man 1,2Man1,6Man (**42**) provided the worst yields. Indeed, both reactions performed at room temperature and at 0°C in 1 h led to the product formation, but in low yields lower than 5% (Table 4.2). Changing the reagents ratio (1 eq of sugar and 0.7 eq of fullerene) resulted in a slight improvement (8% yield). This could be due to the excess of malondiamide chain.

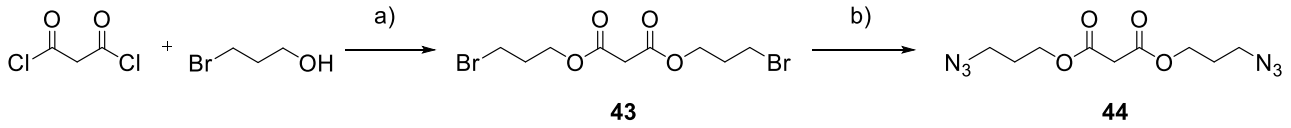
TABLE 4.2: Optimisation of the conjugation of sugar-malondiamide chains with Fullerene

PRODUCT	RATIO (C_{60}:sugar)	TEMPERATURE	TIME	YIELD
Fullerene-Man1,6Man (**39**)	1:1	R.T.	1.5h	10%
	1:1	R.T	1h	12%
	1:1	R.T	30 min	16%
	1:1	R.T	15 min	22%
	1:1	R.T	7 min	24%
	1:1	0°C	20 min	26%
	1:1	0°C	1h	30%
	1:1	0°C	2h	31%
Fullerene-Man1,2Man (**40**)	1:1	0°C	1h	8%
	1:1	-20°C	3h	8%
	1:1	-20°C	1.5h	<5%
	1:1	R.T	3h	11%
	1:1	R.T	1.5h	10%
	1:1	R.T	6h	5%
	1:0.7	R.T	1h	5%
Fullerene-Man1,6Man1,6Man (**41**)	1:1	0°C	1h	12%
	1:1	0°C	20 min	15%
	1:1	0°C	10 min	13%
	1:1	R.T.	15 min	12%
Fullerene-Man1,2Man1,6Man (**42**)	1:1	0°C	1h	<5%
	1:1	R.T.	1h	<5%
	0.7:1	R.T.	3h	8%

4.3. PREPARATION OF GLYCOSYLATED C_{60} MEDIATE BY THE MALONATE ESTER LINKER

The synthesis of the malonate chain was performed by a two-step reaction (Scheme 4.8). Firstly, the malonate bis(3-bromopropyl) malonate (**43**), presenting two terminal bromide was prepared

in 79% yield. Then, a nucleophilic substitution was performed to afford bis(3-azidopropyl) malonate (**44**) in 90% yield.



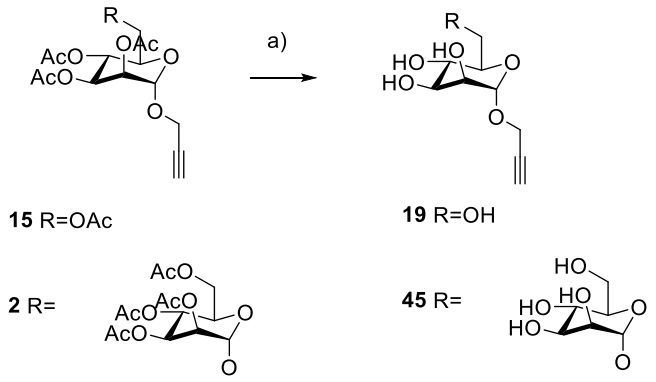
SCHEME 4.8: Synthesis of Bis(3-azidopropyl) malonate (**44**). Experimental conditions: a) Pyridine, DCM, 0°C to r.t., 18h(79%); b) NaN_3, Dry DMF, r.t., 16h(90%).

Using the azido-ester chain **44** two different coupling approaches were investigated for glycosylating C_{60}. The first approach we tested, consisted in linking one malonate chain to the fullerene similarly to that investigated with the malondiamide chain.

In the conjugation with fullerene, malonate chains are much more reactive than that with malondiamide chains. Thus, we have considered a second approach aiming the synthesis of a fullerene functionalized with six malonate ester chains that (after glycosylation) should provide a water-soluble product.

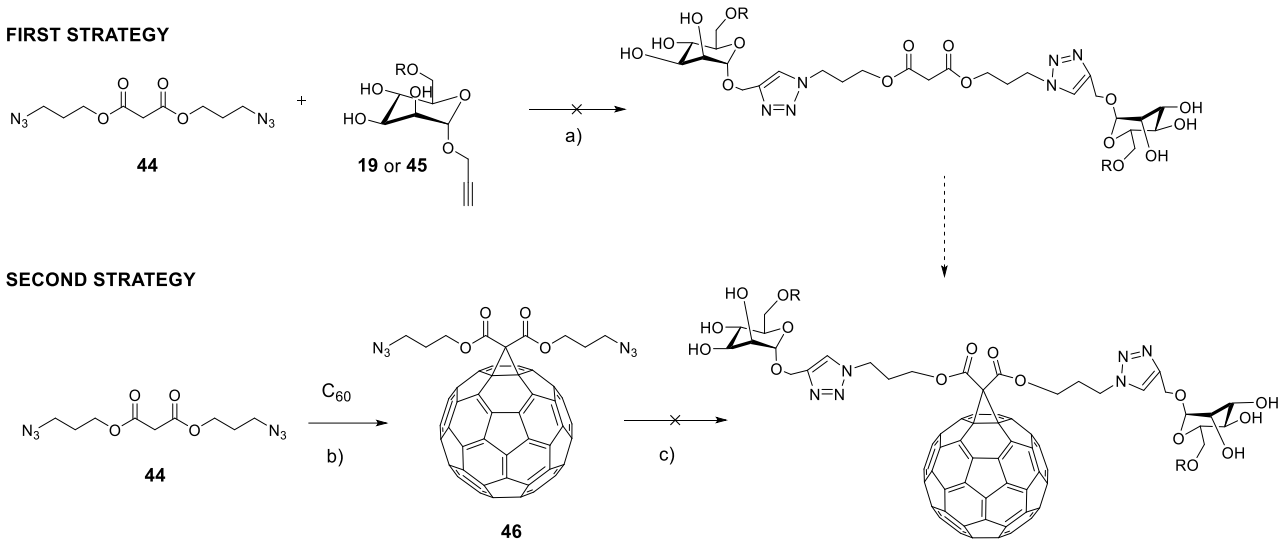
4.3.1 FUNCTIONALIZATION OF C_{60} WITH A SINGLE MALONATE ESTER CHAIN

The main drawback of this approach deals with the use of free sugars that can be managed only in aqueous solvent. Consequently, deprotected mannose **19** and man-α1,6man **45** with the anomeric propargyl group were prepared (Scheme 4.9).The general procedure for removing acetyl group consisted in the use of sodium methoxide, generated in *situ* by mixing dry methanol and metallic sodium (2mg/10ml). The protected sugar (**15** and **2**) was dissolved in this solution and stirred until completion of the reaction was observed (yield over 90%).



SCHEME 4.9: Deacetylation reaction. Experimental conditions: a) Na/MeOH (2mg/ml). r.t. (>90%).

As described for the amide chain, we planned to link a single malonate chain to the fullerene core. Also in this case, two approaches were attempted (Scheme 4.10). We could firstly synthesize the glycosylated linker by click reaction to obtain the 1,2,3-triazole derivatives followed by cyclopropanation with fullerene. Alternatively, the cyclopropanation between the azide-malonate linker **44** and the C_{60} could be carried out before the click reaction with deprotected glycans.



SCHEME 4.10 : Single malonate-ester chain strategies. Experimental conditions : a) $CuSO_4$, Na ascorbate, THF:H_2O; b) DBU, I_2, dry toluene; c) **19**, $CuSO_4$, Na ascorbate, DMSO.

a) First strategy: Glycosylation of the malonate-ester linker

Click reaction was performed with the deprotected monosaccharide, Propargyl α-D-mannopyranoside (**19**) in a mixture THF/H_2O (1:1) with copper sulphate and sodium ascorbate. The solution was checked at mass spectrometry that confirmed the product formation (MS(ESI) m/z = 729.9789 $[M+ Na]^+$). Until the product was in the reaction environment and seemed to be stable, but unfortunately, after purification it degraded.

A purification *via* flash chromatography was performed according to TLC analysis used for reaction monitorization, but the acidic nature of silica gel could be responsible for the degradation of the product (probably due to the low stability of the malonic ester chain).

A Size-exclusion chromatography (LH-20 column) was tested, but also in this case the isolation of the product failed.

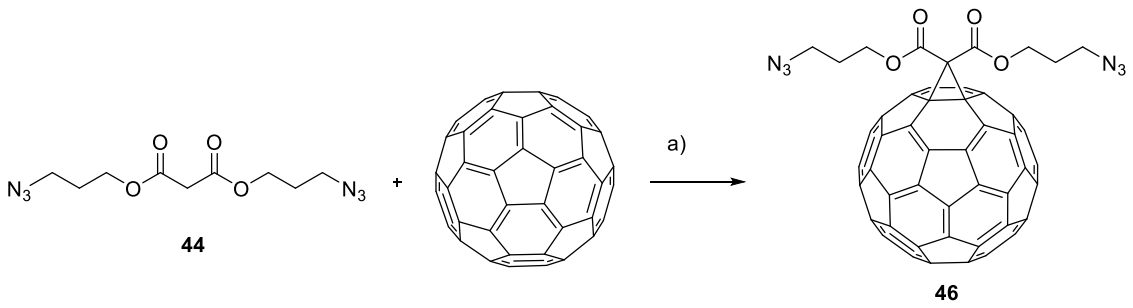
The same problems were encountered when we tried to link a disaccharide Propargyl (α-D-mannopyranosyl-(1→6)- α-D-mannopyranoside (**45**)). By mass spectrometry the glycosylation

product was detected (MS(ESI) m/z = 1054.2286 $[M+ Na]^+$), but also in this case it was not possible to purify it without causing its degradation.

b) Second strategy: fullerene conjugation with a single malonate-ester linker

Under mild condition and in a short reaction time, it is possible to obtain a fullerene conjugation with a single malonate chain [254].

Mixing bis(3-azidopropyl) malonate (**44**), $C_{60,}$ I_2 and DBU at 0°C, the monomalonate Fullerene-$(N_3)_2$ (**46**) was obtained in 42% yield (Scheme 4.11) after 45 min of stirring. The time is the main parameter affecting the outcome of this reaction. Indeed, when the reaction was performed for 1.5 h, fullerenes bearing two or three chains were obtained as main products, instead of the mono-functionalized fullerene.



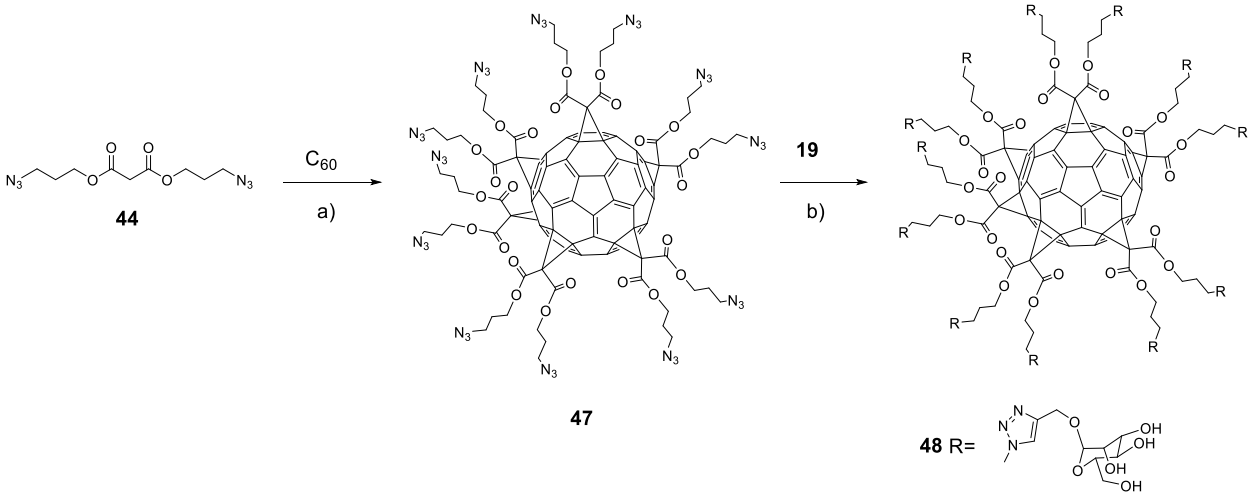
SCHEME 4.11: C_{60}-Single N_3chain (18) synthesis. Experimental condition : a) DBU, I_2 or CBr_4, dry toluene, 0°C to r.t, 45 min(42%).

