Chemical and enzymatic valorization of polyols from biomass

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“Chemical and Enzymatic Valorization of Polyols from Biomass”

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És complicat en un pròleg com aquest poder mostrar l'agraïment adequat a totes les persones que d'alguna manera o altra m'han acompanyat durant tota la tesi doctoral,

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Resum

En les últimes dècades han augmentat els problemes derivats de la sobreproducció i acumulació de residus de la industria, així com els problemes mediambientals i la disminució de fonts de matèries primeres. En aquest sentit, ha incrementant l'interès en reutilitzar-los, per tal de re-valoritzar-los produint productes d'interès, acostant-nos cada cop més al concepte de residu zero.

Un dels majors subproductes de la industria agroalimentària és el conegut amb el terme de biomassa. En aquest treball, ens hem centrat en investigar la re-valorització d'una petita part dels poliols presents en la biomassa, entre ells alguns carbohidrats i el glicerol.

En el primer capítol s'ha estudiat la modificació enzimàtica dels carbohidrats presents en fibres de fruita, per tal de millorar-ne les seves propietats fisicoquímiques, i poder així utilitzar-les com a ingredients alimentaris.

En els següents capítols, s'utilitza el glicerol com a producte de partida per a la obtenció de productes d'interès.

En el segon capítol s'ha estudiat l'obtenció de compostos capaços de reaccionar amb CO_2 o algun dels seus derivats, també subproductes de la industria, per tal d'obtenir productes amb possible interès com a cosmètics, pesticides, productes farmacèutics i productes de química fina.

En el tercer capítol s'han estudiat les alquil monoamides de 1,3-diamino-2-propanol, obtingudes en el segon capítol, ja que varen presentar un comportament referent a punt de ebullició i solubilitat no esperat, que es va relacionar amb la formació de ponts d'hidrogen. Degut a l'interès d'aquest tipus d'interaccions, i la seva importància en el comportament i propietats dels productes, es va dur a terme un estudi de la presencia i influencia d'aquests per FTIR a temperatura variable.

I finalment, en el quart i últim capítol s'ha estudiat l'obtenció de compostos amb un alt contingut de sucres en la seva estructura. Aquest engloba des de l'obtenció de polímers amb possible activitat en camps biomèdics, fins a productes que poden ser utilitzats com a surfactants o per la producció de microarrays.

Resumen

En las últimas décadas han aumentado los problemas derivados de la sobreproducción y acumulación de residuos de la industria agroalimentaria, así como los problemas medioambientales y la disminución de fuentes de materias primas. Incrementando así el interés en reutilizarlos, revalorizándolos produciendo productos de interés, acercándonos cada vez más al concepto de residuo cero.

Uno de los mayores subproductos de la industria es el conocido con el término de biomasa. En este trabajo, nos hemos centrado en investigar la revalorización de una pequeña parte de los polioles presentes en la biomasa, entre ellos algunos carbohidratos y el glicerol.

En el primer capítulo se ha estudiado la modificación enzimática de los carbohidratos presentes en fibras de fruta, para mejorar sus propiedades fisicoquímicas, y poder así utilizarlas como ingredientes alimentarios.

En los siguientes capítulos se utiliza el glicerol como producto de partida para la obtención de productos de interés.

En el segundo capítulo se ha estudiado la obtención de compuestos capaces de reaccionar con CO_2 o alguno de sus derivados, también subproductos de la industria, con el fin de obtener productos con posible interés como cosméticos, pesticidas, productos farmacéuticos y productos de química fina.

En el tercer capítulo se han estudiado las alquil monoamidas de 1,3-diamino-2-propanol, obtenidas en el segundo capítulo, ya que presentaron un comportamiento referente a punto de ebullición y solubilidad no esperado, que se relacionó con la formación de puentes de hidrógeno. Debido al interés de este tipo de interacciones, y su importancia en el comportamiento y propiedades de los productos, se llevó a cabo un estudio de la presencia e influencia de estos por FTIR a temperatura variable.

Finalmente, en el cuarto y último capítulo se ha estudiado la obtención de compuestos con un alto contenido de azúcares en su estructura. Este engloba desde la obtención de polímeros con posible actividad en campos biomédicos, hasta productos que pueden ser utilizados como surfactantes o para la producción de microarrays.

Summary

In the last decades the problems related with overproduction and waste industry accumulation have increased. Causing environmental problems and depletion of raw material sources. Because of that, an increasing interest in the reuse of by-materials to prepare valuable products has grown, helping to be closer to the concept of zero waste.

Biomass is one of the major agroindustrial by-products. In this study, we were focused on adding-value to a small portion of the polyols presents in biomass, including some carbohydrates and glycerol.

In the first chapter, we studied the enzymatic modification of carbohydrates present in fruit fibers in order to improve their physical and chemical properties and their use as food ingredients.

In the following chapters, glycerol is used as the starting material to prepare products of interest.

In the second chapter, we describe the production of compounds capable to react with CO_2 or its derivatives, also products of the industry. The resulting compounds could have likely interest as cosmetics, pesticides, pharmaceuticals and fine chemical products.

In the third chapter, we describe the behavior of alkyl monoamides of 1,3-diamino-2-propanol synthesised in the second chapter. The unexpected behavior of some of the synthesized compounds regarding to their solubility and boiling point could be related to the formation of hydrogen bonds. Due to the interest of such interactions and their importance in the behavior and properties of products, we carried out a study of their presence and influence by variable temperature FTIR.

And finally, the fourth and final chapter was centered in the synthesis of compounds with high sugar content in their structure. These compounds range from the production of polymers, with potential activity in several biomedical fields, to products that can be used as surfactants or microarrays.

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ABBREVIATIONS

AG	Arabinogalactan
ATR	Attenuated total reflectance
ATRP	Atom transfer radical polymerization
BMIM-Br	1-Butyl-3-methylimidazolium bromide
$Br^{-}Ph_3^{+}PPEG_{600}\text{-}P^{+}Ph_3Br^{-}$	Polyethylene glycol functionalized phosphonium salt
ca.	arround
CBP	Carbohydrate binding proteins
CD_3OD	Deuterated methanol
$CDCl_3$	Deuterated chloroform
CEL	Cellulase enzyme
CH_2Cl_2	Dichloromethane
C-Lectin:	Ca^{2+} dependent lectine
CMC	Carboxymethyl cellulose
CRD	Carbohydrate Recognition Domains
CTMS	Chlorotrimethylsilane
CuAAC	Cu-catalysed azide-alkyne cycloaddition
D_2O	Deuterated water
D_6MSO	Deuterated dimethylsulfoxide
DBU	1,8-Diazabicycloundec-7-ene
DC	Dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DC-SIGNR	A DC-SIGN homologue, termed DC-SIGNR (for DC-SIGN related), that exhibits 77% amino acid identity with DC-SIGN.
DE	Degree of methoxylation
DEC-205	Type I cell surface protein expressed primarily by dendritic cells
DF	Dietary fibre
D-Gal*p*A D	Galacturonic acid residues
DHB	2,5-Dihydroxybenzoic acid
DMC	2-Chloro-1,3-dimethylimidazolinium chloride
DMF	Dimethylformamide
DS	Degree of substitution
EBiB	Ethyl α-bromoisobutyrate
ESI-MS	Electrospray ionization mass spectrometry
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
FAME	Fatty acid methyl esters
FER	Ferulase enzyme
FT/IR	Fourier transform infrared spectre
gp120	HIV envelope glycoprotein
GS	Substituted galacturonans
HG	Homogalacturonan
HIV	Human immunodeficiency virus
HOMO	Highest occupied molecular orbital
IL	Ionic liquid

LUMO	Lowest unoccupied molecular orbital
MALDI- TOF	Matrix assisted laser desorption ionization- time of flight
Me_6TREN	Tris[2-(dimethylamino)ethyl]amine
MeOH	Methanol
MGL1	MGL1
MR2	MR2
MW	Microwave
$OPPh_3$	Tryphenyl phosphine oxide
PAMP	Pathogen Associated Molecular Patterns
Pd/C	Palladium over Carbon
PEC A	Pectolytic enzymes type A
PEC B	Pectolytic enzymes type B
PEC	Pectolytic enzymes
PG	Polygalacturonase
PL	Pectin lyase
PME A	Polymethyl esterase type A
PME B	Polymethyl esterase type B
PME	Pectin methyl esterase
PPh_3	Tryphenyl phosphine
RAFT	Reversible addition-fragmentation chain transfer polymerization
RG II	Ramnogalacturonan II
RGI	Ramnogalacturonan I
r.t.	Room temperature
SET-LRP	Single-electron transfer living radical polymerization
SPR	Surface solid plasmon resonance
SWC	Swelling water capacity
TEMPO	2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
THF	Tetrahydrofuran
WHC	Water holding capacity
XG	Xylogalacturonan

INTRODUCTION

I.1a.- BIOBASED ECONOMY.

Over the last years, the term bioeconomy has appeared. It reflects the emergence of the biobased economy, focused on new technology breakthroughs, consumer trends and the growing adoption of industrial biotechnology innovation, improving conventional fuels, producing advanced and cellulosic biofuels, generating renewable chemicals, and spending development of new-purpose energy crops, finding climate change solutions in the manufacturing space.

Bioeconomy is then based on the efforts to build an economy that enable improved living standards around the world and at the same time replace non-renewable petroleum-based materials with cleaner and greener biobased materials.

But, the bioeconomy is not just about biofuels, renewable chemicals also reduce the greenhouse gas emissions, along with other pollutant emissions associated with supply, processing and use of petroleum and petrochemicals. New energy crops and sources are being studied as feedstock, protein source, and production organisms, with applications in fuels, cosmetics and even food.

These new processes appear to be a firm foothold in the global economy, with multiple new technologies for producing renewable chemicals and products finding solutions to the climate change[1].

I.1b.- BIOMASS.

Nowadays, the term biomass can be defined as "the set of organic vegetable, animal or result of their natural or artificial transformation, considered as a waste and susceptible to be reused"[2] (Figure I1).

Under the concept of biomass a large number of products with various origins and characteristics can be identified, obtained from very different sources such as: forestry and agriculture, wastes from agro-forestry industry, wood or paper, energy crops, animal or human waste and used vegetable oils[3-5].

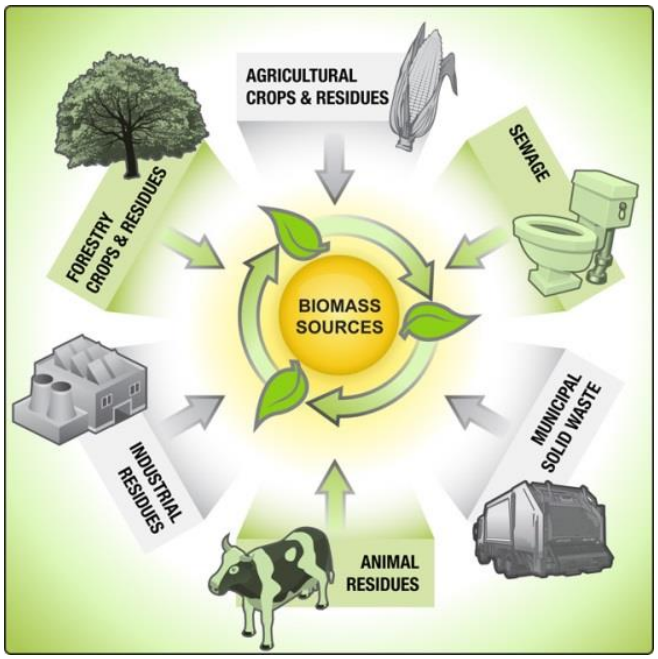


Figure I1: Visual representation of biomass sources. Source: extracted of[6]

Formerly, the term Biomass was referred to plants that were specifically grown in order to produce electricity or heat. That includes the use of sugars and vegetable oils found in arable croups. They were identified as first generation biomass sources. As these sources were based in arable croups, this sort of biomass was limited on its ability to achieve targets for oil-product substitution, climate change mitigation, and economic growth. Besides, their sustainable production was reviewed, because of the possibility of creating undue competition for land and water used for food and fibre production[4].

To avoid the use of food land and sources to produce biomass competing with food industry[3], and to reduce the waste accumulation a second generation of biomass (non-food biomass) was faced up. This was based on taking profit of the crescent accumulation of arable and agroindustrial residues. The interest was focused in the use of this waste as starting material to prepare fuels, chemical compounds and materials[7].

Second generation biomass uses cereal straw, bagasse, forest residues, and purpose-grown energy crops such as vegetative grasses, short rotation forests (agricultural waste), and agroindustrial waste[3-5]. In its most general sense, the term “waste” covers any organic material apart from the primary material for which the plants were originally grown (e.g., corn stubble from maize or lignin from paper pulping). Waste

biomass is perhaps a concept even broader, because it applies to any biomass-derived by-product for which supply greatly exceeds demand. Actually, nearly all wastes currently have some value. For instance, stubble used to improve the cultivable soil, or lignin as a fuel to power paper mills. But, because of the high amount produced, which cause its accumulation and make the prices down, or also because the inefficient way they are reused, the need to develop new applications is growing. For example, glycerol can be a valuable chemical, but it is being generated in increasing quantities by the biodiesel industry, so that it production is expected to grow until six times more by 2020[8]. Consequently, glycerol will become a "waste."

Also, the use of this second generation biomass will help the environment by avoiding accumulation of residues and getting closer to the approach of environmentally sustainable processes with a low carbon fingerprint. Hence, the starting materials could have low or even non-cost.

WASTE BIOMASS AS A ENERGY AND NEW MATERIALS SOURCE.

In the recent years, environmental problems and waste accumulation have led the valorisation of the whole biomass in terms of carbon economy. This approach is based on a zero-waste concept. Indeed, the society currently faces the twin challenges of resource depletion and waste accumulation leading to rapidly escalating raw material costs and increasingly expensive and restrictive waste disposal legislation.

Most of the carbon-based compounds currently manufactured by the chemical industry are derived from petroleum. The rising cost and dwindling supply of oil have been focusing attention on possible routes to making chemicals, fuels, and solvents from biomass (Figure I2).

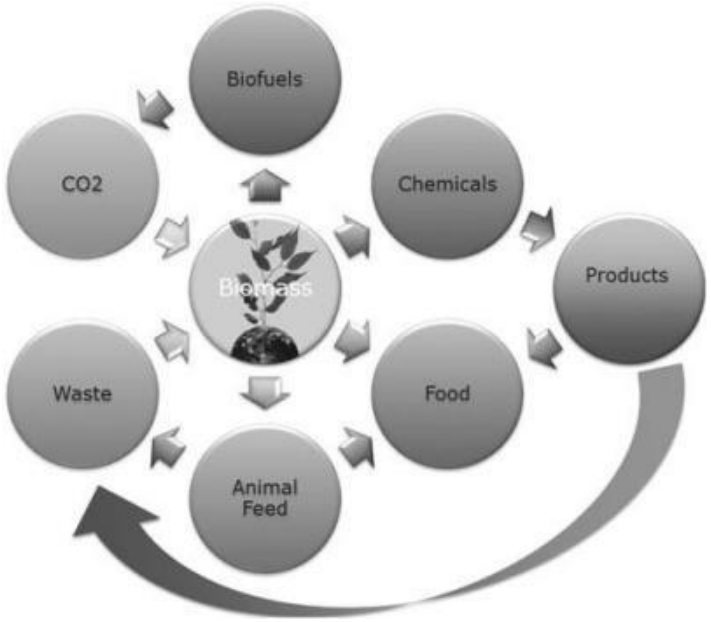


Figure I2: Second generation biomass as a closed cycle[1]**.**

Interest in the biomass-to-chemical value chain raised as:

- The necessity to use renewable carbon sources aimed at compensating the expected decrease of petroleum production.
- The need to decrease greenhouse gas (GHG) emissions, suspected to have a detrimental effect on the global climate, by employing renewable carbon available from biomass.

Also, the use of biomass can play an important role in sustainable development and handling global warming through the use of biomass as base resource for chemicals, materials and products. More specifically for the chemical industry, we can distinguish three main areas:

- The use of renewable raw materials replacing fossil fuel feedstock's, where new enzyme and whole cell systems convert biomass into fermentable sugars and then downstream products.
- Bioprocesses replacing traditional chemical processes to make many organic and other chemicals.
- Development of new products such as chemicals, new plastics, high performance polymers.

- The use of biomass to replace fossil fuels as starting material to prepare chemicals and materials

MAIN COMPOSITION OF BIOMASS.

Biomass sources are so diverse, and their content is a complex and variable mixture of molecules, which separation becomes a key issue. Roughly, the composition of biomass can be defined in terms of four source-independent categories: polysaccharides, lignin, lipids (mainly acylglycerides) and proteins[5].

Considering the huge diversity of sources and products to work with and to investigate as sources for new products, we decided to focus this thesis in the study of some of the polyols present in the biomass.

I.1.1.- POLYOLS FROM BIOMASS: CARBOHYDRATES.

Carbohydrates are the most abundant class of organic compounds found in plant living organisms. It is estimated that approximately 75% of the dry weight of herbaceous and woody biomass is composed of carbohydrates[9]. Hence, residues and products of plant origin are mainly recognized by their carbohydrate composition. Carbohydrates are the primary products from photosynthesis (Scheme I1), an endothermic reductive condensation of carbon dioxide and water requiring light energy and chlorophyll and other pigments.

$$n\ CO_2 + n\ H_2O + \text{energy} \longrightarrow C_nH_{2n}O_n + n\ O_2$$

Scheme I1: Carbohydrate synthesis through photosynthesis.

Carbohydrates perform numerous roles in living organisms, both for plants and for animals, which partially depend on plants for food. As a general idea, carbohydrates serve for energy storage (e.g., starch and glycogen) and as structural components (e.g. cellulose, hemicellulose, pectin….). Other carbohydrates could act in the energy transport compounds (ATP), as recognition site for cell surface and they also are one of the three essential components of DNA and RNA.

A kind of carbohydrate subclass is the polysaccharides. As the name indicates, polysaccharides are large high-molecular weight molecules constructed by joining

monosaccharide units together by glycosidic bonds. They are sometimes called glycans. The most important compounds in this class, cellulose, starch and glycogen are all polymers of glucose.

Cellulose and chitin are examples of structural polysaccharides. Cellulose is used in the cell walls of plants and other organisms, and is said to be the most abundant organic molecule on the Earth. It has many applications such as a significant role in the paper and textile industries, where it is used as a feedstock for the production of rayon (via the viscose process), cellulose acetate, celluloid, and nitrocellulose.

I.1.1.1.- Uses of Carbohydrates from Biomass Sources.

Usually, biomass rich in carbohydrates is subjected to different operations. Initially, high-value chemicals already present in the biomass, such as fragrances, flavouring agents, food-related products and high-value nutraceuticals that provide health and medical benefits, are extracted[10]. Once these relatively valuable chemicals are recovered, plant polysaccharides and lignin are used as feedstocks for bio-derived chemicals, materials and fuels.

I.1.1.1.1.- USE AS A FUEL.

Biomass is widely used as a source of energy so recovering part of the metabolic energy accumulated during their formation.

These processes usually lead to heat, which just could be used in the produced place or close to there. This heat can be used for chemical processing, district heating or to generate steam for power production.

There are basically three conversion processes: physical (e.g. combustion), thermo-chemical (e.g. pyrolysis, gasification and liquefaction) and biological treatments (e.g. anaerobic digestion and fermentation)[4, 5, 7]. Solid, liquid or gaseous fuel can be obtained, which can become substitutes of charcoal, petrol and natural gas, respectively.

This sort of energy have larger spectra and are maybe the easiest way for using biomass, but it is usually not the best energetic yield process in terms of efficiency.

I.1.1.1.1.1.- COMBUSTION.

Direct combustion from biomass rich in carbohydrate is the most obvious way to obtain energy, straightforward and commercially available. Combustion systems come in a wide range of shapes and sizes burning virtually any kind of fuel, not only carbohydrate based biomass. Heat from burning wastes is currently used in space and water heating, industrial processing and electricity generation. One problem with this method is its very low efficiency[11].

I.1.1.1.1.2.- PYROLYSIS.

Pyrolysis has been used for centuries to produce charcoal. The process is the simplest and almost certainly the oldest method of processing one fuel in order to produce a better one. A wide range of energy-rich fuels can be produced by roasting dry wood or even straws. Conventional pyrolysis involves heating the original material (which is often powdered or shredded then fed into a reactor vessel) typically at 300 - 500°C in the near-absence of air, until the volatile matter has been driven off. The residue is the char -more commonly known as charcoal- a fuel that has about twice the energy density of the original and burns at a much higher temperature. Depending on the moisture content and the efficiency of the process, 4 to 10 t of wood are required to produce 1 t of charcoal. If no attempt is made to collect the volatile matter, the charcoal is obtained at the cost of nearly two-thirds of the original energy content[12].

I.1.1.1.1.3.- GASIFICATION.

Gasification based on biomass rich in carbohydrate is performed by partially burning and partially heating the biomass (using the heat from the limited burning) in the presence of charcoal and air[13]. A flammable gas mixture of hydrogen, carbon monoxide, methane and other non-flammable by-products are produced.

I.1.1.1.1.4.- SYNGAS FUELS.

A biomass gasifier, which uses oxygen rather than air, can produce a gas consisting mainly of hydrogen (H_2), carbon monoxide (CO) and carbon dioxide ($C0_2$). The removal

of the $C0_2$ leaves to the synthesis gas (syngas), from which almost any hydrocarbon compound may be synthesized. Hence, pure methane can be synthesized by reacting the H_2 and CO. Another possible product is methanol (CH_3OH), a liquid alcohol with an energy density of 23 GJ/t[14].

I.1.1.1.1.5.- FERMENTATION.

Sugar fermentation can lead to ethanol. Sugars fermentation can also lead to platform molecules, which could be transformed into fuels, chemicals or polymers[7].
The complete process requires a considerable amount of heat, which is usually supplied by crop residues (e.g. sugar cane bagasse or maize stalks and cobs). The energy loss in fermentation is substantial, but this may be compensated for by the convenience and transportability of the liquid fuel, and by the comparatively low cost and well defined technology[15].

I.1.1.1.1.6.- ANAEROBIC DIGESTION.

Some bacteria could decompose biomass rich in carbohydrate in a process that takes place under the absence of air (oxygen) and produce a mixture of methane (CH_4) and CO_2 commonly called 'Biogas'[16].
Anaerobic digestion really could take place in almost any biological material. It is favoured by warm, wet and of course airless conditions.
The detailed biochemistry of the production of biogas is complex, but it appears that a mixed population of bacteria breaks down the organic material into sugars and then into various acids, which are decomposed to produce the final gas, leaving an inert residue whose composition depends on the type of process and the initial feedstock.

I.1.1.1.2.- TRANSFORMATION TO NEW BIO-MATERIALS.

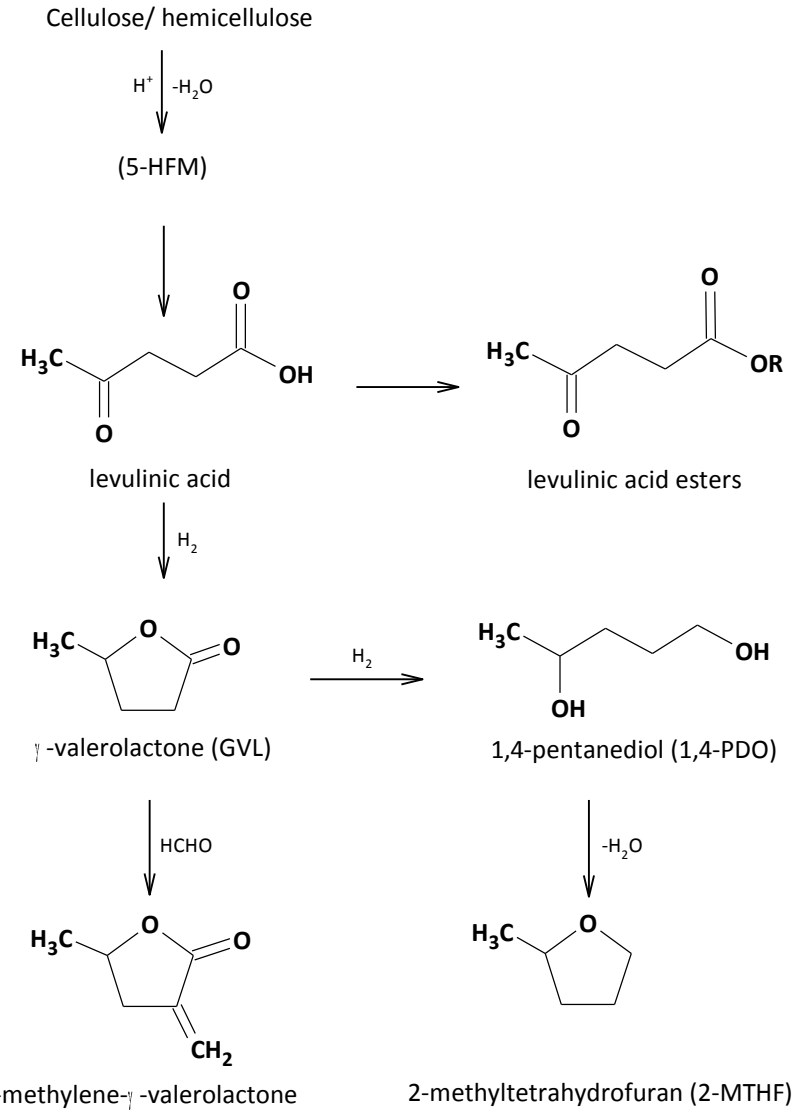
Biomass carbohydrates will provide a viable route to many products, using various conversion strategies. For example, biomass can be converted into platform molecules, which are subsequently employed as building blocks for the synthesis of intermediates, specialties, fine chemicals and polymers[17]. Biopolymers can also be

modified to introduce new functionalities along the polymer backbone so to achieve new properties and applications such as surfactants, lubricants, foams plasticizers, binders, paints, food additives and cosmetics[18-22].

I.1.1.1.2.1.- *BUILDING BLOCKS.*

The aim of this section is not provide a comprehensive list about the conversion of biomass to platform molecules but some examples, which are summarized next.

I.1.1.1.2.1.1.- *Levulinic Acid.*



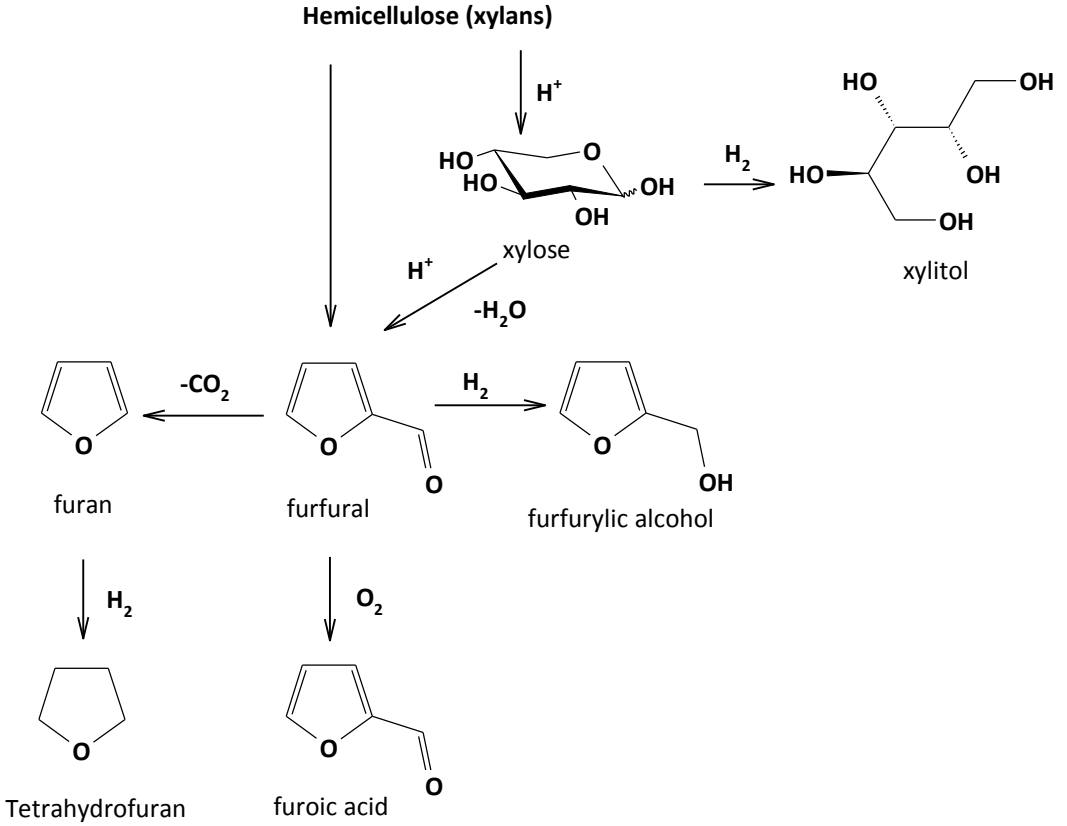
Scheme I2: Levulinic acid and derivatives pathways.

Levulinic acid is produced at industrial scale from the cellulose and hemicellulose present in agricultural or forest residues[23]. The raw material is hydrolysed and

dehydrated by acidic treatments in a first reactor yielding 5-hydromethylfurfural (5-HMF), which is recovered and transformed in a second reactor to levulinic acid. A final yield of 60% with respect to the monomers contained in the starting cellulosic materials has been achieved[24]. This acid can be the starting material to produce several compounds such as γ-valerolactone, 1,4-pentanediol and 2-methyltetrahydrofuran (2-MTHF) (Scheme I2).

I.1.1.1.2.1.2.- *Xylose.*

The acid catalyzed hydrolysis of xylan-type hemicelluloses, present in soft woods and straw, yields C_5 sugars such as xylose, which can be further dehydrated to furfural or reduced to xylitol (Scheme I3).



Scheme I3: Xylose derivatives pathway

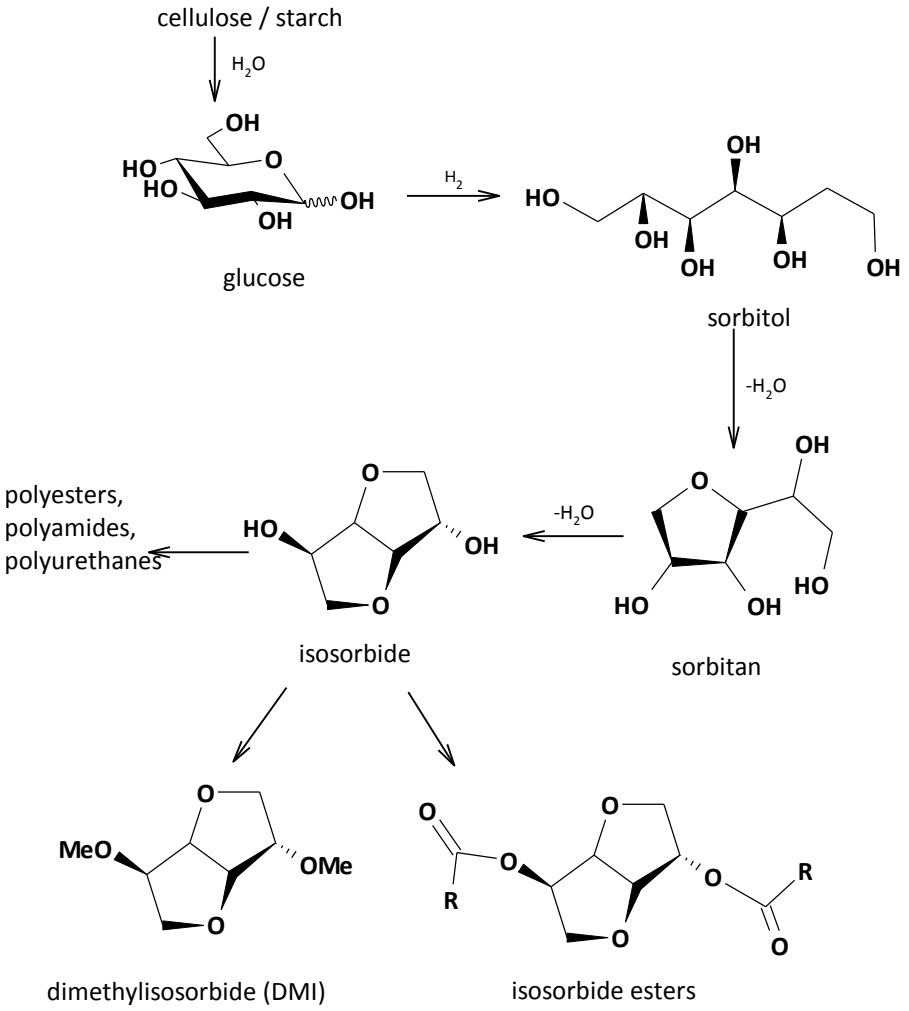
Xylose hydrogenation has mainly been achieved industrially in the presence of Raney nickel catalysts affording up to 98% yield. Nevertheless, the catalysts has been

described to suffer from deactivation due to the leaching of promoters, surface restructuring and poisoning by strongly adsorbed organic molecules[25, 26].

Furfural is produced industrially (*ca.* 250 000 t/ year) by hydrolysis of agricultural or forestry wastes with concentrated sulphuric acid[18]. It is employed as a foundry sand linker, in the refining of lubricating oil, and as an intermediate for furfuryl alcohol, furan and tetrahydrofuran production[18].

I.1.1.1.2.1.3.- *Sorbitol.*

Sorbitol is produced industrially (*ca.* 700,000 t/year) by catalytic hydrogenation of glucose (Scheme I4).



Scheme I4: Sorbitol and derivatives.

Most of the industrial processes rely on batch-wise hydrogenation on Raney nickel catalysts promoted with electropositive metal atoms[27]. Because of the risk of nickel

or metal promoter leaching they tend to be replaced by more active and stable supported ruthenium catalysts. Thus, Ru-catalysts were used in the continuous hydrogenation of glucose in a trickle-bed reactor for up to 596 h on stream yielding 99.5% of sorbitol and experiencing no metal leaching[19]. Sorbitol finds many applications in food, pharmaceutical and cosmetic industries and as additives in many end-products. It has been used as a key intermediate in the synthesis of ascorbic acid (vitamin C)[19], and its fatty acid esters were used as surfactants[20, 21]. A new interest arose for isosorbide (1,4 : 3,6-dianhydro-D-glucitol) prepared by double dehydration of sorbitol[28]. This molecule finds application in pharmaceutical and personal care products and it is used to synthesize polymers, dimethylisosorbide (DMI) or other mono- or di-alkyl ethers, and isosorbide esters.

I.1.1.1.2.1.4.- *Modified Sugars.*

Modified sugars are important constituents of glycoproteins and glycolipids. They are commonly deoxy or amino sugars. In deoxy sugars an -OH is replaced by -H or a -CH_2OH by CH_3. The former typically occurs at C_2 and the latter at C_6. In amino sugars the –OH is replaced by -NH_2, commonly at C_2.

These sugars obtained from polymer degradation by fermentation can be transformed into building-blocks by enzymatic and chemical transformations. The amino group might be easily acetylated to give the N-acetyl derivative. Azido sugars and sugar glycosides can be also prepared, which are products useful for the synthesis of a huge set of products.

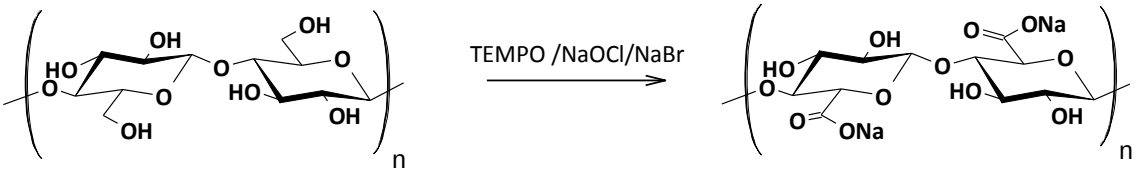
I.1.1.1.2.2.- *MODIFICATION OF BIOPOLYMERS.*

Instead of degrading polysaccharides (cellulose, hemicellulose, starch, inulin, chitin) or other biopolymers (lignin, proteins) into small molecules in an attempt to synthesize chemicals and polymeric materials, similar to those currently produced from fossil resources, a more sustainable use of biomass consists of adding functionalities to the biopolymer backbone. This approach is usually a one-pot process producing functional materials, which fulfil various applications. These processes do not require a number of waste-generating steps and large energy consumption. The one-step reaction is

usually performed at moderate temperature, usually in water, without further separation processes.

I.1.1.1.2.2.1.- Oxidation of Cellulose and Hemicellulose.

The oxidation of cellulose-containing materials can be performed with NaClO or H_2O_2, with or without metal salts or metal complexes added. These are the usual processes for the bleaching of paper pulp and textile[29]. Cellulose oxidation can also lead to reduce its molecular weight, which makes cellulose more amenable to further transformations, or to generate acidic groups along the biopolymer backbone increasing its pH depending properties. The TEMPO-mediated oxidation of cellulose at the C_6 position using NaClO as an oxidizing agent has been extensively studied[30-32](Scheme I5). Thus, cellulose from bleached kraft pulp was converted into materials employed as transparent gas barrier films for packaging or in high tech applications such as flexible display panels[30, 32]. A softwood bleached kraft pulp has been subjected to electro-mediated oxidation in water with TEMPO or 4-acetamido-TEMPO without any chlorine-containing oxidant. The oxidation has yielded both carboxylic and carbonyl functions at the C_6 position[33].



Scheme I5: TEMPO-mediated oxidation of cellulose**.**

I.1.1.1.2.2.2.- Etherification of Hemicellulose.

Etherification of hemicelluloses with cationic agents has been used to prepare flocculants, adhesives or wet-end additives in papermaking. Pinel and co-workers[34] achieved the cationisation of galactomannan and xylan hemicelluloses with 2,3-epoxypropyltrimethylammonium chloride in alkaline media resulting in degree of substitution (DS) values from 0.1 to 1.3 depending on reaction conditions such as the hemicelluloses and epoxide concentrations.

I.1.1.1.2.2.3.- Esterification of Cellulose and Hemicellulose.

Hydrophobic celluloses have been prepared by esterification, fluorination or silylation of surface OH groups with stoichiometric reagents[35]. Water repellent cellulose fibres have been obtained by transesterification of soybean oil with cellulose[36]. Hydrophobic materials have been prepared by grafting limonene and myrcene on cellulose fibres in a solvent-free process at room temperature involving plasma technology[37]. *O*-Acetylgalactomannan reacted with benzyl chloride in water in the presence of a phase transfer catalyst to produce oxygen barrier films for food packaging applications with a low moisture sensitivity[38].

The hydrophobicity of hemicelluloses has also been decreased to make biodegradable films and coatings designed for food packaging, biomedical and coating industries. This hydrophobisation was achieved by esterification with acyl chlorides, fatty acids grafting or other methods[35].

I.1.1.1.2.2.4.- Alkali-Catalyzed Reaction of Cellulose.

Carboxymethyl cellulose (CMC) is a cellulose derivative with carboxymethyl groups (-CH_2-COOH) linked to some of the hydroxyl groups of the glucopyranose monomers. It is often used as sodium carboxymethyl cellulose, which is water soluble. It is synthesized by the alkali-catalyzed reaction of cellulose with chloroacetic acid.

The functional properties of CMC depend on the DS achieved during the addition process, the chain length of the cellulose backbone structure and the degree of clustering of the carboxymethyl substituents.

CMC is used primarily because it has high viscosity, is nontoxic, and is generally considered to be a major hypoallergenic source of fibre. It is also used as a food additive (E466) as a viscosity modifier or thickener and to stabilize emulsions in various products including ice cream. CMC is extensively used in gluten free[22] and fat reduced food products. It is also a constituent of many non-food products, such as toothpaste, laxatives, diet pills, water-based paints, detergents, textile sizing and various paper products. CMC is used as a soil suspension polymer in laundry detergents. It was designed to deposit onto cotton and other cellulosic fabrics,

creating a negatively charged barrier to soils in the wash solution. CMC is used as a lubricant in artificial tears[39].

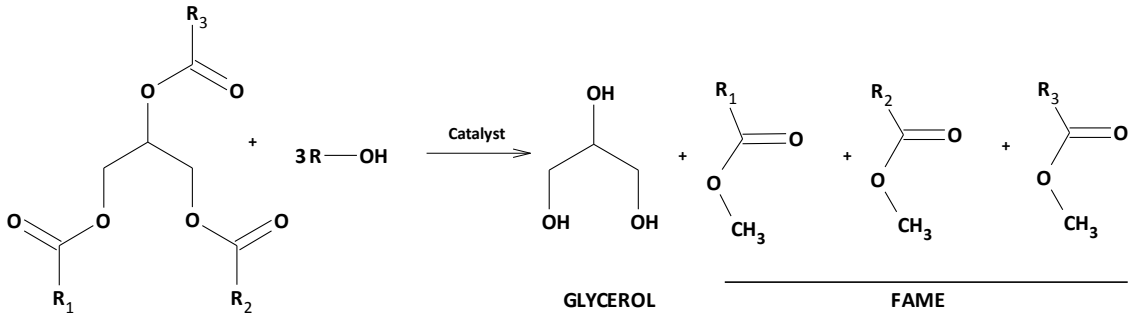
I.1.1.1.2.2.5.- Enzymatic Modification.

Enzymes, as biocatalysts, have been widely used in the transformation of carbohydrates. Glycoside hydrolases are enzymes able to transform disaccharides, oligosaccharides and polysaccharides. Cellulases, amylases, inulinases and invertases belong to this enzyme class. Carbohydrate esterases are involved in the removal of O- (ester) and N-acetyl moieties from carbohydrates. Polysaccharide lyases catalyze the β-elimination reaction on uronic acid glycosides while glycosyltransferases act forming glycosidic bonds using activated sugar donors.

I.1.2.- POLYOLS FROM BIOMASS: GLYCEROL.

I.1.2.1.- Biodiesel Industry.

Environmental concerns and crude oil prices have increased the interest for developing new renewable biofuels as novel alternative fuel sources. Biofuels have been put forward as one of a range of alternatives with lower emissions and a higher degree of fuel security. Moreover, biofuels give potential opportunities for rural and regional communities. Biodiesel, a biofuel, is produced from vegetable oils and animal fats. The process usually implies the transesterification of the acylglycerides into fatty acid methyl esters (FAME), which results in the formation of a non-negligible amount of glycerol ($C_3H_8O_3$) as a by-product, (Scheme I6). On a molar basis, one mole of glycerol is produced for every three moles of FAME. Hence, 10% of the initial reactants are roughly converted to glycerol. With increased production of biodiesel, an excess amount of glycerol is expected in the world market.



Scheme I6: General procedure of biodiesel preparation from triacylglycerides.

I.1.2.2.– Glycerol Applications.

Pure glycerol is physiologically innocuous and it is currently used in a large variety of applications because of its particular combination of chemical and physical properties. Nevertheless, the production of glycerol as a by-product is exponentially increasing resulting in an overproduction of glycerol and causing a nearly saturation of the typical glycerol market (primarily cosmetics and food and beverage industries)[8]. Consequently, the interest in developing new value added uses is increasing.

I.1.2.2.1.- GLYCEROL AS ENERGY SOURCE.

Glycerol can be used as an energy source through various approaches, among them:

I.1.2.2.1.1.- COMBUSTION.

Crude glycerol from biodiesel production has been proposed to replace fossil fuels since it could improve the economics of biodiesel production. Initially, the glycerol combustion offers added advantages of energy integration and fossil fuel substitution. However, challenges to the use of crude glycerol as a boiler fuel include its low energy density, high viscosity, and high auto-ignition temperature. Additionally, the composition of the crude glycerol can change dramatically depending upon the biodiesel feedstock (*e.g.*, vegetable oils or rendered animal fats), the catalyst used, and the degree of post-reaction clean-up (*e.g.*, acidulation and methanol elimination)[40].

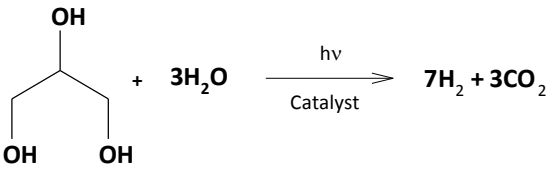
I.1.2.2.1.2.- HYDROGEN OBTENTION.

Glycerol is a potential feedstock to produce hydrogen. Theoretically, 1 mol of glycerol can lead to 4 mol of hydrogen. Hydrogen is mostly used in refinery hydro treating

operations, for ammonia production and in fuel cells. There are some different methods to obtain hydrogen from glycerol, among them:

I.1.2.2.1.2.1.- Photocatalysis.

Scheme I7 shows the heterogeneous photocatalytic H_2 production from aqueous glycerol solutions performed under mild conditions:



Scheme I7: Photocatalytic reaction of glycerol and water.

TiO_2 or gold based catalysts loaded with Pt[41], Pd[42] and CuO_x or Cu[43, 44] are the most used fotocatalyst for this transformation.

I.1.2.2.1.2.2.- Reforming.

Steam reforming is an alternative route for hydrogen production from glycerol. Nevertheless, this process is highly energy dependent and several by-products are formed depending on reaction temperature, reactants ratio, contact time and system pressure[45]. These by-products affect the final purity of the hydrogen produced. Moreover, the process must be performed at low glycerol-to-water molar ratio to minimize the deactivation of the catalysts.

The steam reforming reaction of glycerol proceeds according to the following equation:

Scheme I8: Glycerol steam reforming

I.1.2.2.1.2.3.- Pyrolysis.

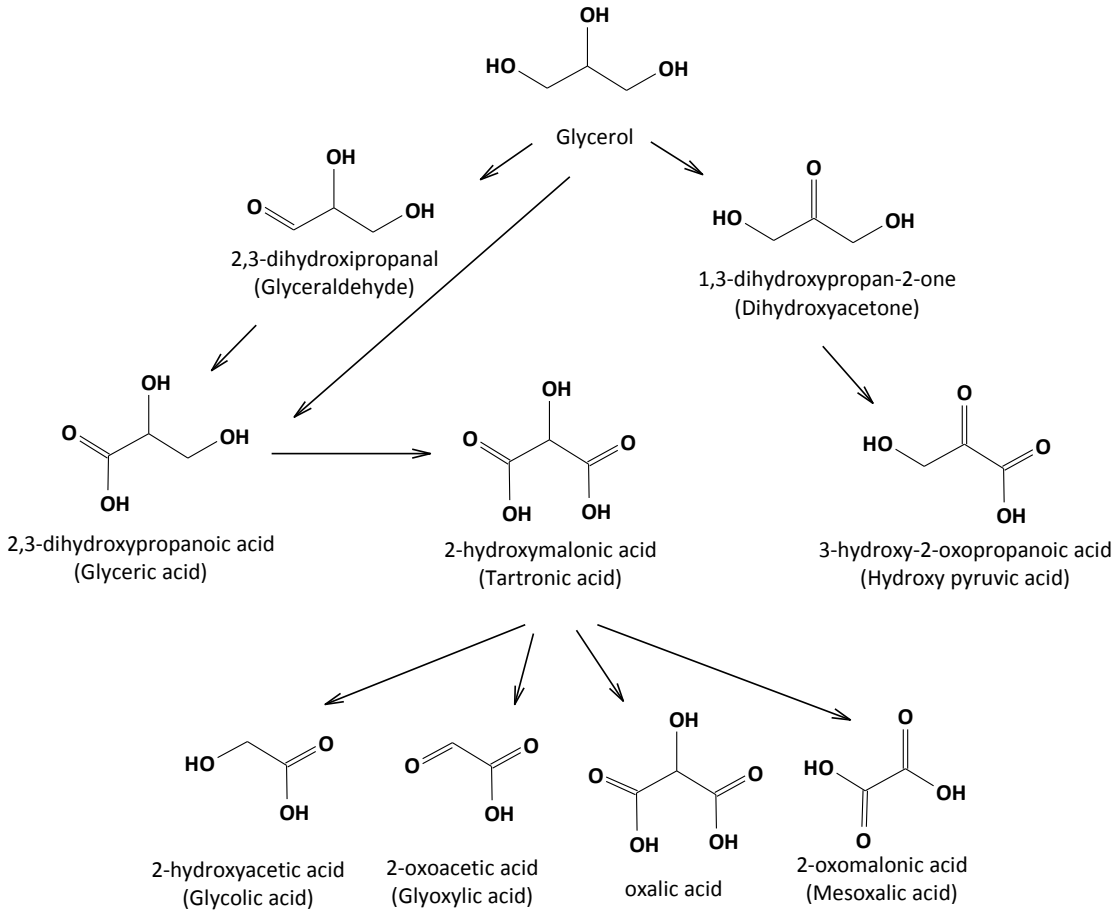
High value-added products such as hydrogen or syngas can be produced from the pyrolysis of glycerol using a fixed bed reactor. The products were mostly gas, essentially consisting of CO, H_2, CO_2, CH_4 and C_2H_4. Temperature, carrier flow rates and particle diameter of packing material have profound effects on the conversion of glycerol as well as the ratio of the final products[46].

I.1.2.2.2.– GLYCEROL AS REAGENT.

I.1.2.2.2.1.- BIOTRANSFORMATION.

Glycerol also could be used in fermentative processes to prepare various products. For example, bacteria can transform glycerol into 1,3-propanediol (PDO)[47], an useful monomer in the polymer industry. Dihydroxyacetone (DHA) can also be prepared from glycerol biotransformation[48].

I.1.2.2.2.2.- OXIDATION PRODUCTS.



Scheme I9: Products derived from glycerol oxidation.

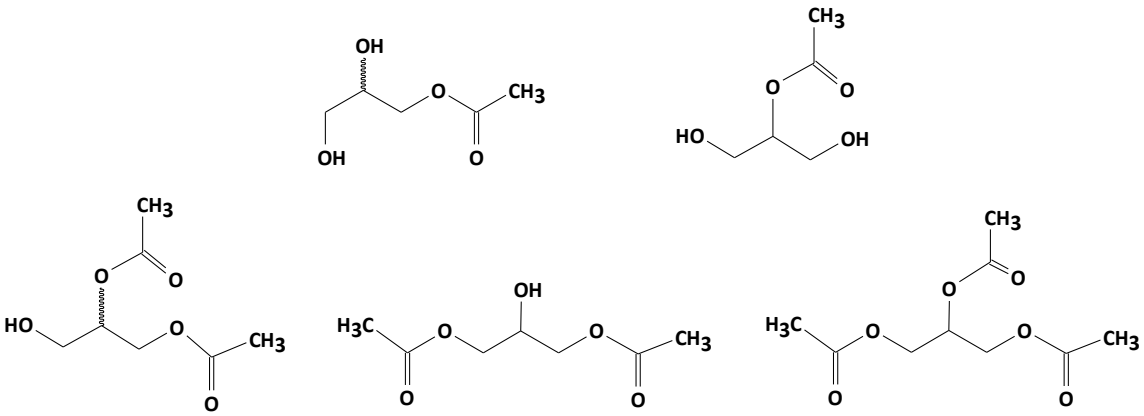
Glycerol oxidation can lead to a large number of products, through complex pathways (Scheme I9). Most of them are useful as intermediate substances or directly valuable fine chemicals. However, up to date they are produced by expensive processes and therefore their utilisation on an industrial scale is still scarce[49]. Depending on the

reaction conditions (pH, temperature, substrate to metal ratio) and the metal employed as catalyst, the reaction pathway can be directed either to the oxidation of the primary or the secondary hydroxyl groups.

I.1.2.2.2.3.– ESTERS.

Glycerol can be esterified to lead compounds such as acylglycerides and glycerol carbonates.

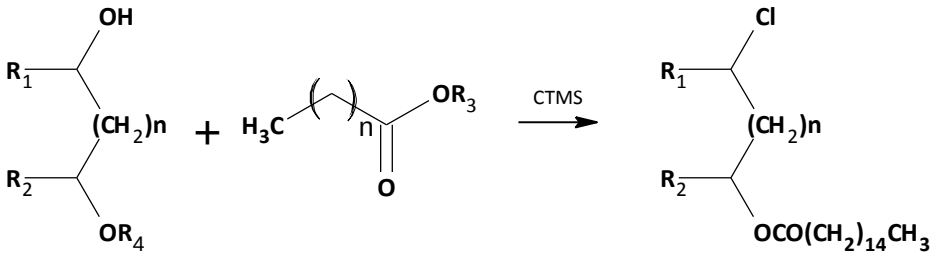
Scheme I10 shows the esterification of glycerol using acetic acid to lead monoacetylglycerols, diacetylglycerols and triacetylglycerols.



Scheme I10: Some examples of glycerol acetyl esters.

I.1.2.2.2.4.- PRIOR CONTRIBUTIOS OF THE RESEARCH GROUP.

Scheme I11 shows the one-pot synthesis of chlorohydrin esters starting from diols or glycerol and chlorotrimethylsilane (CTMS) developed in our research group. The reaction is stereospecific and its regioselectivity depends on the distance between the hydroxyl groups. The reaction is indistinctly accomplished in diols separated up to 6C atoms and using esters or carboxylic acids[50].



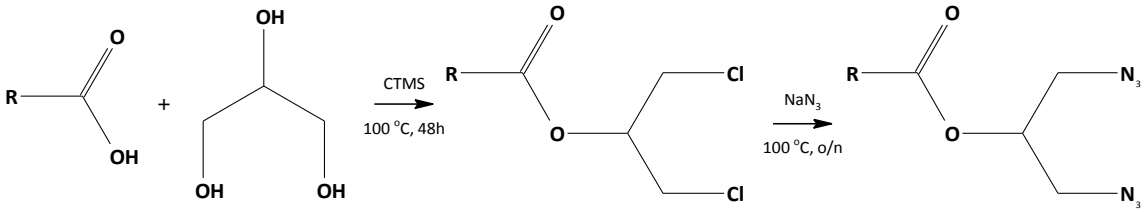
n= 0-4 ; R_1=H, CH_3; R_2= H, CH_3, CH_2CH_3, -O-, CH_2Cl; R_3= H, CH_3; R_4= H, -$(CH_3)_2$-C-

Scheme I11: Chlorohydrin ester synthesis using carboxylic derivatives, polyols and CTMS as reagents.

The alkyl chain structure of the carboxylic acid has a clear influence in the reaction regioselectivity. Long alkyl chains increase regioselectivity whereas it is decreased by electrophilic substituents[51]. Reaction temperature has also a clear influence in the regioselectivity of the reaction[52].

Finally, the influence of the α-substitution on the carboxylic acid has also been studied. Again, an increase on the degree of α-substitution lead to an increase on the regioselectivity of the reaction[53].

These compounds were used for Solarte[54] to prepare the corresponding diazides derivatives (Scheme I12). The substitution process was performed using a previously described methodology[55].



Scheme I12: Azide synthesis from glycerol and a carboxylic acid.

All the azides used as starting material in the present thesis have been synthesised using the methodology described in Scheme I12.

I.2.- AZIDES.

The azide is a functional group in organic synthesis, while, in inorganic chemistry, it is known as the N_3^- anion. This is a linear anion usually forming sodium azide, which is

prepared industrially by the reaction of nitrous oxide with sodium amide in liquid ammonia solvent (Scheme I13).

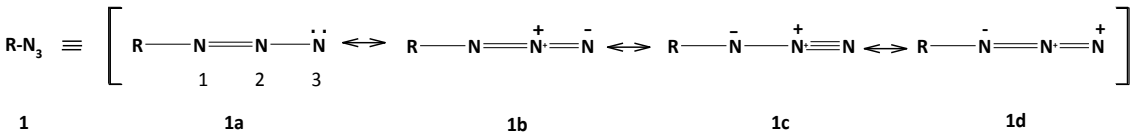
$$N_2O + 2\ NaNH_2 \rightarrow NaN_3 + NaOH + NH_3$$

Scheme I13: Industrial preparation of sodium azide.

Sodium azide, considered as a pseudohalogen compound, generally displaces leaving groups to give the azido compound. Consequently, most of the organic and inorganic azides are directly or indirectly prepared from sodium azide.

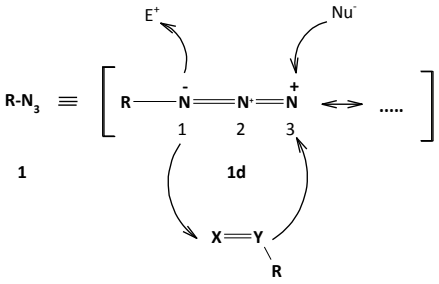
I.2.1.- AZIDES AND THEIR REACTIVITY.

Fenilazide was the first organic azide synthesized by Peter Grieß in 1864[55]. Since then, azides have taken great importance as high versatile reaction intermediates, functional groups in the pharmaceutical industry and in the synthesis of heterocycles.
Nevertheless, azides present an important problem of stability, especially the small ones which are considered explosive. This property is related to the fact that they tend to decompose releasing N_2 when heated or pressed. Hence, inorganic azides are widely used in airbags[55, 56].
The physicochemical properties presented by organic azides can be explained considering their polar mesomeric structures[57]. Scheme I14 shows how the azides tend to be stabilized by a conjugated system.



Scheme I14: Azide stabilization through a conjugated system[55].

The presence of dipolar structures as **1b** and **1c** are used to enlighten the high reactivity of this sort of compounds as 1,3-dipole[58]. This model also can also explain their instability and their easily decomposition to the corresponding nitrene and nitrogen.



Scheme I15: Organic azides reactivity.

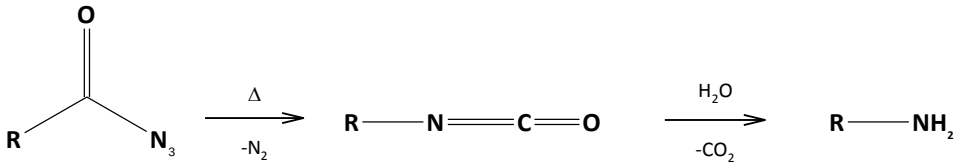
At the same time, this model can explain the regioselectivity of their reactions with nucleophiles and electrophiles. Scheme I15 shows how the mesomeric structure **1d** enlightens that the electro-donor (Nu^-) groups will react over N^3, while the electro-acceptor (E^+) groups will react over N^1.

I.2.2.- REACTIONS OF AZIDES.

I.2.2.1.- Curtius Rearrangment.

The thermal decomposition of carboxylic azides producing an isocyanate is known as Curtis rearrangement[59].

Subsequent reaction product with water leads to amines (Scheme I16).

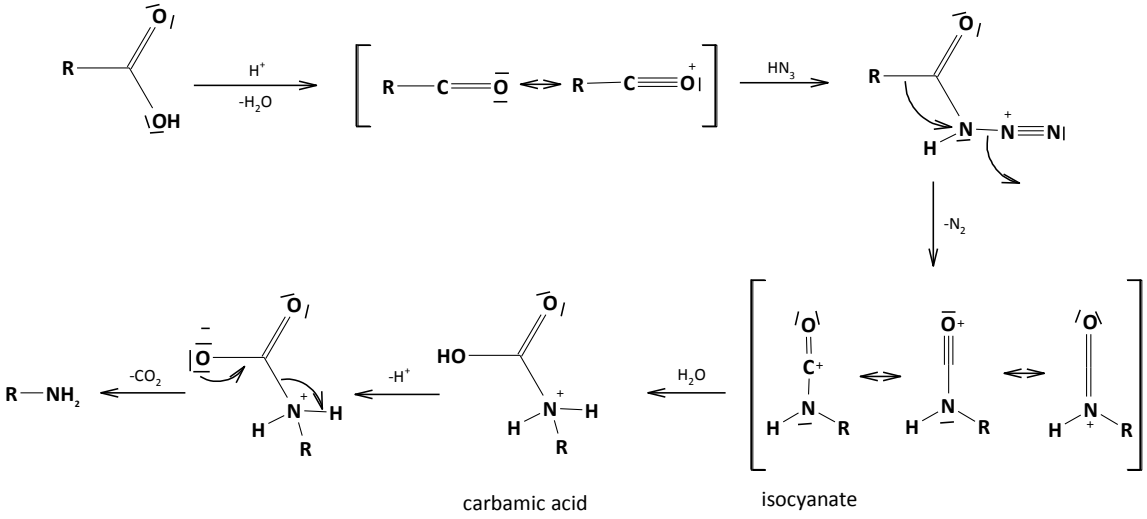


Scheme I16: Curtius rearrangement pathway.

I.2.2.2.- Schmidt Rearrangement.

The Schmidt rearrangement is an acid-catalysed reaction between a hydrogen azide and an electrophile, such as carbonyl compounds, tertiary alcohols or alkenes. Amines, nitriles, amides or imines are produced through the C migration to the azide with the consecutive elimination of N_2. This reaction is similar to the Curtius reaction described above when the starting material in a carboxylic acid, differing only in that the acyl azide in the previous reaction is prepared from the acyl halide and an azide salt.

Scheme I17 shows the mechanism of this reaction. Carboxylic acids lead to acyl azides, which rearrange to isocyanates. These may be solvolysed to carbamates or hydrolysed to carbamic acid, which decarboxylation leads to amines[59].



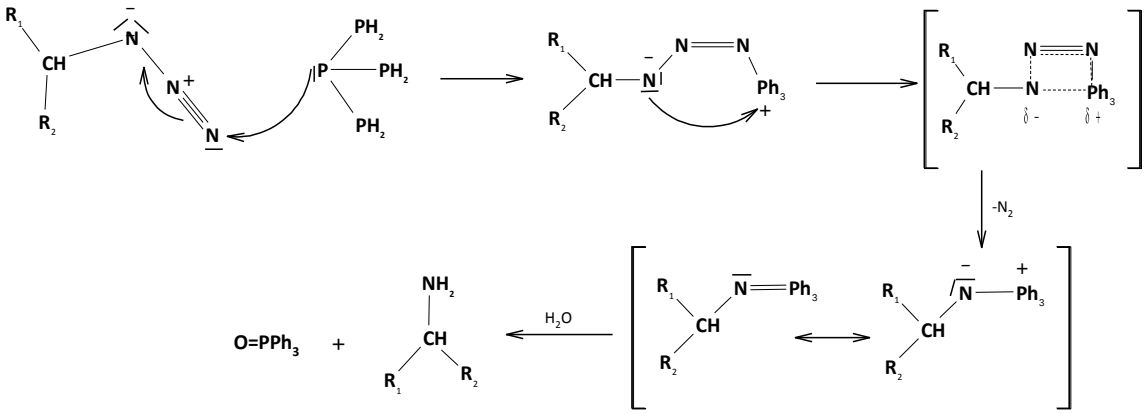
Scheme I17: Schmidt rearrangement reaction starting for a carboxylic acid and HN_3.

I.2.2.3.- Azide Reduction.

Azide reduction is profusely described in bibliography[60]. Sodium borohydride ($NaBH_4$)[61] and lithium aluminum hydride ($LiAlH_4$) are widely used reducing agents. Nevertheless, yields are not high, neither is the selectivity. Reduction yields can be improved by using hydride derivatives[62], but they are still expensive and not easy to manipulate. Metals[63-66], enzymes[67-69], triphenylphosphine (Staudinger reduction)[70-73] and hydrogenation using Pd/C systems[71, 74] are also used to prepare amines from azides.

I.2.2.3.1.- STAUNDINGER REACTION.

The Staudinger reaction is a very mild azide reduction using PPh_3 as a reducing agent. Triphenylphosphine reacts with the azide to generate a phosphazide, that loses N_2 to form an iminophosphorane[75]. Finally, the addition of water yields the corresponding amine (Scheme I18).



Scheme I18: PPh_3 reduction of azides through Staundinger reaction.

I.2.2.3.2.- ENZYMATIC REDUCTION.

Baker's yeast (*Saccharomyces cerevisiae*) has been described as a novel reducing agent of aryl azides to aryl amines in quantitative yield. The reaction is highly stereoselective[67] and is performed in exceptionally mild conditions[68]. Unfortunately, this methodology is not suitable for reducing alkyl azides since now[68].

Lipases have been also described for the chemoselective reduction of various azidoarenes to areneamines in organic media under microwave irradiation[69].

I.2.2.3.3.- METALLIC REDUCTION.

Organic azides can be reduced in the presence of metals. Palladium, platinum, nickel-Raney, Lindlar catalyst and zinc have been the most commonly used metals (0) for converting azides to amines. Although these reductive processes are easy to perform and generally inexpensive, they are poorly chemoselective[57, 76] and unsuited with many functional groups.

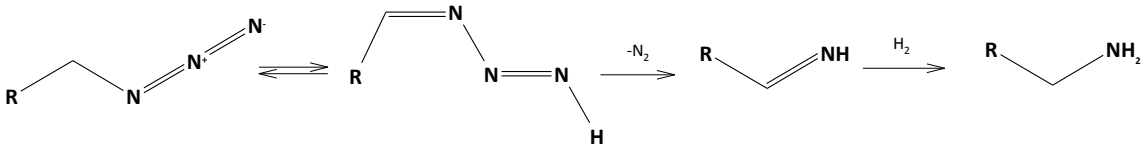
I.2.2.3.3.1.- *METAL (0).*

I.2.2.3.3.1.1.- *Catalytic Hydrogenation.*

Catalytic hydrogenation is highly atom-economic and widely employed in academia and industry. Palladium on activated carbon (Pd/C) is extensively used as a heterogeneous catalyst for hydrogenation in synthetic organic chemistry because of its

high catalyst activity, cost efficiency, easy separation from the reaction mixture, and reusability[74].

Pd/C is an useful methodology to reduce many organic groups, between them alkynes and alkenes, nitro, nitriles, imines and azide (Scheme I19) at atmospheric pressure, and even aromatic systems at high temperature and pressure.



Scheme I19: Reduction system by Pd/C-H_2.

I.2.2.3.3.1.2.- Alkaline Earth Metals.

Mg and Ca have been used in combination with MeOH as effective reducing agents of alkyl and aryl azides. The reaction is expected to be complete in 15-20 min at 0ºC. Catalytic amount of iodine can be used to accelerate the process[77].

I.2.2.3.3.2.- *METAL (0) ASSISTED BY A METAL SALT.*

Metal (0)-metal salt are usually efficient procedures to reduce alkyl, aryl, aroyl and arylsulfonyl azides with excellent yields. The metal salt is an important key factor in the system. Zn-$CoCl_2$ in THF, Zn-$NiCl_2$ in THF, Zn-$FeCl_3$ in EtOH, Fe-$NiCl_2$ in THF, Zn-NH_4Cl in EtOH: H_2O have been used for this purpose[60, 65].

I.2.2.3.3.3.- METAL AND NON-METAL SALTS.

High variety of reactions have been described in this field. A few examples are $LiAlH_4/Et_2O$, modified borohydride agents[62], $FeSO_4/NH_3$[64] and $FeCl_3$/NaI[63]. Each reducing agent gives different selectivity depending on the compound to be reduced.

I.2.2.4.- Triazol Synthesis: 1,3 Dipolar Cycloaddition.

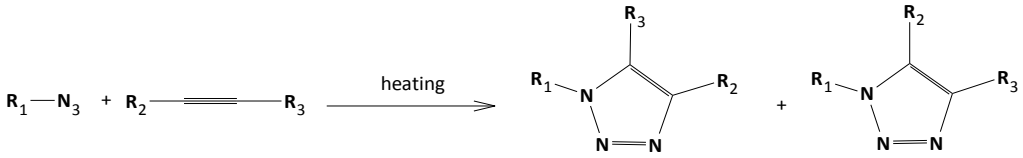
Huisgen 1,3-dipolar cycloadditions are exergonic fusion processes that bind two unsaturated reactants and provide fast access to an enormous variety of five-

membered heterocycles. The cycloaddition of azides and alkynes to give triazoles is arguably the most useful member of this family[78].

This is a kind of reaction that fulfils the criteria of a click reaction (high chemical yield, simple reaction conditions, stereospecificity)[56] and with a wide range of applications[79]. The simplicity of these 'click' reactions offer several advantages under a synthetic point of view for the functionalization or ligation of biological systems, in materials science and in combinatorial chemistry for drug discovery.

I.2.2.4.1.- CLASSICAL HUSGEIN REACTION.

Huisgen's thermal 1,3-dipolar cycloaddition between azides and alkynes is one of the most widely used methods for the synthesis of 1,2,3-triazoles as indicated above. However, the harsh conditions of this uncatalyzed cycloaddition lead to a mixture of 1,4- and 1,5-regioisomers (Scheme I20). These results are supported by the similar values of both azides and alkynes HOMO-LUMO energy levels, both dipole-HOMO- and dipole-LUMO-controlled pathways operate in these cycloadditions. As a result a mixture of both regioisomeric 1,2,3-triazole products is formed, when the alkyne is unsymmetrically substituted.



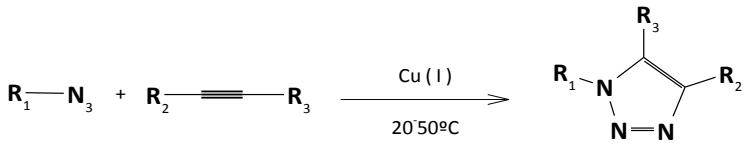
Scheme I20: Husgein's 1,3-dipolar cycloaddition.

Although the reaction is highly exothermic (*ca.* -210 to 272 kJ/mol), its high activation barrier (104.6-108.8 kJ/mol) for methyl azide and propyne[80] results in exceedingly low reaction rates for inactivated reactants even at elevated temperature. The high temperature conditions necessary to achieve the triazol formation also contributes to the synthesis of a mixture of 1,4- and 1,5-regioisomers.

I.2.2.4.2.- CuACC REACTION.

Efforts to control this 1,4- versus 1,5-regioselectivity lead to find copper(I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC). CuAAC allows the synthesis of 1,4-

disubstituted 1,2,3-triazoles as unique regioisomers (Scheme I21). This catalysis also entails an enormous rate acceleration (10^7 to 10^8 comparing to the uncatalyzed process)[80]. The methodology is experimentally straightforward and shows an enormous scope and tolerance to aqueous conditions.



Scheme I21: CuAAC reaction

It is better to prepare the catalyst in situ by reduction of Cu(II) salts ($CuSO_4 \cdot 5H_2O$ serves well). Sodium ascorbate has proved to be an excellent reducing agent. This approach allows the preparation of a broad spectrum of 1,4-triazole products in high yields and purity at 0.25 ± 2 mol% catalyst loading[79]. The reaction is finished after 6 to 36 h at room temperature in a variety of solvents, including aqueous *tert*-butyl alcohol or ethanol and, remarkably, water with no organic co-solvent[79]. Despite the widespread use of copper-catalyzed cycloaddition reactions, the mechanism of these processes has remained difficult to establish due to the involvement of multiple equilibriums between several reactive intermediates.

The Copper-Catalyzed Azide-Alkyne Cycloaddition improves the Huisgen Azide-Alkyne 1,3-Dipolar Cycloaddition in term of yield and regioselectivity. The reaction can be performed, at room temperature in a wide pH range. Many research groups have extended the potential of CuAAC by developing methods for multiple successive cycloadditions, which led to 1,2,3-triazole-oligomers. A common approach for the synthesis of higher order triazole cycloadducts involves the repetition of a two-stage process consisting of the introduction of an azido group in an appropriate substrate, followed by a CuAAC with the suitable alkyne to obtain triazole-based biomimetic oligomers or triazole linked oligonucleotides analogues. Another approach is based upon the successive CuAAC on a single scaffold, containing both the azide and the alkyne moieties[81-83].

I.2.2.4.3.- $Cu(AcO)_2$.

The influence of different copper salts and sodium ascorbate were studied. Combining copper acetate ($Cu(AcO)_2$) and sodium ascorbate allows the reaction to be quantitatively completed within 5 min. Without sodium ascorbate the reaction was completed in 10 min[84]. Sodium ascorbate is very useful as an additive for the reaction of less reactive substrates. The possibility to prepare 1,4-substituted triazoles using $Cu(AcO)_2H_2O$ at room temperature has also been described[85].

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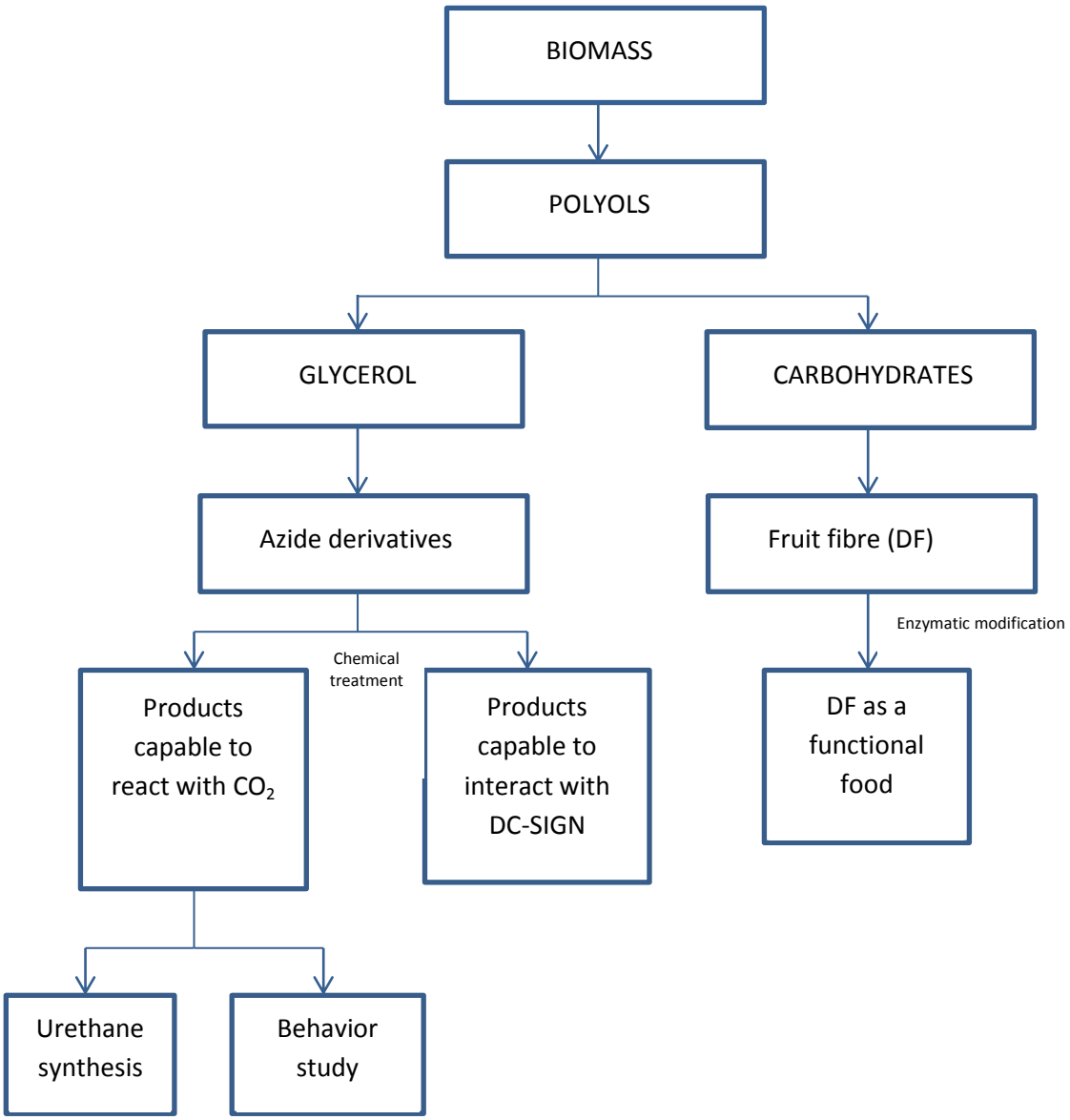
GENERAL OBJECTIVES

The main objectives of this Thesis were to prepare new derivates from polyol present on biomass. Both chemical and enzymatic technologies were used for this pourpose. The next working plan was follow to achieve these aims:



CHAPTER 1: ADDING-VALUE TO FRUIT FIBRE.

1.1.- BACKGROUND.

Every year, the variety of food processes used in food and drink industry generates food wastes on a multi tonne scale[1-5]. Residual products in the food industry are characterized by a high ratio of product-specific co-products[2, 6, 7], resulting from the extraction of the desired components from the raw material. Often, there are other potentially useful components present in the remaining materials, usually derived from seeds, peel and pulp[2, 5, 8, 9]. These materials are characterized by their high content on many valuable substances[1, 3, 6, 8].

Traditionally, several methodologies have been developed for the further utilization of that specific wastes along traditional lines, as they had little commercial value and became a disposal problem[10, 11]. Three general uses of food wastes had been: a) as animal feed (e.g., spent grains, distillery waste)[1, 12] b) as fertilizer (e.g., spent Kieselguhr, carbonation sludge)[5, 8, 13] or c) as an energy source (e.g. heat, fuel production)[10, 14, 15]. Over time, the number of new applications studied for waste and co-products has been considerably increased[16] by the implementation of new technologies capable to either recover the value-added ingredients from the co-products, or to convert them into commercial products such as raw materials for secondary processes (intermediate food ingredients), operating supplies or ingredients for new products[17, 18]

Lleida is an important producer of different fruits. The project described in this chapter has been focused to the valorisation of the fruit fibre derived from the fruit juice industry, which is generally a mixture of peel, seeds and pulp form the fruits[1, 4, 11, 19, 20]. This waste material is one of the most abundant by-products from the fruit industry and can be considered as fibre since it is mainly composed by non-digestible carbohydrates[1, 19].

These fruit fibres have currently been used as an energy source but, as they can be an interesting source of dietary fibre, we decided to study various modifications of these

fibres to improve their functional properties, so that they could be used as food ingredients[3].

1.1.2.- DIETARY FIBRE.

Dietary fibre (DF) is defined as lignin plus the polysaccharide components of plants that are indigestible by enzymes in the human gastrointestinal tract[19]. It must be noted that depending on the fibre source, and therefore the type of fruit in our case, the fibre composition may present great variability[19, 21]. Dietary fibres from cereals are more frequently used than those from fruits, however fruit fibres have better quality due to their higher total and soluble fibre contents, better water holding capacity (WHC) and lower caloric value content[22]. For example, apples are good sources of dietary fibre with a well-balanced proportion between soluble (e.g. pectin, xylose…) and insoluble fraction (e.g. cellulose, hemicellulose…)[23]. Wastes from orange juice extraction are potentially an excellent source of DF, being rich in pectin, cellulose, hemicellulose and lignin[19, 24]. Citrus and apple fibres have better quality than other DF due to the presence of associated bioactive compounds such as flavonoids, polyphenols and carotenes[1, 25]. Consequently, many studies have been performed that show a wide range of likely applications for this sort of products. Finally, the major fraction of peach peel and pulp wastes is insoluble fibre. Its high affinity for water, low caloric value and low amount of fat, protein and ash suggested peach dietary fibre could be used as food ingredient[19].

1.1.2.1.- Dietary Fibre Composition.

As we have mentioned above, dietary fibre is composed by an extensive and heterogeneous group of chemical compounds, generally derived from the plant cell wall.