The click reaction was challenging because of the disadvantage of using deprotected sugars that have a completely different solubility compared with fullerene. Thus, once monosaccharide was deacetylated (**19**), several click chemistry conditions with the monomalonate Fullerene-$(N_3)_2$ (**46**) were experimented.

The main problem was the high instability of **46**. Therefore, the compound was reacted immediately with deprotected monosaccharide (**19**) after its preparation. Several conditions (by changing solvents, ratio and reaction time) were attempted to obtain the desired product but without success because an insoluble black product was obtained. Thus, it was impossible to analyze it by mass spectrometry or NMR analysis.

4.3.2 HOMO-FUNCTIONALIZATION OF C_{60} WITH SIX MALONATE ESTER CHAINS

High glycosylation of C_{60} has the advantage that the final product will be completely soluble in aqueous medium. Thanks to the high reactivity of the malonate chains in the Bingel reaction, six chains can be easily linked to the fullerene in a one-pot reaction [234] allowing the introduction of 12 azido groups. Thus compound **44** and C_{60} in dry dichlorobenzene were treated with CBr_4 and DBU and at room temperature for 72 hours. After purification, hexa-malonate Fullerene-$(N_3)_{12}$ (**47**) was obtained in 60% yield (Scheme 4.12), confirming literature reports[234].



SCHEME 4.12: Synthesis of C60-$(N_3)_{12}$ and click reaction optimization. Experimental conditions: a) DBU, CBr_4, dry ODCB, 72h(60%), b) CuSO4, Na ascorbate, THF/H_2O, 100°C MW, 2h(61.3%).

a) Click glycosylation with propargyl-mannose

After C_{60} functionalization, we investigated the 1,3-Huisgen dipolar cycloaddition between twelve deprotected sugars and the hexa-malonate Fullerene-$(N_3)_{12}$ (**47**). In the literature, there are some studies on this click chemistry reaction between properly functionalized fullerene and monosaccharides[173,234,255]. Several conditions were tried to find the best ones affording the desired product (Table 4.3) using the monosaccharide (**19**) in order to obtained the glycoconjugate C_{60} product **48** (Scheme 4.12).

Following conditions from literature [234,255,256], to the solution of **47** in tetrahydrofuran, a solution of Propargyl α-D-mannopyranoside (**19**) in DMSO was added, followed by the addition of an aqueous solution of copper sulphate and sodium ascorbate. The mixture was stirred at room temperature for 48 hours, but the product was not detected. The changing of solvent (DMSO) and copper source ($CuBrSMe_2$) did not allow to obtain the product.

Because of the different polarity of the two starting material (free sugar extremely hydrophilic conversely functionalized C_{60} high hydrophobic), we hypothesised that this different polarity and so the different solubility could negatively affect the reaction outcome. Probably, the mild conditions tested were not enough to overcome this issue and make them to react.

For this reason, more harsh conditions were attempted.

Following a procedure developed by Munoz *et al*[246], the click reaction was first performed in a mixture of THF/H_2O (1:1) with 15 eq. of sugar, in a microwave reactor at 100°C for 2h. The Fullerene-$(Man)_{12}$ (**48**) was obtained in 61% yield. Longer reaction times and different solvent mixtures did not result in any improvement of the reaction yield (Table 4.3).

Table 4.3: Optimization of twelve monosaccharide conjugation on fullerene

TEST	RATIO (C_{60}:Sugar)	CONCENTRATION	CATALYST	SOLVENT	TIME	TEMPERATURE	YIELD
1	1:13	55 mg C_{60}/ml	$CuSO_4$ 0.1 eq Na Ascorbate 0.3 eq	THF:H_2O: DMSO	48h	r.t.	Failed
2	1:30	8mg C_{60}/0.5 ml	$CuBrSMe_2$	DMSO	48h	r.t.	Failed
3	1:15	55 mg C_{60}/ml	$CuSO_4$ 0.1 eq Na Ascorbate 0.3 eq	THF:H_2O: DMSO	2h	100°C MW	Incomplete
4	1:15	30mg C_{60}/ml	$CuSO_4$ 0.1 eq Na Ascorbate 0.3 eq	THF:H_2O	2h	100°C MW	61.3%
5	1:15	30mg C_{60}/ml	$CuSO_4$ 0.59 eq Na Ascorbate 1 eq	THF:H_2O	2.45h	100°C MW	60%
6	1:20	30mg C_{60}/ml	$CuSO_4$ 0.39 eq Na Ascorbate 1 eq	THF:H_2O	4h	100°C MW	54%
7	1:20	30mg C_{60}/0.45ml	$CuSO_4$ 0.1 eq	THF:H_2O: DCM	2h	100°C MW	22.5%

			Na Ascorbate 0.3 eq				
8	1:15	9mg C_{60}/0.4ml	$CuSO_4$ 0.1 eq Na Ascorbate 0.3 eq	THF:H_2O	2h	100°C MW	38.4%
9	1:15	6mg C_{60}/0.4ml	$CuSO_4$ 0.39 eq Na Ascorbate 1 eq	DMF:H_2O	2h	100°C MW	Incomplete

This reaction was very difficult to monitor. TLC analysis couldn't give us any information (even by using silica C_{18} TLC plates). Therefore, infrared (IR) analysis was employed to confirm completion of the reaction. Indeed, IR can easily detect the presence of the azide bond, indicated by a characteristic peak at 2100 cm^{-1} in the diagnostic region (Figure 4.2). Conversely, ^{1}H-NMR analysis was not very indicative to identify the final product. Moreover, sugar molecule aggregation causes peaks broadening, making even more difficult NMR spectra interpretation. However, once IR shows the completion of the reaction, the formation of the desired product must be confirmed by mass spectrometry analysis and ^{13}C-NMR too.

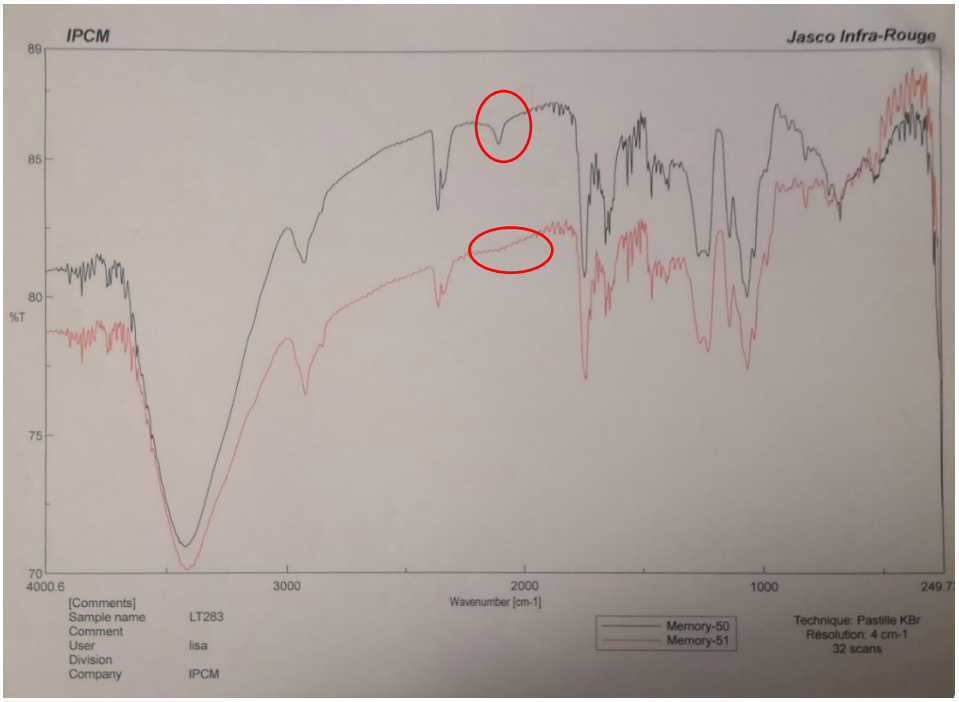


FIGURE 4.2: Analysis of the click chemistry reaction for the synthesis of Fullerene-$(man)_{12}$ **48**: complete (red) VS Reaction Incomplete (black).

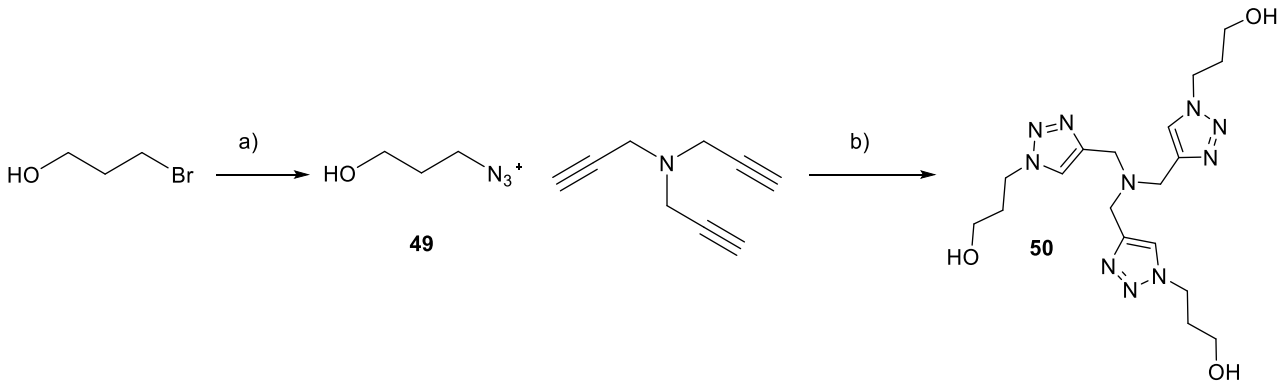
b) Click conjugation with propargyl-man1,6man disaccharide

1,3-Huisgen dipolar cycloaddition was attempted also between fullerene and the disaccharide Propargyl α-D-mannopyranosyl-(1→6)- α-D-mannopyranoside (45).

Following a literature procedure involving the linkage between a properly functionalized fullerene and a propargyl lactose[234], the disaccharide (**45**), dissolved in DMSO, was added to a solution of Fullerene-$(N_3)_{12}$ (**47**) in toluene. $CuBrSMe_2$ was added as copper source and the reaction was stirred for four days. A red solid was collected, but IR analysis showed a large amount of residual azide groups, and the desired product was not detected by mass spectrometry analysis.

More harsh conditions were attempted by using microwave irradiation and higher temperatures, successful conditions in the monosaccharide click reaction. The best result was achieved when the reaction was performed at 80°C, under microwave irradiation, for 3h. Mass spectrometry analysis showed the formation of a mixture of partially-glycosylated C_{60} bearing 5-9 disaccharide moieties. However, longer reaction times led only to product degradation.

According to the literature, the use of ligands able to stabilize Cu(I) may be beneficial to click reaction success. Hence, tris-hydroxypropyltriazolylmethylamine (THPTA), one of the most widely used ligands in click chemistry reaction, was synthesized[257](Scheme 4.13). 3-azidopropan-1-ol (**49**), prepared by carefully mixing 3-bromopropan-1-ol and sodium azide in water at 50°C for 24 hours, was obtained in 90% yield and immediately reacted with tripropargylamine in a mixture H_2O/*t*-BuOH (1:1), in the presence of copper sulphate and sodium ascorbate. After 48 hours, THPTA (**50**) was obtained in 77% yield.



SCHEME 4.13: THPTA (22) synthesis. Experimental conditions: a) NaN_3, H_2O, 50°C, 24h(90%); b) $CuSO_4$, Na ascorbate, H_2O/t-BuOH, r.t., 48h(77%).

The basic tertiary amine of THPTA was supposed to accelerate the catalytic process of the 1,3-Huisgen dipolar cycloaddition by providing additional electron density to the Cu(I) centre.

Unfortunately, neither the addition of ligands, like THPTA, nor different solvent systems allowed the formation of the desired product.

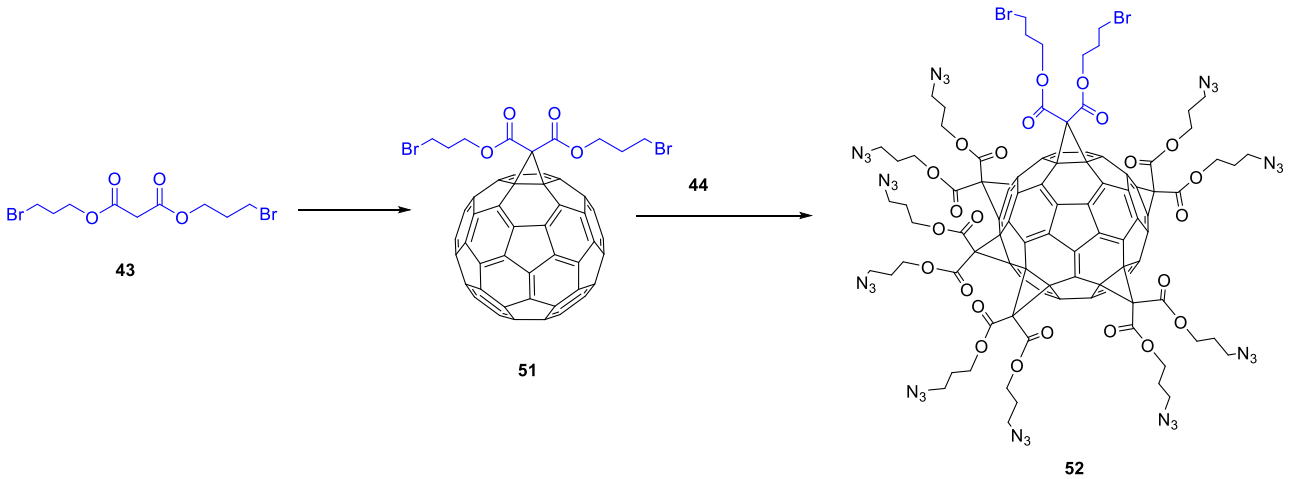
4.3.3 ETERO-FUNCTIONALIZATION OF C_{60} WITH SIX MALONATE ESTER CHAINS

Once Fullerene-$(Man)_{12}$ (**48**) were optimized, we have investigated the hetero-functionalisation of C_{60} in order to make possible the decoration of C_{60} also with peptides. Our strategy (Scheme 4.14) consisted in linking a single terminal *di*-brominated malonate ester chain to fullerene, followed by the attachment of five di-azido malonate chains.

At this point, the azido groups could be exploited for click chemistry reaction to link ten mannoses. Then, previous conversion of the two Br functions into azido, also 1,3-Huisgen dipolar cycloaddition with a peptide baring a propargyl group in the amino terminal amino acid.

Firstly, we focused on the synthesis of double-chain derivatized fullerene (Scheme 4.14) by reaction of C_{60} with bis(3-bromopropyl) malonate (**43**), prepared as described in paragraph 4.3 (scheme 4.8), dry toluene with I_2. DBU was added at 0°C and after the reaction was performed at room temperature for 30 minutes. Malonate C_{60}-$(Br)_2$ (**51**) was obtained in 44% yield in agreement with literature [258].

The intermediate **51**, was then reacted in dry dichlorobenzene with bis(3-azidopropyl) malonate (**44**), CBr_4, and DBU at room temperature for 72 hours to obtain the malonate Fullerene-$(Br)_2(N_3)_{10}$ (**52**) in 72% yield.



SCHEME 4.14: Hetero-functionalisation of C_{60}: Experimental conditions: a) DBU, I_2, dry toluene, 0°C to r.t., 1h(44%); b) DBU, CBr_4, dry ODCB, r.t(72%).

The best conditions found for the conjugation of twelve sugars with the functionalized C60-Azido fullerene (see paragraph 4.3.2) were applied for glycans glycosylation with Fullerene-$(Br)_2(N_3)_{10}$ (**52**) in order to link ten mannose units to the azide chains. Thus, **52** was dissolved in dry tetrahydrofuran, and Propargyl α-D-mannopyranoside (**19**) was dissolved in water and added to the fullerene solution, followed by the final addition of the aqueous solution of copper sulphate and sodium ascorbate. The mixture was stirred at 100°C, under microwave irradiation, for 2h. After purification a red solid was collected, but it was not the desired product (no product detected by mass spectrometry analysis).

Several reasons could be responsible for the reaction failure, like compound instability. Indeed, the terminal *di*-brominated chains could lead to side reactions, under microwave irradiation at 100°C, resulting in product degradation. However, when milder conditions (unsuccessful in the formation of Fullerene-$(N_3)_{12}$ (**48**)) were applied, the desired product was not obtained.

4.4 MATERIALS AND METHODS

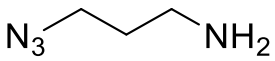
Reactants and chemicals were purchased from commercial sources (Sigma-Aldrich, Alfa Aesar, Fluorochem) and used without further purification. High Resolution Mass spectrometry (HRMS) spectra were recorded on a Bruker microTOF spectrometer, using Tuning-Mix as reference. NMR spectra were recorded on a Brüker Avance II 600 MHz or Brüker AM- 400 MHz. Assignments were aided by COSY and HSQC experiments.

Electrospray-mass spectrometry (ESI-MS) were made at 1 mg/ml. Experiments were carried out using a LTQ-Orbitrap XL-ETD mass spectrometer (Thermo Scientific, San Jose, CA, USA) operated in positive ionization mode with a spray voltage at 3.6 kV. Applied voltages were 40 and 100 V for the ion transfer capillary and the tube lens, respectively. The ion transfer capillary was held at 275°C. Spectra were analyzed using the acquisition software XCalibur 2.1 (Thermo Fisher Scientific, Courtaboeuf, France) without smoothing and background subtraction.

4.4.1 EXPERIMENTAL PART

a) Synthesis of malondiamide derivatives and conjugation with C_{60}

3-azidopropan-1-amine (30)

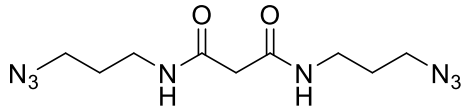


3-azidopropan-1-amine (**30**) was synthesized as previously reported[259].

3-chloropropylamine hydrochloride (6g, 46.147mmol, 1eq.) and NaN_3 (11.4g, 175.358 mmol, 3.8 eq.) was dissolved in H_2O (20ml) and the mixture was stirred at 80°C for 6h.

The reaction was then treated with saturated KOH solution (10ml), added dropwise at 0°C. The aqueous layer was extracted with Et_2O (3 x 25ml). The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo* at room temperature (aliquots of pentane were added and evaporated to extract any Et_2O residue). The desired product was obtain as a yellow oil (4.18 g, 66%). ^1H and ^{13}C NMR were in agreement with previously reported data[259].

N^1,N^3-bis(3-azidopropyl)malondiamide (31)



3-azidopropan-1-amine (**30**) (2.5g, 0.025mol, 1eq.) was dissolved in chloroform (20ml) and a solution of NaOH (2g) in H_2O (4ml) was added. The mixture was cooled to 0°C and a solution of malonyl chloride (1.21 ml, 0.0125mol, 0.5eq.) in chloroform (10ml) was added dropwise, then was stirred for 20 min at 0°C. The mixture was extracted with chloroform, the organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo.*

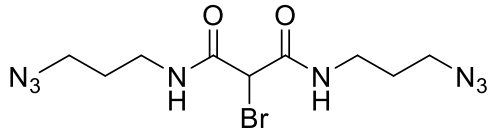
The reaction mixture was monitored by TLC (ethyl acetate 100%. Rf=0.45). Column chromatography (SiO_2, ethyl acetate 100%) gave the desired product (1.5g, 44%) as a white powder.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.47(t, 2H, J= 6.4Hz, N*H*CO), 3.42-3.33 (m, 8H, NHC*H*$_2$CH$_2$CH$_2$N$_3$, NHCH$_2$CH$_2$C*H*$_2$N$_3$), 3.20 (s,2H, COC*H*$_2$CO) 1.82 (q, 4H, J= 6.8 Hz, NHCH$_2$C*H*$_2$CH$_2$N$_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 167.59 (*C*OCH$_2$*C*O), 49.10, 43.12, 37.07, 28.58 (NHCH$_2$*C*H$_2$CH$_2$N$_3$)

MS(ESI) m/z = 291.57 $[M+ Na]^+$ (Calcd = 291,27).

N^1,N^3-bis(3-azidopropyl)-2-bromomalondiamide (31a)



N^1,N^3-bis(3-azidopropyl)malondiamide (**31**) (50mg, 0.186mmol, 1eq.) was dissolved in ethyl acetate (2.5ml). Four aliquots (3.25 µL each one: 13 µL, 0.25mmol, 1.35eq.) of Br_2 was added (at 4 minutes intervals). The orange colour almost completely disappearing each time and after the last aliquot yellow colour remained. After completing the addiction, TEA (35 µL, 0.25mmol, 1.35eq) was quickly added with large amount of salt precipitation.

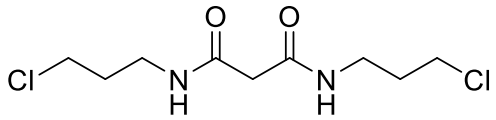
The mixture was filtered to remove the precipitate. The reaction mixture was monitored by TLC (ethyl acetate 100%. Rf=0.67). Column chromatography (SiO_2, ethyl acetate: yclohexane 1:1) gave the desired product (40mg, 62%) as a yellow solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.43 (t, 2H, J= 6.6 Hz, N*H*CO), 4.73 (s,1H, COCHBrCO),3.45-3.38 (m, 8H, NHC*H*$_2$CH$_2$CH$_2$N$_3$, NHCH$_2$CH$_2$C*H*$_2$N$_3$),1.85 (q, 4H, J= 6.6 Hz, NHCH$_2$C*H*$_2$CH$_2$N$_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 165.49 (*C*OCH$_2$*C*O), 49.06, 44.43, 37.91, 28.29 (NHCH$_2$*C*H$_2$CH$_2$N$_3$)

MS(ESI) m/z = 371.53 $[M+ Na]^+$ (Calcd = 370.17).

N^1,N^3-bis(3-chloropropyl)malondiamide (32)



3-chloropropylamine hydrochloride (100mg, 0.769mmol, 1eq.) was dissolved in chloroform (1ml) and was added 0.25 ml of a solution of NaOH (2g) in H_2O (4ml). The mixture was cooled to 0°C and a solution of malonyl chloride (37.4 µL, 0.384 mmol, 0.5eq.) in chloroform (0.5 ml) was added dropwise, then was stirred for 20 min at 0°C.

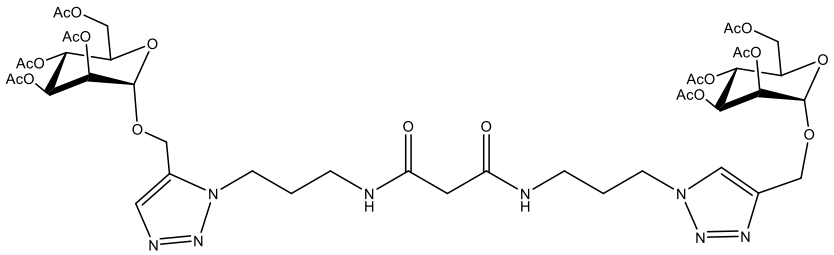
The mixture was extracted with chloroform, the organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo.* The reaction mixture was analysed by TLC (dichloromethane: MeOH 9:1, Rf=0.63). Column chromatography (SiO_2, ethyl acetate: cyclohexane 8:2) gave the desired product (36 mg, 36%) as a white powder.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.51(t, 2H, J=6.4Hz,N*H*CO), 3.59 (t, 4H, J=6.4 Hz, $NHCH_2CH_2CH_2Cl$), 3.43 (q, 4H, J= 6.4Hz, $NHCH_2CH_2CH_2Cl$) , 3.22 (s,2H, $COCH_2CO$), 2.01 (q, 4H, J= 6.5 Hz, $NHCH_2CH_2CH_2Cl$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 167.71 ($COCH_2CO$), 43.17, 42.22, 37.02, 31.92 ($NHCH_2CH_2CH_2Cl$)

MS(ESI) m/z = 277.48 $[M+ Na]^+$ (Calcd = 278.13).