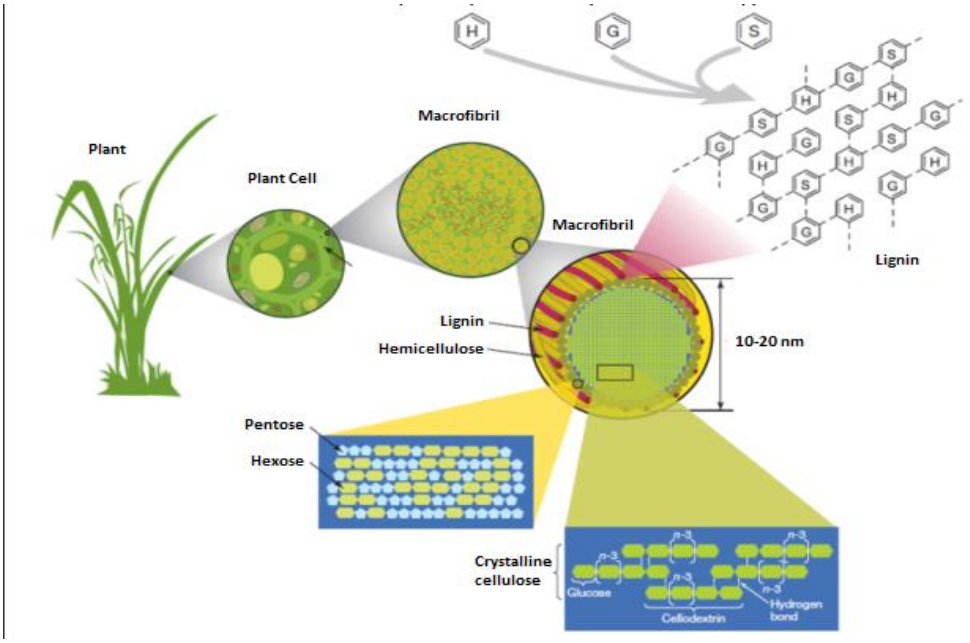


Figure 1.1: Plant cell wall structure. Source:[31].

The main role of these components is to give mechanical strength to plants, and to provide a barrier from external environment[26]. Generally, plant-derived fibres mainly include cellulose, which is usually entangled with hemicellulose, lignin and pectin[5, 9, 19, 27, 28]. Figure 1.1 shows their contribution to the cell wall structure[26, 29, 30]. They confer the main physicochemical properties characteristic of each fibre[29], and their modification can lead to a behaviour changes.

1.2.1.1.1.- CELLULOSE.

Cellulose is an important structural compound of the primary cell wall of green plants, being considered the most abundant organic polymer on Earth[28]. It is formed by glucopyranose monomers linked by β-1→4-glycosidic bonds achieving linear structures[28]. These structures show strong interactions among them through hydrogen bond formation[28] thus allowing the formation of 2D structures that can present two distinct regions, a very highly ordered region (crystalline region) and a less ordered region (amorphous region) (Figure 1.). These structures confer very different properties to the material, being the crystalline region much less accessible to enzymatic and chemical modifications[28].

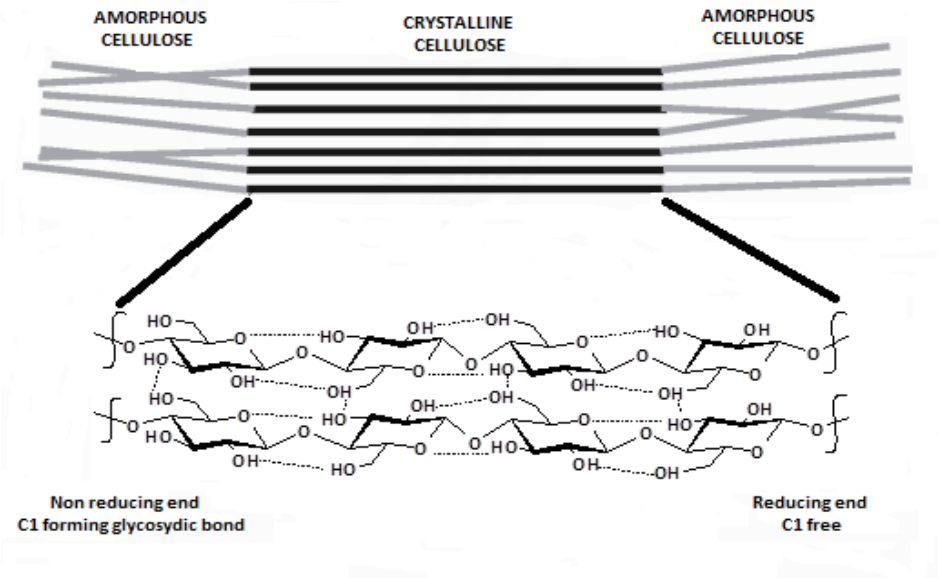


Figure 1.2: Supramolecular structure of cellulose, the solid state is represented by areas of both high order (crystalline) and low order (amorphous): source[32].

Many properties of cellulose also depend on its chain length or degree of polymerization. Cellulose from wood pulp has typical chain lengths between 300 and 1700 units; cotton and other plant fibres as well as bacterial cellulose have chain lengths ranging from 800 to 10,000 units[28].

1.2.1.1.2.- HEMICELLULOSE.

Hemicellulose plays the biological role of contributing to strengthening the cell wall by interacting with cellulose and, in some cases, with lignin[30]. It is a heteropolysaccharide characterized by a β-(1→4)-linked backbone with an equatorial configuration at C1 and C4. There exist some specific sorts of hemicelluloses that also present β-(1→3) bonds, known as β-(1→3,1→4)-glucans[30]. Hemicelluloses include xyloglucans, xylans, mannans and glucomannans and, as stated, β-(1→3,1→4)-glucans[30]. The detailed structure of the hemicelluloses and their abundance varies widely between species and cells. It has a random, amorphous structure difficult to determine that mainly contains D-pentose sugars and, occasionally, small amounts of L-sugars. Among them: xylose, mannose, rhamnose, and arabinose and also some acidified forms of C6 sugars such as glucuronic and galacturonic acids[30]

.

1.2.1.1.3.- PECTIN

The term pectin comprises a family of complex polysaccharides that are found in high amounts in plant primary cell wall. Figure 1. shows the structure of homogalacturonan (HG) ramnogalacturonan I (RGI) and ramnogalacturonan II (RGII) (Scheme 1.3), which are the three big groups that constitute pectin structure[33].

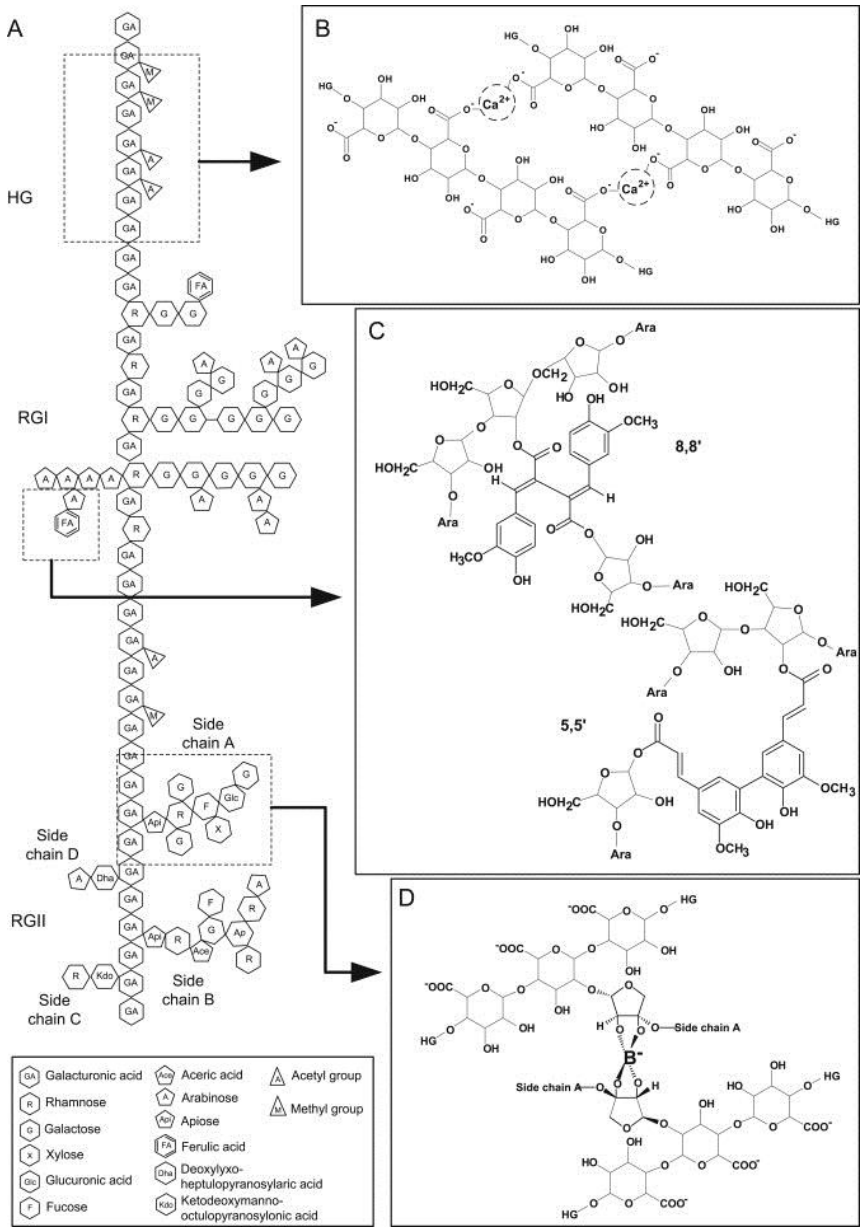


Figure 1.3: Structure of pectin. Basic structures are: HG: Homogalacturonan; RGI: Rhamnogalacturonan I: RGII: Rhamnogalacturonan II. And (A) Structure of pectic polysaccharides backbone and side chains, and three types of covalent cross-links by (B) calcium bridges, (C) ferulic acid oxidation and (D) RGII-borate ester[33].

Homogalacturonans (HG) constitute near to the 65% of the pectin molecule[26]. HG are linear chains of α-(1→4)-linked D-galacturonic acid partially methylesterified at the C-6 carboxyl group. HG may also be *O*-acetylated at *O*-2 or *O*-3[34].

Rhamnogalacturonan I (RG-I) constitute near 20–35% of the pectin molecule[26]. RG-I contains a backbone of the repeating disaccharide: 4-α-D-galacturonic acid α-(1→2)- L-rhamnose with different side chains containing various neutral sugars mainly branching off, from rhamnose residues. These neutral sugars include D-galactose, L-arabinose and D-xylose. Their relative amounts differ with the origin of the pectin[26].

Rhamnogalacturonan II (RG-II) is the third structure present in the pectin molecule. It is a very complex and highly branched polysaccharide less frequent than the ones described above. Rhamnogalacturonan II is classified by some authors within the group of substituted galacturonans since the rhamnogalacturonan II backbone is made exclusively of D-galacturonic acid units[26].

1.2.1.1.4.- LIGNIN.

Lignin is the most abundant naturally occurring aromatic polymer and, following cellulose, the second most abundant organic polymer on Earth. It comprises 20–35% of the dry weight of plant cell wall, depending on the biomass source[35]. It surrounds and crosslinks the cellulose–hemicellulose matrix through lignin–carbohydrate network structures[36]. Lignin is composed of phenylpropanoid units known as monolignols or lignin precursors that are linked together through carbon–carbon and carbon–oxygen bonds with a varying degree of methoxylation[36, 37]. There are three monolignol monomers, methoxylated to various degrees: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol[38]. These monolignols produce respectively *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), phenylpropanoid units when incorporated into the lignin polymer[37-39], with various types of substructures that appear to repeat in a haphazard manner (Figure 1.4).

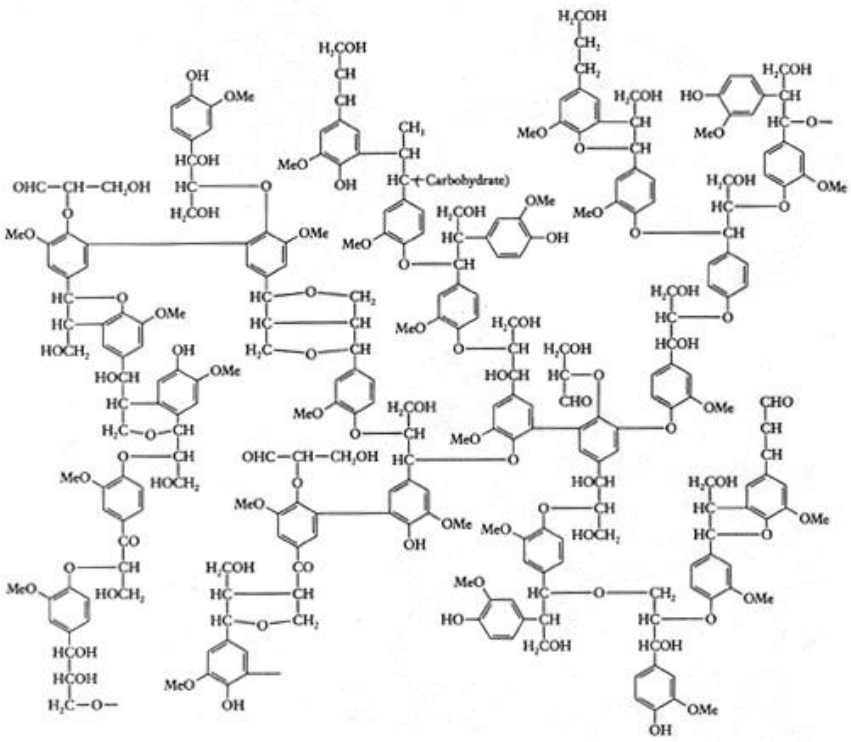


Figure 1.4: An example of a possible lignin structure. Source: [36]

1.2.1.1.5.- FERULIC ACID AS A CELL WALL POLYSACHARIDE CROSS-LINKER.

Ferulic acid is one of the most abundant phenolic acids in plants, varying from 5 g/kg in wheat bran to 9 g/kg in sugar-beet pulp and 50 g/kg in corn kernel[40]. or up to 0.09-0.19 g/kg in oranges and greater than $2.5x10^{-3}$ g/kg in apples[41]. In plants, ferulic acid is rarely found in the free form. It is usually found as ester cross-links with polysaccharides in the cell wall, such as arabinoxylans in grasses, pectin in spinach and sugar beet and xyloglucans in bamboo[40].

Particularly in fruits, ferulic acid is bound to α-L-arabinofuranose and β-D-galactopyranose hydroxyl residues in Rhamnogalacturonan I structure of pectins, and can facilitate a phenolic ester oxidative cross-linking between feruloyl groups on the side chains of RGI[33] (Figure 1.3(C)). Physicochemical properties of pectins and notably their gelation ability rest on different kinds of cross-linking mechanisms involving different structural entities of the pectin molecule(s) as can be the case of phenolic ester oxidative cross-linking between feruloyl groups on the side chains of see (Figure 1.3(C))[33].

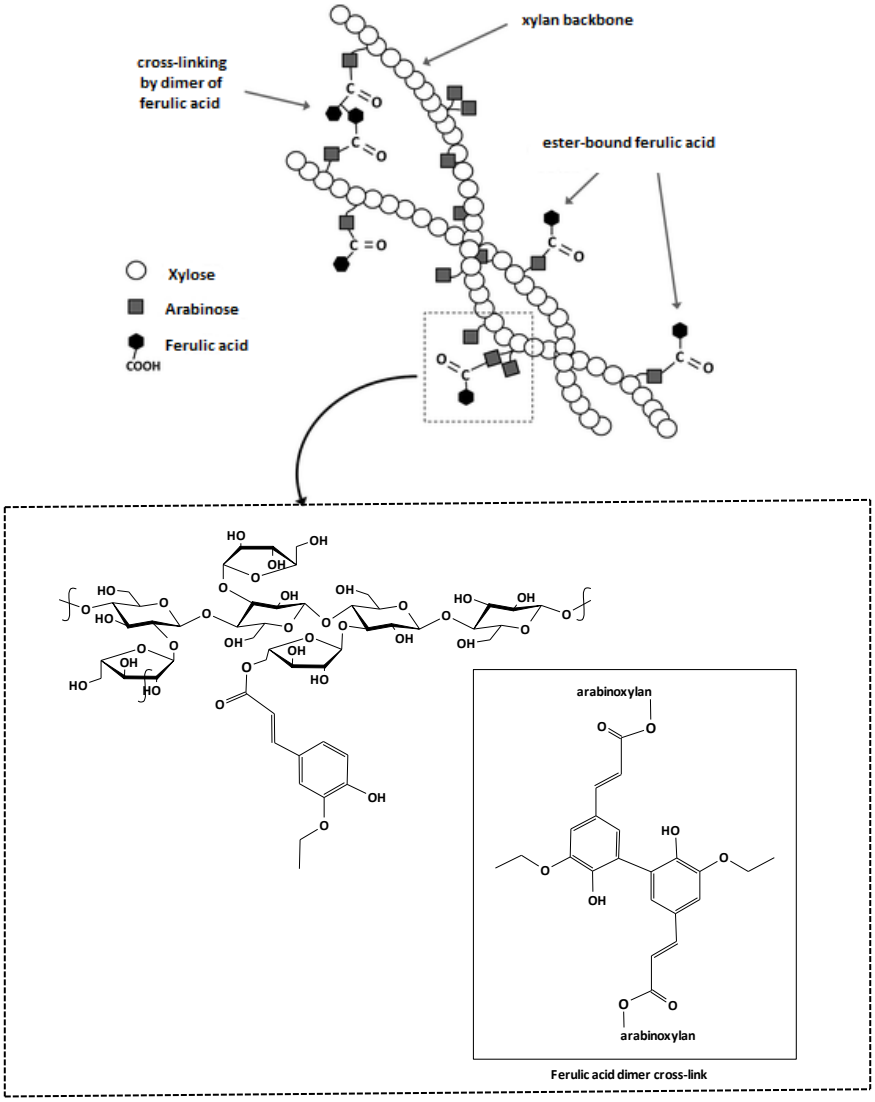


Figure 1.5: Structure of arabinoxylan. The inset shows one type of ferulic acid dimer that can be formed. Source:[45, 46]

Ferulic acid moieties also exist in arabinoxylans, one of the most abundant hemicellulose polysacharide[40, 45, 46]. Ferulic acid moieties usually cross-link the arabinoxylan chains by the formation of ferulic acid dimers (Figure 1.5). Ferulic acid is also involved in the linking of hemicellulose to lignin[43, 44].

1.1.2.2.- Dietary fibre as Food Ingredient.

It is well known that the addition of dietary fibre to food can modify its properties. It can change the consistency, texture, rheological behaviour and sensory characteristics of the final products where it is used as a functional ingredient[47]. The addition of fibre to food can reduce production costs, improve quality, flavour and functional properties, as well as improve shelf life[3, 6, 11, 27, 48]. Therefore, fibres are

ingredients with great interest to the food industry[21, 47] not only due to their possible application as functional ingredients but also because their use in foods has been considered beneficial to health[49] as their consumption has been associated with the prevention, reduction and overcoming of some diseases[49, 50]. In this regarding, an increase in daily consumption of dietary fibre has been recommended (25–30 g/day). Because of this, it is interesting to increase the consumption of foods considered as dietary fibre sources. Fibre incorporation in frequently consumed foods can help to overcome the fibre deficit[11].

However, raw edible fibres not always match the desired technological properties needed for different food applications. Because of that, often raw fibres cannot be added to foodstuffs in relevant amounts. In addition, they frequently tend to impair unpleasant textures and colours making its incorporation a real challenge[47]. Dietary fibre modifications have been studied with the objective to improve its functional properties. Functional properties of fibres can be improved using various treatments such as chemical[52-54], enzymatic[55, 56] and physical[57] ones.

1.1.3.- ENZYMATIC MODIFICATION OF FIBRES.

The use of enzymes instead of other treatments to modify fruit fibres can improve the process and the final product. Indeed, the enzymes are effective under mild reaction conditions, they are easy to inactivate, it is easy to tune their activity by controlling the temperature of the medium, and they can show high effectiveness at relatively low concentrations. There are few studies dealing with enzymatic treatments applied to fruit or vegetable dietary fibres, and they are generally focused on the extraction of one of the compounds present on the main bulk[58, 59]. However, the literature concerned with the effects of enzymatic treatments on the physicochemical properties of fibres is scarce. Moreover, different materials, different measurement methodologies and different process conditions have been used to perform these studies[22], which lead sometimes to contradictory results.

The present chapter aims to study various enzymatic treatments applied to fruit fibres to improve their technological properties and therefore their applicability in the food

industry. The enzymes were selected taking into account its activity and the described composition of dietary fruit fibres.

1.1.3.1.- Selected Enzymes.

As stated, the approximate composition described in the literature for the available fibres was taken into account to select a range of enzymes, which could modify different components of those fibres. The chosen enzymes were the following:

- Pectin methyl esterase (PME).
- Polygalacturonase (PG) and pectin lyase (PL).
- Cellulases (CEL).
- Feruloyl esterase (FER).

1.1.3.1.1.- PECTIN METHYL ESTERASE (PME).

Pectin esterases (EC 3.1.1.11) are hydrolase type enzymes that catalyses the hydrolysis of methyl ester linkages of galacturonan backbone to release acidic pectins and methanol (Figure 1.6).

In this study we have used two PME from two commercial sources, with no side activities. Both companies described the same activity for these enzymes. It must be taken in account that PMEs from different sources usually lead to different demethoxylation patterns[33].

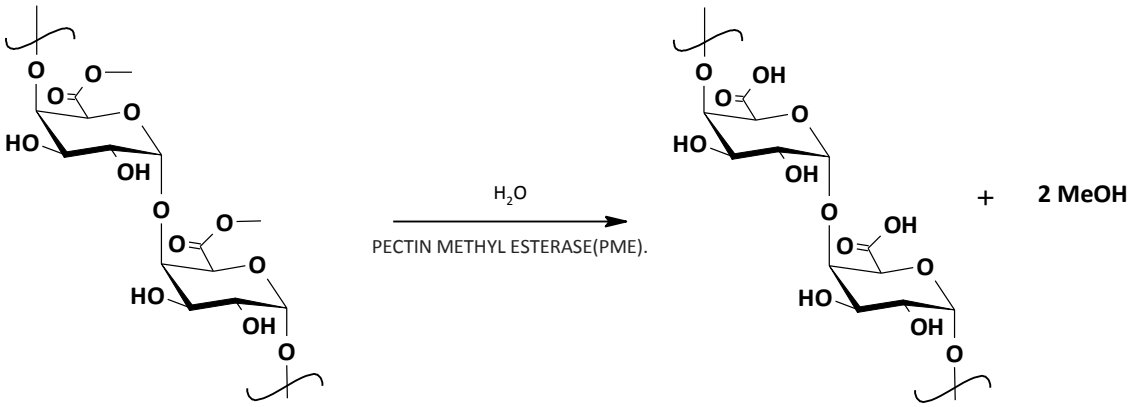


Figure 1.6: PME mode of action

Indeed, the gelling properties of pectins are influenced by the chain length, the pattern and degree of methoxylation (DE)[60], the Ca^{2+} level and the pH. Low methylated (LM) pectins (DE<50%) easily cross-link with Ca^{2+} to form a gelling network while highly methylated (HM) pectins (DE>50%) gel under low pH and high sugar concentration. Demethoxylation of HM pectins has been shown to increase their ability to be cross-linked by Ca^{2+} [33] forming firm gel structures (Figure 1.7).

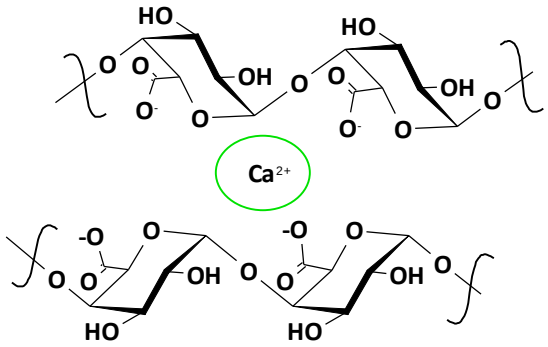
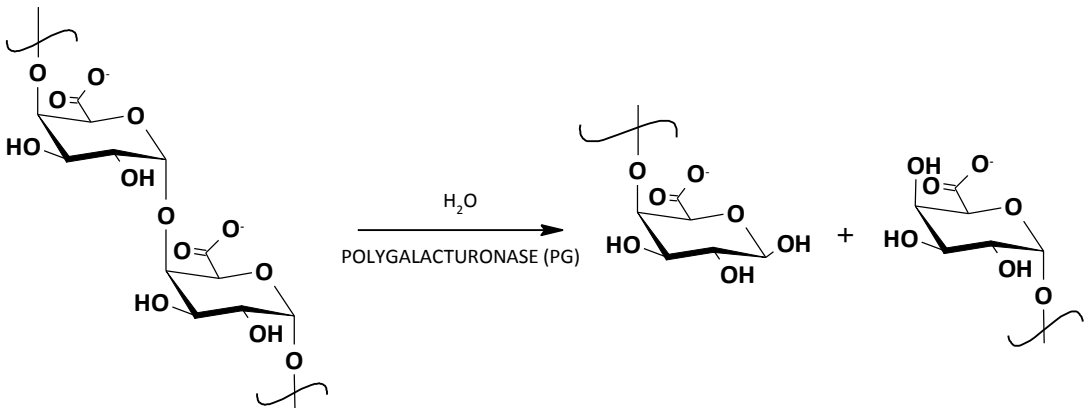


Figure 1.7: LM pectin cross-linking with Ca^{2+}.

1.1.3.1.2.- POLYGALACTURONASE (PG) AND PECTIN LYASE (PL).

These enzymes cleave the α-(1→4) links in the galacturonic acid skeleton of pectins. Nevertheless, pectin lyases (EC 4.2.2.10) perform a non-hydrolytic breakdown characterized by a *trans*-eliminative water split, while polygalacturonases (EC 3.2.1.15) catalyse the hydrolytic cleavage of the polygalacturonic acid chain with the addition of water across the ketal linkage[42](Figure 1.). In this study, endopectinlyases (EC 4.2.2.10) and endopolygalacturonases (EC 3.2.1.15) were used.



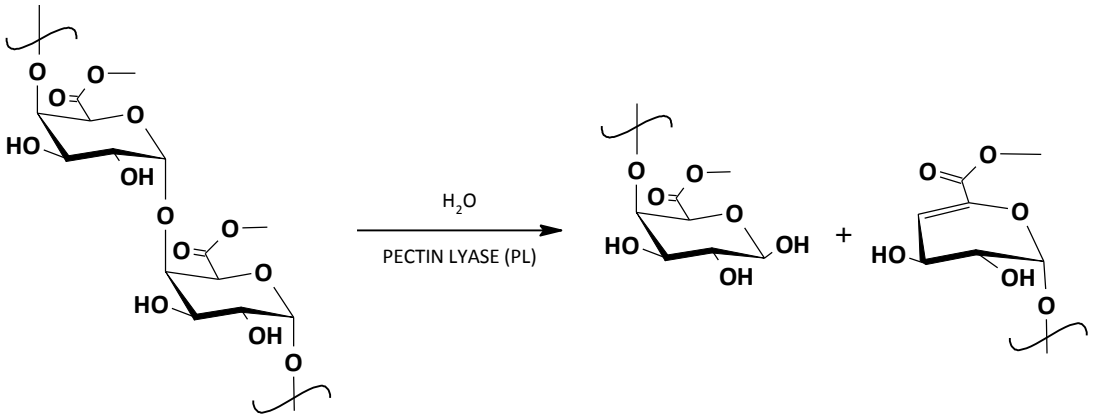


Figure 1.8: Action of depolymerizing enzymes.

1.1.3.1.3.- CELLULASES.

Cellulases (EC 3.2.1.4) are a group of enzymes able to catalyse the hydrolysis of cellulose by breaking their ß-(1→4) glycoside bonds. Commercial cellulases are a mixture of different enzymes with cellulose activity and associated side activities such as cellobiase, ß-glucosidase and ß-glucanase. The complementary activity of these enzymes can result in a complete cellulose breakdown (Figure 1.).

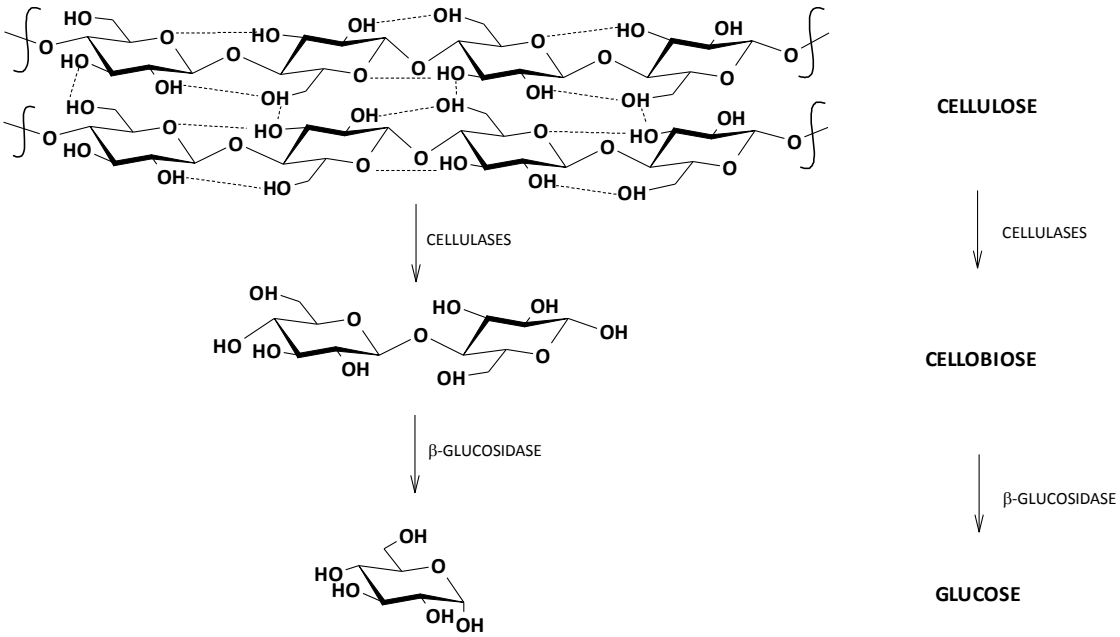


Figure 1.9: Possible reactions of cellulose and side activity enzymes.

1.1.3.1.4.- FERULOYL ESTERASE.

Feruloyl esterases (EC 3.1.1.73) are enzymes that catalyse the hydrolysis of ferulic acid ester side chains attached to the skeleton of pectins and arabinoxylans[33,45].

Feruloyl esterases are involved in the degradation of plant cell walls[45], breaking the cross-links by hydrolyzing the ester bond between ferulic acid and the main chain (Figure 1.10), as for example arabinose, releasing arabinoxylan[45]. Hence, the accessibility of the cell-wall hydrolases to their substrates is increased. Therefore the use of such enzymes may be useful to hydrolyse recalcitrant areas of cell walls favouring the possibility of an increased breakdown of the fibres. Ferulic acid can be used in the production of compounds of commercial interest, e.g. *p*-coumaric acid, which is commonly used in sun creams, or vanillin, a flavouring agent widely used in the food industry[45].

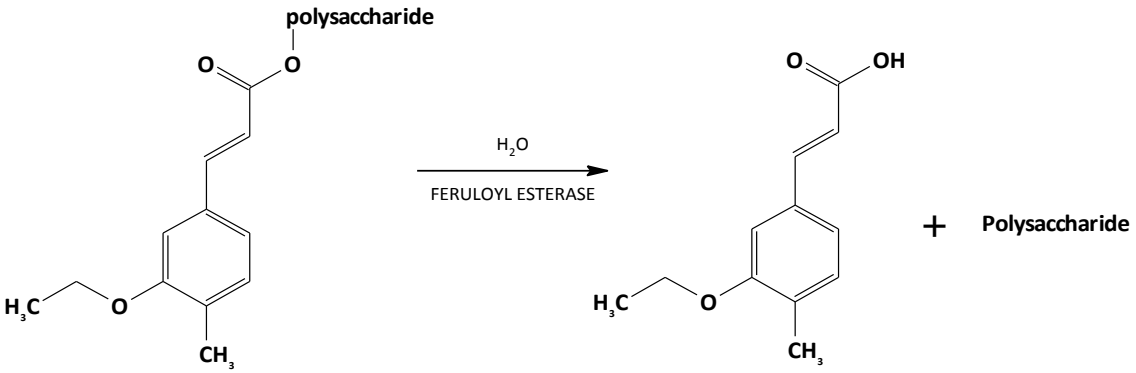


Figure 1.10: Feruloyl enzyme mode of action

1.1.4.- STUDY OF THE PHYSICOCHEMICAL PROPERTIES.

To assess the effect of enzymatic treatments on the technological properties of fibres, water holding capacity (WHC) and swelling water capacity (SWC) are usually determined. These are important parameters to take into account for the implementation of fibres as food ingredients, as they refer to its ability to retain and swell water within the matrix[25]. When fibres increase these properties, viscosity is enhanced or some other organoleptic properties are improved when fibre is added to food[47]. Specifically, WHC is the ability of a moist material to retain water within its matrix when subjected to an external centrifugal gravity force or compression[61]. It

consists of the sum of linked water, hydrodynamic water and physically trapped water, the latter of which most contributes to this capacity[62]. WHC is an important property of dietary fibre from both physiological and technological points of view. Fibres with high WHC have potential applications to products that require hydration, viscosity development, and freshness preservation such as baked foods or cooked meat products.

On the other hand, SWC indicates the fibre matrix swells widespread when water is absorbed.

Hydration properties of dietary fibres depend on factors such as the chemical structure of the polysaccharides present in the fibre[4, 63, 64] the water affinity of its components, the capillary structures of the fibre where water holds as a result of surface tension strength[65] or and the water interaction through hydrogen bonding or dipole forms. Other factors such as porosity, particle size, ionic form, extractive conditions or pH[66] can also affect hydration properties.

1.2.- OBJECTIVES.

The aim of this study was:

-To evaluate various treatments applied to fruit fibre in order to modify the fibre and improve its functional properties and therefore its applicability in the food industry.

1.3.- MATERIALS AND METHODS.

1.3.1.- REAGENTS AND EQUIPPMENT.

Raw materials were fibres resulting from the fruit juice extraction process performed by a local juice industry. Fruit fibres were from citrus (citrus N, citrus M), apple (apple N, apple M) and peach (peach N).

Six different food grade enzymes were selected, all of them supplied by the fruit company and selected among those they use in their processes. Two of them were pectin methylesterases: PME A from *Aspergillus niger* (100 U/g) and PME B (7 U/g) from food grade *A. oryzae modified with* the *A. aculeatus* gene encoding this pectin esterase. Also, two pectinases PEC A from *Aspergillus sp* (2,200 U/g), and PEC B from *Aspergillus sp*. (600 U/g), were used, both with endogalacturonase activity, but with different poligalacturonase: pectin lyase ratios (higher in PEC A). Finally, a cellulase (CEL) (1,500 U/g) activity from *Trichoderma sp.* and a ferulic acid esterase (FER) (36 U/g) from *Humicola sp.* were used.

FT-IR spectra were recorded on a Jasco FT/IR-6300, in a range 600 to 4000 cm^{-1}, prepared to take 60 spectra /second with 16 cm^{-1} resolution and working as attenuated total reflectance (ATR).Water activity (a_w) was recorded in an AQUA LAB at 25°C with an accuracy of ±0.0003 a_w while pH was measured with a pHmeter model 210, Crison 2002

1.3.2.- METHODS .

1.3.2.1.- Enzymatic Treatments.

1.3.2.1.1.- ASSAYS WITH VARIOUS ENZYMES.

Enzymatic solutions were prepared to meet the working concentration recommended by each manufacturer.

Enzymatic reactions were conducted using a 15 g of dry fibre suspension in 150 mL of the previously prepared enzymatic solution. The suspension was stirred to obtain a homogeneous mixture and the pH was measured. Reaction time and temperature were selected according to each enzyme manufacturer's recommendation. The suspension was kept under agitation at 140 rpm in an orbital shaker during the time selected in each case.

After the enzymatic treatment, the enzymes were inactivated by heat during the time and at the temperature recommended by the manufacturer. The pH was measured again after the enzyme inactivation.

Treated fibre suspensions were frozen at -80 ° C for 24h and lyophilized for 72 h to remove the water. Modified fibres were crushed and sieved to obtain a homogenous particle size. Each physicochemical analysis was performed in triplicate.

Finally, water activity and FT-IR spectra were measured for each sample.

Blank samples were obtained following the same protocol but without the addition of enzyme.

1.3.2.1.2.- PRELIMINARY TEST TO EVALUATE THE EFFECT OF Ca^{2+}.

Enzymatic solutions of PME were prepared to meet the working concentration recommended by the manufacturer in four different solvents: deionized water, tap water, and water containing 10 g/100 mL or 20 g/100 mL of $CaCl_2$.

Enzymatic reactions in each solvent were conducted using a suspension of 6 g of dry fibre in 40 mL of the previously prepared enzymatic solution. The suspension was stirred to obtain a homogeneous mixture and the pH was measured. Temperature was set at 50ºC according to manufacturer's recommendations. The suspension was kept under agitation at 140 rpm in an orbital shaker for 24 and 60 h.

After the enzymatic treatment, the enzymes were inactivated by heat during the time and at the temperature recommended by the manufacturer. The pH was measured again after the enzyme inactivation.

Treated fibre suspensions were centrifuged at 5000 g for 10 min, the supernatant decanted, frozen at -80°C for 24 h, and then lyophilized for 72 h until to remove the water.

Modified fibres were crushed and sieved to obtain a homogenous particle size. Each physicochemical analysis was performed in triplicate.

1.3.2.2.- Characterization of Fruit Fibres. Technological Properties.

1.3.2.2.1.- WATER HOLDING CAPACITY (WHC .)

WHC was determined according published methods with some modifications[1, 63].

Fibres (1 g) were hydrated with 30 mL distilled water in a 50 mL Falcon tube at room temperature. After equilibration (18 h), samples were centrifuged (3,000 x g; 20 min). The supernatant was decanted, the sample transferred to a weighed Petri dish and the fresh weight of the sample was recorded. The sample was dried in an oven at 60ºC to constant weight. WHC was calculated as the amount of water retained by the pellet (g/g) as shown in Equation 1.1.

$$\mathrm{WHC(g/g)} = \frac{\text{Residue fresh weight} - \text{Residue dry weight}}{\text{Residue fresh weight}}$$

Equation 1.1: Determination of the WHC value.

In order to compare the effect of enzymatic treatments, % of WHC modification was calculated as stated in equation 1.2.

$$\%\text{WHCmodification} = \frac{\text{WHCenzymatically treated} - \text{WHCnon} - \text{enzymatically treated}}{\text{WHCnon} - \text{enzymatically treated}} x100$$

Equation 1.2: Equation to calculate % of WHC modification.

1.3.2.2.2.- SWELLING WATER CAPACITY (SWC).

SWC was determined according to published methods with some modifications[1, 63]. Fibres (0.1 g) were hydrated with 10 mL distilled water in a 15 mL calibrated Falcon tube (1.5 cm diameter) at room temperature. After equilibration (18 h), the wet volume was recorded and expressed as volume/g original fibre dry weight. SWC was calculated as shown in Equation 1.3.

$$\text{SWC (mL/g)} = \frac{\text{Volume occuped by the sample}}{\text{Origianl sample dry weight}}$$

Equation 1.3: Determination of SWC values.

In order to compare the effect of enzymatic treatments, % of SWC modification was calculated as stated in equation 1.4.

$$\%\text{SWCmodification} = \frac{\text{SWCenzymatically treated} - \text{SWCnon} - \text{enzymatically treated}}{\text{SWCnon} - \text{enzymatically treated}} x100$$

Equation 1.4: Equation to calculate % of SWC modification.

1.3.2.2.1.- FTIR SPECTRA.

FTIR spectra were recorded for all samples once completely dried. The spectra manager software was used to calculate the peak area between adjacent valleys.

1.4.- RESULTS AND DISCUSSION.

1.4.1.- SELECTED FIBRES.

The study was conducted using 5 fibres. APPLE N, PEACH N and CITRUS N were obtained directly after the juice extraction without any further treatment. They were fruit fibres from apple, peach and a mixture of citric fruit fibres. APPLE M and CITRUS M were physically modified fibres. The modification was previously carried out by the fruit juice company before being supplied. These fibres were from apple and a mixture of citric fruit fibres, respectively.

Table 1.1 shows the WHC and SWC for the fibres used in this project as received from the fruit juice company.

Table 1.1: WHC and SWC of the fibres used in this project.

	WHC (g water/g dry fibre)	SWC (mL/g)
CITRUS N	11.10 ± 0.68	9.60 ± 0.64
CITRUS M	14.77 ± 0.75	16.28 ± 0.47
APPLE N	13.75 ± 0.68	12.26 ± 0.70
APPLE M	14.70 ± 0.51	13.72 ± 0.42
PEACH N	14.38 ± 0.83	10.47 ± 0.58

Peach showed the highest WHC value of the native (N) fibres studied. This values is similar to those found in the literature[19]. Apple fibre shows higher value than citrus. Although it may seem contrary to what it is found in the literature[19], we should state that published data usually refers to orange fibre while the citrus fibre used in this project was a mixture of different citrus fruits.

The other two supplied fibres, APPLE M and CITRUS M modified obtained by the company after a physical treatment, presented higher values than their non-modified counterparts. CITRUS M showed an important increase in WHC value compared to CITRUS N while the difference between apple N and M was smaller.

SWC relative values were quite similar to those of WHC. Values for native (N) fibres were lower than those of physically modified (M) ones indicating that whatever the physical treatment the company applies, those two properties improve compared to

the values of native fibres. Again, citrus fibre showed a greater improvement than apple fibre in SWC after this physical treatment.

1.4.2.- ENZYMATIC TREATMENTS.

The effect of these treatments was assessed by determining the technological properties (WHC, SWC) of the modified fibres. These results were compared with those of the corresponding fibres treated under the same conditions but without including the enzyme in the treatment and the final result was given as the degree of modification obtained for each property in each case.

The pH control performed at the beginning and at the end of the treatment. Shows a pH maintenance within the working range throughout the treatments with very little modifications.

Water activity of the fibres was also determined at the beginning and after the lyophilisation step done at the end of each treatment. Similar values were obtained for each fibre under each treatment.

1.4.2.1.- Evaluation of the Effect of Ca^{2+} on PME Treatments.

The effect of the treatments with PME A was described by the manufacturers to be influenced by divalent ions such as Ca^{2+}. Thus, various assays were performed controlling the presence of Ca^{2+} in order to evaluate its effect.

Initially, the effect of PME A was assayed with CITRUS. Tables 1.2 and 1.3 show the results for WHC and SWC values. Once the preliminary treatments were performed, the best operating conditions were selected and the study with PME A controlling the Ca^{2+} content was carried out with all fibres.

Table 1.2: WHC after the modification of CITRUS M fibres at 50°C with PME A in 4 reaction media.

WHC (g water/g dry fibre)	24h	60h
DEIONIZED WATER	15.7 ± 0.66	16.66 ± 1.02
TAP WATER	15.83 ± 0.34	17.66 ± 0.37
DEIONIZED WATER + 0.6M calcium chloride	10.15 ± 1.12	12.33 ± 0.28
DEIONIZED WATER + 1.2M calcium chloride	10.99 ± 1.00	13.21 ± 0.32

Table 1.3: SWCafterr the modification of CITRUS M fibre at 50°C with PME A in 4 reaction media.

SWC (mL/g)	24h	60h
DEIONIZED WATER	16.81 ± 1.13	17.40 ± 1.05
TAP WATER	14.69 ± 0.28	13.29 ± 0.21
DEIONIZED WATER + 0.6M Calcium Chloride	5.84 ± 0.26	5.47 ± 0.28
DEIONIZED WATER + 1.2M Calcium Chloride	4.17 ± 0.04	4.12 ± 0.32

As can be seen in both tables, the addition of Ca^{2+} clearly lead to lower WHC and SWC values at any tested reaction time, when comparing with the samples where deionized or tap water was used. Deionized and tap water showed similar results in WHC values while SWC where slightly higher for deionized water. Reaction time did not induce any remarkable difference in SWC values. In contrast, WHC increases with time, especially when calcium chloride was added.

It should be noted that the fibres treated $CaCl_2$ aqueous solutions presented a clear decrease the pH of the medium at the beginning of the treatments. This is not observed in samples treated with deionized or tap water. Once the treatment was finished and the enzyme inactivated, the final pH of the solutions showed a slight variation (around 0.6-0.7) in each case compared to the initial pH (samples with added calcium kept their lower values).

On the other hand, it must also be remarked that, as a result of the enzymatic treatment, the viscosity of the solution clearly increased. Thus, instead of being centrifuged, samples were directly frozen, then lyophilized and grinded.

These results led us to discard the use of $CaCl_2$.

1.4.2.2.- Treatments with PMEs.

Table 1.4 show the results corresponding to the % of WHC modification obtained for each fibre treated with PME A or PME B under various time and temperature conditions and in various solvents.

Table 1.4: % of WHC modification in fruit fibres treated with PME.

WHC	PME A								PME B			
	DEIONIZED WATER				TAP WATER				DEIONIZED WATER		TAP WATER	
	30°C		50°C		30°C		50°C		50°C		50°C	
	1/2h	2h	1/2h	2h	1/2h	2h	1/2h	2h	24h	60h	24h	60h
CITRUS N	38.4	2.9	30.6	27.5	19.6	4.2	12.9	23.8	1.0	0.9	1.2	-
CITRUS M	11.9	5.6	15.9	6.9	8.2	8.1	6.9	13.1	11.1	16.4	13.0	23.4
APPLE N	15.6	14.0	36.7	8.5	4.59	28.3	10.42	12.3	4.0	-23.70	-6.40	-
APPLE M	-4.7	3.8	11.04	22.5	0.8	-14.4	37.0	28.0	30.2	31.4	21.1	53.2
PEACH	4.2	-19.9	-0.8	-7.4	-5.6	0.5	4.6	4.7	3.7	6.4	12.3	-

These results show that treatments with PME A caused important changes in WHC in the case of CITRUS N and APPLE N fibres. CITRUS N fibres showed a greater modification than CITRUS M fibres at each temperature and solvent and for most of reaction times. Similar results were obtained with apple fibres when treated at lower temperature. On the other hand, WHC for PEACH N fibre presented little or negative improvement after being treated with PME A. This is a foreseeable result as peach fibre has low pectin content and PME action is against ester groups of pectin.

Regarding to the reaction time, most treatments gave higher modifications at short reaction periods. This behaviour could be a consequence of some modifications in the structure of pectins due to the fact that longer reaction times give an increased number of free carboxyl groups that could interact, especially at low pH, providing lower interaction with water and thus lower WHC improvement.

The effect of using deionized or tap water did not show a clear pattern. Probably the differences can be explained in relation to diverse factors that include the degree of demethylation, which in turn depends on the pectin content of the fibre and the effectiveness of the PME treatment and, on the other hand, the available calcium from the fruit and also from the water in the case of using tap water.

Results with PME B were quite different from those of PME A. Reaction times were much longer than with PME A as it was indicated by the manufacturer. WHC improvements were better at 60 h than 24 h treatment in this case, which indicates that the enzymatic activity of PME B was lower than PME A. Also contrary to what happened with PME A, the physically modified fibres (CITRUS M and APPLE M) showed a greater improvement in WHC than their non-physically modified counterparts (CITRUS N and APPLE N). In the case of CITRUS N, a nearly negligible improvement was obtained. Peach fibre showed similar results with both PME, A and B.

Deionized water showed better improvement in WHC when reaction time was 24 h but tap water gave the best results in the long term. Similarly to what happened with PME A, the viscosity of the solution increased notably after treatment with PME B.

Following, Table 1.5 shows the results corresponding to the SWC modification obtained with the enzymatic treatments with PME A and B.

Table 1.5: % of SWC modification in fruit fibres treated with PME.

SWC	PME A								PME B			
	DEIONIZED WATER				TAP WATER				DEIONIZED WATER		TAP WATER	
	30°C		50°C		30°C		50°C		50°C		50°C	
	1/2h	2h	1/2h	2h	1/2h	2h	1/2h	2h	24h	60h	24h	60h
CITRUS N	8.5	23.7	48.8	16.4	24.2	33.5	37.0	23.4	-11.0	0.2	-11.0	-
CITRUS M	5.6	21.0	19.5	-3.5	42.8	9.4	0.8	3.5	38.3	30.5	69.3	56.5
APPLE N	28.9	-9.9	-8.5	-2.2	5.0	-20.5	-15.1	11.3	29.2	73.9	4.1	-
APPLE M	16.0	23.1	-16.1	43.2	29.5	1.8	6.4	31.5	21.5	43.6	8.7	-3.1
PEACH N	3.2	4.1	9.7	-7.8	-10.9	-15.3	-18.8	-8.8	21.9	41.3	21.8	-

Regarding to the SWC properties, better results were found with in tap water than in deionized water. These results could be consequence of the presence of Ca^{2+} ions in the tap water, which can allow the fibre to swell better. Other than that, there were not any clear patterns that can explain the results neither in deionized nor in tap water. Citric and apple fibres performed better than the peach ones, which gave the worst results. Results with PME B showed notable % of modification for SWC for all the fibres less for CITRUS N.

1.4.2.3.- Treatments with PECs.

Two pectinases with different activities were used. PEC A with a high polygalacturonase (PG) : pectin lyase (PL) ratio and also with arabanase activity. On the other hand, PEC B with low PG : PL ratio with low PE activity and high arabanase activity.

Tables 1.6 and Table 1.7 show the % modification in WHC and SWC respectively, obtained with those enzymes.

Treatments with PEC A were first assayed with native fibres at the highest temperatures (50 and 55ºC). APPLE N and CITRUS N presented an increase of the percentage with time, while PEACH N did not. APPLE N gave the greatest modification in WHC. On the other hand, CITRUS N showed a little increase on the percentage of WHC. Whereas a slight depolymerisation may facilitate the interaction with water, excessive depolymerisation could lead to the loss of WHC. Thus, longer reaction times could provide excessive depolymerisation to keep and improved WHC. PEC B, with low polygalacturonase (PG) : pectin lyase (PL) ratio and arabanase activity, showed the same tendency to increase SWC with longer treatments both with APPLE N and with PEACH N

Physically modified fibres were assayed including a lower temperature (30 and 50°C). The values for % WHC modification were higher for them than for non-modified fibres in both cases, but the effect of temperature was different in each case, whereas APPLE M presented a little increase with the temperature, CITRUS M WHC modification showed a small decrease with the temperature.

Results with PEC B also showed that APPLE N gave the better modification regarding to WHC properties, giving the longer reaction times the best results. In the case of CITRUS N and PEACH N the modification was lower than for APPLE N, with no clear pattern related to temperature and time treatments.

With PME A, SWC modification presented quite a similar pattern as WHC related with physically modified fibres, being physically modified fibres results better than those results for non-modified ones. Also with the non-modified, where APPLE N showed the bestsr results

While, PME B generally presented the improved results at lower times and temperatures, being PEACH N and APPLE N the ones who present the better results.

Table 1.6: % of WHC modification in fruit fibres treated with PEC.

WHC	PEC A						PEC B				
	30°C	50°C		55°C			50°C			55°C	
	1/2h	1/2h	1h	1/2h	1h	4h	8h	16h	4h	8h	16h
CITRUS N	-	2.5	4.5	-2.0	13.9	17.0	-0.9	-4.9	8.7	11.4	-3.6
CITRUS M	40.7	33.9	-	-	-	4.2	-	-	-	-	-
APPLE N	-	32.9	36.9	38.1	-4.4	14.3	17.6	31.0	12.4	12.4	34.2
APPLE M	63.4	75.5	-	-	-	41.5	-	-	-	-	-
PEACH N	-	10.1	0.8	3.5	-7.1	9.31	8.1	24.4	1.3	6.3	13.9

Table 1.7: % of SWC modification in fruit fibres treated with PEC.

SWC	PEC A						PEC B				
	30°C	**50°C**		**55°C**			**50°C**			**55°C**	
	1/2h	**1/2h**	**1h**	**1/2h**	**1h**	**4h**	**8h**	**16h**	**4h**	**8h**	**16h**
CITRUS N	-	9.6	-11.4	-32.5	3.5	23.3	27.4	10.5	8.9	6.8	-37.0
CITRUS M	114.9	77.8	-	-	-	-8.0	-	-	-	-	-
APPLE N	-	25.4	40.8	-6.6	14.4	38.1	44.3	50.0	27.1	33.7	92.1
APPLE M	93.3	101.4	-	-	-	30.5	-	-	-	-	-
PEACH N	-	25.5	6.6	-9.9	11.3	37.6	30.6	60.4	23.3	42.5	25.9

Contrary to what happened with PMEs, the viscosity of the solution did not increase when fibres were treated with PECs, which is in accordance with the fact that PECs activity focuses on the polygalacturonase chains shortening their length and so rendering fragments that develop less viscosity in solution than in the case of PMEs, which do not have the ability of shorten polymer chains.

It is also noticeable that peach fibres showed a considerable modification both in WHC and SWC parameters. Although peach fibre has lower pectin content than apple or citrus fruits, two aspects should be taken into account. On the one hand, although the pectin content in peach fibre is lower that in the other fibres, it could be easily attacked by the enzyme rendering important changes in the structure that modify the WHC and SWC properties. On the other hand, the used enzymes presented side

arabanase activities, which could modify other molecules than pectins rendering notorious changes in structure and consequently in fibre properties.

1.4.2.4.- Treatment with Cellulase (CEL).

The cellulase used in this assay is suited not only for cellulose degradation but other structural polysaccharides as well. It presents side activities as cellobiase, beta-glucosidase and beta-glucanase that can result in complete cellulose breakdown. Among other applications it is used in liquefaction and maceration of many fruits and vegetables. It was used at doses and conditions indicated for partial hydrolysis.
Table 1.8 and Table 1.9 show for the fruit fibres after treatment with the enzyme CEL the results corresponding to the % modification of WHC and SWC, respectively.

Table 1.8: % of WHC modification in fruit fibres treated with CEL.

WHC	CEL					
		60°C			65°C	
	2h	6h	18h	2h	6h	18h
CITRUS N	-11.3	1.0	16.5	-5.0	-2.6	11.1
CITRUS M	-	22.6	-	-	-	-
APPLE N	39.8	13.5	12.4	24.0	13.1	12.8
APPLE M	-	-12.6	-	-	-	-
PEACH N	12.8	9.0	2.6	13.9	10.3	2.8

Table 1.9: % of SWC modification in fruit fibres treated with CEL.

SWC	CEL					
		60°C			65°C	
	2h	6h	18h	2h	6h	18h
CITRUS N	-8.4	0.8	11.6	-4.4	-1.3	-39.0
CITRUS M	-	92.1	-	-	-	-
APPLE N	19.7	30.3	30.7	22.7	32.7	46.0
APPLE M	-	-33.2	-	-	-	-
PEACH N	48.2	39.4	42.5	35.9	55.5	51.3

Table 1.8 shows that CEL generally led to an improvement of %WHC. This improvement is lower at higher temperature except for PEACH fibre and for longer time except for CITRUS N where %WHC increases with time. APPLE N showed the best results with an increase of nearly 40% in WHC for the lowest time and temperature assayed. According to the literature, apple fibre tends to be the richest in cellulose compared to citrus and peach fibres[67-69]. Consequently, it should be expected a greater effect of cellulases on this fibre compared to the other native ones.

CEL also led to an improvement of SWC. Fibres tend to increase their SWC with the temperature and the time, being 6 h of treatment the one that generally achieves the best results, especially at the highest tested temperature. However, in the case of CITRUS N, SWC decreases with increasing temperature.

1.4.2.5.- Treatment with Ferulase (FER).

The ferulic acid esterase used in this project is commercially used to treat recalcitrant parts of the plant cell wall. As a result of its action, flavours, colorants, sugars and phenolic acids are released from the matrix structure. As stated before, ferulase cleaves ferulic acid ester linkages among pectins and arabinoxylans those loosening the structure of the fibre.

Table 1.10 and Table 1.11 show the results obtained after the treatment of the fruit fibres with FER.

Table 1.10: % of WHC modification in fruit fibres treated with FER.

WHC	FER			
	45°C		65°C	
	24h	72h	24h	72h
CITRUS N	-1.8	-1.5	-4.7	-0.9
CITRUS M	10.5	4.0	6.8	5.4
APPLE N	-4.7	2.9	2.9	19.9
APPLE M	-8.8	0.4	10.9	6.0
PEACH	-5.5	-6.1	13.8	2.5

Table 1.11: % of SWC modification in fruit fibres treated with FER.

SWC	FER			
	45°C		65°C	
	24h	72h	24h	72h
CITRUS N	15.4	20.2	4.2	-18.1
CITRUS M	13.3	-12.7	-3.5	-27.0
APPLE N	-8.3	0.7	-3.1	-15.8
APPLE M	-26.7	-18.3	48.9	-16.3
PEACH	6.3	-2.0	-16.8	-18.7

FER did not improve the WHC of CITRUS N, neither of most of the other fibres. In general, higher temperatures led to better results but there is not a clear tendency with various reaction times. Physically modified fibres gave better improvements that the native counterparts and in this case, shorter reaction times led to better results.

Generally, fibres treated with FER diminished their SWC especially with highest temperature and time treatments. Results were clearly worst for the physically modified fibres than for the native fibres. It could be hypothesised that after enzymatic treatment of fibres, the degree of hydrolysis is so high that the resulting fibres loss their ability to properly interact with water molecules.

1.4.3.- FOLLOW UP OF ENZYMATIC TREATMENTS BY FT-IR.

In order to gather some information on the major changes introduced by enzymatic treatments, the FT-IR spectrum of the fibres, before and after enzymatic treatment, was recorded. To simplify the study of the big data volume obtained, only the enzymatic treatments that gave better results related with %WHC modification were analysed.

Initially, some characteristic bands were selected to monitor the likely modifications in the polymeric structure caused by each enzymatic treatment. Specifically:

(1) The stretching vibration of O-H corresponding to the sugar residues was assessed to the 3600 and 3200 cm^{-1} band.
(2) Csp^3-H stretching corresponding to the sugar rings characteristic of polysaccharides[70] was assessed to the band with the maximum around 2923 cm^{-1}.
(3) The band with the maximum absorption around 1730 cm^{-1} was reported as the absorbance of pectin methyl ester group and acetyl ester groups of hemicellulose[71].
(4) The band that appeared around 1623 cm^{-1} was described as the carboxylate functional group[71].
(5) A peak at 893 cm^{-1} was assigned as indicative of a possible beta-glycoside linkage between the neutral sugars[72].

For those fibres that showed the greatest modification after each treatment as well as for the fibres before any enzymatic treatment, the area of the above bands (1-5) was

calculated. The ratio between the areas of specific bands was determined and tabulated.

Tabble 1.12: Ratios between the areas of the bands studied for non-enzymatically treated fibres.

	(1)/(2)	(3)/(4)	(5)/(2)	(3)/(2)	(4)/(2)
CITRICS N	28.9±4.1	0.4±0.0	31.7±8.9	2.0±0.3	5.3±0.5
CITRICS M	27.0±3.8	0.4±0.0	28.9±4.7	2.1±0.4	4.6±0.6
APPLE N	23.0±3.8	0.6±0.1	23.0±3.8	1.1±0.1	1.8±0.3
APPLE M	15.0±3.4	0.9±0.0	15.0±3.4	1.1±0.2	1.2±0.1
PEACH N	31.1±9.4	0.5±0.0	30.0±11.3	1.9±0.7	4.1±1.3

Table 1.12 shows the results the ratios between the areas for the non-modified fibres. It clearly shows a big variability for the band related to the beta-glycoside linkage, probably due to the many contributions this region can have. Because of the big variability presented with this band, we decided not to work with it.

Figure 1. shows the characteristic FTIR spectra of the different fruit fibres used in this study before any enzymatic treatment.

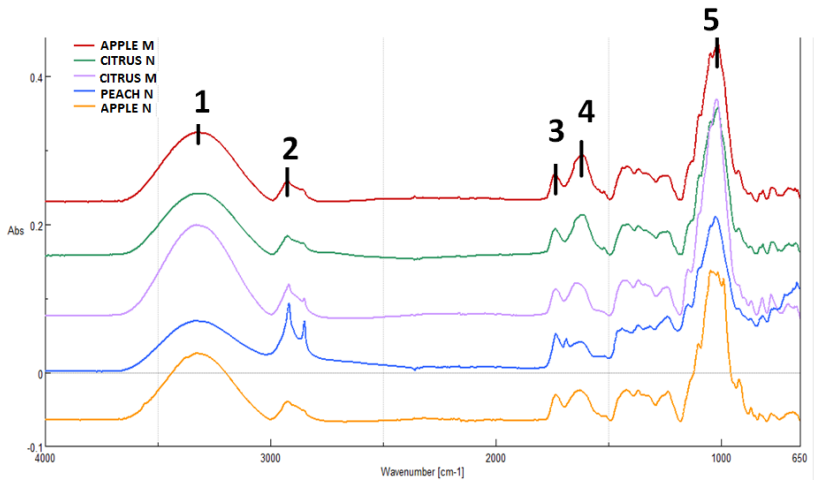


Figure 1.3: FTIR-ATR of non-treated fibres. Numbered peaks are assigned to different atom bond vibrations.

1.4.3.1.- Treatments with PMEs.

Table 1.13 shows the relationship among pairs of selected bands for the native fibres and the same fibres after the enzymatic treatments.

Table 1.13: Ratios between the areas of the bands studied

SOURCE	FT-IR BANDS	(3)/(2)	(3)/(4)	(4)/(2)
CITRIC N	NON-ENZYMATIC	2.0±0.3	0.4±0.1	5.3±0.5
	PME A	1.8	0.3	5.0
	PME B	1.5	0.4	4.3
CITRIC M	NON-ENZYMATIC	2.1±0.4	0.5±0.1	4.6±0.6
	PME A	2.1	0.4	5.4
	PME B	1.7	0.4	4.5
APPLE N	NON-ENZYMATIC	1.1±0.1	0.6±0.1	1.8±0.3
	PME A	1.0	0.6	1.8
	PME B	0.9	0.9	2.5
APPLE M	NON-ENZYMATIC	1.1±0.2	0.9±0.1	1.2±0.1
	PME A	1.1	0.6	1.7
	PME B	1.3	0.5	2.6
PEACH N	NON-ENZYMATIC	1.9±0.7	0.5±0.1	4.1±1.3
	PME A	1.7	0.5	3.6
	PME B	2.1	0.5	4.6

The FTIR spectra recorded corresponds to: CITRUS N: PMEA 30°C 1/2h,PMEB 50° 24h; CITRUS M: PMEA 50°C 1/2h, PMEB 50°C 60h; APPLE N: PMEA 30°C 2h,PMEB 50°C 60h; APPLE M: PMEA 50°C 2h,PMEB 50°C 60h; PEACH N: PMEA 50°C 1/2h, PMEB 50°C 24h. The NON-ENZYMATIC data correspond to the mean of all the FTIR recorded for these fibres.

Table 1.13 shows the decrease of the pectin ester band (3) relative to the carboxylic acid band (4) or the Csp^3-H band (2) after treatment with the PME A and B. This would confirm the demethylation of pectins performed for these enzymes. This effect seems more remarkable in the case of PME B, especially for apple fibres.

In the case of peach fibre, the poorest in pectins content among those fibres included in this study, we can see little modifications in all these relationships.

1.4.3.2.- Treatments with PECs.

Table 1.14 shows the results corresponding to the ratio among band areas for the fibres after and before treatment with PECs.

Table 1.14: Ratios between bands of the FTIR-ATR spectra of the fibres treated with the PEC enzymes

SOURCE	FT-IR BANDS	(1)/(2)	(3)/(4)	(5)/(2)
CITRUS N	NON-ENZYMATIC	28.9±4.1	0.4±0.1	31.7±9.0
	PEC B	44.5	0.3	38.0
	PEC A	33.2	0.3	28.0
CITRIUS M	NON-ENZYMATIC	27.0±3.8	0.5±0.1	28.9±4.7
	PEC B	38.5	0.4	35.6
	PEC A	27.8	0.4	29.7
APPLE N	NON-ENZYMATIC	23.0±3.8	0.6±0.1	23.0±3.8
	PEC B	32.5	0.7	32.5
	PEC A	26.2	0.8	26.2
APPLE M	NON-ENZYMATIC	15.0±3.4	0.9±0.1	15.0±3.4
	PEC B	11.9	0.8	11.9
	PEC A	23.1	0.7	23.1
PEACH N	NON-ENZYMATIC	31.1±9.4	0.5±0.1	30.0±11.3
	PEC B	27.1	0.4	38.5
	PEC A	42.5	0.4	39.1

The FTIR spectra recorded corresponds to: CITRUS N: PECA 55°C 1h,PECB 50° 4h; CITRUS M: PECA 30°C 1/2h,PECB 50° 4h; APPLE N: PECA 55°C 1/2h,PECB 55° 16h; APPLE M: PECA 50°C 1/2h,PECB 50° 4h; PEACH N: PECA 50°C 1/2h,PECB 50° 16h. The NON-ENZYMATIC data correspond to the mean of all the FTIR recorded for these fibres.

Table 1.14 shows the intensity of the OH band (1) in front of the Csp3-H (2) in treated fibres increases when comparing with the non-treated fibres. That would indicate an increase in the number of hydroxyl groups, which would be expected since the hydrolysis of glycosidic bonds releases two OH groups for each split bond. In the case of citrus fibres and APPLE N, this increase is noticeably higher for PEC B than for PEC A. In contrast, APPLE M and PEACH N fibres present higher values for PEC A.

The intensity of the ester band (3) in front of the carboxylic acid band (4) tends to decrease in the treated fibres in front the non-treated fibres in all samples except for the APPLE N fibre. Nevertheless, the variations were low considering the standard errors determined on the non-treated fibres.

1.4.3.3.- Treatments with CEL.

Table 1.15 shows the results corresponding to the ratios between some FT-IR bands in the spectra of fibres treated with cellulase and non-treated fibres

Table 1.12: Ratios between bands of the FTIR-ATR spectra of the fibres treated with the CEL enzyme

SOURCE	FT-IR BANDS	(3)/(2)	(3)/(4)	(4)/(2)	(1)/(2)
CITRUS N	NON-ENZYMATIC	2.0±0,3	0.4±0.0	5.3±0.5	28.9±4.1
	CEL	0.9	0.1	6.2	49.6
CITRUS M	NON-ENZYMATIC	2.1±0.4	0.5±0.0	4.6±0.6	27.0±3.8
	CEL	2.1	0.4	5.4	38.6
APPLE N	NON-ENZYMATIC	1.1±0.1	0.6±0.1	1.8±0.3	23.0±3.8
	CEL	1.5	0.5	2.8	43.3
APPLE M	NON-ENZYMATIC	1.1±0.2	0.9±0.1	1.2±0.1	15.0±3.4
	CEL	1.5	0.6	2.5	25.8
PEACH N	NON-ENZYMATIC	1.9±0.7	0.5±0.0	4.1±1.3	31.1±9.4
	CEL	1.6	0.4	3.7	34.0

The FTIR spectra recorded corresponds to: CITRUS N: CEL 60°C 18h, CITRUS M: CEL 60°C 6h; APPLE N: CEL 60°C 2h, APPLE M: CEL 60°C 6h; PEACH N: CEL 65°C 2h. The NON-ENZYMATIC data correspond to the mean of all the FTIR recorded for these fibres.

A clear increase in the relationship among OH band (1) and Csp3-H band (2) is observed, which clearly support an increase of the OH groups in the media. These results completely match with the expected hydrolytic activity for the enzyme used in this treatments.

The other relationships shown in the table, (3)/(2), (3)/(4) and (4)/(2), indicate a decline in ester groups parallel to an increase to acid groups. That could indicate some side effect due to the pool of enzymes included in the commercial product, some of them could have esterase activity.

1.4.3.4.- Treatments with FER.

Table 1.16 shows the ratios between the areas of some FT-IR spectra bands corresponding to fibres treated with ferulase and non-treated fibres.

Similarly to what was commented for the enzymatic treatment with FER (1.4.2.5), there is not a clear pattern for the relationship among the selected bands areas. These

results would confirm that this enzyme has a recalcitrant activity, which can lead to various changes in the interactions between constitutional polysaccharides

Table 1.13: Ratios between bands of the FTIR-ATR spectra of the fibres treated with the FER enzymes

SOURCE	FT-IR BANDS	(3)/(2)	(3)/(4)	(4)/(2)	(1)/(2)
CITRUS N	NON-ENZYMATIC	2.0±0.3	0.4±0.0	5.3±0.5	28.9±4.1
	FER	2.0	0.4	4.9	28.3
CITRUS M	NON-ENZYMATIC	2.1±0.4	0.5±0.0	4.6±0.6	27.0±3.8
	FER	1.8	0.5	4.0	26.2
APPLE N	NON-ENZYMATIC	1.1±0.1	0.6±0.1	1.8±0.3	23.0±3.8
	FER	1.2	0.6	2.2	21.6
APPLE M	NON-ENZYMATIC	1.1±0.2	0.9±0.1	1.2±0.1	15.0±3.4
	FER	1.1	0.8	1.5	15.1
PEACH N	NON-ENZYMATIC	1.9±0.7	0.5±0.0	4.1±1.3	31.1±9.4
	FER	2.0	0.5	3.7	28.7

The FTIR spectra recorded corresponds to: CITRUS N: FER 65°C 72h, CITRUS M: FER 45°C 24h; APPLE N: FER 65°C 72h, APPLE M: FER 65°C 24h; PEACH N: FER 65°C 72h. The NON-ENZYMATIC data correspond to the mean of all the FTIR recorded for these fibres.

1.5.- CONCLUSIONS.

In general, technically feasible results have been accomplished with the studied enzymes considering the purpose of the study. WHC and SWC increased in most of the fibres and even in some treatments viscosity increased. These results help to add value to by-products of the juice industry, as it a simple enzymatic treatment can render a fibre with improved functional properties.

The expected activity for each type of enzyme was demonstrated. The polymeric structure has been modified in some extent for most of the tested enzymes and conditions. These modifications allow the achievement of better interaction of fibres with water molecules and therefore more water could be retained in the structure. FT-IR allows the confirmation of several effect of the used enzymes.

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CHAPTER 2.- PREPARATION OF CYCLIC URETHANE

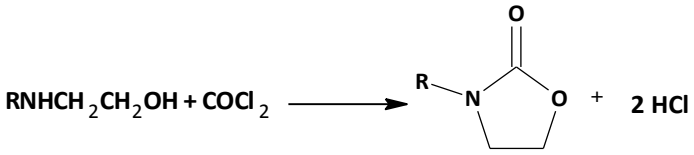
2.1.- BACKGROUND.

Following our interest to use agroindustrial waste as raw materials, we decided to reduce the azides prepared from glycerol using the procedure previously developed in our group (Introduction, Scheme I12). The reduction of these products allows the preparation of amidoalcohol or diamine derivatives. Compounds that have been described as CO_2 capturers yielding cyclic urethanes or substituted urea[1, 2].

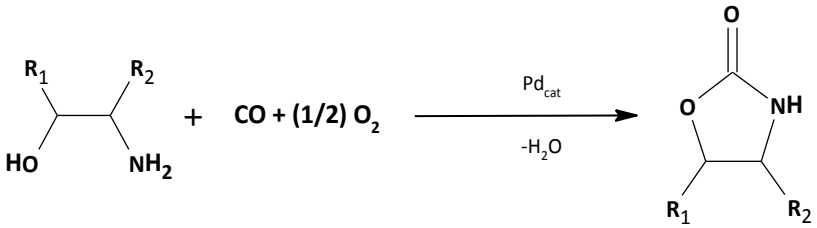
2.1.1.- INTERESTS OF URETHANES.

Urethanes are an important class of products with many applications. Cyclic urethanes have interest as synthetic intermediates of different products such as fine chemicals, pharmaceuticals, cosmetics and pesticides[2-7].

The synthesis of these compounds can be achieved by the reaction of aminoalcohols, 1,2-diamines or aziridines (a predehydrated form of ß-aminoalcohol) with reagents such as: phosgene[8] (Scheme 2.1), a mixture of carbon monoxide and oxygen via oxidative carbonylation[9] (Scheme2.2), dialkyl carbonates[8, 10], cyclic carbonates[1], urea[2, 11, 12] or CO_2[2, 13, 14].



Scheme 2.1: Cyclic urethane synthesis using phosgene as reagent.



Scheme2.2: Cyclic urethane synthesis using oxidative carbonylation.

Nevertheless, these approaches usually include the use of large amounts of catalysts or dangerous reagents. For example, the use of phosgene or oxidative carbonylation are not eco-friendly due to risks associated with explosion hazards, environmental pollution and equipment corrosion as is widely known[12]. Although dialkyl carbonates

are extensively used, it should be noted that they are currently produced by phosgenation and oxidative carbonylation routes[15], although cyclic carbonates can also be synthesised from CO_2 and urea[16, 17], which is prepared from CO_2.

In this chapter, we have studied the synthesis of new compounds using amidoaminealcohols and CO_2, urea or cyclic carbonates as starting materials. The influence on the behaviour of the process by the reaction conditions and the alkyl chain of the amide have been determined.

2.1.2.- CO_2 AND ITS DERIVATIVES AS CARBON SOURCES TO PREPARE URETHANES OR UREA DERIVATIVES.

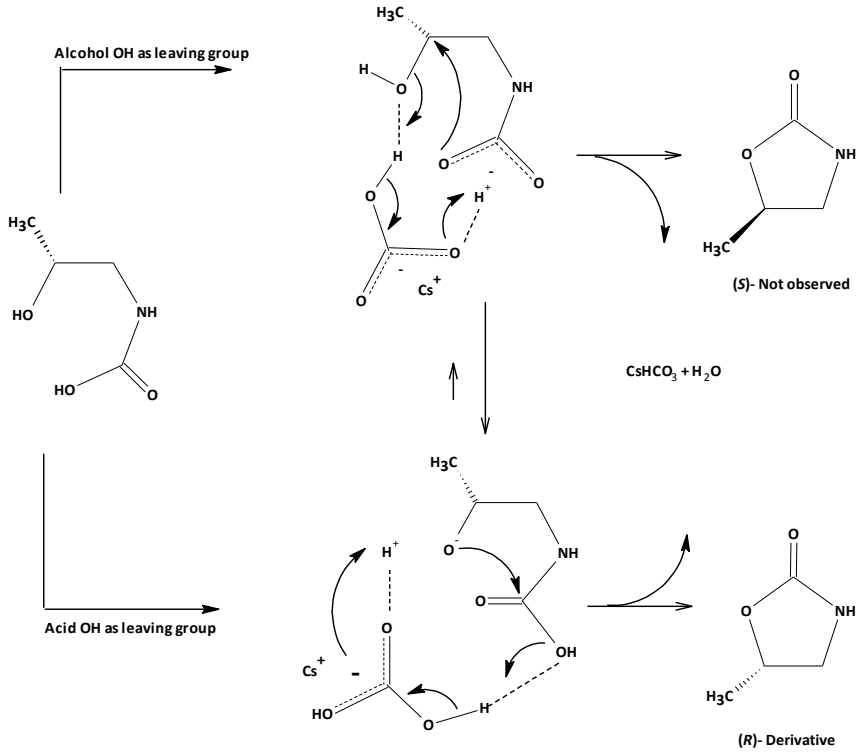
2.1.2.1.- CO_2 Used as Carbon Source.

CO_2 is the end-product of the largest-volume and most globally applied chemical reaction: the combustion of hydrocarbons and biomass for power generation, public electricity and heat production[18]. Due to its large production as by-product of many industries, nowadays CO_2 is being accumulated in high quantities. The increase in CO_2 emission has resulted in a clear increase of atmospheric CO_2 during the last 200 years. This increase is thought to cause atmospheric warming, which is associated with a global climate change and planetary temperature increase[19]. There is an on-going research to find ways to reduce CO_2 emissions into the atmosphere.

One way to reduce atmospheric carbon dioxide level involves the development of new processes where this renewable carbon source can be used in an environmentally friendly manner. One of the possibilities is the use of CO_2 as a chemical reagent. The efficient transformation of carbon dioxide into useful chemicals is very attractive in view of resource utilization and pollution prevention. Exploiting CO_2 as a carbon feedstock for the production of useful organic compounds will allow the reduction of its concentration in the atmosphere[20].

As described above he synthesis of cyclic urethanes and substituted urea using carbon dioxide and ß-amino alcohols or diamines has been performed using various catalysts and/or conditions[2, 20-30]. Nevertheless, a clean, simple and catalyst free approach for the oxazolidinone synthesis starting from ß-amino alcohol has yet to be

established. Among the causes for this, we can find thermodynamic reasons and catalyst deactivation caused by the water co-produced during the reaction[19, 31]. In order to find a solution to the deactivation produced by the water production, several attempts have been conducted to shift the equilibrium towards the products. The use of a dehydrating agent to trap the co-produced water[21, 29, 32] and the use of phosphorylating agents, as in Mitsunobu reaction[20, 26-28], are the most straightforward and used alternatives to avoid these problems. Nevertheless, it must be noted that these approaches need the presence of a strong base as DBU.They also need the presence of trialkylamines like guanidine, which leads to the production of many by-products. For example, it is difficult to completely remove the alkylphosphine oxide formed during the process.

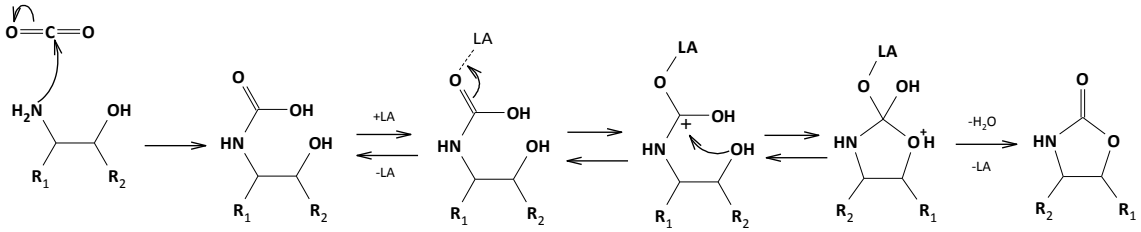


Scheme 2.3: Mechanism proposed by Foo et al.[24] for the synthesis of chiral oxazolidones.

Catalysts such as Cs_2CO_3[24] and ionic liquids (IL) with alkali metal promoter[21] have been developed to overcome these drawbacks. For example, *Foo et al*.[24] reported the synthesis of oxazolidones from amino alcohols and carbon dioxide, without using special dehydrating agents, being Cs_2CO_3 the agent supposed to promote the dehydration process. The reaction was performed between 1 and 5 atm. Scheme 2.3

shows the proposed reaction mechanism, which implies that the OH of the amino alcohol can act as both nucleophile and leaving group during the final cyclization step. A complete retention of the parent chiral centre bearing the methyl substituent was observed, with the exclusive formation of the (*R*)-isomer [24].

Fujita et al.[21] described the synthesis of cyclic urethanes using CO_2 and ionic liquids. They proposed a two-component catalytic system of BMIM-Br and K_2CO_3. Scheme 2.4, shows the proposed mechanism for this reaction.



LA= imidazolium cation

Scheme 2.4: Proposed mechanism for the formation of cyclic urethane from amino alcohol using IL[21].