Man malondiamide chain (33)



Propargyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**15**) (86.38 mg,0.224mmol, 3eq.) was dissolved in THF (8ml) and H_2O (8ml). N^1,N^3-bis(3-azidopropyl)malondiamide (**31**) (20 mg, 0.0746mmol, 1eq.) then copper sulphate pentahydrate (186.2mg, 0.746mmol, 10eq.) and sodium

ascorbate (252mg, 1.268mmol, 17eq.) were added and the mixture was stirred overnight at r.t. The reaction was extracted with dichloromethane, and the organic layer was washed with saturated $NaHCO_3$ solution, then dried over $MgSO_4$, filtered and concentrated *in vacuo.*

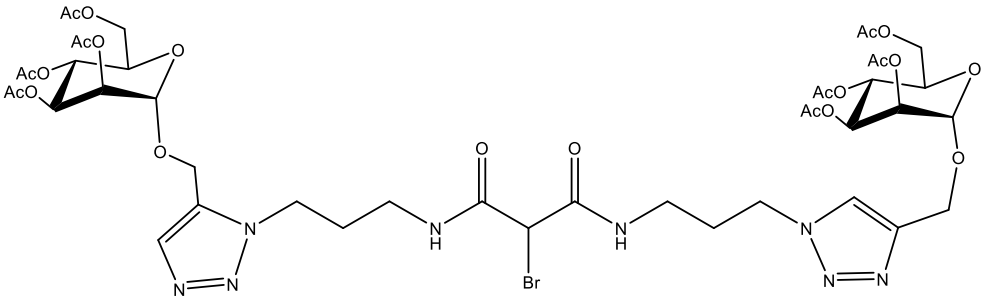
The reaction mixture was analysed by TLC (dichloromethane/MeOH 95:5, Rf= 0.31). Column chromatography (SiO_2, dichloromethane/MeOH 95:5) gave the desired product (62mg, 80%) as white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.74 (s, 2H, NHCO), 7.18 (s, 2H, NCH=C), 5.33 (m, 4H, H-3, H-4), 5.25 (s,2H, H-2), 4.99 (s, 2H, H-1), 4.79 (dd, 4H, J= 11.5 Hz, 63.1 Hz, triazole-C*H*$_2$O), 4.47 (t, 4H, J= 5.6 Hz, NHCH$_2$CH$_2$C*H*$_2$-triazole), 4.31 (dd, 2H, J=4.9 Hz, 12.2 Hz, H-6a), 4.17-4.06 (m, 4H, H-6b, C-5), 3.35 (s, 4H, NHC*H*$_2$CH$_2$CH$_2$-triazole), 3.16 (s, 2H, COC*H*$_2$CO), 2.25-2.18 (m, 4H, NHCH$_2$C*H*$_2$CH$_2$-triazole), 2.17, 2.14, 2.06, 2.00 (4s, 24H, $COCH_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 170.73, 170.10, 169.69 (8 x CH_3*C*OO), 167.80 (*C*OCH2*C*O), 143.52(Cq), 123.66 (N*C*H=C), 96.86 (C-1), 69.40 (C-4), 69.16(C-2), 68.72 (C-5), 66.02 (C-3), 62.40 (C-6a, C-6b), 60.93 (triazole-*C*H$_2$O), 47.92 (NHCH$_2$CH$_2$*C*H$_2$-triazole), 42.84 (CO*C*H$_2$CO), 36.51 (NH*C*H$_2$CH$_2$CH$_2$-triazole), 29.96 (NHCH$_2$*C*H$_2$CH$_2$-triazole), 20.87, 20.78, 20.69 (8x *C*H$_3$COO).

HRMS: m/z = 1063.3716 [M+ Na]$^+$(calculated 1063.3714) err[ppm]=0.2.

Brominated malondiamide-Man chain (33a)



Man malondiamide chain (**33**) (48 mg, 0.046mmol, 1eq.) was dissolved in ethyl acetate (2ml). Four aliquots (0.79 µL each one: 3.19 µL, 0.0621mmol, 1.35eq.) of Br_2 was added (at 4 minutes intervals). The orange colour almost completely disappearing each time and after the last aliquot a yellow colour remained.

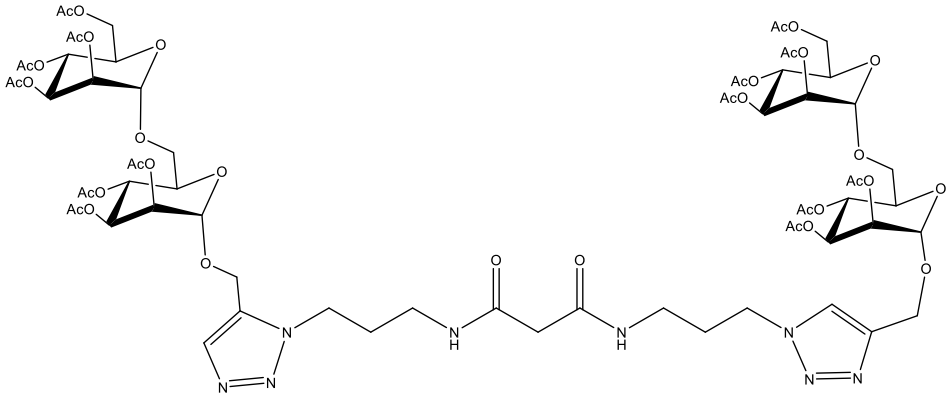
After completing the addiction of Br_2, TEA (8.63 µL, 0.0621mmol, 1.35eq) was quickly add ed with large amount of salt precipitation and return to a colourless mixture. The mixture was filtered to remove the precipitate.

Column chromatography (SiO_2, ethyl acetate: MeOH 9:1, Rf=0.63)gave the desired product (19mg, 37%) as a yellow solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.71 (d, 2H, J= 5.2 Hz,N*H*CO), 7.31 (s, 2H, NCH=C) 5.35-5.31 (m, 4H, H-3, H-4), 5.25 (s,2H, H-2), 4.98 (d, 2H, J=1.8 Hz,ff H-1), 4.79 (dd, 4H, J= 11.5 Hz, 63.1 Hz, triazole-C*H*$_2$O), 4.67(s, 1H, COCHBrCO) 4.47 (t, 4H, J= 5.6 Hz, NHCH$_2$CH$_2$C*H*$_2$-triazole), 4.31 (dd, 2H, J=4.9 Hz, 12.2 Hz, H-6a), 4.17-4.06 (m, 4H, H-6b, C-5), 3.35 (m, 4H, NHC*H*$_2$CH$_2$CH$_2$-triazole), 2.28-2.21 (m, 4H, NHCH$_2$C*H*$_2$CH$_2$-triazole), 2.17, 2.14, 2.06, 2.00 (4s, 24H, $COCH_3$).

MS(ESI) m/z = 1143.1912 $[M+ Na]^+$ (Calcd = 1142.87).

Man1,6Man malondiamide chain (34)



Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-d-mannopyranoside (**2**) (37.6 mg, 0.0558 mmol, 3eq.) was dissolved in THF (2.5 ml) and H_2O (2.5 ml). N^1,N^3-bis(3-azidopropyl) malondiamide (**31**) (5mg, 0.0186 mmol, 1eq.) then copper sulphate pentahydrate (46.4 mg, 0.186 mmol, 10eq.) and sodium ascorbate (63 mg, 0.316 mmol, 17eq.) were added and the mixture was stirred overnight at r.t. The reaction was extracted with dichloromethane, and the organic layer was washed with saturated $NaHCO_3$ solution, then dried over $MgSO_4$, filtered and concentrated *in vacuo.* The reaction mixture was analysed by TLC (dichloromethane/MeOH 95:5. Rf=0.28). Column chromatography (SiO_2, dichloromethane/MeOH 95:5) gave the desired product (25 mg, 83%) as white solid.

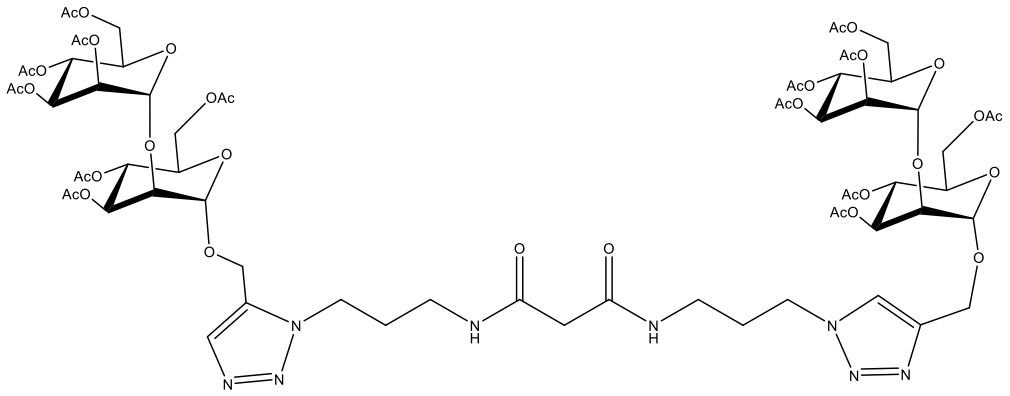
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.74 (s, 2H, NCH=C), 7.23 (s, 2H, NHCO), 5.29-5.15 (m, 12H, H-2, H-2', H-3, H-3', H-4, H4'), 4.87 (s, 2H, H-1), 4.83(d, 2H, J= 1.7Hz, H-1'), 4.69 (d, 4H,J= 94.7 Hz, triazole-C*H*$_2$O), 4.39 (s, 4H, NHCH$_2$CH$_2$C*H*$_2$-triazole), 4.21 (dd, 2H, J= 5.3 Hz, 12.2 Hz, H-6'a), 4.08 (dd, 4H, J= 2.4 Hz, 12.2 Hz, H-6'b), 4.04 (ddd, 2H, J= 2.4 Hz, 5.2 Hz, 9.7 Hz, H-5'), 4.01-3.96 (m, 2H, H-5), 3.74

(dd,2H, J= 5.3 Hz, 11.0 Hz, H-6a), 3.54 (dd, 2H, J= 2.2 Hz, 11.1 Hz, H-6b), 3.25 (s, 4H, NHC*H*$_2$CH$_2$CH$_2$-triazole), 3.07 (s, 2H, COC*H*$_2$CO), 2.16-2.10 (m, 4H, NHCH$_2$C*H*$_2$CH$_2$-triazole), 2.09, 2.08, 2.04, 1.98, 1.97, 1.91, 1.90 (7s, 42H, COCH$_3$).

^{13}C-NMR (100 MHz, CDCl$_3$): δ = 170.65, 170.21, 170.02. 170.02, 169.81, 169.77, 169.77 (14C x CH$_3$*C*OO), (14 C x CH$_3$*C*OO), 167.69, (*C*OCH2*C*O), 97.56 (C-1'), 96.60 (C-1), 69.56, 69.42, 69.36, 69.19, 68.74, 66.62, 66.52 (C-6a, C-6b), 65.98, 62.49 (C-6'a, C-6'b), 60.67 (triazole-*C*H$_2$O), 47.99 (NHCH$_2$CH$_2$*C*H$_2$-triazole), 42.66 (CO*C*H$_2$CO), 36.68 (NH*C*H$_2$CH$_2$CH$_2$-triazole), 29.74 (NHCH$_2$*C*H$_2$CH$_2$-triazole), 20.86, 20.77, 20.74, 20.71, 20.68, 20.65 (14 x *C*H$_3$COO).

HRMS: m/z = 1639.5405 [M+ Na]$^+$(calculated 1639.5405) err[ppm]=0.0.

Man1,2Man malondiamide chain (35)



Propargyl 2',3',4',6'-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranoside (**4**) (71 mg, 0.105 mmol, 3eq) was dissolved in THF (3 ml) and H$_2$O (3 ml).

N^1,N^3-bis(3-azidopropyl) malondiamide (**31**) (9.4 mg, 0.0351 mmol, 1eq.) then copper sulphate pentahydrate (118.8 mg, 0.351 mmol, 10eq.) and sodium ascorbate (87.64 mg, 0.596 mmol, 17eq.) were added and the mixture was stirred overnight at r.t. The reaction was extracted with dichloromethane, and the organic layer was washed with saturated NaHCO$_3$ solution, then dried over MgSO$_4$, filtered and concentrated *in vacuo.*

The reaction mixture was monitored by TLC (dichloromethane/MeOH 95:5, Rf=0.28). Column chromatography (SiO$_2$, dichloromethane /MeOH 95:5) gave the desired product (43.7 mg, 77%) as white solid.