Tamura et al.[22, 23] proposed using CeO as heterogeneous catalysis for the synthesis of cyclic urethanes. The procedure was performed at 49.3atm (5000 kPa) and the products were synthesised with good yields.

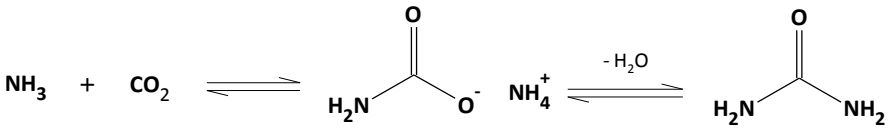
Although these catalytic reactions are likely approaches to prepare cyclic urethanes from CO_2, it is preferable to avoid the use of catalysts to improve the whole process avoiding catalyst separation and catalyst recycling procedures.

There are few studies describing the synthesis of urethanes without the use of catalysts[2, 12]. *Bhanage et al*.[2] have described the synthesis of oxazolidones using carbon dioxide and a diamine or aminoalcohols with a pressurized system without the use of any catalyst. However, the yields are not very high. Consequently, the development of a simple, highly efficient and environmentally friendly methodology for the synthesis of cyclic urethanes is still a challenge.

2.1.2.2.- Urea Used as Carbon Source.

The industrial production of urea using CO_2 is around 100 million t/year[33]. The production of urea is carried out in two steps at elevated pressure (15,000 to 25,000 kPa) and temperature (150 to 200°C). The first step consists on the formation of ammonium carbamate from ammonia and CO_2. The second step implies the dehydration of the carbamate to urea (Scheme 2.5).

Nowadays, urea is used as a chemical fertilizer, as animal feed additive, and to prepare urea and urea-melamine resins.



Scheme 2.5: Industrial production of urea from ammonia and CO_2.

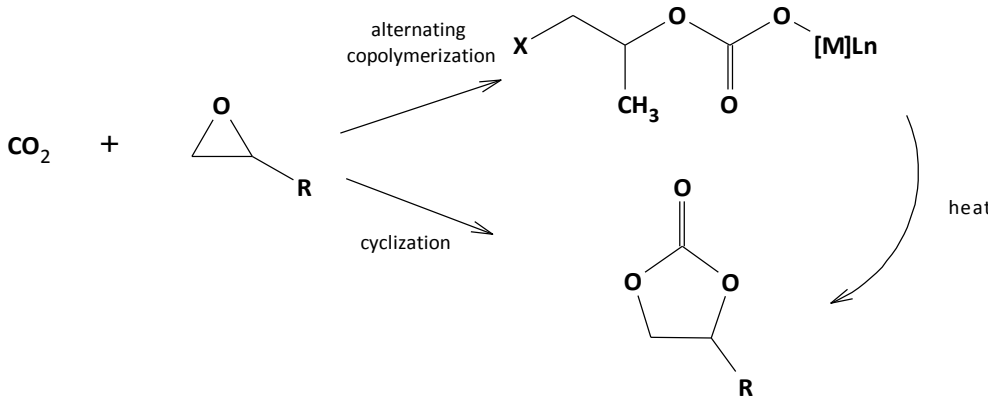
There are few reports on the synthesis of cyclic urethanes starting from urea and aminoalcohols. These reactions have been performed in a solvent-free media without using any catalyst nor any solvent[34, 35]. Nevertheless, the yields were moderate and high temperatures required, which could cause polyurea formation.

Because the formation of ammonia was reported as an inhibitor of the reaction process, *Bhanage et al.*[12] improved the methodology using dynamic evacuation to remove the ammonia co-generated during the reaction. This methodology allowed the synthesis of cyclic urethanes using aliphatic aminoalcohols in a solvent-free media. The reaction of aromatic aminoalcohols needs to be carried out using DMF as solvent since this reaction carried out without solvent results in a clear decrease of the yield.

Urea is an attractive reagent for the synthesis of cyclic urethanes, reaction yields and selectivity are higher than for the analogous reactions using carbon dioxide without the use of a catalyst[2, 12].

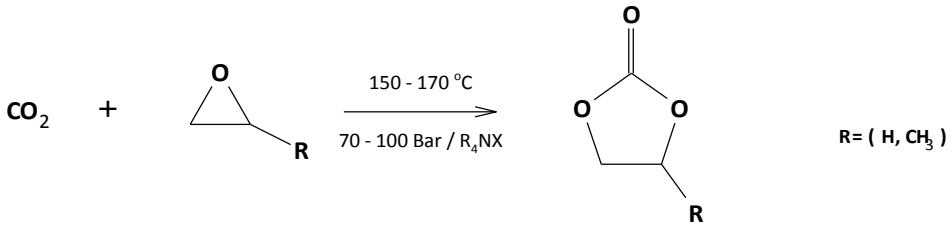
2.1.2.3.- Cyclic Carbonates Used as Carbon Source.

The reaction between oxiranes and CO_2 produces cyclic carbonates or polymeric carbonates as Scheme 2.6 shows:



Scheme 2.6: Reaction with oxiranes and CO_2 can lead to cyclic or polymeric carbonates.

The current industrial production of ethylene and propylene carbonate from CO_2 is performed by the reaction with epoxides in the presence of quaternary ammonium halides (Scheme 2.6). The halide salts are also suitable catalyst as they are soluble in cyclic carbonates, which avoids the use of solvents during the reaction[17].



Scheme 2.7: Synthesis of ethylene and propylene carbonate from their respective oxiranes and CO_2[1].

Cyclic urethanes can be prepared from ß-aminoalcohols and cyclic carbonates using various catalysts. For example, 2-oxazolidones can be prepared with good yields under mild conditions using $Br^{-}Ph_3^{+}PPEG_{600}\text{-}P^{+}Ph_3Br^{-}$ as catalyst[13]. However, this catalyst is very expensive and its consistent only up to 5 cycles[13].

Five-membered cyclic carbonates and ß-aminoalcohol in organic solvents yielded cyclic urethanes with good yields in presence of K_2CO_3 under mild conditions[1]. Dialkyl carbonates are also used for the synthesis of 2-oxazolidinones and cyclic ureas[10]. However, the problem is the way how dialkyl carbonates are prepared[15],as we have mentioned above (section 2.1.1).

2.2.- OBJECTIVES

The aim of this chapter was:

-To study the synthesis of cyclic urethane starting from glycerol and CO_2 or CO_2 derivatives.

2.3.- MATERIALS AND METHODS.

2.3.1.- REAGENTS AND EQUIPMENT.

2,2-Dimethylbutyric acid (96% Fluka), diphenylacetic acid (99% Acros organics), caprylic acid (>98% Fluka), stearic acid (97% Probus), glycerol (99.5% Fluka), CTMS (ACROS, 98%), sodium azide (99%, Aldrich), Amano-lipase PS-LM (Immobilized on diatomaceous earth, Sigma), Lipozyme IM from *Mucor miehei* (Fluka), Lipase acrylic resin from *Candida antarctica* (Sigma), Amano lipase PS from *Pseudomonas cepacia* (30.000 μ/g Aldrich), *R. oryzae* (produced in the own laboraroty), Baker's yeast (commercial), $FeSO_4 \cdot 7H_2O$ (99%,Probalo), Zn (Probus), NH_4Cl (99.5% Panreac), $(PPh_3)_3RuCl_2$ (98% Strem chemicals), triphenylphosphine (97% Fluka), Pd/C immobilized on charcoal (10% Aldrich), urea (Panreac), 1,3-dioxolan-2-one (98%, Aldrich) Novozyme 435 (Novozymes) were used as reagents and catalysts. Solvents were purchased from Sigma-Aldrich, Aldrich, Fluka, Supelco and Across and were used without further purification. Diazides (**1a**-**1d**) were synthesised from glycerol and the corresponding carboxylic acid according the procedure described by our research group[36]. The crude reaction mixture was not purified, due to its instability, and directly used in further reactions. Purification of the final resulting solid compounds was performed by crystallization.

^{1}H and ^{13}C NMR spectra were recorded on Varian AS400 MERCURYplus (^{1}H, 400MHz and ^{13}C, 100 MHz), using $CDOD_3$, $CDCl_3$ and D_6MSO as solvent. The spectra were recorded at 30°C or 55°C depending on the product solubility and 20 s of relaxing time. Chemical shifts (δ) were reported in ppm relative to the solvent used. Spin multiplicities were reported as a singlet (s), doublet (d), or triplet (t) with coupling constants (*J*) given in Hz, or multiplet (m).

The melting points were measured by open capillary tubes in Gallenkamp equipment. They are uncorrected.

IR spectra were recorded on a Jasco FT/IR-6300 equipment, in a range between 400 to 4000 cm^{-1}. The equipment is set to take 60 spectra/second with a resolution of 16 cm^{-1} with ATR.

High resolution mass spectra were recorded by direct infusion in a Agilent G6510AA Q-TOF mass spectrometer using ESI ionization.

2.3.2.- METHODS.

a) General procedure for the synthesis of 2a-2d:

a.1) Using baker's yeast.

In a typical experiment, a preincubated suspension of Baker's yeast (4 g) in phosphate buffer solution at pH 7.2 (20 mL) was added to a solution of **1a** (100 mg, 0.23mmol) in aqueous ethanol (50%). The mixture was stirred vigorously for the appropriate reaction time. Finally, EtOAc (60 mL) was added to the reaction mixture. The organic phase was recovered, dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum. The final residue was analysed by ^{1}H NMR.

a.2) Using enzymes and fungi.

In a typical experiment, a solution of **1a** (100 mg, 0.023 mmols) in MeOH (8mL) was placed in a round flask (50 mL) fitted with a reflux condenser. The biocatalyst (0.05 g) was added and the mixture was magnetically stirred at 50°C. On completion of the reaction time the mixture was allowed to cool and $CHCl_3$ (20 mL) was added. The mixture was filtered and the filtrate was washed with H_2O. The organic solution was dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum. The final residue was analysed by ^{1}H NMR.

a.3) Using enzymes with MW heating.

In a typical experiment, lipase (0.05 g) was added to a stirred solution of **1a** (100 mg, 0.023 mmols) in MeOH (10 mL) placed in the reaction vessel reactor. The mixture was magnetically stirred and MW irradiated for 5 min at 50°C. The mixture was allowed to cool at room temperature, $CHCl_3$ (10 mL) was added and the solution was filtered and washed with H_2O. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum. The final residue was analysed by ^{1}H NMR.

a.4) Using $FeSO_4 \cdot 7H_2O$ system.

In a typical experiment, $FeSO_4 \cdot 7H_2O$ (0.3 g, 0.1 mmol) and 25% ammonia solution (0.25 mL) were added to a stirred solution of **1a** (100 mg, 0.023 mmols) in dichloromethane (10 mL). This reaction mixture was continuously stirred at room temperature. After the

appropriate reaction time, the mixture was diluted with dichloromethane and filtered through a Celite bed. The filtrate was washed with water and dried over anhydrous Na_2SO_4. The organic layer was filtered and evaporated under vacuum. The final residue was analysed by ^1H NMR.

a.5) Using an NH_4Cl/Zn system.

In a typical experiment, zinc powder (0.26 mg, 0.04 mmol) was added to a stirred solution of **1a** (100 mg, 0.023 mmols) and ammonium chloride (0.32 mg, 0.06 mmol) in EtOH:H_2O (3:1) (10 mL). The mixture was stirred vigorously at reflux temperature. After the appropriate reaction time, AcOEt (50 mL) and aqueous ammonia (2 mL) were added. The mixture was filtered, and the filtrate was washed with brine and dried over anhydrous Na_2SO_4. The organic layer was filtered and evaporated under vacuum. The final residue was analysed by ^1H NMR.

a.6) Using a Ruthenium catalyst.

In a typical experiment, **1a** (100 mg, 0.023 mmols) and K_2CO_3 (10% molar) were added into a two-necked round-bottom flask equipped with a condenser and a magnetic stirring bar. The system had previously been purged with Ar. Acetone (5mL) (containing 0.6% of water) was added through a syringe and the resulting solution was refluxed under Ar atmosphere. After the appropriate reaction time, the reaction mixture was filtered through Celite and dried over anhydrous Na_2SO_4. The organic layer was filtered and evaporated under vacuum. The final residue was analysed by ^1H NMR.

a.7) Using the Staudinger reaction.

In a typical experiment, PPh_3 (1.44 g, 0.03 mmol) was added to a stirred solution of the diazide **1a** (0.4g, 0.1 mmol) in MeOH (15 mL). The resulting suspension was stirred until the triphenylphosphine was completely dissolved. Water (5 µL, 0.3 mmol) was added to the reaction vessel. The mixture was stirred at room temperature overnight. After removal of solvent under vacuum, the final residue was analysed by ^1H NMR. The purification of the product by crystallization was studied using various solvents.

a.8) Using the Staudinger reaction in acidic media.

In a typical experiment, an Et_2O: AcOEt (1: 1) solution (10mL) of PPh_3 (1.44 g, 0.03 mmol) was added dropwise to a stirred solution of the diazide **1a** (0.4g, 0.1 mmol) in 5% HCl (15 mL) at 0°C. The mixture was stirred at 0°C for 1 h and then 24 h at room temperature. The organic layer was discarded and the aqueous layer was washed with CH_2Cl_2. A base was added to the aqueous layer and it was extracted with CH_2Cl_2. The organic solution was dried over anhydrous Na_2SO_4 and after removal of solvent under vacuum, the final residue was analysed by ^{1}H NMR.

a.9) Pd/C Hydrogenation.

In a typical experiment, a suspended mixture of 10% Pd/C (10 wt%) in MeOH (1.0 mL). was added to a stirred solution of the diazide (**1a**-**1d**) (1.0 mmol) in MeOH (1 mL). The system was sealed with a septum. After two vacuum/H_2 cycles, the mixture was vigorously stirred at 25°C under ordinary hydrogen pressure (balloon) for 72 h. The reaction mixture was filtrated through Celite. The solution was concentrated under vacuum to yield white powders for **2b**-**2d** products. Compound **2a** was a colourless oil. The solid crudes were further purified by crystallization and analysed by ^{1}H NMR.

b) General procedure for the synthesis of 6a-6d.

b.1) Method using CO_2.

In a typical experiment, a solution of **2c or 2d** (0.15 mmol) was introduced into the 25 mL steel reaction vessel. Catalyst was added if convenient. The reactor was flushed three times with CO_2 **(3)** and finally CO_2 **(3)** was introduced up to the gas bottle pressure. The reactor was heated to the established temperature for each reaction trial, and then the pressure was measured. Reaction was magnetically stirred keeping the established pressure. After the appropriate reaction time, the reactor was cooled to room temperature and depressurized. The solvent was removed under vacuum and the final residue was analysed by ^{1}H NMR.

b.2) Method using urea.

In a typical experiment, **2a-2d** (3 mmol), urea (**4)** (1.2-5.8 mmol) and the desired amount of catalyst were introduced into the Schlenk flask equipped with a water-cooled condenser. The reaction mixture was magnetically stirred under vacuum (30 kPa) at 150°C for the appropriate reaction time. The reaction was cooled to room temperature. The product was purified by column chromatography on silica gel using CH_2Cl_2: MeOH as eluent. The final chromatographic fractions were analysed by ^1H NMR.

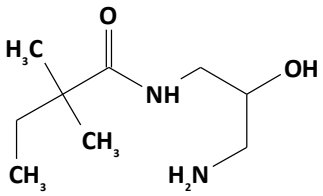
b.3) Method using cyclic carbonate.

In a typical experiment **2c** (1mmol), cyclic carbonate **(5)** (1.2 mmol) and K_2CO_3 (0.01 mmol) or Novozyme435 (10%) in DMF or *tert-butanol* (5 mL) were placed into a 25 mL round-bottomed flask equipped with a magnetic stirrer. Reaction was performed at 80°C at the established reaction time. The reaction mixture was filtered through Celite. Once the solvent was removed under vacuum, the final residue was analysed by ^1H NMR.

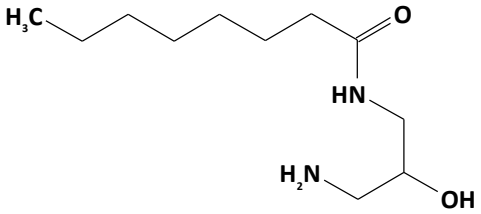
c) General procedure for the synthesis of 8d.

A stepwise addition of $NaHCO_3$ (4.21 mmol, 0.3540 g) and subsequently di-*tert*-butyl dicarbonate (**9)** (1.68 mmol, 0.3683 g) was performed to a stirred solution of **2d** (1.40 mmol, 0.4949 g) in THF: H_2O (1: 1, 30 mL) cooled in an ice bath. The reaction mixture was stirred for 30 min at 0°C and left to reach room temperature. The solvent was evaporated under vacuum, and the crude product was purified by column chromatography on silica gel. The final chromatographic fractions were analysed by ^1H NMR.

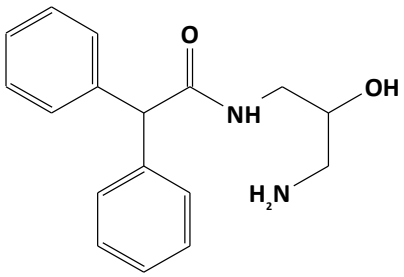
2.3.3.- EXPERIMENTAL DATA.



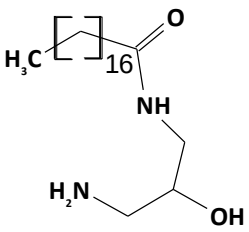
N-(3-Amino-2-hydroxypropyl)-2,2-dimethylbutanamide (**2a)** was synthesized as a colourless oil. Yield = 93%. ^{1}H NMR (400 MHz, $CDCl_3$)δ 6.14 (1 H, s), 3.60 – 3.50 (1 H, m), 3.32 (2 H, dddd, *J* = 25.7, 20.3, 9.4, 4.8), 2.65 (2 H, ddd, *J* = 19.9, 12.6, 5.8), 1.53 – 1.42 (2 H, q), 1.15 – 1.03 (6 H, m), 0.83 – 0.72 (3 H, t). ^{13}C (101 MHz, $CDCl_3$) δ 178.95, 71.18, 44.68, 43.16, 42.45, 33.82, 24.96, 9.20. IR (ATR/ν) 3342.03, 2966.95, 1632.45 .HRMS (ESI) m/z calcd. for $C_9H_{21}N_2O_2$ $[M+H]^+$ 189.1598 , found 189.1598.



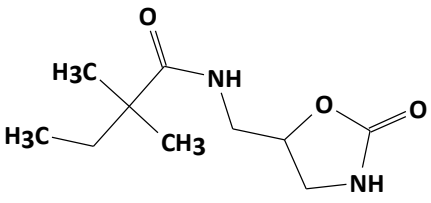
N-(3-Amino-2-hydroxypropyl)octanamide (2b) was synthesized as a white solid. Yield = 60%. M_p(°C)=114.8-119.0. ^{1}H NMR (400 MHz, CD_3OD) δ 3.67 (1 H, dtd, *J* =7.5, 5.8, 4.2), 3.27 – 3.17 (2 H, m), 2.67 (2 H, ddd, *J* =20.7, 13.2, 5.8), 2.23 – 2.16 (2 H, t), 1.64 – 1.55 (2 H, t), 1.36 – 1.25 (8 H, m), 0.90 (3 H, t, *J* =6.9).^{13}C NMR (101 MHz, CD_3OD) δ 175.51, 69.81, 43.55, 42.45, 35.60, 31.49, 28.92, 28.68, 25.55, 22.25, 12.99. IR (ATR/ν): 3354.57, 2916.81, 1639.2. HRMS (ESI) m/z calcd. for $C_{11}H_{25}N_2O_2$ $[M+H]^+$ 217.1911, found 217.1912.



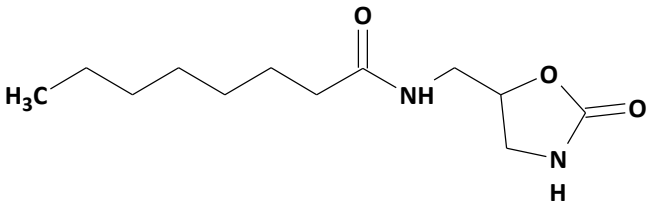
N-(3-Amino-2-hydroxypropyl)-2,2-diphenylacetamide (2c) was synthesized as a white power. Yield = 68%. M_p(°C)=99.7-103.8. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.21 (10 H, m), 6.10 (1 H, s), 4.92 (1 H, s), 3.60 (1 H, ddd, *J* =13.7, 6.9, 4.1), 3.36 (2 H, dddd, *J* =13.9, 11.9, 6.4, 3.9), 2.67 (2 H, ddd, *J* =19.8, 12.7, 5.7). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.74, 139.33, 128.81, 128.68, 127.20, 70.93, 59.23, 44.69, 43.51. IR (ATR/ν) 3315.97, 2924.23, 1644.12. HRMS (ESI) m/z calcd. for $C_{17}H_{21}N_2O_2$ $[M+H]^+$ 285.1598, found 285.1600.



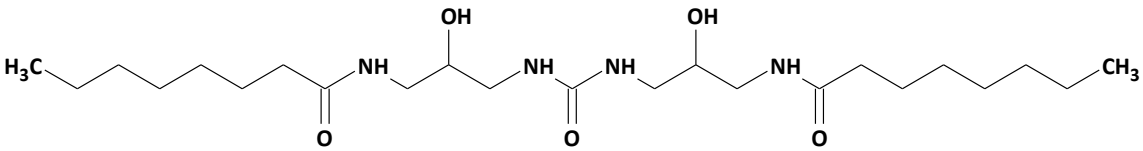
N-(3-Amino-2-hydroxypropyl)octadecanamide (2d) was synthesized as a white solid. Yield = 63%. M_p(°C)=114.9-118.1. ^{1}H NMR (400 MHz, CD_3OD) δ 3.65 – 3.56 (1 H, m), 3.26 – 3.14 (2 H, m), 2.61 (2 H, ddd, *J* =20.3, 13.2, 5.7), 2.19 (2 H, t, *J* =7.1), 1.59 (2 H, m), 1.30 (28 H, d, *J* =14.0), 0.89 (3 H, t, *J* =6.7). ^{13}C NMR (101 MHz, CD_3OD) δ 175.35, 70.53, 42.53, 35.68, 31.56, 29.26, 29.12, 28.94, 25.52, 22.21, 12.89. IR (ATR/ν): 3299.91, 2921.96, 1636.40. HRMS (ESI) m/z calcd. for $C_{21}H_{44}N_2O_2$ $[M+H]^+$ 357.3476, found 357.3472



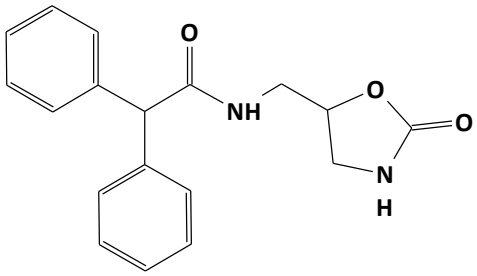
2,2-Dimethyl-N-[(2-oxo-1,3-oxazolidin-5-yl)methyl]butanamide (6a) was synthesized as a yellow oil.Yield = 3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.29 (1 H, t, *J* =5.9), 5.99 (1 H, s), 4.75 (1 H, tdd, *J* =9.6, 6.5, 3.2), 3.67 (2 H, dtd, *J* =12.4, 6.4, 3.4), 3.52 – 3.44 (1 H, m), 3.35 – 3.31 (1 H, m), 1.58 – 1.50 (2 H, q, *J* =7.4), 1.19 – 1.12 (6 H, m), 0.88 – 0.77 (3 H, t , *J* =7.4). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.67, 159.25, 75.82, 43.03, 42.59, 42.26, 33.73, 24.92, 24.90, 9.16. IR (ATR/ν): 3199.33, 2935.13, 1735.62. HRMS (ESI) m/z calcd. for $C_{18}H_{19}N_2O_3$ $[M+H]^+$ 311.1383, found. 311.1390.



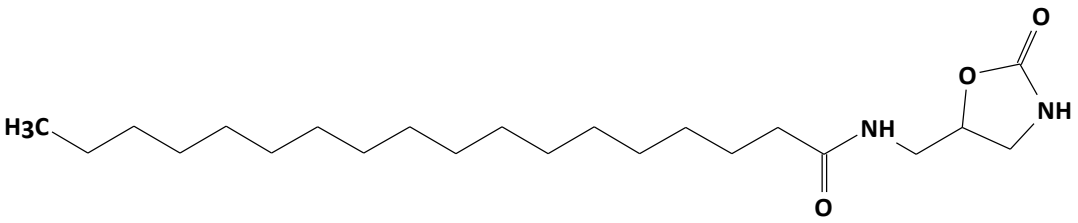
N-[(2-Oxo-1,3-oxazolidin-5-yl)methyl]octanamide (6b) was synthesized as a white solid. Yield = 10%. M_p(°C)=90.3-91.5, ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.07 (1 H, s), 5.35 (1 H, s), 4.82 – 4.71 (1 H, m), 3.73 – 3.65 (2 H, m), 3.50 (1 H, dt, *J* 14.6, 6.2), 3.36 (1 H, ddd, *J*= 8.9, 6.6, 0.8), 2.21 (2 H, dd, *J* =16.2, 8.3), 1.63 (3 H, dd, *J*= 14.9, 7.5), 1.36 – 1.20 (9 H, m), 0.89 (3 H, dd, *J* =8.4, 5.5).^{13}C NMR (101 MHz, $CDCl_3$) δ 173.96, 158.99, 75.73, 42.92, 41.94, 36.59, 31.65, 29.20, 28.96, 25.65, 22.59, 14.05. IR (ATR/ν): 3305.39, 2922.59, 1752.01, 1726.94. HRMS (ESI) m/z calcd. for $C_{12}H_{22}N_2O_3Na$ $[M+Na]^+$ 265.1630, found.265.1677



Compound 7b was synthesized as a white solid. Yield = 42%. M_p(°C)= 118-120ºC ^{1}H NMR (400 MHz, D_6MSO) δ 7.73 (1 H, t, *J* =5.7), 5.95 (1 H, t, *J* =5.7), 4.99 (1 H, d, *J*= 4.8), 3.44 (1 H, q, *J* =11.0, 4.9), 3.08 – 2.94 (3 H, m), 2.94 – 2.81 (1 H, m), 2.07 (2 H, t, *J*= 7.5), 1.51 (2 H, m,), 1.51 – 1.09 (8 H, m), 0.86 (3 H, t, *J*= 6.9) ^{13}C NMR (101 MHz, D_6MSO) δ 172.86, 159.57, 69.80, 43.08, 35.86, 31.63, 29.11, 28.92, 25.75, 22.53, 14.41. IR (ATR/ν): 3417.24, 3333.36, 3257.18, 2921.63, 1634.38, 1609.31. HRMS* (ESI) m/z calcd. for $C_{23}H_{46}KN_4O_5$ $[M+K]^+$ 497.3139, found 497.3100. *just confirmed with one trial.



N-[(2-Oxo-1,3-oxazolidin-5-yl)methyl]-2,2-diphenylacetamide (6c) was synthesized as a white solid. Yield = 13%. M_p(°C)=174.2-175.1. ^{1}H NMR (400 MHz, D_6MSO) δ 8.62 (1 H, t, *J* =5.8), 7.47 (1 H, s), 7.34 – 7.18 (10 H, m), 5.02 (1 H, s), 4.63 – 4.52 (1 H, m), 3.47 (1 H, t, *J*= 8.8), 3.38 – 3.26 (2 H, m), 3.11 (1 H, dd, *J*= 8.6, 6.8). ^{13}C NMR (400 MHz, D_6MSO) δ 172.15, 158.95, 140.71, 140.64, 128.91, 128.87, 128.67, 127.06, 74.58, 56.69, 42.83, 42.20. IR (ATR/ν): 3262.97, 3084.58, 1743.33, 1649.80. HRMS (ESI) m/z calcd. for $C_{18}H_{19}N_2O_3$ $[M+H]^+$ 311.1370 found. 311.1390



N-[(2-Oxo-1,3-oxazolidin-5-yl)methyl]octadecanamide (6d) was synthesized as a white solid. Yield = 8%. · M_p(ºC)=116.7-118.2. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.90 (1 H, s), 3.55 (1 H, td, *J* =7.2, 3.7), 3.45 – 3.05 (1 H, m), 2.65 (1 H, ddd, *J* =20.1, 12.7, 5.8), 2.13 (1 H, t, *J* =7.7), 1.60 – 1.49 (1 H, m), 1.20 (7 H, d, *J* =13.4), 0.81 (1 H, t, *J* =6.9). ^{13}C NMR (101 MHz, $CDCl_3$) 172.8481, 160.9660, 77.30, 41.91, 36.62, 31.91, 30.91, 29.68, 29.64, 29.60, 29.46, 29.34, 29.30, 29.25, 25.64, 22.67, 14.10. IR (ATR/ν): 3296.71, 2916.81.96, 1729.83, 1640.16,. HRMS (ESI) m/z calcd. for $C_{22}H_{43}N_2O_3$ $[M+H]^+$ 383.3285, found 383.3268.

2.4.- RESULTS AND DISCUSION.

2.4.1.- AZIDE REDUCTION.

The 1,3-diazidopropyl esters were synthesised in two steps as is described in the Introduction section(I.1.2.2.2.3, Scheme I12). The first step consisted on the reaction of glycerol, chlorotrimethylsilane and the corresponding carboxylic acid to yield dichloropropyl esters[18, 36]. Subsequently, sodium azide was used to substitute the chlorine atoms to yield the 1,3-diazopropyl derivatives[37, 38].
1,3-Diazidopropan-2-yl 2,2-dimethylbutanoate **(2a)** was used to perform the initial reduction studies. Once the suitable reaction conditions were determined, we proceeded to synthesize the other derivatives.

2.4.1.1.- Enzymatic reduction.

The use of biomass as staring material is related to the concept of industrial biotechnology. Industrial biotechnology seeks to apply biotechnological processes, usually cleaner than the chemical ones, to synthesize the desired compounds. Among other biotechnological processes, the employment of biocatalysts for organic synthesis has become an attractive alternative to conventional chemical methods. In fact, enzymes quite often display high chemo-, regio-, and enantioselectivity. In addition, biocatalytic processes are usually less hazardous, less polluting, and less energy-consuming than conventional-chemistry-based methodologies.

The use of *Baker's yeast* has been reported as a useful procedure to reduce azides under mild conditions[39, 40]. Following the described methodology (methodology, a.1), two experiments we performed using yeast that had been previously incubated in a phosphate buffer (pH 7.2). Table 2.1 shows the conversion results obtained when yeast was mixed with the corresponding azide in a 50% EtOH solution at room temperature. The first reaction was conducted for 24 h (entry 1.6) and the second for 48 h (entry 1.7). None of them showed any conversion of the starting material. Similar results using different alkyl azides had already been described[39].

We decided to try with other biocatalysts. These biocatalysts were selected from enzymes that had previously been used for long in our laboratory and also some fungi, that were grown in our laboratory and known to contain a mixture of likely useful lipases.

The heterogeneous reactions were performed in MeOH using the optimal pH and temperature conditions described for each enzyme (entries 1.1-1.3) or fungi (entry 1.4). Table 2.1 summarizes the conditions assayed. Enzyme or fungi were separated form the media by filtration and MeOH was eliminated by evaporation under vacuum. The presence of starting material as a single compound was determined in all final crude residues by FT-IR and ^{1}H NMR. MW irradiation and Amano lipase PS[41] also yielded starting material alone (entry 1.5).

Table 2.1: 1,3-diazidopropan-2-yl 2,2-dimethylbutanoate enzymatically reduction trials.

Entry	Biocatalyst	Solvent	Reaction time	Temperature (°C)	Conversion (%)
1.1	Amano-lipasa PS-LM	MeOH	24 h	50	0%
1.2	Lipozyme IM rom mucor miehei	MeOH	24 h	50	0%
1.3	Lipase acrylic resin from candida antarctica	MeOH	24 h	50	0%
1.4	R. Oryzae	MeOH	24 h	50	0%
1.5	Amano lipasa PS, form pseudomonascepacia	MeOH	5 min	50(MW)	0%
1.6	*Baker's yeast* (pH 7.2)	EtOH 50%	24 h	r.t.	0%
1.7	*Baker's yeast* (pH 7.2)	EtOH 50%	48 h	r.t.	0%

In summary, the diazide derivatives cannot be reduced using the studied biocatalysts.

2.4.1.2.- Reduction using a metal as catalyst.

We decided to study various metals or metal salts as catalysts to reduce the diazide compounds. The first trials were conducted using iron sulphate ($FeSO_4 \cdot 7H_2O$)[42]. The reaction was performed adding the iron sulphate suspended in a 25% NH_3 aqueous solution into a solution in CH_2Cl_2 of the desired diazide. The reaction was stirred for 4 h, at room temperature. Although the colour of the reaction mixture changed from green to yellow, which could indicate the oxidation of iron II to iron III, only starting material was present in the final crude reaction mixture (entry 2.1, Table 2.2). Equivalent results were obtained when the reaction was repeated at the same conditions for 24 h (entry 2.2).

Considering the results using iron sulphate, we decided to study the same reduction reaction using ammonium chloride and zinc[43]. The reaction was performed under reflux conditions (entry 2.3) and stopped when the Zn metal completely disappeared observing the formation of a white precipitate. Nevertheless, the starting material was the only compound present in the reaction crude mixture. The reaction was repeated continuing for a longer time after the Zn solid disappeared, but the starting material was again the only compound detected (entry 2.4).

We decided to study the performance of a ruthenium catalyst[44], since it has been described as a very efficient catalysis for the reduction of azides. Two trials were performed, one at room temperature and the other in refluxing MeOH. Table 2.2 shows just starting material as recovered compound (entries 2.5 and 2.6, respectively).

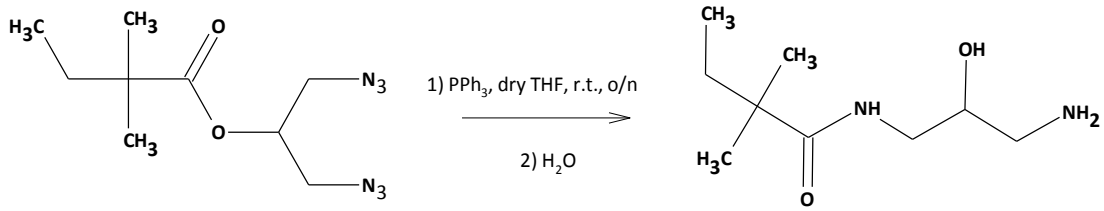
Table 2.2: 1,3-diazidopropan-2-yl 2,2-dimethylbutanoate metal catalysed reduction trials.

Entry	Catalyst	Solvent	Reaction time	Temperature (°C)	Conversion (%)
2.1	$FeSO_4 \cdot 7H_2O + NH_3$	CH_2Cl_2	4 h	r.t.	0 %
2.2	$FeSO_4 \cdot 7H_2O + NH_3$	CH_2Cl_2	24 h	r.t	0 %
2.3	NH_4Cl/Zn	EtOH: H_2O (3: 1)	5 h	Reflux	0 %
2.4	NH_4Cl/Zn	EtOH: H_2O (3: 1)	48 h	Reflux	0 %
2.5	$(PPh_3)_3RuCl_2$	Acetone	o/n	r.t.	0 %
2.6	$(PPh_3)_3RuCl_2$	Acetone	o/n	Reflux	0 %

o/n = over night

Considering these results, we decided to study a standard method for azide reduction.

2.4.1.3.- Staundinger Reaction.



1a **2a**

Scheme 2.8: 1,3-diazidopropan-2-yl 2,2-dimethylbutanoate reduction using PPh_3.

Table 2.3, entry 3.1, shows the results obtained in the first study conducted with PPh_3 according the standard Staundinger reaction[45]. PPh_3 was added into a solution of the desired azide in dry THF. The reaction was exothermic and the formation of a white gas was observed. The reaction was kept overnight at room temperature, then water was added and the formation of a white precipitate was observed. The product was

extracted with an organic solvent. The organic solution was dried, filtered and the organic solvent was evaporated under vacuum. The final product was not the expected diamine derivative but N-(3-amino-2-hydroxypropyl) 2,2-dimethylbutanamide (**2a**). The reaction led to an O-to-N acyl rearrangement to yield the amide instead of the expected diamine product. Scheme 2.8 shows the proposed transformation occurred in this reaction. A similar rearrangement had been described for products containing an azide and a carboxyl group in the same molecule[46]. Considering that **2a** still has two functional groups able to react with CO_2, we decided to follow with the planned synthesis.

Nevertheless, the remaining PPh_3 and the by-product $OPPh_3$ were really difficult to completely remove from the reaction media. Consequently, the purification of the final product was hardly to achieve.

The use of MeOH: H_2O instead of THF as solvent was also studied to perform the azide reduction[47]. The reaction performed in a similar manner in this more eco-friendly solvent (Table 2.3, entry 3.2). Unfortunately, once again, it was very difficult to completely remove the $OPPh_3$ formed and the excess of PPh_3 used. The high solubility shown by both $OPPh_3$ and PPh_3 in most of the solvents[48] hampered the purification of the product by crystallization. Even the purification by column chromatography yielded a product with remaining triphenylphosphine and triphenylphosphine oxide.

The synthesis in acid media was also studied[49, 50]. The final product expected to be soluble in the aqueous layer whereas triphenylphosphine and triphenylphosphine oxide would remain in the organic layer. Compound **1a** was dispersed in 5% HCl and PPh_3 in Et_2O/AcOEt was added (entry 3.3). The organic layer was discarded and the aqueous layer was washed with CH_2Cl_2 to remove the remaining $OPPh_3$ and non-ionic organic components. Afterwards the pH of the aqueous solution was adjusted to 8-9 by addition of 0.1M NaOH. Dichloromethane was used to extract the aqueous solution only recovering the 1,3-diamine-2-propanol. These results indicated that a hydrolytic process had occurred during the process.

The reaction was repeated using $NaHCO_3$ instead of NaOH as base to deprotonate the amine group (entry 3.4). 1-Butanol was used as extracting solvent and **2a** was recovered from the aqueous layer. Unfortunately, once again, a small amount of $OPPh_3$ and PPh_3 still remained in the final product.

Considering the observed difficulties in the final product purification process, we decided to study an alternative process to prepare the desired product.

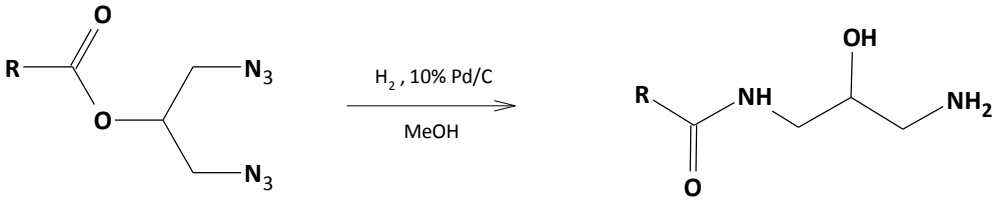
Table 2.3: Staundinger reaction trials.

Entry	Solvent	Reaction time	Acid media	Basic neutralization	1a conversion (%)[1]	2a Yields (%)[1]
3.1	THF	o/n	No	-	100	65
3.2	MeOH: H_2O	o/n	No	-	100	75
3.3	Et_2O/AcOEt	1 h (0°C) + 24 h r.t.	HCl 5%	NaOH	100	0
3.4	Et_2O/AcOEt	1 h (0°C) + 24 h r.t.	HCl 5%	$NaHCO_3$	100	71

[1] Yield determined by ^{1}H NMR

2.4.1.4.- Pd/C Hydrogenation.