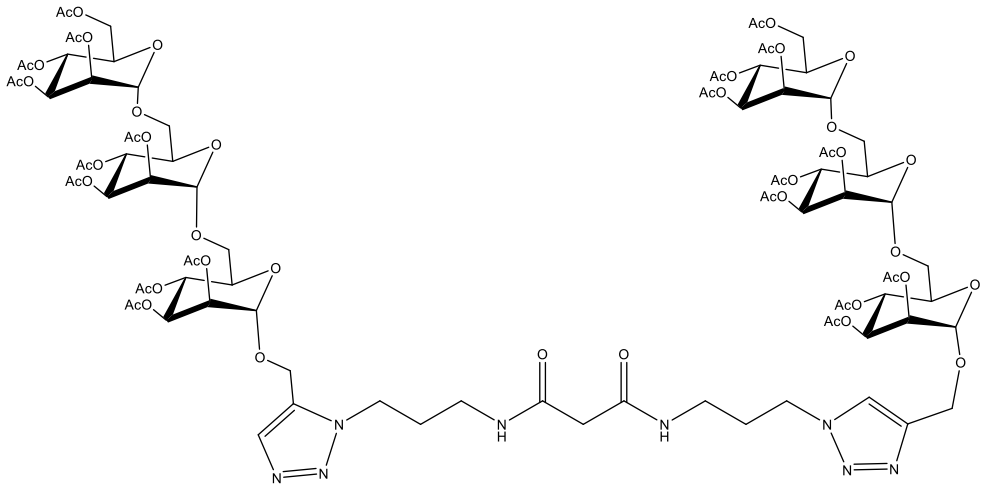
^{1}H-NMR (400 MHz, CDCl$_3$): δ = 7.75 (s, 2H, NCH=C), 7.37 (s, 2H, NHCO), 5.43-5.23 (m, 10H, H-2', H-3, H-3', H-4, H4'), 5.14 (s, 2H, H-1), 4.92 (s, 2H, H-1'), 4.83 (s, 2H, triazole- C*H*$_2$O), 4.68 (s, 2H,

triazole- C*H*$_2$O), 4.46 (s, 4H, NHCH$_2$CH$_2$C*H*$_2$-triazole), 4.27-3.96 (m,14H, H-6a, H-6b, H-6'a, H-6'b, H-5, H5'b, H-2), 3.32 (s, 4H, NHC*H*$_2$CH$_2$CH$_2$-triazole), 3.18 (s, 2H, COC*H*$_2$CO), 2.23-2.16 (m, 4H, NHCH$_2$C*H*$_2$CH$_2$-triazole), 2.15, 2.14, 2.10, 2.07, 2.05, 2.02, 2.01 (7s, 42H, COCH$_3$).

^{13}C-NMR (100 MHz, CDCl$_3$): δ = 170.94, 170.64, 170.45, 169.82, 169.68, 169.43, 169.33 (14 x CH$_3$*C*OO), 167.72, (*C*OCH2*C*O), 99.12 (C-1'), 97.76 (C-1), 70.20, 69.73, 69.14, 68.76, 68.38, 66.34, 66.18, 62.42, 62.10, 60.95 (triazole-*C*H$_2$O), 53.43, 47.80 (NHCH$_2$CH$_2$*C*H$_2$-triazole), 42.61 (CO*C*H$_2$CO), 36.56 (NH*C*H$_2$CH$_2$CH$_2$-triazole), 29.88 (NHCH$_2$*C*H$_2$CH$_2$-triazole), 20.84, 20.77, 20.70, 20.68, 20.66, 20.64, 20.62(14 x *C*H$_3$COO).

HRMS: m/z = 1639.5405 [M+ Na]$^+$(calculated 1639.5405) err[ppm]=0.1.

Man1,6Man1,6Man malondiamide chain (36)



Propargyl 2",3",4",6"-*Tetra*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2',3',4'-*tri*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranoside (**6**) (32 mg, 0.0336 mmol, 3eq.) was dissolved in THF (2 ml) and H$_2$O (2 ml). N^1,N^3-bis(3-azidopropyl) malondiamide (**31**) (3 mg, 0.0112 mmol, 1eq.) then copper sulphate pentahydrate (27.9 mg, 0.112 mmol, 10eq.) and sodium ascorbate (37.9 mg, 0.190 mmol, 17eq.) were added and the mixture was stirred overnight at r.t.. The reaction was extracted with dichloromethane, and the organic layer was washed with saturated NaHCO$_3$ solution, then dried over MgSO$_4$, filtered and concentrated *in vacuo.* The reaction mixture was monitored by TLC (dichloromethane /MeOH 95:5, Rf=0.30). Column chromatography (SiO$_2$, dichloromethane/MeOH 95:5) gave the desired product (18.5 mg, 75%) as white solid.

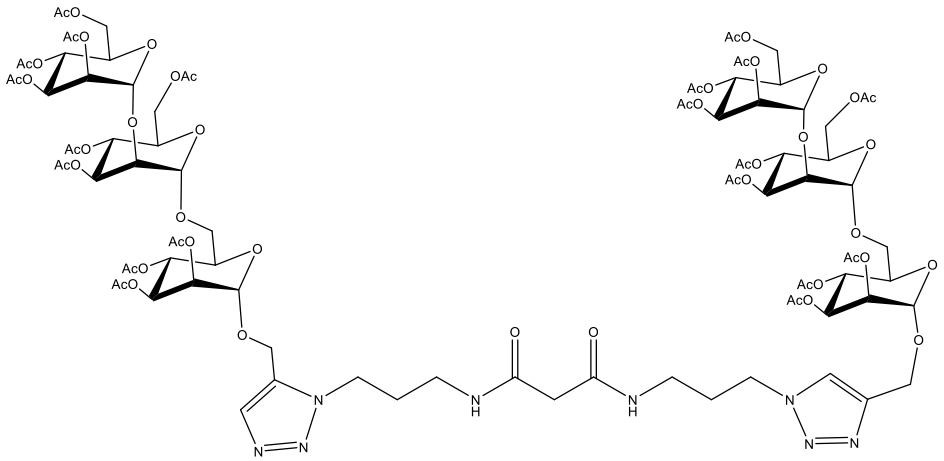
^{1}H-NMR (400 MHz, CDCl$_3$): δ = 7.70 (s, 2H, NCH=C), 7.18 (s, 2H, NHCO), 5.32-5.19 (m, 16H, H-2', H-2'', H-3, H-3', H-3'', H-4, H-4', H-4''), 5.16 (s, 2H, H-2), 4.88 (s, 2H, H-1), 4.81 (s, 4H, H-1', H-1''), 4.77

(s, 2H, triazole- CH_2O), 4.62 (s, 2H, triazole- CH_2O), 4.38 (t, 4H, J=4.3 Hz, NHCH$_2$CH$_2$CH_2-triazole), 4.21 (dd, 2H, J= 3.6 Hz, 8.3 Hz), 4.06 (dd, 2H, J=1.5 Hz, 8.3 Hz), 4.02-3.93 (m, 6H, H-5, H-5', H-5''), 3.77 (dd, 2H, J= 3.3Hz, 7.5 Hz), 3.73(dd, 2H, J= 3.3Hz, 7.5 Hz), 3.56-3.50 (m, 4H), 3.31-3.19 (m, 4H, NHCH_2CH$_2$CH$_2$-triazole), 3.06 (s, 2H, COCH_2CO), 2.15-2.11 (m, 4H, NHCH$_2$CH_2CH$_2$-triazole), 2.10, 2.09, 2.08, 2.05, 1.99, 1.99, 1.98, 1.91, 1.90, 1.90 (10s, 60H, COCH$_3$).

^{13}C-NMR (150 MHz, CDCl$_3$): δ =170.63, 170.36, 170.24, 169.07, 169.94, 169.84, 169.79, 169.76, 169.73, 169.62 (20 x CH$_3$*C*OO), 167.72 (*C*OCH2*C*O), 97.76, 97.65 (C-1', C.1''), 96.54 (C-1), 69.68, 69.48, 69.41, 69.39, 69.27, 69.24, 69.08, 68.66, 66.58, 66.49, 66.38, 66.21, 65.95, 62.43, 60.74 (triazole-*C*H$_2$O), 47.80 (NHCH$_2$CH$_2$*C*H$_2$-triazole), 42.67 (CO*C*H$_2$CO), 36.64 (NH*C*H$_2$CH$_2$CH$_2$-triazole), 29.76, 29.68 (NHCH$_2$*C*H$_2$CH$_2$-triazole), 20.86, 20.79, 20.76, 20.73, 20.72, 20.69, 20.64 (20 x *C*H$_3$COO).

HRMS: m/z = 2216.7143 [M+ Na]$^+$(calculated 2216.7128) err[ppm]=1.4.

Man1,2Man1,6Man malondiamide chain (37)



Propargyl 2",3",4",6"-*Tetra*-*O*-acetyl- α-D-mannopyranosyl-(1→2)-3',4',6'-*tri*-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-*tri*-*O*-acetyl-α-D-mannopyranoside (**8**) (13.3 mg, 0.0138 mmol, 3eq.) was dissolved in THF (1 ml) and H_2O (1 ml). N^1,N^3-bis(3-azidopropyl) malondiamide (**31**) (1.23 mg, 0.0046 mmol, 1eq.) then copper sulphate pentahydrate (11.48 mg, 0.112 mmol, 10eq.) and sodium ascorbate (15.5 mg, 0.234 mmol, 17eq.) were added and the mixture was stirred overnight at r.t..

The reaction was extracted with dichloromethane, and the organic layer was washed with saturated $NaHCO_3$ solution, then dried over $MgSO_4$, filtered and concentrated *in vacuo.* The

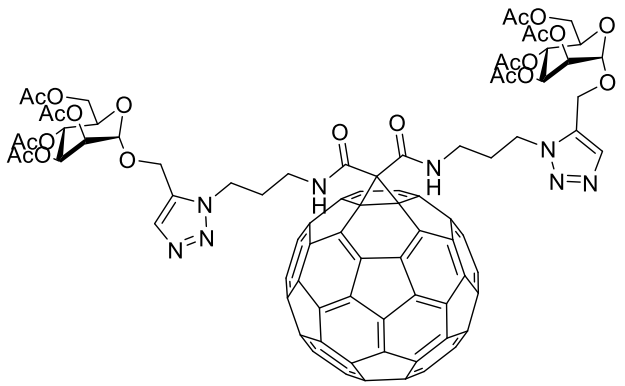
reaction mixture was analysed by TLC (dichloromethane/MeOH 95:5, Rf=0.30). Column chromatography (SiO_2, dichloromethane/MeOH 95:5) gave the desired product (6.5 mg, 65%) as white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.80 (s, 2H, NCH=C), 7.50 (s, 2H, NHCO), 5.39-5.12 (m, 16H), 4.94 (s, 2H, H-1), 4.87 (s, 4H, H-1', H-1''), 4.79 (s, 2H, triazole- CH_2O), 4.60 (s, 2H, triazole- CH_2O), 4.42 (s, 4H, J=4.3 Hz, NHCH$_2$CH$_2$CH_2-triazole), 4.19-4.02 (m, 12H), 3.98 (d, 4H, J= 10.3 Hz), 3.77(dd, 2H, J= 5.1Hz, 10.6 Hz), 3.53 (d, 2H, J=10.7 Hz), 3.26 (s, 4H, NHCH_2CH$_2$CH$_2$-triazole), 3.13 (s, 2H, COCH_2CO), 2.14 (s, 4H, NHCH$_2$CH_2CH$_2$-triazole), 2.08, 2.08, 2.07, 2.03, 1.99, 1.99, 1.98, 1.94, 1.94, 1.91 (10s, 60H, $COCH_3$).

^{13}C-NMR (150 MHz, $CDCl_3$): δ =170.93, 170.53, 170.34, 169.13, 170.05, 169.89, 169.72, 169.72,169.46, 169.46 (20C, CH_3*C*OO), 168.01 (*C*OCH2*C*O), 99.25, 98.23 (C-1', C.1''), 96.69 (C-1), 70.27, 69.81, 69.59, 69.33, 69.20, 68.75, 68.45, 66.85, 66.50, 66.37, 65.90, 62.47, 62.21, 53.44 (triazole-*C*H$_2$O), 48.29 (NHCH$_2$CH$_2$*C*H$_2$-triazole), 42.31 (CO*C*H$_2$CO), 36.62 (NH*C*H$_2$CH$_2$CH$_2$-triazole), 29.66 (NHCH$_2$*C*H$_2$CH$_2$-triazole), 20.87, 20.78, 20.74, 20.74, 20.74, 20.69, 20.69, 20.69, 20.67, 20.63 (20C, *C*H$_3$COO).

HRMS: m/z = 2216.7147 [M+ Na]$^+$(calculated 2216.7128) err[ppm]=1.4.

Fullerene-Man (38)



C_{60} (7 mg, 0.0096mmol, 1eq.), compound **33** (10mg, 0.0096mmol, 1eq.) and I_2 (3mg, 0.0117mmol, 1.22 eq.) were dissolved in dry toluene (4.5ml). DBU (3 µL, 0.0216mmol, 2.25 eq.) was added to the mixture under argon at 0°C and the reaction was stirred at R. T. for 45 min. The reaction mixture was monitored by TLC (dichloromethane/Acetone 1:1. Rf=0.77). The solvent was removed

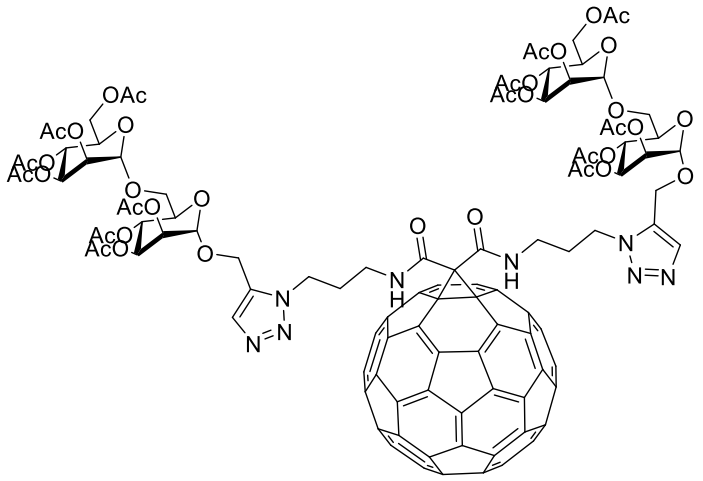
under reduced pressure and a column chromatography (SiO_2, dichloromethane/Acetone 1:1) gave the desired product (7mg, 41%) as a black-brown solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 9.00 (s, 2H, NHCO), 7.85 (s, 2H, NCH=C), 5.39-5.30 (m, 4H, H-3, H-4), 5.27 (s, 2H, H-2), 4.98 (s, 2H, H-1), 4.89- 4.61 (m, 8H, J= 11.5 Hz, triazole-C*H*$_2$O, NHCH$_2$CH$_2$C*H*$_2$-triazole), 4.28 (m, 4H, J= 5.6 Hz, H-6a, H-6b), 4.10 (s, 2H H-5), 3.54 (s, 4H, NHC*H*$_2$CH$_2$CH$_2$-triazole) 2.55-2.35 (m, 4H, NHCH$_2$C*H*$_2$CH$_2$-triazole), 2.17, 2.14, 2.06, 2.00 (4s, 24H, $COCH_3$).

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 170.31, 170.27, 169.68 (8C x CH_3*C*OO), 163.83 (*C*OCH2*C*O), 146.18-136.56 (C- C_{60}), 123.69 (N*C*H=C), 96.66 (C-1), 69.51 (C-4), 69.22(C-2), 69.00 (C-5), 65.98 (C-3), 62.43 (C-6a, C-6b), 60.96 (triazole-*C*H$_2$O), 53.86, 47.81 (NHCH$_2$CH$_2$*C*H$_2$-triazole), 37.23 (NH*C*H$_2$CH$_2$CH$_2$-triazole), 31.94, 31,76 ,29.71(NHCH$_2$*C*H$_2$CH$_2$-triazole), 29.32, 29.16, 20.91, 20.87, 20.74, 20.74 (8x *C*H$_3$COO).

HRMS: m/z = 1782.3555 $[M+ Na]^+$ (calculated 1782.3590) err[ppm]=1.8.

Fullerene-Man1,6Man (39)



C_{60} (6.24 mg, 0.00866 mmol, 1eq.), Compound **34** (14 mg, 0.00866 mmol, 1eq.) and I_2(2.68 mg, 0.0105 mmol, 1.22 eq.) were dissolved in dry toluene (4.5ml). DBU (2.9 µL, 0.0195 mmol, 2.25 eq.) was added to the solution under argon at 0°C and the reaction was stirred at 0°C for 1 hour. The reaction mixture was monitored by TLC (dichloromethane/Acetone 6:4. Rf=0.75). The solvent was removed under reduced pressure and a column chromatography (SiO_2, dichloromethane/Acetone 6:4) gave the desired product (6 mg, 30 %) as a black-brown solid.

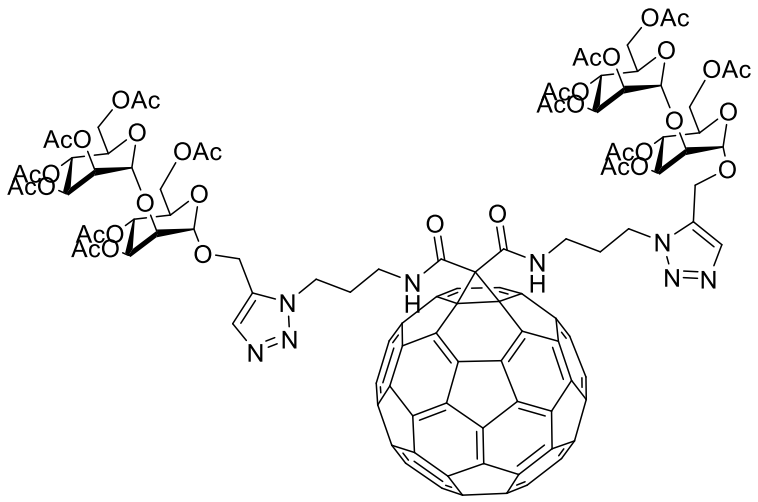
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.88 (s, 2H, NHCO), 7.22 (s, 2H, NCH=C), 5.29-5.15 (m, 12 H, H-2, H-2', H-3, H-3', H-4, H-4'), 4.86 (s, 2H, H-1), 4.81 (d, 2H, J= 2.7 Hz, H-1'), 4.79-4.61 (m, 4H, triazole-

C*H*$_2$O), 4.50 (t, 4H, J= 6.6 Hz, NHCH$_2$CH$_2$C*H*$_2$-triazole), 4.20 (dd, 2H, J= 5.3 Hz, 12.2 Hz, H-6'a), 4.09 (dd, 2H, J= 2.4 Hz, 12.3 Hz, H-6'b), 4.06-4.03 (m, 2H, H-5'), 4.00-3.96 (m, 2H, H-5), 3.74 (dd, 2H, J= 5.8 Hz, 11.1 Hz, H-6a), 3.53 (dd, 2H, J= 2.6 Hz, 11.1 Hz, H-6b), 3.50 (t, 4H, J= 6.7 Hz, NHC*H*$_2$CH$_2$CH$_2$-triazole), 2.34-2.24 (m, 4H, NHCH$_2$C*H*$_2$CH$_2$-triazole) 2.08, 2.04, 1.97, 1.90 (5s, 42H, COCH$_3$).

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 170.70, 170.34, 170.09, 170.02, 169.78 (8C x CH$_3$*C*OO), 163.48 (*C*OCH2*C*O), 145.34-137.77 (C- C$_{60}$), 123.70 (N*C*H=C), 97.53 (C-1'), 96.45 (C-1), 73.79, 69.60, 69.52, 69.39, 69.22, 69.17, 68.75, 66.63, 66.55 (C-6a, C-6b), 65.99, 62.51 (C-6'a, C-6'b), 60.65 (triazole-*C*H$_2$O), 53.87, 47.57 (NHCH$_2$CH$_2$*C*H$_2$-triazole), 37.60 (NH*C*H$_2$CH$_2$CH$_2$-triazole), 33.41, 31.92, 29.76 (NHCH$_2$*C*H$_2$CH$_2$-triazole), 29.65-29.24 (C$_{60}$), 20.90, 20.83, 20.77, 20.73, 20.70, (14 x *C*H$_3$COO).

HRMS: m/z = 2358.5283 $[M+ Na]^+$(calculated 2358.5281) err[ppm]=1.0.

Fullerene-Man1,2Man (40)



C$_{60}$ (4.4 mg, 0.00618 mmol, 1eq.), Compound **35** (10 mg, 0.00618 mmol, 1eq.) and I_2 (1.9 mg, 0.00754 mmol, 1.22 eq.) were dissolved in dry toluene (4 ml). DBU (2.1 µL, 0.0139 mmol, 2.25 eq.) was added to the solution under argon at 0°C and the reaction was stirred at r.t. for 1.5 hours. The reaction mixture was monitored by TLC (dichloromethane/Acetone 6:4. Rf=0.73). The solvent was removed under reduced pressure and a column chromatography (SiO_2, dichloromethane/Acetone 6:4) gave the desired product (1.4 mg, 10 %) as a black-brown solid.

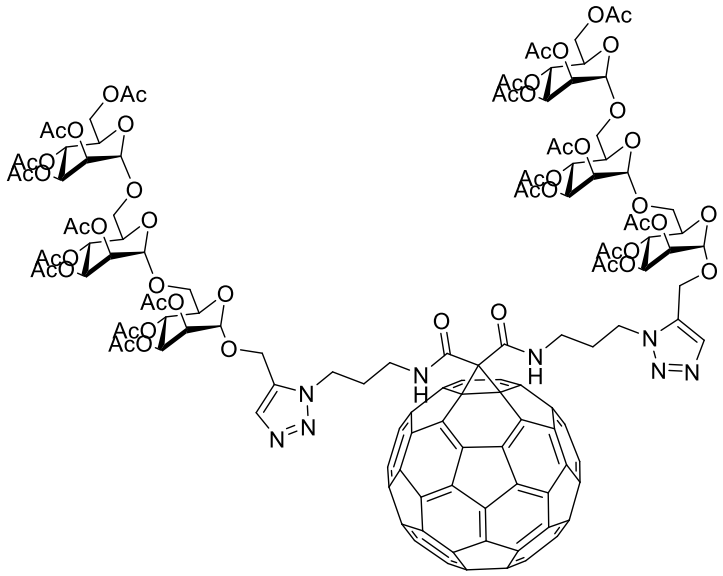
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 8.00 (s, 2H, NCH=C), 7.74 (s, 2H, NHCO), 5.44-5.25 (m, 10H, H-2', H-3, H-3', H-4, H4'), 5.15 (s, 2H, H-1), 4.94 (d, 2H, H-1'), 4.87 (d, 2H, triazole- C*H*$_2$O), 4.73 (d, 2H, triazole- C*H*$_2$O), 4.56 (m, 4H, NHCH$_2$CH$_2$C*H*$_2$-triazole), 4.27-3.99 (m,14H, H-6a, H-6b, H-6'a, H-6'b,

H-5, H5'b, H-2), 3.61 (m, 4H, NHC*H*$_2$CH$_2$CH$_2$-triazole), 2.38-2.35 (m, 4H, NHCH$_2$C*H*$_2$CH$_2$-triazole), 2.18, 2.17, 2.13, 2.09, 2.07, 2.04, 2.03 (7s, 42H, COCH$_3$).

^{13}C-NMR (150 MHz, CDCl$_3$): δ = 170.95, 169.75 (CH$_3$*C*OO), 145.34-137.77 (C- C$_{60}$), 70.29, 69.68, 66.55, 66.19, 62.75, 62.30, 31.92, 29.76 (NHCH$_2$*C*H$_2$CH$_2$-triazole), 29.65-29.24 (C-C$_{60}$), 20.91- 20.72 (*C*H$_3$COO).

HRMS: m/z = 2358.5225 [M+ Na]$^+$ (calculated 2358.5281) err[ppm]=3.2.

Fullerene-Man1,6Man1,6Man (41)



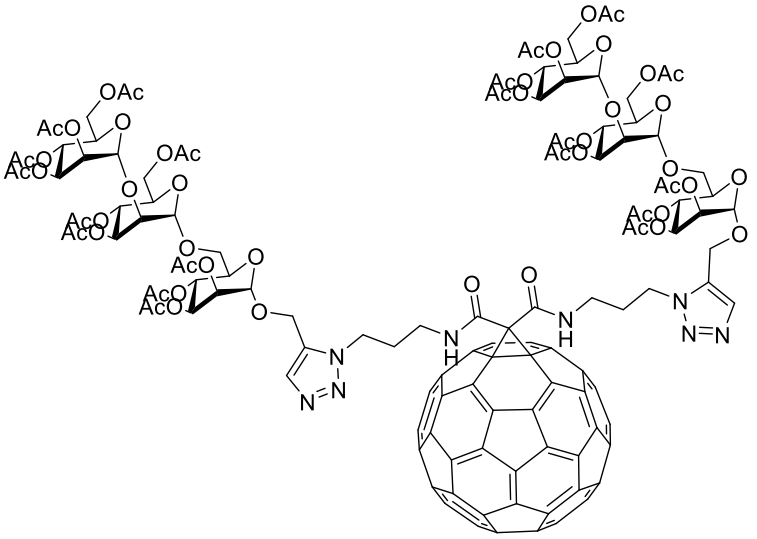
C$_{60}$ (5 mg, 0.007 mmol, 1eq.), Compound **36** (15.5 mg, 0.007 mmol, 1eq.) and I$_2$ (2.18 mg, 0.00854 mmol, 1.22 eq.) were dissolved in dry toluene (4 ml). DBU (2.4 µL, 0.0157 mmol, 2.25 eq.) was added to the mixture under argon at 0°C and the reaction was stirred at r.t. for 1.5 hours. The reaction mixture was monitored by TLC (dichloromethane/Acetone 6:4, Rf=0.74). The solvent was removed and a column chromatography (SiO$_2$, dichloromethane/Acetone 6:4) gave the desired product (3 mg, 10 %) as a black-brown solid.