A hydrogenation reaction at atmospheric pressure using Pd/C as a catalyst is an alternative for the reduction of azides[46, 51]. The reaction was performed mixing the azide with dry methanol. Two vacuum/H_2 cycles were performed afterwards to replace the air inside the vessel with hydrogen. Finally, Pd/C suspended in dry methanol was added into the sealed system through a septum. The reaction was carried out for 72 h under H_2 atmosphere. After 48 h reaction 1% of Pd/C was added to avoid the poisoning of the catalyst (Scheme 2.9). Once finished, the reaction mixture was filtered through a Celite bed to remove the catalyst. Solvent was evaporated under vacuum and the residue was analysed by ^{1}H NMR.



1a-1i **2a-2i**

R= $CH_3CH_2CH(CH_3)_2$; $(C_6H_5)_2CH$; $CH_3(CH_2)_6$; $CH_3(CH_2)_{16}$

Scheme 2.9: Derivative hydrogenation over Pd/C.

Although the reaction allowed the preparation of the desired compound avoiding the presence of phosphorus derivatives, the reaction is time-consuming and it did not perform properly when we tried to scale-up the process, even with the addition of a bigger amount of catalyst. We have hypothesised that as the balloon volume increase, the reaction pressure could slightly decrease hampering the process and lowering the

final yield. Unfortunately, we do not have pressure reactor facilities in our university to study this hypothesis.

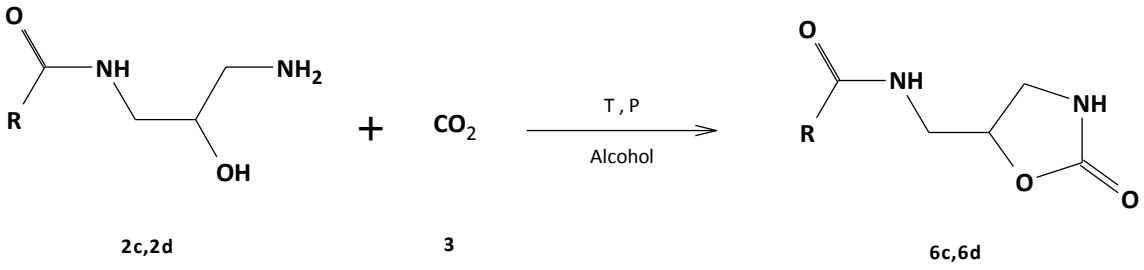
Finally, the diphenyl, C8 and C18 derivatives (**2c**, **2b** and **2d**) were synthesized. Surprisingly, the stearic derivate (**2d**) showed unexpected solubility, as it was non-soluble in most of the organic solvents. Consequently, the synthesis of compounds **2e-2h** was performed, which could allow the study of the influence of the alkyl chain in the observed behaviour. The results of this study are described in chapter 3.

2.4.2.- PREPARATION OF CYCLIC URETHANES.

Once the monoamides of the 1,3.diamine-2-propanol were prepared, the synthesis of cyclic urethanes was studied.

2.4.2.1.- Pressurized Carbon Dioxide as C Source.

Compounds **2c** and **2d** were selected as models to establish the reaction conditions (Scheme 2.10). This selection was performed considering that compound **2d** was the easiest to purify and compound **4d** absorbs at the UV light and it is easily analysed by TLC.



2c,2d **3** **6c,6d**

R=(C_6H_5)CH, $CH_3(CH_2)_{16}$

Scheme 2.10: Preparation of cyclic urethanes using pressurized CO_2.

2.4.2.1.1.- PROCESS WHITHOUT CATALYSTS.

The reaction between the selected compounds with pressurized CO_2 was initially studied using similar conditions than those already described[2]. Table 2. shows the results obtained using various conditions. Assays were performed in a 25 mL reactor following the methodology described in b.1.

The first trial was conducted at 85°C (bath temperature) and 7000 kPa for 14 h (entry 4.1). A white solid was recovered, which was neither the starting material nor the desired product. Unfortunately, the solid was very insoluble and could not be identified. The second trial was conducted for 24 h at 95°C (bath temperature) and 7200 kPa (entry 4.2). The ^{1}H NMR of the crude product did not show any signal corresponding to the desired product.

Consequently, we decided to work at higher temperatures. The third trial was performed at 125°C to reach higher pressure in the system. Reaction was performed at 8000 kPa for 24 h (entry 4.3). The ^{1}H NMR of the crude product mixture showed characteristic signals of the desired product (**6d**). Nevertheless, the final yield calculated by NMR was 4±1%. Considering this low yield, we decided to perform a new experiment at 150°C and 9000 kPa (entry 4.4). The yield of **6d** determined by ^{1}H NMR was 7±1 %.

Because we expected some effect of the amide chain in the product formation, we decided to repeat some of the trials using the diphenyl compound (**2c**). The first trial was performed at 120°C and 7400 kPa for 24 h (entry 4.5). The ^{1}H NMR of the crude product mixture did not show any signal corresponding to the desired product (**6c**). In this case, the presence of 40% of the starting material (**2c**) was observed in the crude reaction mixture. Considering this result and the previous ones, the reaction was performed at 150°C and 9000 kPA (entry 4.6). Product **6c** was synthesised with a 9±1% yield determined by ^{1}H NMR.

These poor results led us to study alternative ways to improve the yields of the desired compounds.

Table 2.4: Results of the reactions of the monoamides of the aminoacohols with CO_2 in methanol and the absence of catalyst.

Entry	R	Substrate mmols	Solvent volume	Pressure (KPa)	Temperature (°C)	Reaction time	6 Yield[1](%)	Conversion[1] (%)
4.1	$CH_3(CH_2)_{16}$	0.16	3 mL	7000	85	14 h	n.d.	100
4.2	$CH_3(CH_2)_{16}$	0.15	3 mL	7200	95	24 h	n.d.	100
4.3	$CH_3(CH_2)_{16}$	0.15	3 mL	8000	125	24 h	4±1	100
4.4	$CH_3(CH_2)_{16}$	0.14	3 mL	9000	150	24 h	7±1	100
4.5	$(C_6H_5)_2CH$	0.13	3 mL	7400	120	24 h	n.d.	60
4.6	$(C_6H_5)_2CH$	0.12	3 mL	9000	150	24 h	9±1	100

[1]Yield determined by ^{1}H NMR. n.d.= no detected by ^{1}H NMR.

Two approaches could be adopted to improve the usual moderate yields in the preparation of cyclic urethanes from aliphatic or aromatic amino alcohols[2]. One

consists on the employment of homogeneous catalysts[24, 29, 32]. The other approach consisted in the use of dehydrating reagents in stoichiometric ratio[52]. Nevertheless, this last approach was considered too much expensive in terms of CO_2 recovering.

2.4.2.1.2.- USING CATALYSTS.

Table 2.5 shows all the performed attempts using Cs_2CO_3 as catalyst. The general idea was to increase the nucleophile strength of the OH group. Nevertheless, all the performed conditions at high temperature and pressure (entries 5.1 to 5.3) yielded the desired compound with yields lower than 10%. .

A next experiment was performed using DMSO instead of alcohol as solvent (entry 5.4). Reaction was performed at atmospheric pressure using Cs_2CO_3 as catalyst[24]. The ^{1}H NMR of the crude reaction mixture determined the only presence of starting material.

The reaction was conducted in EtOH as solvent using ionic liquid and K_2CO_3 as catalysts[21] (entry 5.5). The ^{1}H NMR of the crude reaction mixture showed that the desired product **6d** has been synthesized with a 6% yield (yield assessed by ^{1}H NMR), but the yield was considered not high enough to purify the desired product by column chromatography, taking into account the added difficulty to completely remove the IL.

Finally, the synthesis of **2c** was studied using MeOH as solvent and Cs_2CO_3 as catalyst (entry 5.6). The formation of the desired product (**6c**) could be observed, also with a low yield (12%). Moreover, the deprotonation of the α-carbon of the alkyl chain was observed in some extend. This deprotonation process should be favoured by the basic medium.

In summary, the use of a basic catalysis did not highly improve the formation of the corresponding cyclic urethanes.

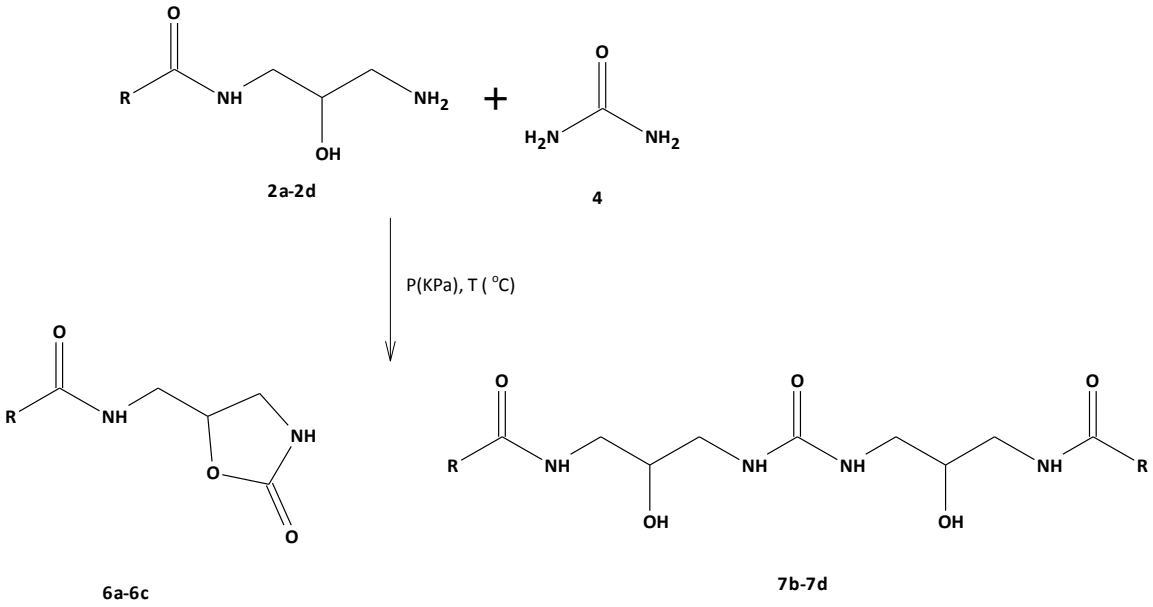
Table 2.5: Results of the reactions of aminoacohols with CO_2 with catalyst use.

Entry	R	Solvent	Pressure (KPa)	Temperature (°C)	Catalyst	2 Conversion (%)[1]	6 Yield (%)[1]
5.1	$CH_3(CH_2)_{16}$	MeOH	6800	128	Cs_2CO_3 (10%)	100	4±1
5.2	$CH_3(CH_2)_{16}$	2-Propanol	7400	150	Cs_2CO_3 (10%)	70	10±1
5.3	$CH_3(CH_2)_{16}$	*tert*-BuOH	6400	140	Cs_2CO_3 (10%)	72	n.d.
5.4	$CH_3(CH_2)_{16}$	DMSO	101	150	Cs_2CO_3 (10%)	0	n.d.
5.5	$CH_3(CH_2)_{16}$	EtOH	7000	100	BMIM·PF_6 / K_2CO_3	100	6±1
5.6	$(C_6H_5)_2CH$	MeOH	9400	160	Cs_2CO_3	100	12±1

[1] Yield determined by ^{1}H NMR

2.4.2.2.- Using Urea as Reagent.

2.4.2.2.1.- FIRST TRIALS.



R= $(C_6H_5)_2CH_3$; $CH_3(CH_2)_6$; $CH_3(CH_2)_{16}$; $CH_3CH_2C(CH_3)_2$

Scheme 2.11: Synthesis of cyclic urethanes using urea as reactant.

We decided to perform the synthesis of the urethane derivatives using urea as a reagent (Scheme 2.11). We used the conditions already described in the literature[12]. Table 2.6 shows that the starting conditions (entry 6.1) yielded a mixture of products. The desired compound (**6c)** was present in this mixture with a 41% yield as was determined by ^{1}H NMR. Trying to improve this yield for **6c,** we performed the reaction under the same conditions but increasing the urea content. We expected that a higher urea ratio would improve the **6c** yield (entry 6.2) by a dilution effect. Nevertheless the yield dropped to 21% and the formation of several unidentified by-products was observable. Considering that the use of small amount of reagents could hinder an

increase in the reaction yield, we decided to scale-up the reaction using the initial conditions (entry 6.3). The final yield (43%) was very similar to the previous one.

Still trying to dilute the media concentration, we decided to perform the reaction using 0.1 mL of IL as a solvent (entry 6.4). The other reaction parameters were maintained as the starting ones. However, we were not able to completely remove the IL from the crude reaction mixture. Consequently, we were not able to determine the presence of the desired compound in this mixture.

First of all, considering that the reaction was a solvent-free process and that both reagents were solids, we decided to grind together the two reagents (entry 6.5). Nevertheless, the yield was not improved despite the contact between the reagents had been increased by mixing.

Subsequently, the reaction was conducted at lower temperature (entry 6.6). The presence of the starting compound (**2c**) corroborated the need for higher temperatures in order to transform the limiting reagent (**2c**).

Finally, the reaction was performed for 5 h with a higher amount of urea and grinding the two reagents before stating the reaction (entry 6.7). The increase of the reaction time slightly improved the yield when comparing with entry 6.2. Moreover, the yield was lower than entries 6.1, 6.3 and 6.5 with a higher presence of unidentified by-products.

Table 2.6: Results of the reactions of aminoalcohols with urea in a solvent-free process at 80KPa.

Entry	R	4 mols	Temperature (°C)	Catalyst	Reaction time	Conversion (%)	6 Yield (%)[1]
6.1	$(C_6H_5)_2CH$	1.2	150	-	3h	100	41
6.2	$(C_6H_5)_2CH$	5.8	150	-	3h	100	21
6.3	$(C_6H_5)_2CH$	1.2	150	-	3h	100	43
6.4	$(C_6H_5)_2CH$	1.2	150	$BMIM{\cdot}PF_6$	3h	-	-
6.5	$(C_6H_5)_2CH$	1.2	150	-	3h	100	44
6.6	$(C_6H_5)_2CH$	1.2	110	-	3h		
6.7	$(C_6H_5)_2CH$	5.0	150	-	5h	100	28

[1:] Yield determined by ^1H NMR.

Considering these results some of the experiments were repeated using **2d**.

Table 2.7 shows that all the yields were lower for **2b** than for **2c** using an equivalent procedures. The reaction with an IL (entry 7.3) as catalyst presented the same problem than using **6c.** The IL was not removed from the reaction mixture and we were not able to assess the presence of the desired compound in the crude reaction mixture.

The use of Cs_2CO_3 as a catalyst to enhance the acidity of the alcohol group in order to increase the cyclic urethane yield (entry 7.4) was not successful. In contrast, the yield was very low and the presence of the limiting reagent was detected.

The best yields were reached by grinding the two reagents together before heating starts (entries 7.2 and 7.5).

Table 2.7: Results of the reactions of aminoalcohols with urea in a solvent-free process at 80KPa.

Entry	R	mols (4)	Temperature (°C)	Catalyst	Reaction time	Conversion (%)	6 yield (%)[1]
7.1	$CH_3(CH_2)_{16}$	1.2	150	-	3h	100	24
7.2	$CH_3(CH_2)_{16}$	4.6	150	-	3h	100	29
7.3	$CH_3(CH_2)_{16}$	4.6	150	BMIM PF_6	3h	-	-
7.4	$CH_3(CH_2)_{16}$	1.2	150	Cs_2CO_3	3h	70	3
7.5	$CH_3(CH_2)_{16}$	1.2	150	-	5h	100	28

[1]Yield determined by ^{1}H NMR.

2.4.2.2.1.- SCOPE OF THE UREA REACTION.

Once the methodology was established, the reaction scope was studied using urea and **2a-2d** derivatives as reagents.

Table 2.8 shows the formation of the desired compounds **6a** to **6d** with yields up to 59% (yields determined by ^{1}H NMR). The two shortest alkyl chains yielded the lowest percentages of the desired products (**6b** and **6d**). The highest yield was reached using the biphenyl derivative (entry 8.3) confirming the results described above.

The purification of each reaction crude mixture allowed the isolation of the pure cyclic urethanes although the yields were lower than the expected. Nevertheless, 6c was the product isolated with the highest yield.

Chromatographic purification of the reaction crude mixture resulting from **2b** yielded the urea derivative **7b** (40%isolation yield). The equivalent urea derivative for **2d** was isolated in a very low yield (entry 8.4). The equivalent compound was not isolated when the alpha-substituted carboxamides (**2a** and **2c**) were used as starting materials (entries 8.3 and 8.4), showing the clear influence in the reaction of the side chain.

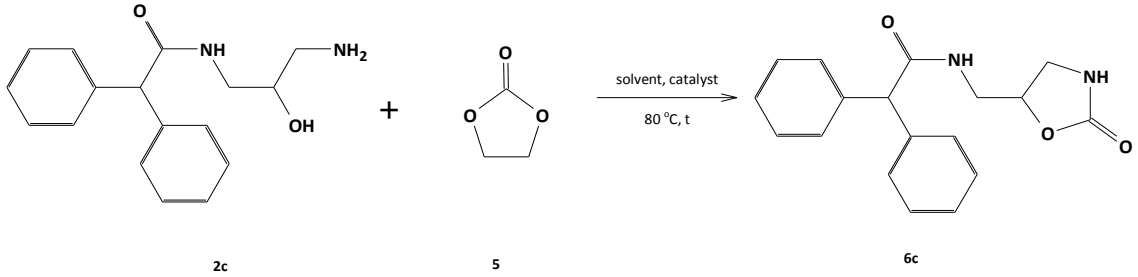
Table 2.8: Results of the reactions of aminoalcohols with urea in a solvent-free media at 80 kPa, 150°C.

Entry	R	6 Yield (%)[1]	6 Yield (%)[2]	7 Yield (%)[2]	Conversion (%)
8.1	$CH_3(CH_2)_{16}$	25	8	<1	100
8.2	$CH_3(CH_2)_6$	16	10	42	100
8.3	$(C_6H_5)_2CH$	59	13	n.d.	100
8.4	$CH_3CH_2C(CH_3)_2$	14	3	n.d.	100

n.d=no detected. [1]Yield determined by ^{1}H NMR. [2]Yield after column purification

2.4.2.3.- Using 1,3-Dioxolan-2-one as Reagent.

Finally, we decided to study the formation of the cyclic urethanes using cycle-carbonates as a reactive resulting from CO_2.



2c 5 6c

Scheme 2.12: Synthesis using a cyclic carbonate as reagent.

Table 2.9 shows the results obtained in the various experiments performed using cyclic carbonate (Scheme 2.12). The first trial (entry 9.1) was performed at 80°C for 5 h using DMF as solvent and K_2CO_3 as catalyst[1]. The ^{1}H NMR of the crude reaction mixture shows the presence of starting material and a non-identified mixture of products. The reaction was then conducted for 24 h (entry 9.2) using the same solvent, temperature and catalysit. Although ^{1}H NMR showed that there was not starting material left, a non-identified mixture of compounds was also obtained.

The next reaction was performed using the enzyme Novozyme 435 as catalyst instead of K_2CO_3 (entry 9.3). Nevertheless, the starting material was the only compound present in the crude reaction mixture. The reaction was repeated using *tert*-butanol as solvent (entry 9.4) instead of DMF. This approach was performed seeking to improve the behaviour of Novozyme, which is expected to perform much better in *tert*-butanol than in DMF. The ^{1}H NMR showed a non-identified product. Considering that this was the major product of the reaction and no signals corresponding to the desired product were observed. Thus, we decided to not follow with the assays to prepare cyclic urethanes.

Table 2.9: Results of the reactions of aminoalcohols with 1,3-dioxolan-2-one.

Entry	Solvent	5 mols	Temperature (°C)	Time	Catalyst	Product
9.1	DMF	1.2	80	5h	K_2CO_3	starting material
9.2	DMF	1.2	80	24h	K_2CO_3	No identified
9.3	DMF	1.2	80	5h	Novozyme 435	starting material
9.4	*tert*-Butanol	1.2	80	5h	Novozyme 435	No identified

[1]Yield determined by ^{1}H NMR

2.5.- CONCLUSIONS.

The reduction of the synthesized diazide was achieved using PPh_3 and H_2 over Pd/C. The approach using PPh_3 yielded crude reactions mixtures hard to purify. A rearrangement process was observed inboth reaction systems yielding the corresponding monoamide of 1,3-diamino-2-propanol.

Cyclic urethanes were synthesised with low yield using pressurized CO_2. Yields were improved using urea as reagent. The influence of the alkyl amide chain in the behaviour of the reaction was also obvious.

Symmetric substituted ureas were also isolated using some reagents, which have been described as products with important applications in agriculture, medicinal chemistry and chemical processes[53-55]. The influence of the amide alkyl chain was also evident in the formation of the symmetric ureas.

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CHAPTER 3: UNEXPECTED SOLUBILITY OF ALKYL MONOAMIDES OF 1,3-DIAMINE-2-PROPANOL.

3.1.- BACKGROUND.

During the synthesis of the alkyl monoamides of 1,3-diamine-2-propanol (see chapter 2), we observed a special behaviour shown by some of these compounds. The low solubility presented for some of them prompted us to study the likely hydrogen bonds intermolecular interactions, which might take place in these compounds.

3.1.1.- HYDROGEN BONDS.

The importance of hydrogen bonding cannot be overemphasized. It may best be summarized using a definition for hydrogen bonding given in the Penguin dictionary: "*A weak electrostatic chemical bond which forms between covalently bonded hydrogen atoms and a strongly electronegative atom with a lone pair of electrons … Life would be impossible without this type of bond*"[1].

The importance of hydrogen bonding is also thoroughly demonstrated by the number of publications containing the terms "hydrogen bond" or "hydrogen bonding". For the period 2006–2008, there is an average of at least one paper published and indexed in SciFinder every hour[2].

3.1.1.1.- Physical forces involved in the hydrogen bond.

All the initial examples for hydrogen bonding had FH, OH, or NH as donors. These three elements F, O, and N are among the most electronegative in the periodic table. Hence, an H atom attached to any of these three atoms will have a significant partial positive charge. As H has only one electron and it was already participating in the covalent bond formed with F, O, or N, it was originally thought that it could not have another covalent bond.

This led *Pauling*[3] to conclude that the hydrogen bond is electrostatic (ionic) in nature. But, electrostatic interactions do play a crucial role in hydrogen bonding but cannot explain several important experimental observations including the lengthening of the X–H bond with a resultant red-shift in the experimental X–H stretching frequency. Here, electrostatic is taken to mean the interaction between rigid dipoles. Thus, defining a hydrogen bond as "no more than a particularly strong type of directional dipole-dipole interaction"[4] is certainly incomplete. As *Buckingham* wrote

“The hydrogen bond results from inter-atomic forces that probably should not be divided into components, although no doubt electrostatic and overlap interactions are the principal ingredients”[5].

In fact, *Pauling* himself estimated that hydrogen bonding in O–HO contacts could have about 5% covalent nature[3]. This was based on the HO distance of about 1.8 Å compared to the O–H distance of 1.0 Å which was taken to be 100% covalent. Several authors[6-8] have highlighted the importance of a partial covalent nature in the hydrogen bond. The partial covalent nature of the hydrogen bond has been experimentally verified during the last decade by NMR spin–spin coupling[9] and Compton scattering[10] measurements.

Today, it is well accepted that hydrogen bonding has contributions from electrostatic interactions between permanent multipoles, polarization, or induction interactions between permanent and induced multipoles, dispersion arising from instantaneous multipoles-induced multipoles, charge-transfer-induced covalency, and exchange correlation effects from short-range repulsion due to overlap of the electron distribution. The contribution from the individual forces mentioned above varies depending on the hydrogen bond donor, acceptor, and the environment.

To sum up, the hydrogen bond is often described as an electrostatic dipole-dipole interaction. However, it also has some features of covalent bonding: it is directional and strong, produces shorter interatomic distances than the sum of the van der Waals radii, and usually involves a limited number of interaction partners, which can be interpreted as a type of valence. These covalent features are more substantial when acceptors bind hydrogen from more electronegative donors.

3.1.1.2.- Hydrogen bond definition.

In 2011, an IUPAC Task Group recommended a modern evidence-based definition of hydrogen bonding, which was published in the IUPAC journal *Pure and Applied Chemistry.* This definition specifies that: “*The hydrogen bond is an attractive*

interaction between a hydrogen atom from a molecule or a molecular fragment X–H in which X is more electronegative than H, and an atom or a group of atoms in the same or a different molecule, in which there is evidence of bond formation"[11].
A typical hydrogen bond may be depicted as X–H···Y–Z, where the three dots denote the hydrogen bond. X–H represents the hydrogen bond donor. The acceptor may be an atom or an anion Y, or a fragment or a molecule Y–Z, where Y is bonded to Z.

In more specific cases, X and Y are the same and X–H and Y–H distances are the same as well leading to symmetric hydrogen bonds. In any event, the acceptor is an electron rich region such as, but not limited to, a lone pair of Y or π-bonded pair of Y–Z.
The evidence for hydrogen bond formation may be experimental or theoretical, or ideally, a combination of both. Some criteria useful as evidence and some typical characteristics for hydrogen bonding, not necessarily exclusive, include between others for a hydrogen bond X–H···Y–Z[11]:

- The forces involved in the formation of a hydrogen bond include those of an electrostatic origin.
- The atoms X and H are covalently bonded to one another and the X–H bond is polarized.
- The HY bond strength increase with the electronegativity increase of X
- The length of the X–H bond usually increases on hydrogen bond formation leading to a red shift in the infrared X–H stretching frequency and an increase in the infrared absorption cross-section for the X–H stretching vibration
- The X–H···Y–Z hydrogen bond leads to characteristic NMR spectra changes that typically include pronounced proton deshielding for H in X–H, through hydrogen bond spin–spin couplings between X and Y, and nuclear Overhauser enhancement.

3.1.2.- MOLECULAR EFFECTS OF HYDROGEN BONDS.

Hydrogen bond attractions can occur between molecules (*intermolecular*) or within different parts of a single molecule (*intramolecular*). This type of bond can occur in inorganic molecules such as water and in organic molecules like DNA or proteins.
Intermolecular hydrogen bonding is responsible for the high boiling point of water (100 °C) compared to the other 16 hydrides of the group on the periodic table that have no hydrogen bonds. Intramolecular hydrogen bonding is partly responsible for the secondary and tertiary structures of proteins and nucleic acids. It also plays an important role in the structure of polymers, both synthetic and natural.

Several studies have been involved in defining the effect of hydrogen bonds in the behaviour of polyamides and polyurethanes[12, 13]. The amide group has been described as both hydrogen donor and acceptor[14] thus giving an extra stability to the intermolecular interactions. *Zaffalon et al*[15] reported a study about surfactants presenting low solubility, which was related to the presence of hydrogen bonds[15].

3.1.3.- SPECTROSCOPIC EVIDENCE.

Spectroscopy has played a crucial role in the detection of the hydrogen bonds[16]. There are some methods that allow the study of these intermolecular interactions. Among the most commonly used we can find: FTIR spectroscopy, Raman spectroscopy, NMR spectroscopy and X-Ray diffraction, among others[17]. In our case, where compounds shows a really low solubility in a widely range of solvents the hydrogen bond study through NMR was limited in a short interval of temperatures. Also was X-Ray diffraction, because the difficulty to prepare crystals from molecules with high degrees of freedom. Was because of these reasons, that we selected to work with FTIR technique.
FTIR spectroscopy is very sensitive to the formation of hydrogen bonds[17]. As it is known, when an hydrogen bond is achieved, a charge transfer appears from the proton acceptor (Y) to the proton donor (X-H), resulting in a weakening of both, X-H bond and, Y with it adjacent bond. It results in a bond elongation, which is reflected in a decreasing of the X-H vibration frequency comparing to the non-interacting specie.

The degree of intermolecular interaction by hydrogen bonds depends on temperature and concentration. There are different systems to study them through FTIR spectroscopy, one consists in varying the concentration, and the other in modifying the temperature. As some of the components are not soluble at room temperature we decided to study they behaviour by temperature variation.

Our aim with the present chapter is to demonstrate the presence of intermolecular hydrogen bonding interactions in the monoamide synthesised, which provides different physico-chemical properties to these amides. The influence of the alkyl chain of the corresponding amide in these properties will be studied using different techniques and properties.

3.2.- OBJECTIVES.

The aim of this chapter was:

-To study and demonstrate the presence of a multicomponent hydrogen bonding system in the monoamides synthesised. These hydrogen bonds will have a clear influence on the monoamides behaviour.

3.3.- MATERIALS AND METHODS.

3.3.1.- REAGENTS AND equipment.

Tridecanoic acid (≥98% Fluka), myristic acid (>99% Sigma), pentadecanoic acid (99% Acros organics), palmitic acid (98% Aldrich), margaric acid (≥98% Sigma-Aldrich), glycerol (99.5% Fluka), CTMS (ACROS, 98%), sodium azide (99%, Aldrich), Pd on charcoal (Pd/C) (10% Aldrich), boc_2O (≥98% Fluka). Solvents were purchased from Sigma-Aldrich, Aldrich, Fluka, Supelco and Across and were used without further purification. Diazides (**1a**-**1d**) were synthesised from glycerol and the corresponding carboxylic acid according the procedure described by our group[18]. The crude reaction mixture was not purified, due to its instability, and directly used for the further hydrogenation reactions. The purification of the final resulting solid compounds was performed by crystallization.

^{1}H and ^{13}C NMR spectra were recorded on Varian AS400 MERCURYplus (^{1}H, 400MHz and ^{13}C, 100 MHz), using CD_3OD and $CDCl_3$ as solvents. The spectra were recorded at 30°C or 55°C depending on the product solubility and 20 s of relaxing time. The chemical shifts (δ) are reported in ppm relative to the solvent used. Spin multiplicities are reported as a singlet (s), doublet (d), or triplet (t) with coupling constants (*J*) given in Hz, or multiplet (m).

The melting points were measured by open capillary tubes in Gallenkamp equipment. They are uncorrected.

IR spectra were recorded on a Vertex 70 equipment in a spectral range between 400 to 4000 cm^{-1}. The equipment is prepared to take 60 spectra/second with a resolution of 16 cm^{-1} with attenuated total reflectance (ATR) and thermal control between 25°C to 200°C. The infrared spectra of the samples was recorded at room temperature, and then at a temperature interval between 40°C and 100°C. After reaching 125°C samples were cooled to 40°C and the spectrum was recorded again, to ensure the product stability. FTIR spectra with a Jasco FT/IR-6300 equipment was also recorded using ATR, in a range between 600 to 4000 cm^{-1}.

High resolution mass spectra were recorded by direct infusion to a mass spectrometer Agilent G6510AA Q-TOF using ESI ionization.

3.3.2.- METHODS.

a) General procedure for the synthesis of 2e-2i: Pd/C Hydrogenation.

The hydrogenation was performed following the procedure described at Chapter 2, section2.3.2,subsection a.9.

b) General procedure for the synthesis of 9d: BOC2O protection.

To a stirred solution of **2d** (1.40 mmol, 0.4949 g) in THF: H_2O (1: 1, 30 mL) in an ice bath, $NaHCO_3$ (4.21 mmol, 0.3540 g) and subsequently di-*tert*-butyl dicarbonate (1.68 mmol, 0.3683 g) were added stepwise. The reaction mixture was stirred for 30 min at 0°C and left to reach room temperature. The solvent was evaporated under vacuum, and the crude product was purified by column chromatography on silica gel. Compound **9d** was obtained as a white solid.

3.3.2.1.- Solubility study.

In a previously calibrated vial, a known weight of product was added. After that, a known volume of the desired solvent was added. The vial was sealed, and the mixture was magnetically stirred until reflux temperature, were it was kept for 30'.

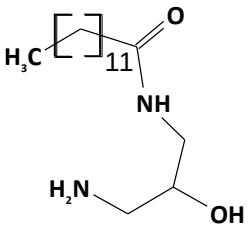
c.1) Reflux solubility studies

Using a micropipette 0.8 mL of supernatant were extracted and placed on a previously calibrated vial. The vial was weighed once it was at room temperature. The solvent was evaporated using a N_2 stream until constant weight. Considering the weight of the solution and the weight of the residue, the weight of the solvent and the product were calculated. Finally, the solubility was calculated using the corresponding density for each solvent.

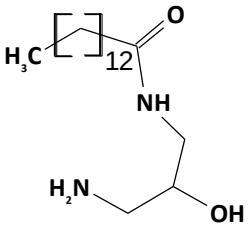
c2) Room temperature solubility studies.

Once the vial was at r.t., 0.5 mL of the supernatant transferred to a previous weighed vial. The vial was weighed and the solvent was evaporated using a N_2 stream until constant weight. Starting from these two data, the same procedure described in c1 was used to determine the solubility at room temperature.

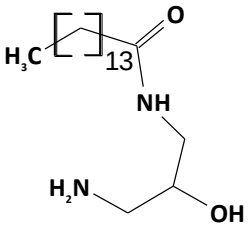
3.3.3.- EXPERIMENTAL DATA.



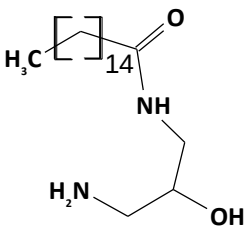
N-(3-Amino-2-hydroxypropyl)tridecanamide (2e) was synthesized as a white solid. Yield = 57%. M_p(°C)=114.11-115.9. ^{1}H NMR (400 MHz, CD_3OD) δ 3.63 (1 H, ddd, *J* =11.6, 8.0, 5.1), 3.21 (2 H, tt, *J* =11.1, 6.7), 2.61 (2 H, ddd, *J* =20.4, 13.2, 5.7), 2.22 – 2.16 (2H, t), 1.64 – 1.54 (2 H, t), 1.30 (18 H, m, *J* =11.1), 0.89 (3 H, t, *J* =6.8).^{13}C NMR (101 MHz, CD_3OD) δ 175.35, 70.52, 43.76, 42.42, 35.11, 31.33, 29.21, 25.42, 22.28, 12.92. IR (ATR/ν): 3343.00, 2953.45, 1638.23. HRMS (ESI) m/z calcd. for $C_{16}H_{35}N_2O_2$ $[M+H]^+$ 287,2693, found 287,2687.



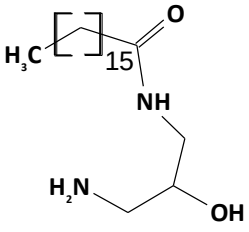
N-(3-Amino-2-hydroxypropyl)tetradecanamide (2f) was synthesized as a white solid. Yield = 99%. M_p(°C)=112.9-115.5. ^{1}H NMR (400 MHz, $CDCl_3$ δ 5.97 (s, 1H), 3.55 (m, 1H), 3.40-3.11 (2H, ddd, *J* = 14.4, 6.4, 3.7 Hz), 2.84-2.60 (2H, dd, *J* = 12.7, 7.4, 4.1 Hz), 2.19 (2H, t, *J* = 7.7 Hz), 1.69 – 1.55 (2H, t), 1.25 (20H, s,), 0.88 (3H, t, *J* = 6.9).^{13}C NMR (101 MHz, $CDCl_3$) δ 175.35, 70.64, 44.16, 42.39, 35.63, 31.65, 29.35, 29.33, 29.21, 29.05, 28.93, 25.61, 22.31, 13.01. IR (ATR/ν): 3356.5, 2966.95, 1593.88. HRMS (ESI) m/z calcd. for $C_{17}H_{37}N_2O_2$ $[M+H]^+$ 301.2850, found 301.2876



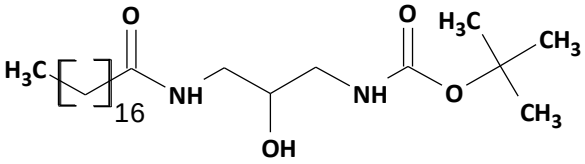
N-(3-Amino-2-hydroxypropyl)pentadecanamide (2g) was synthesized as a white solid. Yield = 75%. M_p(°C)= 115.0-117.4. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.90 (1 H, s), 3.55 (1 H, td, *J* =7.2, 3.7), 3.45 – 3.05 (2 H, m), 2.65 (2 H, ddd, *J* =20.1, 12.7, 5.8), 2.13 (2 H, t, *J* =7.7), 1.61 – 1.49 (2 H, t), 1.20 (22 H, m, *J* =13.4), 0.81 (3 H, t, *J* =6.9).^{13}C NMR (101 MHz, $CDCl_3$) δ 174.14, 70.97, 44.56, 43.04, 36.74, 31.91, 29.66, 29.63, 29.60, 29.47, 29.34, 29.29, 25.74, 22.68, 14.12. IR (ATR/ν): 3310.21, 2956.34, 1646.91. HRMS (ESI) m/z calcd. for $C_{18}H_{39}N_2O_2$ $[M+H]^+$ 315.3006 , found 315.3004.



N-(3-Amino-2-hydroxypropyl)hexadecanamide (2h) was synthesized as a white solid. Yield = 97%. M_p(°C)= 112.1-114.9. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.90 (1 H, s), 3.55 (1 H, q, *J* =7.2, 3.7), 3.45 – 3.05 (2 H, dd), 2.65-2.13 (2 H, ddd, *J* =20.1, 12.7, 5.8), 2.13 (2 H, t, *J* =7.7), 1.60 – 1.49 (2 H, m), 1.20 (24 H, d, *J* =13.4), 0.81 (3 H, t, *J* =6.9).^{13}C NMR (101 MHz, CD_3OD) δ 175.39, 70.81, 44.23, 42.39, 35.64, 31.65, 29.36, 29.33, 29.31, 29.20, 29.05, 28.92, 25.61, 22.31, 13.02. IR (ATR/ν): 3356.5, 2966.95, 1638.23. HRMS (ESI) m/z calcd. for $C_{19}H_{41}N_2O_2$ $[M+H]^+$ 329.3163, found 329.3165.



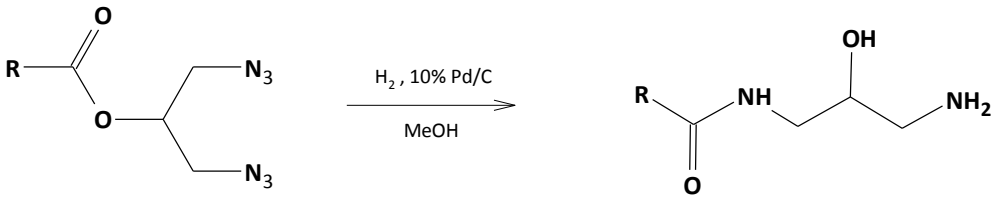
N-(3-Amino-2-hydroxypropyl)heptadecanamide (2i) was synthesized as a white solid. Yield = 69%. M_p(°C)= 109.6-112.1. ^{1}H NMR (400 MHz, CD_3OD) δ 3.70 – 3.60 (1 H, m), 3.26 – 3.16 (2 H, m), 2.66 (2 H, ddd, *J* =20.5, 13.1, 5.8), 2.19 (2 H, t, *J* =15.5, 7.8), 1.60 (2 H, m), 1.28 (26 H, s), 0.89 (3 H, t, *J* =6.8). ^{13}C (101 MHz, CD_3OD) δ 175.52, 69.28, 48.20, 47.99, 47.85, 47.78, 47.64, 47.57, 47.39, 47.35, 47.14, 46.93, 43.53, 42.42, 35.60, 31.64, 29.35, 29.20, 29.04, 28.94, 25.57, 22.30, 13.00. IR (ATR/ν): 3319.18, 2924.17, 1646.03. HRMS (ESI) m/z calcd. for $C_{20}H_{43}N_2O_2$ $[M+H]^+$ 343.3319 , found 343.3318.



***Tert*-Butyl [2-hydroxy-3-(octadecanoylamino)propyl]carbamate (9d)** was obtained as a white solid.Yield= 78%. M_p(°C)=114.9-118.1. ^{1}H NMR (400 MHz, CD_3OD) δ 3.65 – 3.56 (1 H, m), 3.26 – 3.14 (2 H, m), 2.61 (2 H, ddd, *J* =20.3; 13.2; 5.7), 2.46 – 2.39 (2 H, t, *J* =7.1), 2.19 (2 H, t, *J* =14.8; 7.0), 1.59 (1 H, m), 1.30 (10 H, d, *J* =14.0), 0.89 (3 H, t, *J* =6.7). ^{13}C NMR (101 MHz, CD_3OD) δ 175.35, 70.53, 42.53, 35.68, 31.56, 29.26, 29.12, 28.94, 25.52, 22.21, 12.89. IR (ATR/ν): 3299.91; 2921.96; 2853.52; 1636.40; 1552.90; 1469.40. HRMS (ESI) m/z calcd. for $C_{21}H_{44}N_2O_2$ $[M+H]^+$ 357.3476, found 357.3472

3.4.- RESULTS AND DISCUSSION.

The study was designed considering the likely influence on the solubility of the lateral chain of the monoamides. We have appreciated a clear unexpected behaviour, related with the solubility and the melting point, in the chapter 2 showed by the compound **2d,** and partially by **2c** meanwhile **2a** and **2b**, did not show it. Consequently, new compounds were synthesised to prepare a set of compounds presenting the same hydrophilic part of the molecule but with various acyl amide chains. These modifications included: π-systems, alkyl chains with different lengths, odd and par alkyl chains, and α-substituted acyl chains. With this purpose we have synthesized the products **2e-2i,** using the methodology already described in the chapter 2, using glycerol and the corresponding carboxylic acid as starting materials. To carry out the reductions of the azides we did a catalytic hydrogenation under mild conditions using Pd/C (Scheme 3.1)[19]. Moreover, the functional groups capable to interact through hydrogen bonding have been also modified. The work was completed with a study through temperature variable FTIR, which is a powerful tool to study the presence of hydrogen bonds[17].



1a-1i **2a-2i**

R= $CH_3CH_2CH(CH_3)_2$; $(C_6H_5)_2CH$; $CH_3(CH_2)_6$; $CH_3(CH_2)_{16}$; $CH_3(CH_2)_{11}$ $CH_3(CH_2)_{12}$; $CH_3(CH_2)_{13}$; $CH_3(CH_2)_{14}$; $CH_3(CH_2)_{15}$

Scheme 3.1: Synthesis of the monoamides 2e-2i by the hydrogenation of the corresponding diazides 1a-1i.

3.4.1.- SOLUBILITY AND MELTING POINT STUDY.

Table 3.1 shows the melting point and solubility of compounds **2a**-**2i.** Melting points are anomalous high compared with products of similar molecular weight and expected polarity[15]. These values would agree with the presence of intermolecular H-bonds, which can supply an extra stability to the interaction between the individual

molecules. This increase on the stability should be reflexed in an increase of the melting point according to previous studies[15].

Compound **2b** showed low solubility in most of the solvents studied, being completely soluble only in protic solvents at r.t. and a high melting point. These observations could be explained in terms of the ease with which this molecule achieves π-π interactions between the aromatic rings-such interactions can also increase the stability of the intermolecular interactions. This observation contrasts with **2d** or **2e**, compounds with a similar molecular weight but no aromatics. The behaviour of **2f** is also of interest; this compound with an odd chain, showed less solubility and a higher melting point than **2g**, which had a higher molecular weight. This behaviour could be attributable to the packaging capacity of the odd chain. Finally, **2h**, also with an odd chain, and **2i** showed the lowest solubility of all the compounds studied. Particularly remarkable was the behaviour of **2i**, which showed low or non-existent solubility in the organic solvents tested at r.t. and under reflux. In fact, this compound was soluble only in refluxing methanol.

Table 3.1: Reaction yields and physico-chemical properties of the synthesised products **2a-2i** .

	R	Conversion (%)	Yield (%)	m.p (°C)	Solubility (%)[a]	Conditions
2a	$CH_3CH_2C(CH_3)_2$	93.1	-	-	polar (>10)	r.t.
2b	$CH_3(CH_2)_6$	87.4	59.9	114.8-119.0	wide(>10)	r.t.
2c	$(C_6H_5)_2CH$	79.8	68.2	99.7-103.8	protic(>10)	r.t.
2d	$CH_3(CH_2)_{16}$	96.1	62.9	114.9-118.1	MeOH(>3)	Reflux
2e	$CH_3(CH_2)_{11}$	95.7	56.6	114.4-115.9	wide(>10)	r.t.
2f	$CH_3(CH_2)_{12}$	98.9	-	112.9-115.5	wide(>10)	r.t.
2g	$CH_3(CH_2)_{13}$	88.3	74.7	115.0-117.4	polar(>10)	Reflux
2h	$CH_3(CH_2)_{14}$	96.8		112.1-114.9	polar(>10)	r.t.
2i	$CH_3(CH_2)_{15}$	95.7	69.1	109.6-112.1	polar(>10)	Reflux

[a]Polar stands for: acetone, ethyl acetate, dichloromethane and chloroform. Protic stands for MeOH and EtOH. Wide implies a wide range of solvents including the previous described and also hexane and diethyl ether. Percentage in w/v.

Considering these results, we decided to deeply study the behaviour of **2d**. Table 3.2 shows the solubility of this compound in a wide range of solvents. Solubility was determined at room and reflux temperatures. The study confirms the low solubility of the compound in most of the solvents studied at reflux conditions, and the low or even inexistent solubility at room temperature conditions.

Table 3.2: Solubility studies for **2d.**

Solvent	Solubility at RT (%)[a]	Solubility at REFLUX (%)[a]
Toluene	<1	<1
Acetone	<1	<1.5
THF	<1	<1.5
can	<1	<1
MeOH	1.2	3.2
$CHCl_3$	<1	<1
H_2O [b]	decomposes	-
CH_2Cl_2	<1	-
EtOH	1.0	<2.0
AcOEt	<1	<1
Et_2O	<1	-
Hexane	<1	<1

[a]: Percentage in w/v [b]H_2O cloudy solution and appearance of gas bubbles

3.4.2.- 2D INFRARED STUDIES.

FTIR spectroscopy is a very sensitive technique to detect the formation of hydrogen bonds[17]. As described before, the charge transfer between the proton acceptor (Y) and the proton donor (X-H) results in a bond elongation, which is reflected in a decreasing of the X-H vibration frequency comparing to the non-interacting specie.

The degree of intermolecular interaction by hydrogen bonds depends on temperature and concentration. Nevertheless, **2d** is only soluble in hot methanol as already mentioned. Consequently the FTIR studies were performed with the solid using a thermostated ATR system. This system allowed us recorded the FTIR spectra from room temperature to 125°C.

3.4.2.1.- General Overview.

A general overview of the whole spectra at various temperatures led us to a first approximation about the presence of hydrogen bonds. Figure 3.1 shows that as the temperature increases the N-H stretching band (from 3200 to 3500 cm^{-1}[13]) shifts to high wavenumbers. If we focus in the carbonyl stretching bands region (amide I band, from 1620 to 1760 cm^{-1}[13]), we can observe the emergence of a new band also at high wavenumbers when the temperature increase. Although these are the two most important spectral regions to study, other regions are also clearly affected by the temperature variation. For example, the amide II band, related to the N-H in-plane bending vibration which appears near 1540 cm^{-1} at room temperature could be

studied. However, this region can also have contributions of C-N stretching and C-C stretching vibrations in this spectral area[13].

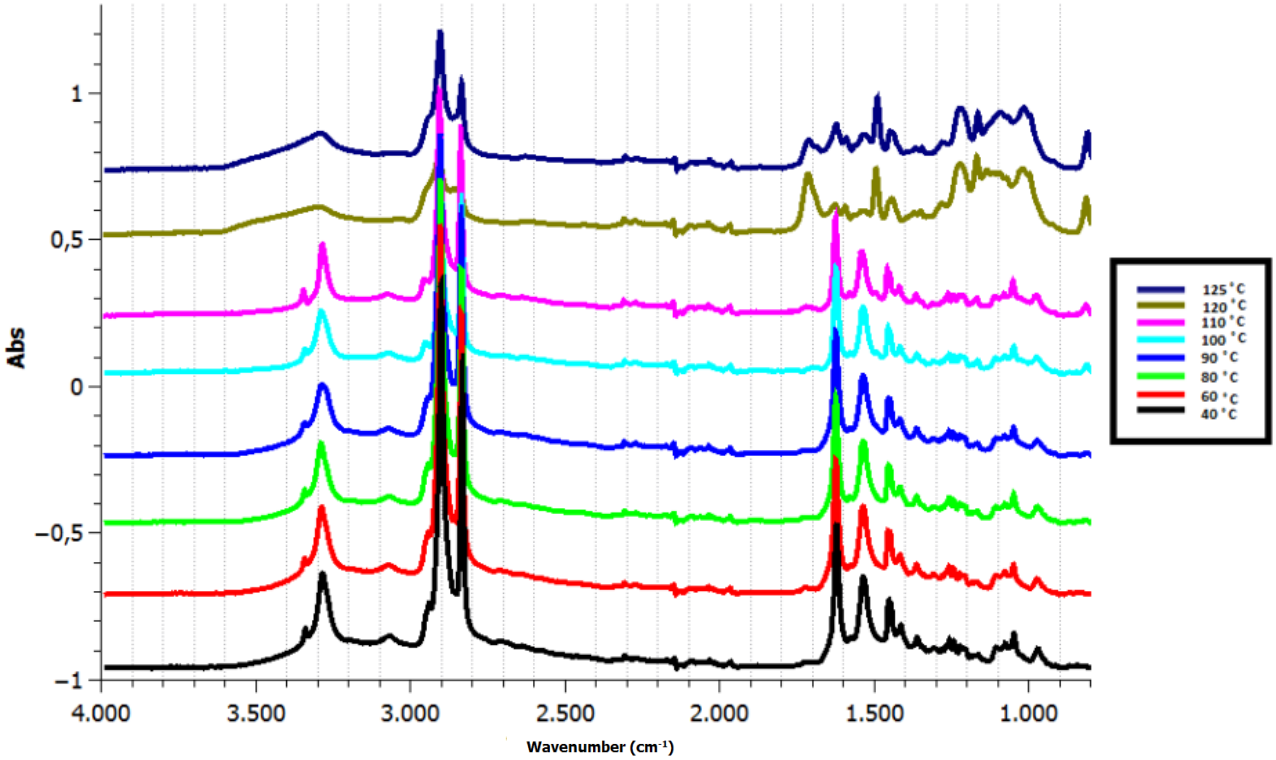


Figure 3.1: FTIR spectra of **2d** at temperatures ranging from 40°C to 125°C.

There are also the regions corresponding to the amide III and amide V, but they are also difficult to analyse because of the highly mixed regions, also the amide V is highly mixed mode containing a significant contribution from the N-H out-of-plane deformation, and they are not easy to analyses.

3.4.2.2.- 3200 to 3500 cm^{-1}. N-H/O-H Stretching Bands.

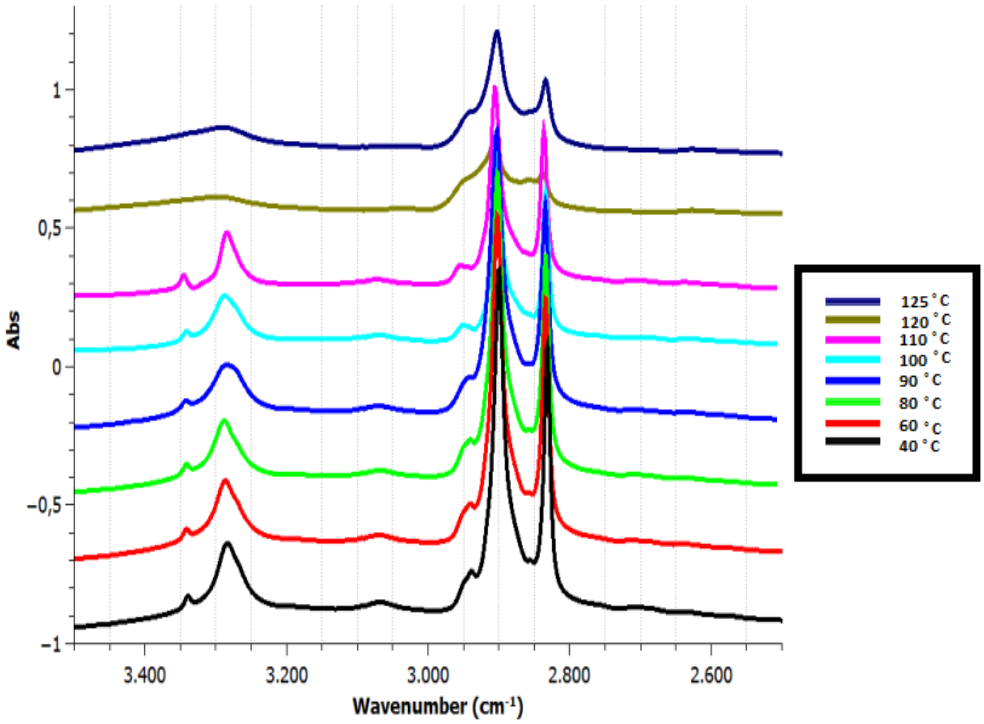


Figure 3.2: Superposition of the FTIR spectra of the O-H and N-H stretch vibration region of **2d** at various temperatures

Figure 3.2 clearly shows the changes on the region of N-H/O-H stretching bands when the temperature increases. The absorbance corresponding to the stretch vibration of the O-H and N-H becomes weak in intensity and shifts to higher wavenumbers as the temperature increases. This confirms the presence of intermolecular interactions, which become weaker as the temperature increases. As we have mentioned above, the decrease in intermolecular interactions directly affects the strength of the N-H or O-H bond, which is reflected in the increase of the wavenumber corresponding to the stretch vibration of these bonds. Is also noteworthy, the presence of two peaks at low temperatures, which could be assigned to the free H-bonded N-H groups and to the H-bonded N-H groups. Only one peak emerges at high wavenumber as temperature increases, which confirms the decrease or even disappearance of the H-bonded groups. We can also observe a widening of the band, which corresponds with the decreasing in the number of hydrogen bondings[17]. Anyway, this region includes a mix of bands resulting from the stretching vibration of three functional groups (amide, amine and alcohol), making it difficult to reach more deeply conclusions.

3.4.2.3.- Carbonyl Stretching Bands (amide I band), from 1620 to 1760 cm^{-1}.

Figure 3.3 shows the effect of temperature the carbonyl stretching bands.

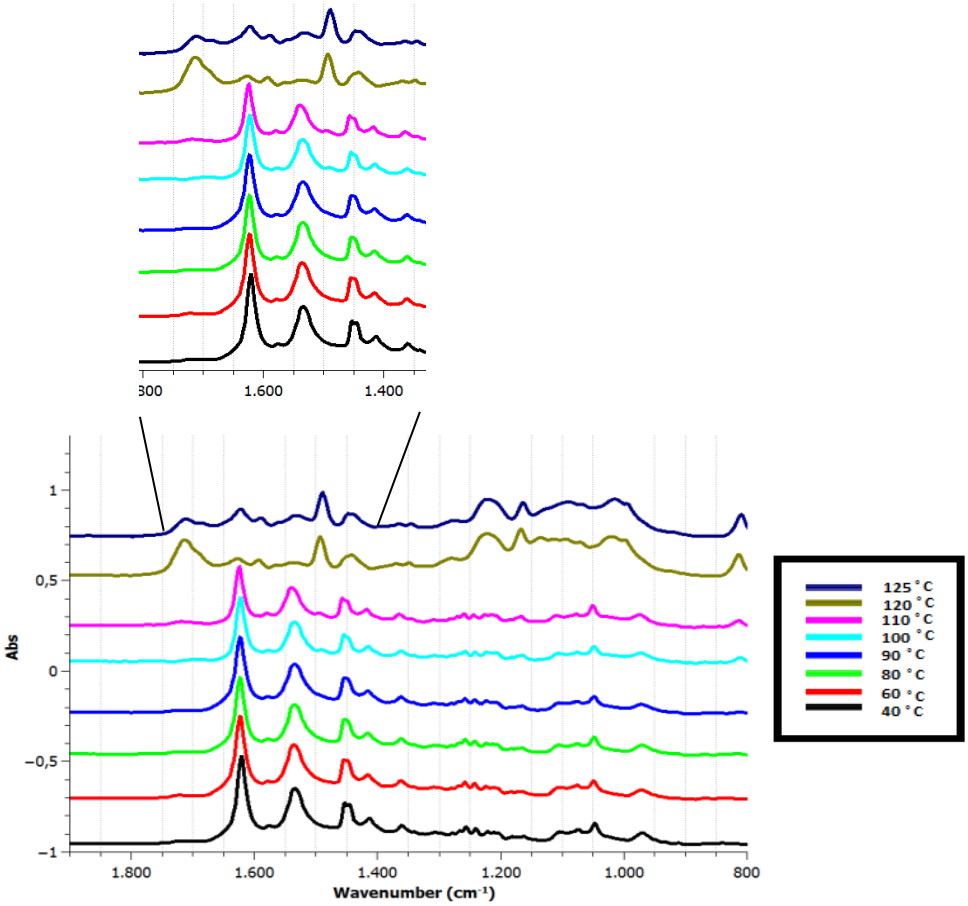


Figure 3.3: Superposition of the FTIR spectra of the carbonyl regionof **2d** at various temperatures.

The increase of temperature directly reverts into two clear effects. First of all, an intensity decrease of the band at 1634 cm^{-1}, which corresponds to the hydrogen bond to carbonyl group. Secondly, the clear emergence of a new peak at 1728 cm^{-1}, whose intensity has a direct dependence with the temperature increament. These facts could indicate that the inter-associated hydrogen bonds become weak and the number of hydrogen bond decreases with the temperature increment. A temperature increment lowes the intermolecular interactions consequence of the H-bonds. Consequently, the carbonyl bond gets stronger, which is reflexed with an increase of the wavenumber of the bond stretching.

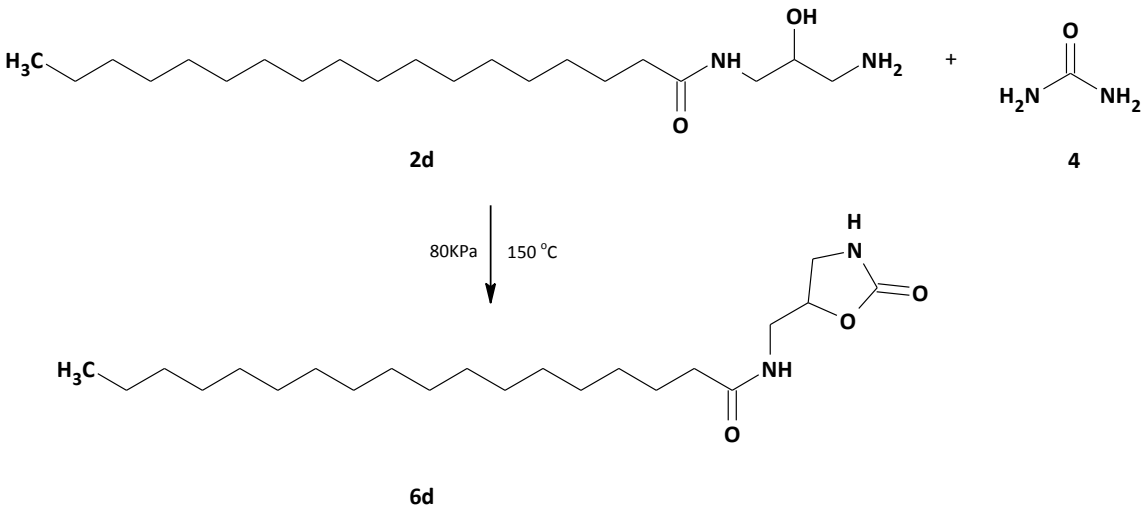
3.4.3.- CONTRIBUTION OF ALCOHOL AND AMINE GROUPS IN THE STUDIED BEHAVIOUR STUDIED.

A part of the amide group, hydroxyl and amine functional groups which are also present in **2d**, can participate in the intermolecular interactions. Consequently, we decided to perform a deeply study on the influence of these two functional groups in the behaviour of **2d**.

3.4.3.1.- Amine and Alcohol Group Derivation.

The influence of those two functional groups into the intermolecular interaction was first studied using the cyclic urethane derivate from **2d** (Scheme 3.2) prepared in the chapter 2. The cyclic urethane **6d** is a white solid, which solubility is very different than the solubility of the starting reagent **2d**. Indeed, **6d** is soluble in $CHCl_3$ and slightly soluble in MeOH at room temperature. This different behaviour could be explained by the disappearance of functional groups capable to easily made inter-molecular hydrogen bonds, which will be reflected on the solubility improvement in $CHCl_3$. The lost of solubility in MeOH can be explained by the same behaviour.

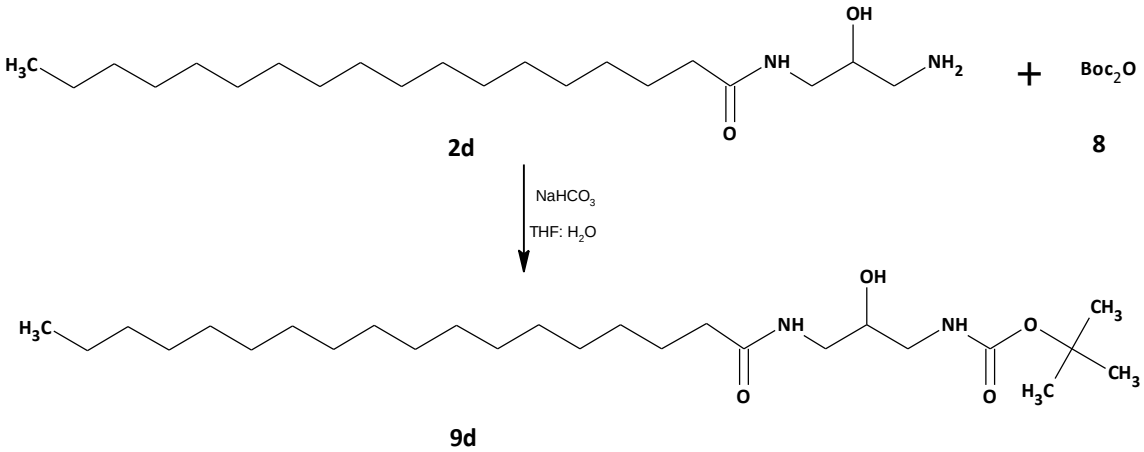
In the other hand, the melting point was still high. Nevertheless, this high melting point can be explained considering that the new cycle present in the molecule improve its package capacity, which hampered the decrease of the melting point of **6d**. In fact, a decrease in the melting point is observed in the carbamate **9d** described below.



Scheme 3.2: Synthesis of the urethane **6d** by reaction of urea with **2d**

3.4.3.2.- Amine Group Derivation.

We synthesize the carbamate derivate from **2d**[21] to support the participation of the amine group in the intermolecular H-bond interaction. Scheme 3.3 shows the synthesis of **9d** using **2d,** di-*tert*-butyl dicarbonate (Boc_2O) and triethylamine. The *tert*-butoxycarbonyl (Boc) group was bonded to the amine group yielding the compound **9d**, which was a white solid.



Scheme 3.3: 9d synthesis pathway using **2d**, di-*tert*-butyl dicarbonate (Boc_2O) and triethylamine

The properties of **9d** changed completely compared to **2d.** The compound was fairly soluble in MeOH and EtOH at room temperature, and slightly soluble in $CHCl_3$ at room temperature, but highly soluble at reflux temperature. It must be noted a clear change in the melting point of **9d**, which was clearly lower than **2d** with more than 40°C of difference. This behaviour clearly supports the participation of the free amine group in the intermolecular interactions that define the physic-chemical properties of **2d.**

3.4.4.- CRYSTALLIZATION ATTEMPTS.

Several attempts have been performed to prepare a solid crystal of **2d** to perform an X-Ray study. Unfortunately, any of these attempts allow the preparation of a suitable crystal observing the decomposition of the compound studied during some of these attempts. Indeed, the methanol ester of the corresponding acid was obtained from a methanol solution of 2d kept several days in the bench at room temperature.

3.5.- CONCLUSIONS.

In summary, a new set of monoamides of the 1,3-diamine-2-propanol has been synthesised. Moreover, the influence of intermolecular hydrogen bonds and the amide chain on the unexpected solubility behaviour of some of these monoamides has been proved. Regarding this, the importance of the amine group in the intermolecular interactions of these molecules has been supported by chemical derivation of this group.

3.6.- REFERENCES.

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CHAPTER 4.- PREPARATION OF MOLECULES WITH CAPACITY TO INTERACT WITH DC-SIGN

Part of this was performed at the Warwick University under the supervision of the Dave Haddleton.

4.1.- BACKGROUND.

Carbohydrates are one of the most widespread materials in nature. They are found not only in cellulose, starch, sucrose, etc. as cell wall constituents' of plants, but also in cell membrane. Cells carry a sugar coating formed by glycoproteins and glycolipids, which are involved in highly specific recognition events between cells and proteins, hormones, antibodies or toxins[1, 2].

The discovery of these diverse biological roles shown by oligosaccharides and glycoconjugates boosts the interest for the study of their interactions. Actually it is well known that many pathogens hide their presence from the immune system or interact with their host cells by displaying normal eukaryotic saccharides on surface glycoproteins or glycolipids[6]**.** The study of these interactions is a chance to develop new anti-infective, anticancer, and anti-inflammatory strategies[2, 6-8] through the comprehension of the mechanisms involved in these processes.

Consequently, the development of chemical compounds able to block their formation, function and/or interactions are becoming more and more interesting. Two general types of inhibitors are being sought: those that block glycoconjugate biosynthesis and those that interfere with glycoconjugate recognition. Effective inhibitors of various biosynthetic steps in glycoconjugate assembly have the potential to transform our understanding of carbohydrate function. By blocking the production of specific glycoconjugates, their biological roles can be ascertained. Similarly, antagonists that prevent glycoconjugate recognition can enlighten the function of the natural interactions[6]. Such metabolic interferences can block the expression of oligosaccharides or alter the structures of the sugars present on cells. Collectively, these chemical approaches are contributing in the great insight into the myriad biological functions of oligosaccharides[7].

4.1.1.- GLYCAN-PROTEIN INTERACTIONS.

Carbohydrate–protein interactions are involved in cell-to-cell interaction, ligand–receptor recognition, blood group typing, transport of biological macromolecules and in the immune recognition processes[9] (Figure 4.1). For example, cell surface proteins play a critical role in pathogen-cell interactions, being involved in the infection mechanisms of many bacteria and viruses.

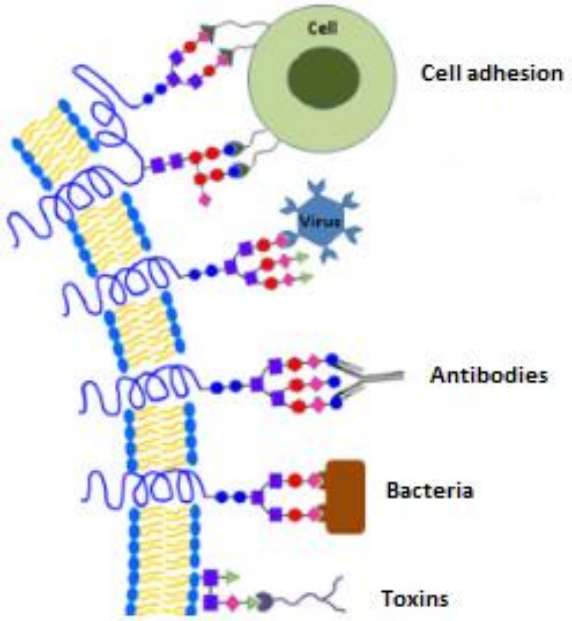


Figure 4.1: Multivalent protein–carbohydrate interactions at the cell surface[3].

Typically, cell-surface proteins act as cell receptors, interacting with sugar moieties from the virus or bacteria activating then the immune response and reaching its ultimate destination in the cell or organism.

4.1.1.1.- C-Lectin Type Protein: DC-SIGN.

Carbohydrate-binding proteins known as C-type (Ca^{2+}-dependent) lectin family are a large group of proteins that include DC-SIGN, mannose receptor, DEC-205 or langerin, among others. This sort of cell surface proteins is specialized in the recognition of carbohydrate structures present on cellular and viral proteins. They are implicated in several processes such as cell adhesion and antigen presentation[10].

DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) is expressed by specific cell populations such as dendritic cells (DCs) and macrophage cells[11]. They play a key role in the activation of the innate and adaptive immune responses. They present the Carbohydrate Recognition Domains (CRD) (Figure 4.2), which are highly specific domain to bind sugar moieties. Specifically, DC-SIGN on macrophages recognizes and bind mannose type carbohydrates, a class of Pathogen Associated Molecular Patterns (PAMPs), commonly found on viruses, bacteria and fungi envelopes such as gp120 in HIV. It especially acts as receptor for several viruses such as HIV and Hepatitis C[5, 12, 13]. These cells capture and process foreign antigens for presentation to T cells enabling, theoretically, an efficient host defence and immunological memory[14]. However, binding to DC-SIGN can promote HIV and Hepatitis C virus to infect T-cell from dendritic cells[12]. Thus binding to DC-SIGN is an essential process for HIV infection[15].

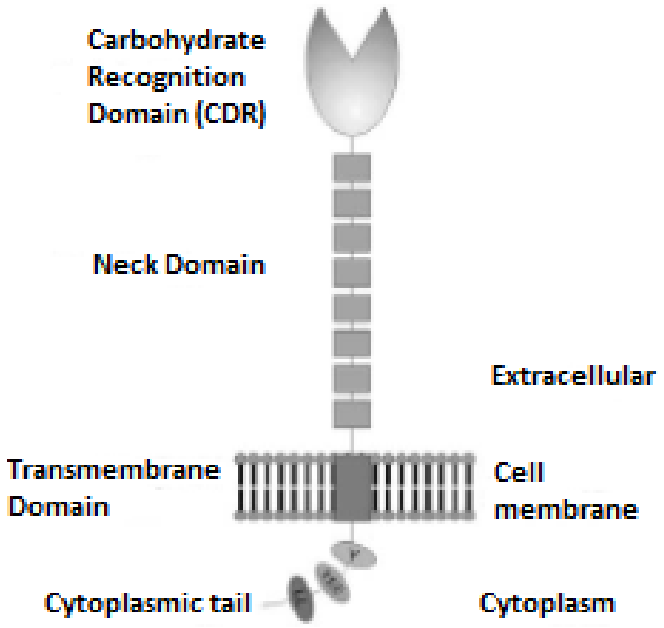


Figure 4.2: Structure of DC-SIGN protein[5].

4.1.2.- NEW APROACHES TO TERAPHEUTIC VACCINES.

The diverse roles attributed to DC-SIGN increases the interest to identify ligands that can be used to explore its different functions. Consequently, the development of new

compounds, capable to inhibit specific carbohydrate-protein binding, allows the study of these interactions and can lead to the discovery of new therapeutic agents.

4.1.2.1.- Carbohydrate–Lectin Binding Studies.

Synthesis of sugar arrays can be a powerful tool to understand the mechanism of the interaction process between cells and other molecules.

Sugar arrays can be used as simple and readily accessible methods for high-throughput analysis, allowing the quick screening of large compounds libraries with small amounts of these materials[16, 17].

Few approaches for carbohydrate immobilization have been developed based on surface coupling chemistry. For example, a Diels-Alder reaction was used to immobilize carbohydrate-cyclopentadiene conjugates to a monolayer that present benzoquinone groups displayed on a gold surface[18, 19]. It has also been described the attachment of maleimide-linked carbohydrates to a glass slide coated by thiol groups[20], between others.

1,3-Dipolar cycloaddition between azides and alkynes have also been described to attach oligosaccharides to a C_{13}-C_{15} hydrocarbon chain that non-covalently sticks within the polystyrene surface to a microliter plates, achieving good stability after repeated aqueous washings. Hence, these devices become functional in biological screens[21, 22].

4.1.2.2.- Strategies for the Design of Therapeutic Vaccines.

Under a therapeutic perspective, each step in the HIV life cycle provides an opportunity for pharmaceutical intervention. Being the initial steps in HIV infection (gp120-C-lectin binding) maybe one of the most interesting points.

Focused in this interaction as intervention point, there are various ways described to block that interaction. The action over the interaction can be approached by different ways, being some of the most important the following ones:

On one hand, some reports suggest that targeting the oligomannose regions of gp120 can potentially produce inhibitors capable of inactivating HIV infection. This could be achieved by different inhibitors. For example, through carbohydrate binding proteins (CBP), they have been suggested as potential candidates for the prevention of HIV infection. Among them, proteins such as the naturally occurring non-human lectin protein (MBL)[23], the cyanovirin-N (CV-N)[24] or the griffithsin[25], which bind the high-mannose region found on gp120. However, the high cost of production and purification of these lectins, added to their low-stability and plausible immunogenic response[26], may limit their use as a microbicides[27].

Another option to inhibit gp120-lectin binding could be through non-carbohydrate inhibitors. For example, a polymeric synthetic lectin based on benzoboroxole (BzB), which exhibits weak affinity ($\sim$25 M^{-1}) for non-reducing sugars was described, similar to those found on the HIV envelope[28]. Nevertheless, these systems bind to a single glycan-binding domain[29], as they are monovalent ligands.

The other main approach is to disrupt the lectin-gp120 interaction using as competition molecules mannose containing agents. Multivalent ligands can have a large effect disrupting or competing to bind lectins. For example, linear polymers have been demonstrated as effective lectin binders by several research groups[30-33].
The design of these molecules must be performed taking into account the detailed research conducted over the preference of DC-SIGN binding to monosaccharides, that has suggested that DC-SIGN binds with monosaccharide by recognition of C-3 and C-4 hydroxyl groups as other C-type ligands do[4]. It also suggested that DC-SIGN shows preference for axial C-2 substituents. It have also been demonstrated that inhibition by mannose is more effective than inhibition by glucose and galactose, but less effective than inhibition through L-fucose[4]. Table 4.1 shows the reported inhibition constants for monosaccharide binding to extracellular domain fragments of DC-SIGN[4].

Table 4.1: Inhibition constants (K_I) for each monosaccharide were determined by solid-phase competition assay. Obtained from *Mitchell et al.*[4].

Monosaccharide	K_I	$K_{I,sugar}/K_{I,Man}$
Mannose	13.1±0.4	1
Methyl α-mannoside	12.5±0.5	1.0±0.1
N-acetylmannosamine	8.7±0.2	0.7±0.1
Galactose	72±5	6.7±0.5
Methyl α-galactose	270±10	27±3
Glucose	23±1	1.9±0.3
Methyl α-glucose	32±1	2.6±0.3
N-acetylglucosamine	32±4	2.5±0.3
2-Deoxyglucose	28±4	2.9±0.6
L-Fucose	6.7±0.5	0.5±0.03

4.1.3.- SYNTHESIS OF DRUG-LIKE MOLECULES.

The development of efficient strategies for conjugate saccharides or oligosaccharides with compounds such as those with alkyl chains or proteins are of interest. Many methods are available in the literature to prepare carbohydrate mimic structures**,** including the glycosylation of diols[34] or the cycloaddition of azide and alkyne under modified Huisgen "click" reaction conditions[35, 36] and also the coupling of alkynyl glycosides[37]. Hence, the synthesis of 1,2,3-triazole as building blocks is one of the most popular binding unit, as it is a facile and efficient methodology. These rings are prepared via an intermolecular 1,3-dipolar cycloaddition "click" reaction between azides and terminal alkynes.

4.1.3.1.- Sugar Functionalization.

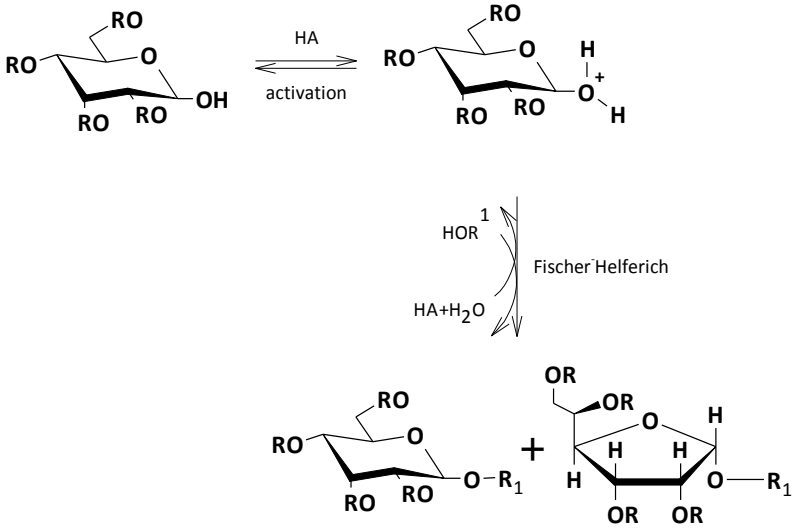
To preparer oligosaccharides capable to develop as drug-like molecules, it must be taking into account that most of the carbohydrates found in nature exist as polysaccharides, glycoconjugates or glycosides. Where the sugar units are attached through O-glycosidic bonds or N-glycosidic bonds. The stereoselective formation of these bonds is an important process in most of these syntheses. Since the first glycoside syntheses by *Michael*[38] and *Fischer*[39]*.* a very large number of glycosidation methods have been developed. The chemical synthesis of glycosides usually involves the transformation of a sugar into a fully protected glycosyl donor with a leaving group at its anomeric centre, followed by the glycosylation of a suitably protected glycosyl acceptor, which generally contains only one free hydroxyl group. An useful alternative is to undergo to the direct anomeric O-alkylation of totally

unprotected sugars, which can become a very convenient method for the glycoside-bond formation. Then it is interesting to find pathways with a minimum number of synthetic steps to specifically functionalize sugars with different groups, through its anomeric carbon.

4.1.3.1.1.- SYNTHESIS OF FUNCTIONALIZED SUGARS.

A method to prepare *O*-glycosides through a direct O-alkylation from unprotected carbohydrates was described by Emil Fischer in 1893. This method is known as Fischer glycosylation reaction[39, 40]. This synthesis of glycosides uses alcohols and mineral acids (H_2SO_4, HCl, etc.) as catalyst. The reaction takes place on the hemiacetal group of the unprotected monosaccharide. Being conceptually this the simplest way to obtain glycosides, the process presented some drawbacks such as the need of large quantities of alcohol and strong mineral acids. Moreover, the reaction takes place at reflux conditions for relatively long reaction times. Consequently, the final product typically results into on equilibrium of isomeric species (Scheme).

Several alternatives or modifications over Fischer type glycosylation procedure have been described. One of them was the acid catalysts supported onto silica gel[41]. For example, silica-H_2SO_4 is a useful form of H_2SO_4 acid easier to handle and remove after reaction. It has been proved as an improved alternative to typical Fischer reaction achieving better conditions as lower reaction time and the use of less alcohol equivalents[41].

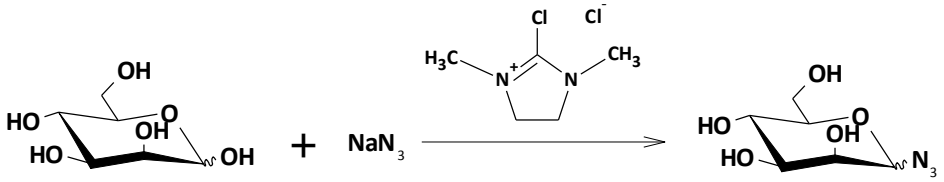


Scheme 4.1: Fischer- Helferich glycosilation[40, 42].

4.1.3.1.1.- SYNTHESIS OF AZYDE FUNCTIONALIZED SUGARS.