^{1}H-NMR (400 MHz, CDCl$_3$): δ = 8.50 (s, 2H, NCH=C), 5.50-5.21 (m, 18H), 5.07-4.56 (m, 12H), 4.30 (dd, 4H J= 5.2 Hz, 12.3 Hz), 4.21-3.96 (m, 10H), 3.83 (ddd, 4H, J= 6.2 Hz, 11.2 Hz, 15.9 Hz), 3.61 (d, 6H, J= 10.5 Hz), 2.47-2.34 (m, 4H, NHC*H*$_2$CH$_2$CH$_2$-triazole), 2.18, 2.17, 2.16, 2.13, 2.08, 2.08, 2.07, 1.99, 1.99, 1.99 (10s, 60H, COCH$_3$).

^{13}C-NMR (150 MHz, CDCl$_3$): δ =170.65, 170.31, 170.11, 169.99, 169.82, 169.79, 169.78, 169.66 (20 C, CH$_3$*C*OO), 163.43 (*C*OCH2*C*O), 145.62-137,67 (C- C$_{60}$) (97.81, 97.66 (C-1', C.1''), 96.67 (C-1), 69.82, 69.53, 69.44, 69.40, 69.36, 69.35, 69.30, 69.10, 68.68, 66.51, 66.32, 66.20, 65.96, 62.43,

60.30 (triazole-*C*H_2O), 53.87, 47.74 (NHCH$_2$CH$_2$*C*H$_2$-triazole), 37.60 (NH*C*H$_2$CH$_2$CH$_2$-triazole), 32.52, 31.93, 29.76 (NHCH$_2$*C*H$_2$CH$_2$-triazole), 29.24 (C_{60}), 20.69, 20.89, 20.84, 20.78, 20.68 (30C, *C*H$_3$COO). HRMS: m/z = 2934.6875 $[M+ Na]^+$(calculated 2934.6971) err[ppm]=1.9.

Fullerene-Man1,2Man1,6Man (42)



C_{60} (4.5 mg, 0.0063 mmol, 0.7eq.), Compound **37** (20 mg, 0.0091 mmol, 1eq.) and I_2 (2.8 mg, 0.011 mmol, 1.22 eq.) were dissolved in dry toluene (4 ml). DBU (3 µL, 0.02 mmol, 2.25 eq.) was added to the mixture under argon at 0°C and the reaction was stirred at r.t. for 2 hours.

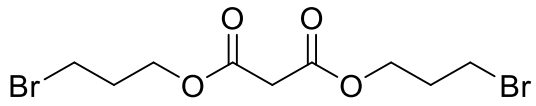
The reaction mixture was monitored by TLC (dichloromethane/Acetone 6:4. Rf=0.74). The solvent was removed and a column chromatography (SiO_2, dichloromethane/Acetone 6:4) gave the desired product (1.6 mg, 8%) as a black-brown solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 8.44 (s, 2H, NCH=C), 5.50-5.21 (m, 18H), 5.52 (s, 4H), 4.96 (s,2H), 4.87 (s, 4H), 4.30-4.00 (m, 18H), 3.87 (s, 4H), 3.77-3.53 (m, 6H), 3.61 (d, J= 10.5 Hz), 2.35-2.28 (m, 4H, NHC*H*$_2$CH$_2$CH$_2$-triazole), 2.18, 2.18, 2.18, 2.08, 2.08, 2.07, 2.06, 2.03, 2.03, 2.00 (10s, 60H, $COCH_3$).

HRMS: m/z = 2934.6972 $[M+ Na]^+$ (calculated 2934.6971) err[ppm]=0.9

b) Synthesis of malonic esters derivatives and conjugation with C_{60}

Bis(3-bromopropyl) malonate (43)



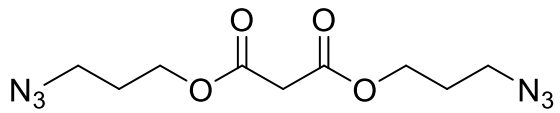
Bis(3-bromopropyl) malonate (**43**) was synthesized as previously reported[249].

Malonyl dichloride (105 µL, 1.079mmol, 0.5eq.) was added to a solution of 3-bromopropan-1-ol (300mg, 2.158mmol, 1eq.) and pyridine (174.36 µL, 2.158mmol, 1 eq.) dissolved in 15ml of dichloromethane at 0°C under argon atmosphere. After 1h, the solution was allowed to warm to r.t. and stirred for 18h and the solvent was evaporated under reduced pressure.

The reaction mixture was monitored by TLC (dichloromethane/Cyclohexane 8:2. Rf=0.60).

Column chromatography (SiO_2, dichloromethane/Cyclohexane 8:2) gave the desired product (420mg, 79%) as a yellow oil. NMR was in agreement with previously reported data[249].

Bis(3-azidopropyl) malonate (44)

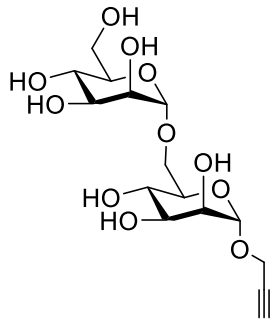


Bis(3-azidopropyl) malonate (**44**) was synthesized as previously reported[249].

NaN_3 (314 mg, 4.84mmol. 4eq.) was added to a solution of bis(3-bromopropyl) malonate (420mg, 1.21mmol, 1 eq.) in dry DMF (8ml) and the mixture was stirred at r.t. for 16h under argon atmosphere. The mixture was diluted with Et_2O, washed with water and brine, dried, filtered and and concentrated *in vacuo.* The reaction mixture was monitored by TLC (dichloromethane 100% or Cyclohexane/ethyl acetate 7:3, Rf=0.57). Column chromatography (SiO_2, dichloromethane 100%) gave the desired product (290mg, 90%) as colourless oil.

NMR was in agreement with previously reported data[249].

Propargyl α-D-mannopyranosyl-(1→6) -α-D-mannopyranoside (45)



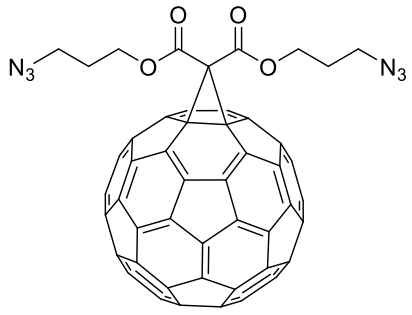
Propargyl 2′,3′,4′,6′-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (**2**) (58mg, 0.086 mmol) was stirred 20min in 1ml solution of Na/dry MeOH (2mg/ml) under argon atmosphere. The reaction mixture was monitored by TLC (dichloromethane/MeOH 5:1,Rf=0.01). The solution was filtered over a short column of Dowex® 50WX8-200 (H^+ resin form). The resin was washed with MeOH (15 mL) and
H2O (15 mL) and the fractions containing the product were concentrated under reduced pressure to afford the desired product (30 mg, 96%) as a white solid.

^{1}H-NMR (400 MHz, CD_3OD): δ = 4.95 (d, 1H, J= 1.7 Hz, H-1), 4.27 (dd, 2H, J= 1.6 Hz, 2.4 Hz, OC*H*$_2$C≡CH), 3.93 (dd, 1H, J= 5.1 Hz, 11.0 Hz), 3.88 (dd, 1H, J= 1.8 Hz, 3.4 Hz), 3.87-3.80 (m, 2H), 3.78-3.62 (m, 9H), 2.88 (t, 1H, J= 2.4 Hz, $OCH_2C{\equiv}C$*H*).

^{13}C-NMR (100 MHz, CD_3OD): δ = 100.08, 98.69 (C-1, C-1'), 78.61, 74.65, 73.00, 72.21, 71.26, 70.70, 70.52, 67.16, 66.96, 65.88, 61.48, 53.55.

MS(ESI) m/z =403.6404 $[M+ Na]^+$ (Calcd = 403.34).

Fullerene-$(N_3)_2$ (46)



C_{60} (34.8 mg, 0.048 mmol, 1eq.), Compound 2 (13 mg, 0.048 mmol, 1eq.) and I_2 (14.69 mg, 0.058 mmol, 1.22 eq.) were dissolved in dry toluene (15 ml). DBU (16 µL, 0.108 mmol, 2.25 eq.) was added to the mixture under argon at 0°C. The reaction was stirred at r.t. for 45 min.

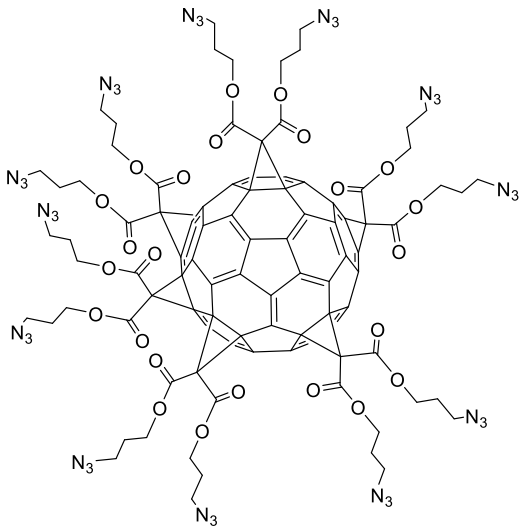
The reaction mixture was monitored by TLC (Cyclohexane/dichloromethane 6:4, Rf= 0.61).

The solvent was removed and a column chromatography (SiO_2, cyclohexane/ dichloromethane 7:3) gave compound 15 (20 mg, 42 %) as a black-brown solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 4.63 (t, 4H, J=6.0 Hz, OC*H*$_2$CH$_2$CH$_2$N$_3$), 3.55 (t, 6H, J=6.0 Hz, OCH$_2$CH$_2$C*H*$_2$N$_3$, COC*H*$_2$CO), 2.15 (q, 4H, J= 6.0 Hz, OCH$_2$C*H*$_2$CH$_2$N$_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 163.42, 162.71, 162.68 (*C*OCH$_2$*C*O), 145.34-138.25 (C_{60}), 70.68, 64.23, 63.52, 63.49, 48.01, 47.30, 47.28, 28.17, 27.47, 27.44, 26.92, 26.22, 26.19.

Fullerene-$(N_3)_{12}$ (47)



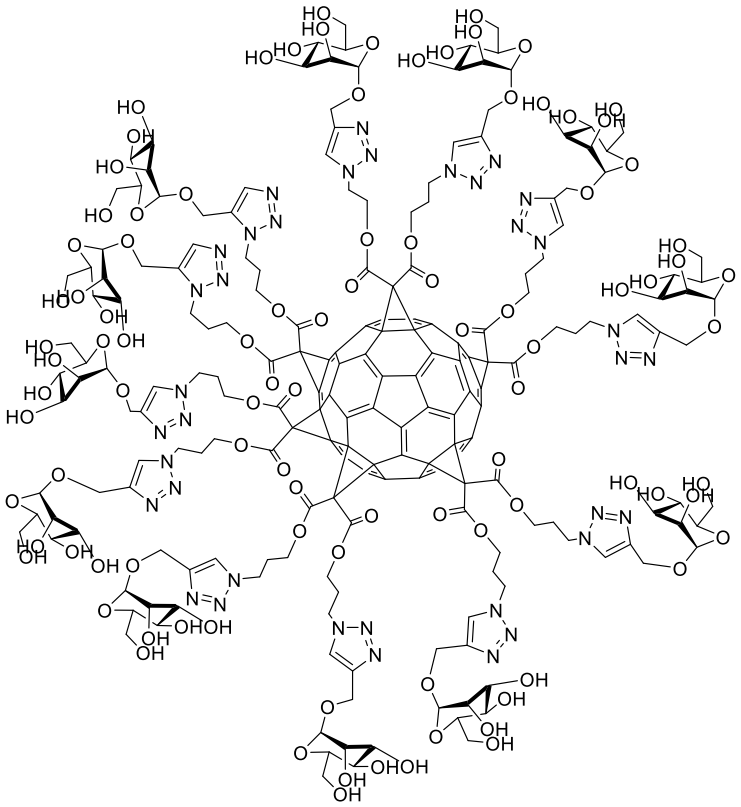
Fullerene-$(N_3)_{12}$ (**47**) was synthesized as previously reported[249].

C_{60} (40 mg, 0.055 mmol, 0.1eq.), Compound 2 (150 mg, 0.55 mmol, 1eq.) and CBr_4 (1.8 g, 5.5 mmol, 10 eq.) were dissolved in dry toluene (15 ml). DBU (164.5 µL, 1.1 mmol, 2 eq.) was added to the mixture under argon at 0°C. The reaction was stirred at r.t. for 72 hours.

The reaction mixture was monitored by TLC (dichloromethane/Acetone 6:4). The mixture was filtered through SiO2 (cyclohexane to remove impurities then dichloromethane/EtOAc 1:1 to recover the crude product). The solvent was removed and a column chromatography (SiO_2, Cyclohexane: EtOAc 6:4) gave compound 16 (76 mg, 60%) as a glassy red solid.

^{1}H-NMR and ^{13}C-NMR were in agreement with previously reported data[249].

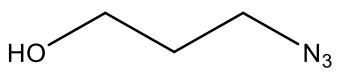
Fullerene-$(Man)_{12}$ (48)



To a solution of Fullerene-$(N_3)_{12}$ (**47**) (30 mg, 0.0128 mmol, 1 eq.) dissolved in THF (0.5 ml) was added a solution of Propargyl α-D-mannopyranoside **(19)** (42 mg, 0.192 mmol, 15 eq.) in H_2O (0.25 ml). Then a solution of copper sulphate (1.88 mg, 0.005 mmol, 0.39 eq.) and sodium ascorbate (2.54 mg, 0.0128 mmol, 1 eq.) was added and the mixture was stirred and heated 2h under microwave irradiation (100°C). The product was precipitated by addition of acetone. The precipitate was washed three times with acetone and three times with MeOH. Gel permeation chromatography (Sephadex G-50, H_2O) gave the desired product (39 mg, 61.3%) as red/orange solid.

^{1}H-NMR,^{13}C-NMR and IR analysis were in agreement with previously reported data[234].

3-azidopropan-1-ol (49)

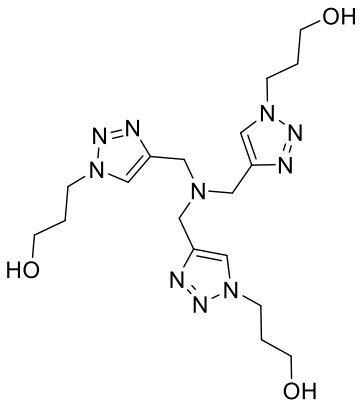


3-azidopropan-1-ol (**49**) was synthesized modifying slightly the protocol reported by Navarro L. A. et al. [257]. 3-bromo-1-propanol (0.91 ml, 10 mmol, 1 eq.) was dissolved in H_2O (10 ml). NaN_3 (1.30 g, 20 mmol, 2 eq.) was added to the emulsion and the reaction was stirred at 50°C for 24h.

The aqueous reaction mixture was extracted four times with dichloromethane. The organic the organic layer dried over $MgSO_4$, filtered and concentrated *in vacuo* at room temperature. The desired product (980 mg, 90%) was obtained as colourless oil.

^{1}H-NMR was in agreement with previously reported data [257].

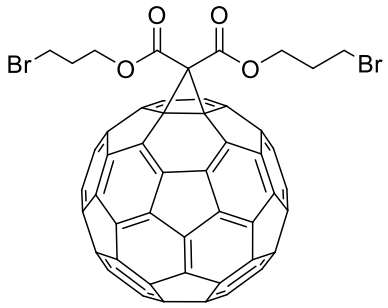
THPTA (50)



THPTA (**50**) was synthesized modifying slightly the protocol reported by Pradere U. et al.[260]. To a solution of 3-azidopropan-1-ol **49** (980 mg, 9.7 mmol, 4 eq.) and tripropargylamine (0.343 ml, 2.425 mmol, 1 eq.) in 5 ml H_2O/t-BuOH (1:1), copper sulphate (60.54 mg, 0.97 mmol, 0.1 eq.) and sodium ascorbate (96 mg, 1.94 mmol, 0.2 eq.) were added. The mixture was stirred at r.t. for 48 hours, then evaporated under reduced pressure. The reaction mixture was monitored by TLC (dichloromethane /MeOH 9:1. Rf=0.12). Column chromatography (SiO_2, dichloromethane/MeOH 9:1 to 5:5) gave the desired product (811 mg, 77%) as yellow solid.

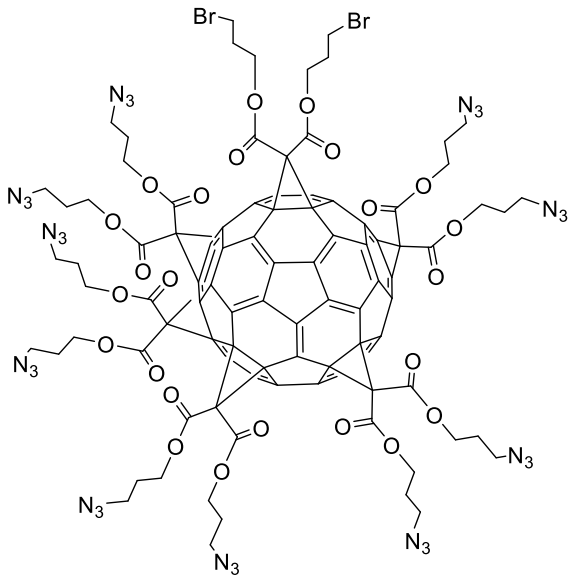
^{1}H-NMR was in agreement with previously reported data [257].

Malonate C_{60}-$(Br)_2$ (51)



Malonate C_{60}-$(Br)_2$ (**51**) was synthesized as previously reported[258]. C_{60} (14.6 mg, 0.0202 mmol, 1eq.), Compound 1 (7 mg, 0.0202 mmol, 1eq.) and I_2 (6.26 mg, 0.0246 mmol, 1.22 eq.) were dissolved in dry toluene (8 ml). DBU (6.8 µL, 0.045 mmol, 2.25 eq.) was added to the mixture under argon at 0°C and the reaction was stirred at r.t. for 30 min. The reaction mixture was monitored by TLC (Cyclohexane/dichloromethane 7:3 or Cyclohexane/toluene 1:1. Rf=0.28). The solvent was removed under reduced pressure and a column chromatography (SiO_2, Cyclohexane/dichloromethane 7:3) gave the desired product (10.8 mg, 50 %) as a red-brown solid. ^{1}H-NMR and ^{13}C-NMR were in agreement with previously reported data[258].

Fullerene-$(Br)_2(N_3)_{10}$ (52)



Malonate C_{60}-$(Br)_2$ (51) (7.7 mg, 0.00658 mmol, 0.14 eq.), Bis(3-azidopropyl) malonate (**44**) (13.6 mg, 0.047 mmol, 1eq.) and CBr_4 (169.8 mg, 0.47 mmol, 10 eq.) were dissolved in dry ODCB (5 ml). DBU (18.3 µL, 0.113 mmol, 2.4 eq.) was added to the mixture under argon at 0°C and the reaction was stirred at r.t. for 72 hours.

The reaction mixture was monitored by TLC (dichloromethane/Et_2O 9:1). The mixture was filtered through SiO_2 (cyclohexane to remove impurities then dichloromethane/ Et_2O 9:1 to recover the crude product). The solvent was removed under reduced pressure and a column chromatography (SiO_2, dichloromethane/Et_2O 9:1) gave the desired product (12 mg, 70%) as a glassy red-orange solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 4.54-4.30 (m, 24H, OC*H*$_2$CH$_2$CH$_2$Br, OC*H*$_2$CH$_2$CH$_2$ N$_3$), 3.52-3.37 (m, 24H, OCH$_2$CH$_2$C*H*$_2$Br, OCH$_2$CH$_2$C*H*$_2$N$_3$), 2.27 (m, 4H, OCH$_2$C*H*$_2$CH$_2$Br), 2.04-1.89 (m, 20H, OCH$_2$C*H*$_2$CH$_2$ N$_3$)

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 163.50 (*C*OC*C*O), 145.79, 141.09 (C_{60}), 69.07, 63.90 (CO*C*CO), 47.88 (O*C*H$_2$CH$_2$CH$_2$N$_3$), 45.25 (OCH$_2$CH$_2$*C*H$_2$N$_3$), 31.30 (OCH$_2$*C*H$_2$CH$_2$Br)., 29.01 (OCH$_2$CH$_2$*C*H$_2$Br), 28.01 (OCH$_2$*C*H$_2$CH$_2$N$_3$).

MS(ESI) m/z = 2427.947 [M+ Na]$^+$ (Calcd = 2428,814).

CHAPTER 5: CONCLUSIONS

In this project we have investigated two different ways to synthesize potential glycoconjugate vaccines for Tuberculosis: the first approach involves an antigenic nano protein, the second one employs fullerene as a carrier.

In order to reach this purpose, we started to design and synthesize analogue of natural polymannan (since the mannosylation could improve antigen uptake by APCs). Therefore, we have prepared di- and tri-mannan oligosaccharides with α1,6man and α1,2man motifs (compounds **1**-**8**), bearing in anomeric position a reactive linker suitable for conjugation with protein (thiocyano methyl group) and fullerene (propargyl group).

Once prepared thiocyanomethyl sugars were activated to IME-group and linked an elastin-like recombinamer, α-E50I60. This new material includes in its sequence the antigenic peptide of Tuberculosis (an Ag85C epitope), and it is able to self-assemble in a micelle exposing the antigenic portions.

The protein glycosylation reaction was first investigated on a soluble protein (RNasi A) and we found the conditions for obtaining complete glycosylation of this protein via Thiocyano methyl linker with all the tested glycans.

The α-E50I60 protein contain only one lysine that is included in the epitope sequences and, consequently, the reactivity of this protein is much lower than RNAse. However in this case the process was designed in order to glycosylate 40-60% of α-E50I60 proteins to obtain, after self-assembly, a suitable number of non-glycosylated epitopes on the nanostructure surface.

Biological evaluation of these compounds is in progress. In particular, the evaluation of the binding between MR (using the Concanavalin A model of the receptor) and the *neo*glycoproteins will be evaluated by SPR (in Complutense university of Madrid) comparing the binding affinity of the soluble RNAse glycoconjugates with the glycosylated nano-protein derivatives prepared with α-E50I60.

In case of positive results, α-E50I60 glycoderivatives could be tested by *ex-vivo* and *in-vivo* to evaluate the suitability of these products for developing a new class of glycoconjugate nano-vaccines against TB.

The other sugars bearing propargyl groups were employed in fullerene strategy, in order to synthesize glycosylated fullerene scaffold suitable for future conjugations with an antigenic peptide. Because of the particular reactivity of C_{60}, this part of the project given different problems. We focused on the conversion of fullerene into methanofullerenes (by Bingel reaction), exploiting malonate or malondiamide bearing a terminal azide as reactive linker, suitable for Huisgen 1,3-dipolar cycloaddition with the sugars.

Thanks to the high stability of amide group, we were able to work in organic solvent linking acetylated mono, di- and tri- saccharides on fullerene (compounds **38**-**42**).

When the malonate ester chain was used as linker, the deacetylation of the glycans was performed prior to the conjugation by click chemistry since the esters of malonate used as linker are reactive in the condition used for deacetylation. This feature required of working in aqueous medium to handle non–protected sugars, with, consequently, problems arising from the low solubility of C_{60} in aqueous medium.

Thanks to the high reactivity of the malonate ester chains in the Bingel reaction, six chains with azido groups can be easily linked to the fullerene. The following click reaction with twelve propargyl mannoses (**19**) was optimized to reach the desired full glycosylated product (**48**). When the same reaction was tested with disaccharides, the reaction failed by all tested conditions.

This work has shown the advantages and disadvantages in working with fullerene but also its potentiality. Further studies will be carried on in amide-linker strategy in order to link a second chain bearing an epitope of Ag85B.

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