Methodologies to prepare *azide*-glycosides are also attractive in sugar synthesis. Being the direct O-azidation from unprotected sugar one of the most interesting pathways, as it lead to good results in the synthesis in few steps of N-linked products.

Typically, the introduction of an azide moiety onto the anomeric center requires protection and deprotection of the other hydroxyl groups. Nevertheless, the direct nucleophilic attack of an inorganic azide over the hydroxyl group of the anomeric carbon has been described[43, 44]. The use of DMC allows the direct activation of the anomeric hydroxyl group from unprotected sugars in aqueous media. This reaction ,takes advantage of the different pKa values showed by the hemiacetal anomeric hydroxyl groups in water, which are lower than those of other hydroxyl groups in water[45]. Hence, the DMC selectively activates the anomeric hydroxyl group allowing the nucleophilic attack of the azide to the anomeric carbon (**Error! No s'ha trobat**

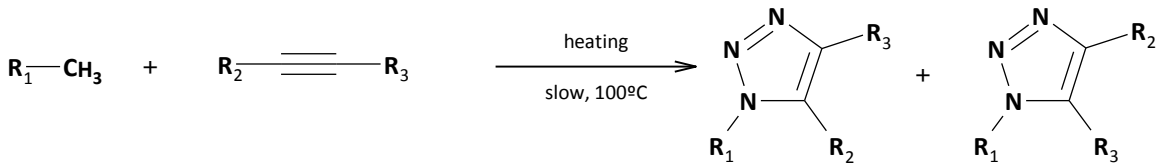


'origen de la referència.4.2).

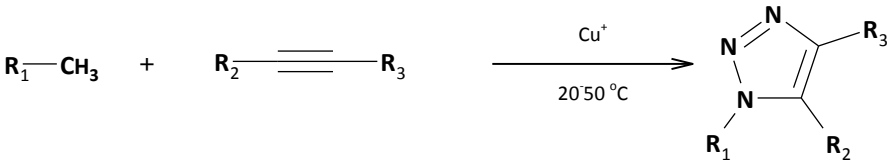
4.1.3.2.- Click Chemistry: 1,3- Dipolar Cycloadditions.

One of the most useful reactions to link azides and terminal alkynes is the triazol formation through the Huisgen's 1,3-dipolar cycloaddition. It is the most direct route to 1,2,3-triazoles. However, these cycloadditions are often very slow even at elevated temperatures (80-120°C for 12-24 h) because of their high activation energy (ca. 100.4-108.8 kJ/mol). Consequently, the reaction lead to mixtures of regioisomers, namely 1,4- and 1,5-substituted-1,2,3-triazoles (Scheme 4.3A)[46].

A) Huisgen's 1,3-dipolar cycloaddition



B) CuAAC reaction

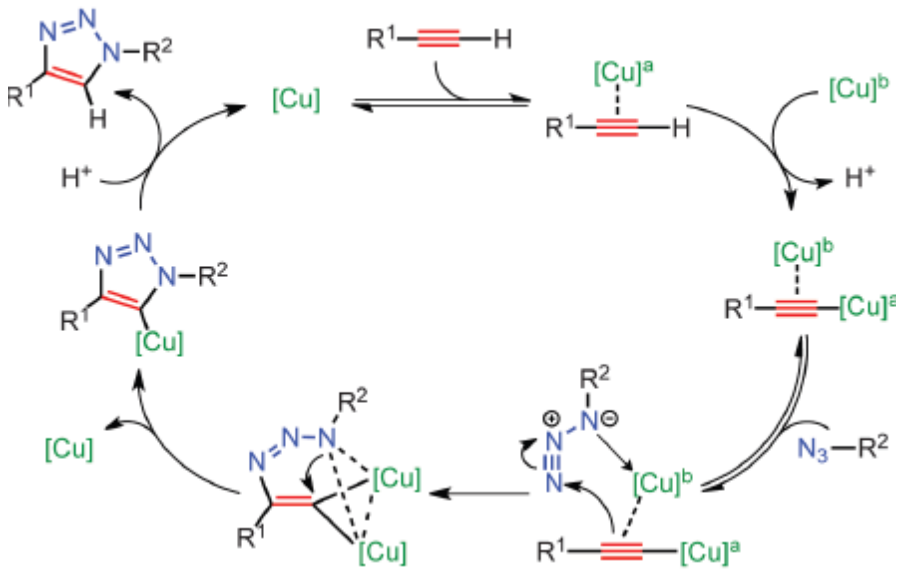


1,4-isomer only

Scheme 4.3: Triazol synthesis through click reaction. A) Husgein classic reaction. B) One-pot synthesis of 1,4-disubstituted 1,2,3-triazoles via a copper(I)-catalysed three component reaction.

The major drawbacks of the azide-alkyne cycloaddition were successfully overcome in 2002 when Cu(I)-catalysed Huisgen cycloaddition, also called Cu-catalysed azide-alkyne cycloaddition (CuAAC), was first reported[47]. CuAAC is an useful methodology affording quantitatively and selectively 1,4-disustituted-1,2,3- triazoles at room temperature (Scheme 4.3B). The reaction performs in presence of many functional groups.

Several studies have been conducted to elucidate the mechanism of the CuAAC with terminal alkynes[48, 49]. Moreover, the experimental evidence of the possible involvement of polynuclear copper(I) intermediates[48, 50, 51] has been supported by theoretical studies[52, 53]. Nevertheless, the whole mechanism is difficult to elucidate, as it involves multiple unstable equilibriums, whit non-isolable and highly reactive intermediates within the catalytic cycle of the CuAAC. Scheme shows the latest proposed mechanism with a dinuclear copper intermediate[54]. Consequently, the whole process leading to the triazol ring would need two copper atoms within the active cycloaddition complex.



Scheme 4.4: Proposed catalytic model for CuACC by *Worrell et al.*[54].

4.1.5.- GLYCOPOLYMERS.

Glycopolymers are synthetic macromolecules featuring sugar moieties, which have shown different bio-related applications[55] such as therapeutic and drug delivery, multivalent recognitions with lectins and signal transduction[7, 30, 56, 57].

Synthetic glycopolymers are important biological materials and play a vital role in many cell-cell communication events, such as preventing the interactions of human DC-SIGN with the HIV envelope glycoprotein gp120.

4.1.5.1.- Strategies for Glycopolymer Synthesis.

Glycopolymer synthesis have generally been performed as either direct polymerization of glycomonomers or post-glycosylation of pre-formed polymers[56]. A third approach exists, where a simultaneous living radical polymerization and the triazol formation are performed at the same time. This approach can be considered as a hybrid of the two previous strategies[55, 56, 58, 59].

4.1.5.1.1.- POLYMERIZATION OF GLYCOMONOMERS.

Ring-opening polymerization is a widely used strategies for the polymerization of various functional cyclic monomers[60]. Nevertheless, this strategy shows some

limitations in direct polymerization of carbohydrate-containing cyclic monomers[61, 62]. Also exist the Reversible addition-fragmentation chain transfer polymerization (RAFT), which seems to be one of the most robust and effective polymerization strategies. Despite many advances in this field, some aspects of the kinetics of RAFT polymerisation still require further investigation.

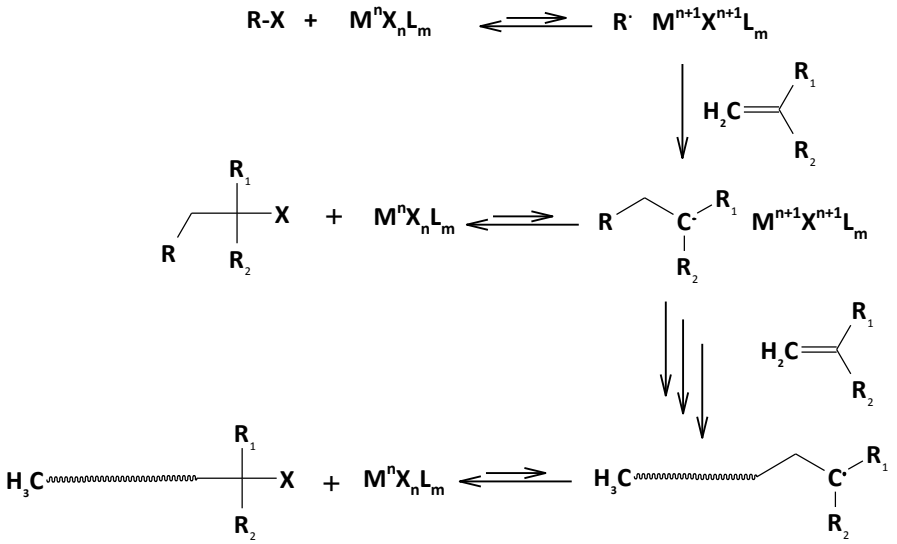
4.1.5.1.1.1.- COOPER MEDIATED LIVING RADICAL POLYMERIZATION.

The metal-catalyzed living radical polymerization is mostly considered to proceed via reversible activation of the carbon−halogen terminals of the organic halide (initiator) by a metal complex (catalyst or activator). In this reaction, one polymer chain is synthesised per molecule of initiator. The activation of the carbon−halogen bond in the organic halide (R−X) is considered to be achieved via one-electron oxidation of the metal center ($M^nX_nL_m$) to form an initiating radical specie (R•) and an oxidized metal compound ($M^{n+1}X_{n+1}L_m$). The R• reacts with the halogen on the oxidized metal to regenerate R−X or adds to the monomer to generate a new radical specie [$R-CH_2-C(R^1)(R^2)•$]. This radical is sooner or later transformed into the adduct [$R-CH_2-C(R^1)(R^2)-X$] of R−X and the monomer via abstraction of a halogen atom from $M^{n+1}X_{n+1}L_m$. The carbon−halogen bond of the adduct is subsequently activated by the metal complex, similarly to R−X, to result in a similar carbon−halogen bond at the polymer terminal via a repetitive set of the reactions (Scheme 4.5).

The effective metal complexes include various late transition metals such as ruthenium, copper, iron, nickel, etc., while the initiators include haloesters, (haloalkyl)benzenes, sulfonyl halides, etc. Generally, they can control the polymerizations of various monomers including methacrylates, acrylates, styrenes, etc., most of which are radically polymerizable conjugated monomers. It must be noted that cooper catalysts have been the most popular within the transition-metal catalysts, which showed to be easily handled and highly efficient catalysts[63].

One of the most used systems is the *Atom transfer radical polymerization* (ATRP), which utilized lower oxidation state cooper (I) halide and nitrogen-based ligand

complexes as catalysts. But, it was reported that even the most active CuX complexes failed to polymerize some products under ATRP systems[64-67].



Scheme 4.5: Metal-Catalysed Living Radical Polymerization general[68].

These problems were solved by the Single-electron transfer living radical polymerization (SET-LRP)[69, 70]. Technique which has proven to be a robust and versatile method for the ultrafast synthesis of well-defined polymers from a large diversity of monomers including acrylates[70-72], acrylamides[73-75] and methacrylates[70, 71, 76, 77] with different polarity profiles and of perfectly functional homopolymers, block copolymers and high-order multiblock copolymers even at complete monomer conversion using incredibly low catalyst loadings under mild reaction conditions[70, 71].

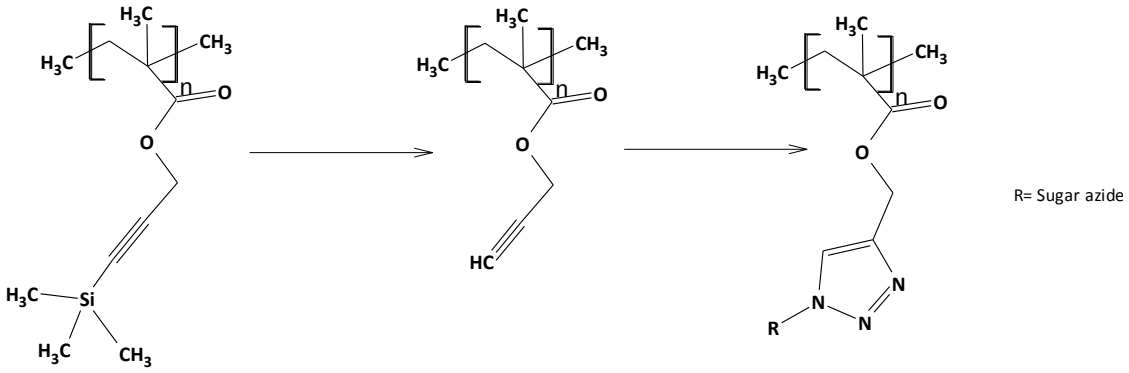
In SET–LRP various forms of copper metal, such as powder[78, 79], wire[80, 81], or nascent Cu(0) produced from the *in situ* disproportionation of Cu(I)X[82, 83], can be used as heterogeneous catalysts with excellent control of molecular weight evolution distribution, and perfect retention.

The Reversible Addition-Fragmentation chain Transfer polymerization (RAFT) is becoming one of the most popular living polymerization processes as it is tolerant of a wide variety of functional monomers and reaction conditions. Most of these polymerizations are conducted under 60-80ºC in aqueous systems with a certain ratio

of organic solvents[84-86], to solubilize the RAFT agents and radical sources, although aqueous RAFT has already been reported[87] even at room temperature[88].

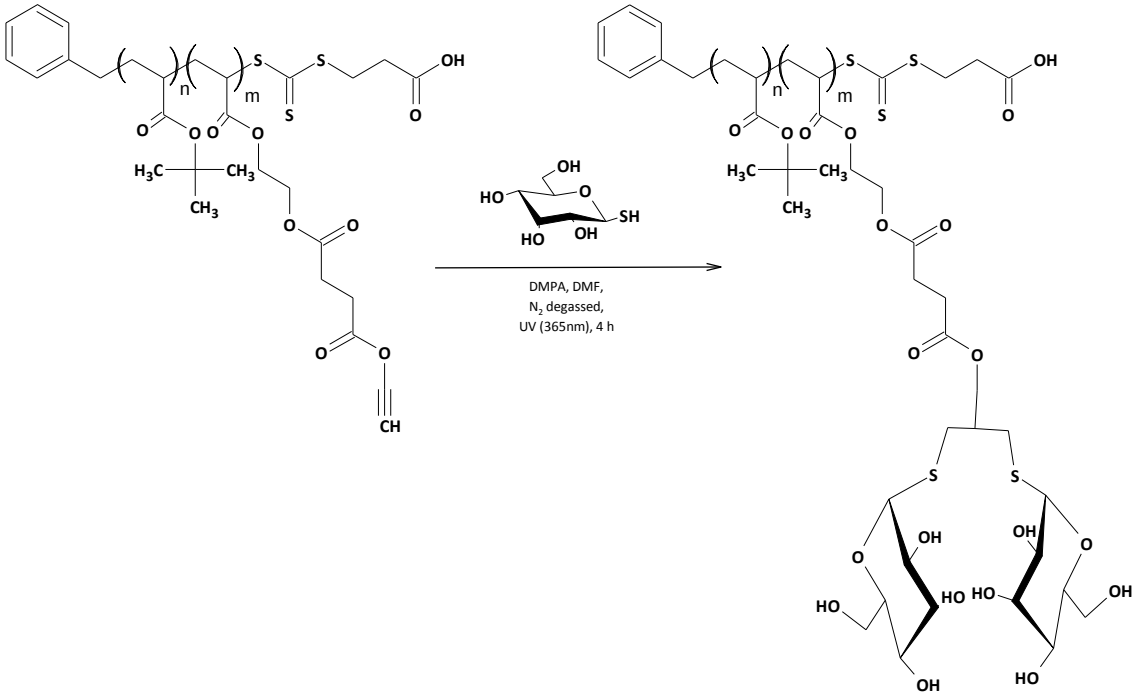
4.1.5.1.2.- POST-GLYCOSILATION OF PRE-FORMED POLYMERS.

In this synthetic approach, the polymer is first synthesized and after that functionalized, allowing "on demand" functionalization of the polymer with either low molecular weight motifs or sequence-defined oligopeptides. There exists a wide range of methodologies that can be used, one of them thorough CuAAC reactions to post-glycosylate the pre-formed polymers. For example, Haddleton and coworkers reported the synthesis of alkylnyl polymers with the alkyne group protected using trimethylsilyl (TMS). After a mild deprotection, they can be functionalized using the desired sugar azides through a CuAAC click reaction[89] (Scheme 4.6). This methodology avoids the use of hazardous azide-functionalised monomers and utilizes diversity of well-documented azido functional sugars[89].



Scheme 4.6: Haddleton's strategy to functional glycopolymers[89].

Another possibility is the reaction through thiol groups which can react with many species with high yields under mild conditions and thus many thiol-related reactions are considered as click type reactions[90]. For example, thiol-yne coupling reaction is versatile, robust and can tolerate different functional groups due to its radical nature. It allows facile addition of two thiols to one alkyne group (Scheme 4.7) which suits for construction of complex polymer structure such as network, dendrimers and hyper-branched polymers[90, 91].



Scheme 4.7: Synthesis of glycopolymers via thiol-alkyne reaction.

4.2.- OBJECTIVES.

The aims of this chapter were using glycerol as starting material:

-Synthesize new small molecules able to interact with DC-SIGN through microarrays.
-Find a new way to synthesize sugar monomers able to polymerize achieving more sugar molecules per chain.

4.3.- MATERIALS AND METHODS.

4.3.1.- REAGENTS AND EQUIPPMENT.

D-Mannose (99%, Sigma-Aldrich), L-fucose (98%, Sigma-Aldrich), D-glucose (99.5%, Sigma-Aldrich), propargyl alcohol (99%, Sigma-Aldrich, The H_2SO_4-silica catalyst was prepared according to the procedure described in the literature[41]. Sodium (+)-L-ascorbate (99%, Acros), $CuSO_4 \cdot 5H_2O$ (98%, Panreac.), glycerol (99.5%, Fischer Scientific), CTMS (98%, ACROS), acetic acid (98.5%, Panreac), caprylic acid (98%, Fluka), palmitic acid (98%, Aldrich), myristic acid (99%, Sigma), stearic acid (98%, Probus), sodium azide (99%, Aldrich), sodium hydroxide (97%, Sigma- Aldrich), acryloyl chloride (97%, Aldrich), 2-chloro-1,3-dimethylimidazolium chloride (DMC) (95%, Carbosynth), triethylamine (99.5%, Fluka), $CuBr_2$.(99%, Aldrich), ethyl α-bromoisobutyrate (EBiB) (98%, Alfa Aesar), tris[2-(dimethylamino)ethyl]amine (Me_6TREN)(98%, TCI), DMSO (99%, Fluka), HCl (37%, Panreac), copper wire (diameter=0.25 mm) which was pre-treated by washing in hydrochloric acid for 15 min and rinsed thoroughly with MiliQ water, dried under nitrogen and used immediately. Silica Gel 0,040-0.063 (Merk), Amberlite Resin IR-120H Hydrogen form (Aldrich), Membrane dialysis (1K MWCO) was obtained from Spectrum Laboratories.

The diazides (1b, 1d, 1f, 1h) and 1,3-dichloro-2-propanol (16) were synthesised from glycerol and the corresponding acid according the procedure previously described[92]. The reaction crude from diazides derivatives was not purified, due to their instability, and directly used for further reactions. Solvents were purchased in Aldrich, Fluka and Across and were used without further purification, if it is not specified.

^1H and ^{13}C were recorded on Varian AS400 MERCURYplus (^1H, 400MHz and ^{13}C, 100 MHz), using CD_3OD, D_6MSO and D_2O as solvents. The spectra were recorded at 30°C and 20 s of relaxing time. The chemical shifts (δ) are reported in ppm relative to the solvent used. Spin multiplicities are reported as a singlet (s), doublet (d), or triplet (t) with coupling constants (J) given in Hz, or multiplet (m).

The melting points were measured by open capillary tubes in a Gallenkamp equipment. They are uncorrected.

IR spectra were recorded on a Jasco FT/IR-6300 equipment, in a range between 600 to 4000 cm^{-1}, prepared to take 60 spectras/second with a resolution of 16 cm^{-1} working with ATR and in a Bruker VECTOR-22 FTIR spectrometer using Golden Gate diamond attenuated total reflection cell.

SPR sensograms were recorded in a BioRad ProteOn XPR36 SPR biosensor (Biorad, Hercules CA). Soluble C-lectine ligands were immobilized to 6000 response units (RU) on discrete channels within Biorad GLC sensor chips. Soluble-phase analytes were prepared in 25mM HEPES pH 7.4, 150mM NaCl, 3mM $CaCl_2$, 0.05% TWEEN-20 and flowed over the immobilized materials at a rate of 25µL/min at 25ºC.

ESI-MS was recorded on a Thermo Finigan LQC Deca quadrupole ion trap mass spectrometer equipped with an atmospheric pressure ionization source operating in the nebulizer assisted electrospray mode and was used in positive ion mode. High resolution mass spectra were recorded by direct infusion to a mass spectrometer Agilent G6510AA Q-TOF using ESI ionization in positive ion mode and data was acquired in the 50-1000 *m/z* range. MALDI-TOF/TOF data was recorded on an Ultraflex III Bruker, with a DHB matrix with and without deflexion at 800 Da.

4.3.2.- METHODS.

a) General procedure for the synthesis of 14j, 14k and 14l[41].

In a typical experiment, a suspension of sugar (**10**-**12**) (70 mmol), propargyl alcohol (**10**) (340 mmol) and H_2SO_4-silica (340 mg) was stirred at 65ºC overnight. After cooling to room temperature, the reaction mixture was transferred to a silica gel column and eluted with CH_2Cl_2: MeOH (8: 1) in the case of **14j** and **14k** and with CH_2Cl_2: MeOH (10: 1) in the case of **14l**. The silica gel column allowed the elimination of the propargyl alcohol excess used in the reaction.

b) General procedure for CuAAC Click reaction.

The reactions were carried out according the methodology previously described by *Zhang et al*.[33] with some modifications.

b1) For the synthesis of alkyne functional sugars (15x-18x, were x= j,k,l).

In a typical experiment a diazido derivative (**1b**, **1d**, **1f**, **1h**) (0.4 mmol) and propargyl sugar(**14j-14l**) (1 mmol) were added into a MeOH: H_2O (2: 1, 12 mL) solution. An aqueous solution of $CuSO_4 \cdot 5H_2O$ (7.5%) and sodium (+)-L-ascorbate (10% w/w) were sequentially added. The mixture was stirred at room temperature for 24 h. Methanol was removed by vacuum and the residue mixture was freeze dried to remove water. The final green solid was purified by silica gel column chromatography using CH_2Cl_2: MeOH (10: 1) as eluent.

b2) For the synthesis of D-Mannose glycomonomer (28k).

In a typical experiment the azido sugar (**19k**) (1 mmol) and the alkyl ester (**27**) (0.8 mmol) were added into a THF: H_2O (2: 1, 12 mL) solution at 50ºC. An aqueous solution of hydroquinone (12%), $CuSO_4 \cdot 5H_2O$ (7.5%) and sodium (+)-L-ascorbate (10% w/w) aqueous solutions were sequentially added. The mixture was stirred at 50ºC for 24 h. THF was removed by vacuum and the residue mixture was freeze dried to remove the water. The final green solid was purified by silica gel column chromatography using CH_2Cl_2: MeOH (2: 1) as eluent.

c) General procedure for D-Mannopyranosyl azide synthesis (19k).

The reaction was carried out following a previously described procedure[44]. 2-Chloro-1,3-dimethylimidazolinium chloride (11.6 g, 68.6 mmol) was added to a solution of D-mannose (**11**) (4.24 g, 23.5 mmol), triethylamine (32.5 mL, 233 mmol) and sodium azide (**20**) (12.16 g, 186.9 mmol) dissolved in water (110 mL). After stirring for 1 h at 0°C, the reaction mixture was let to react at room temperature overnight. After that, it was concentrated under vacuum and ethanol (200 mL) was added. The resulting solid was removed by filtration. The filtrate was concentrated under vacuum, the residue dissolved in water (70 mL) and the aqueous solution washed several times with dichloromethane. The aqueous solution was passed through a short column of acidic Amberlite® IR-120, previously activated with 1 M sodium hydroxide. The resulting aqueous solution was freeze-dried overnight to lead to the desired product (**19k**).

d) General procedure for the synthesis of 1,3-bis(prop-2-yn-1-yloxy)propan-2-ol (25).

The reaction was carried following a previously described methodology[93]. 1,3-Dichloropropan-2-ol (**24**) (5.14g, 39 mmol) was added dropwise to a mixture of propargyl alcohol (**13**) (9.0 mL, 155.7 mmol.) and sodium hydroxide (1.10 g, 27.6 mmol) in water (50 mL). The mixture was heated at reflux for 3 h, cooled and neutralized with 2M HCl. After extraction with CH_2Cl_2 (3 x 60 mL), the organic layers were dried and concentrated under vacuum. The residue was purified by silica-gel column chromatography (CH_2Cl_2) to lead **25** as a clear yellow oil.

e) General procedure for the synthesis of 1,3-bis(prop-2-yn-1-yloxy)propan-2-yl prop-2-enoate (27).

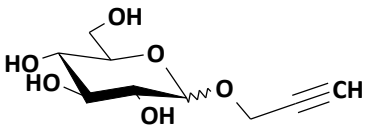
The procedure was carried out following a general previously described procedure with slightly modifications[94]. 1,3-Bis(prop-2-yn-1-yloxy)propan-2-ol (**25**) (1.8 g, 11 mmol) and Et_3N (2.0 mL, 14.3 mmol) were dissolved in 80 mL of CH_2Cl_2 anhydrous and the solution was cooled at 0°C under argon atmosphere. A solution of acryloyl chloride (**26**) (1.5 mL, 18.5 mmol) in CH_2Cl_2 (15 mL) was added dropwise. The resulting mixture was stirred overnight at room temperature, then poured into 50 mL of cold water, and

finally extracted with CH_2Cl_2 (3 x 50 mL). The combined organic phases were dried over Na_2SO_4. The solvent was removed under vacuum. The product was obtained as a yellow oil with enough purity, which led to the desired compound without any further purification needed.

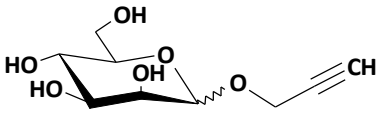
f) Homopolymerization of D-mannose acrylate glycomonomer via SET-LRP (30k).

The procedure was carried out following a general previously described procedure[33]. All polymerizations were carried out using standard Schlenk techniques under an inert atmosphere of oxygen-free nitrogen. Mannose glycomonomer (**28k**) (1mmol), $CuBr_2$ (0.01 mmol) and DMSO (3 mL) were added to a Schlenk tube fitted with a magnetic stir bar and a rubber stopper. Nitrogen was bubbled into the mixture for 15 min. Pre-degassed Me_6TREN (0.018 mmol) and EBiB (**29**) (0.1 mmol) were sequentially added via gas tight syringe. Finally, pre-activated copper wire was carefully added under nitrogen protection. The Schlenk tube was sealed and the light green solution was allowed to polymerize at 25°C. Samples of the reaction mixture were carefully taken at suitable time periods for analysis. The sample for ^{1}H NMR analysis was directly diluted with D_6MSO. After 24 h, the reaction was stopped via exposure to the air and the mixture was diluted with water and dialysed against water for two days. Finally, the glycopolymer was recovered by freeze drying.

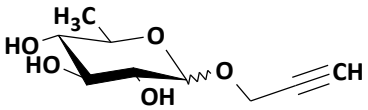
4.3.3.- EXPERIMENTAL DATA.



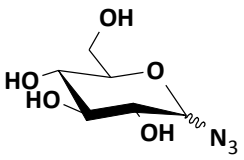
D-Glucopyranoside, 2-propyn-1-yl (**14j**) was obtained as an yellow oil after drying under vacuum. Yield = 53% (5: 1 in α: ß ratio). ^{1}H NMR (400 MHz, CD_3OD) δ 5.00 (1 H, d, *J*=5.9), 4.49 – 4.45 (0.1 H, d, *J* =1.3), 4.32 (0.9 H, d, *J* =1.3), 3.91 – 3.27 (6 H, m, residues of glucose), 2.87 (1 H, d, *J* =2.3). ^{13}C NMR(101 MHz, CD_3OD) δ 102.13, 98.62, 78.03, 75.00, 74.89, 74.11, 73.32, 71.67, 71.62, 62.75, 62.53, 56.57, 55.25. IR (ATR/ν): 3370.96, 2938.02, 2117.46. ESI-MS *m/z*: calcd for $C_9H_{14}O_6Na$ $[M+Na]^+$, 241.1; found 240.9.



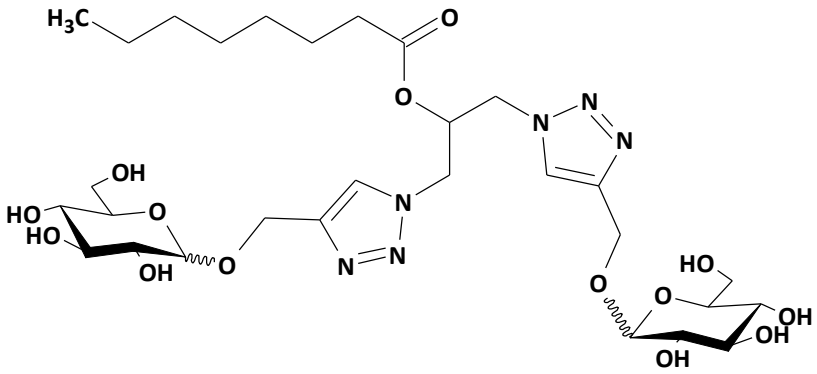
D-Mannopyranoside, 2-propyn-1-yl (**14k**) was obtained as an yellow oil after drying under vacuum. Yield=42% (4: 1 in α: ß ratio). ^{1}H NMR (400 MHz, CD_3OD) δ 4.86 (1 H, s), 4.33 (0.2 H, d, *J* =2.3), 4.18 (0.8 H, d, *J* =2.4), 3.82 – 3.34 (6 H, m, residues of mannose), 2.77 (0.2 H, t, *J* =2.4), 2.75 (0.8 H, t, *J*= 2.4). ^{13}C NMR (101 MHz, CD_3OD) δ 99.85, 99.34, 78.49, 75.26, 75.11, 72.51, 72.39, 72.04, 68.49, 62.84, 56.37, 54.84.IR (ATR/ν): 3361.32, 2942.84, 2117.46. ESI-MS *m/z*: calcd. for $C_9H_{14}O_6Na$ $[M+Na]^+$, 241.1; found 240.9.



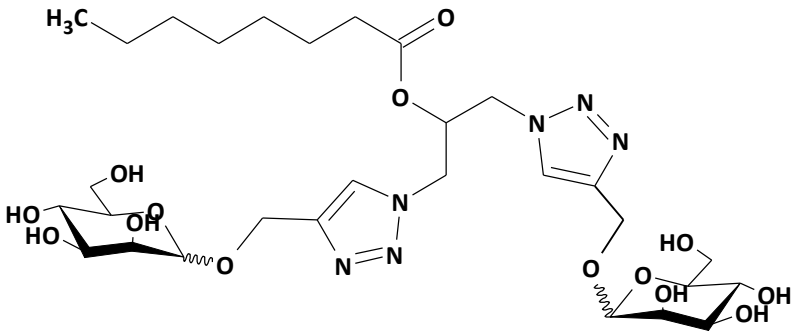
D-Fucopyranoside, 2-propyn-1-yl (14l) was obtained as an yellow oil after drying under vacuum. Yield=57% (5: 2 in α: ß ratio) ^{1}H NMR (400 MHz, CD_3OD) δ 4.96 (0.15 H, d, *J* =3.4), 4.38 (0.85 H, t, *J*=1.9), 4.28 (1 H, d, *J*=2.4), 4.02 – 3.29 (7 H, m, residues of fucose), 1.29 (0.15 H, d, *J*=6.4), 1.23 (0.85 H, d, *J* 6.6). ^{13}C NMR (101 MHz, CD_3OD) δ 102.53, 99.29, 75.11, 73.63, 72.07, 71.58, 69.77, 68.04, 56.36, 55.55, 16.51. IR (ATR/ν): 3266.82, 2931.27, 2119.39. ESI-MS *m/z*: calcd. for $C_9H_{14}O_6Na$ $[M+Na]^+$, 225.1; found 224.9.



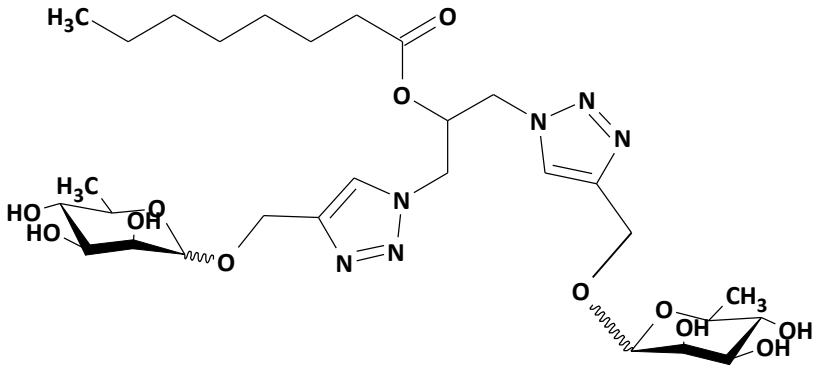
D-Mannopyranosyl azide (**19k**) was obtained as a white solid. M_p(°C)= decomposes at 141.5°C before melt. Yield = 77%. ^{1}H NMR (400 MHz, D_2O) δ 5.32 (1H, d, *J* =5.6), 3.81 – 3.48 (6H, m, residues of mannose). ^{13}C NMR (101 MHz, D_2O) δ 89.68, 74.58, 69.77, 69.71, 66.33, 60.76. IR (ATR/ν): 3424.96; 3293.82; 2960.20, 2128.06. The NMR signals correspond to a previously described compound[95, 96]



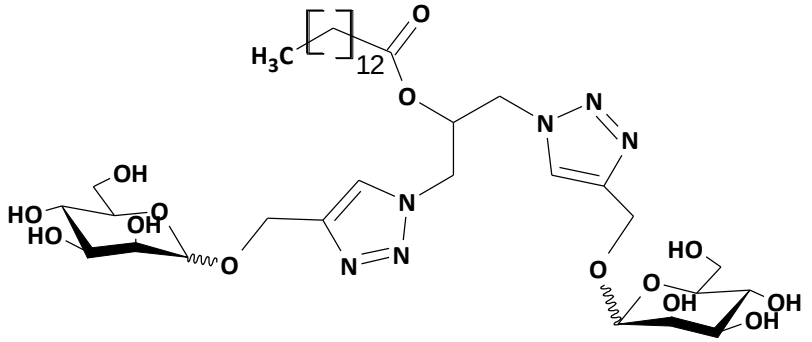
1-(4-(D- Glucopyranosyl)-1H-1,2,3-triazol-1-yl)-3-(4-(D- Glucopyranosyl)-1H-1,2,3-triazol-1-yl)propan-2-yl octanoate (**15j**) was obtained as a white solid, Yield = 55%, ^{1}H NMR (400 MHz, CD_3OD) δ 8.13 – 8.03 (2 H, s), 5.74 – 5.65 (1 H, m), 4.74 – 4.63 (4 H, m), 3.98 – 3.35 (12 H, m), 2.27 (2 H, t, *J* =7.4), 1.51 – 1.42 (2 H, m), 1.38 – 1.14 (8 H, m), 0.91 (3 H, t, *J* =6.9). ^{13}C NMR (101 MHz, CD_3OD) δ 173.73, 145.82, 126.52, 103.47, 99.56, 78.07, 78.00, 75.06, 74.07, 73.49, 71.82, 71.65, 71.22, 62.74, 61.33, 51.67, 34.67, 32.83, 30.01, 25.70, 23.69. IR (ATR/ν): 3329.45, 2924.10, 1739.29, 1638.39, 1227.22, 1019.64. ESI-MS *m/z*: calcd. for $C_{29}H_{48}N_6O_{14}Na$ $[M+Na]^+$, 727.3; found 727.3.



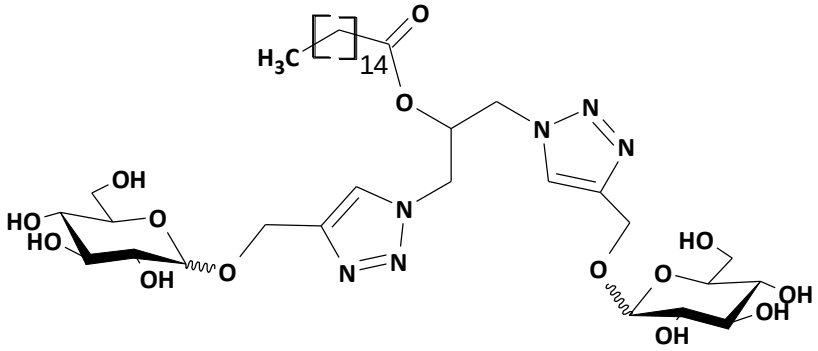
1-(4-(D- Mannopyranosyl)-1H-1,2,3-triazol-1-yl)-3-(4-(D- Mannopyranosyl)-1H-1,2,3-triazol-1-yl)propan-2-yl octanoate (**15k**) was obtained as a white solid, Yield = 41%, ^{1}H NMR (400 MHz, CD_3OD) δ 7.98 (2 H, s), 5.59 (1 H, dt, *J*= 11.0, 3.6), 4.70 – 4.44 (4 H, m), 3.80 – 3.40 (12 H, m), 2.19 – 2.06 (2 H, m), 1.38 – 1.27 (2 H, m), 1.25 – 1.01 (8 H, m), 0.80 (3 H, t, *J*= 6.9). ^{13}C NMR (101 MHz, CD_3OD) δ 173.73, 145.62, 126.53, 100.79, 75.04, 72.54, 72.03, 71.17, 68.64, 63.02, 60.62, 51.68, 34.66, 32.82, 30.02, 25.71, 23.69. IR (ATR/ν): 3330.45, 2925.10, 2855.99, 1738.87, 1639.45, 1227.43, 1024.56. ESI-MS *m/z*: calcd. for $C_{29}H_{48}N_6O_{14}Na$ $[M+Na]^+$, 727.3; found 727.3.



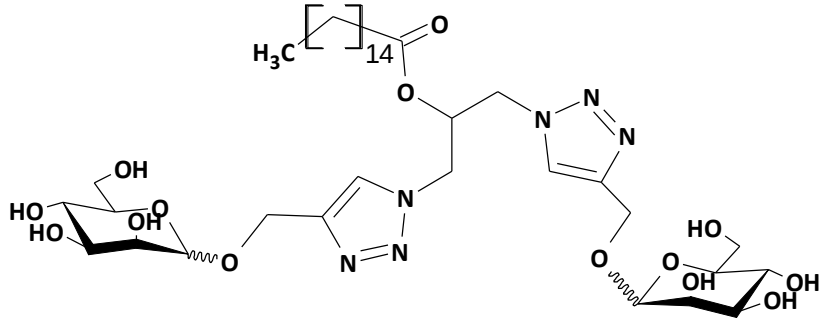
1-(4-(D- Fucopyranosyl)-1H-1,2,3-triazol-1-yl)-3-(4-(D- Fucopyranosyl)-1H-1,2,3-triazol-1-yl)propan-2-yl octanoate (**15l**) was obtained as white solid, Yield = 55%, ^{1}H NMR (400 MHz, CD_3OD) δ 8.07 (2 H, s), 5.69 (1 H, dt, *J* =11.2, 3.8), 4.81 (2 H, s), 4.67 (4 H, dd, *J* =16.7, 4.9), 4.02 – 3.65 (12 H, m), 2.26 (2 H, t, *J* =7.4), 1.51 – 1.41 (2 H, m), 1.31 – 1.12 (8 H, m), 0.92 (3 H, t, *J* =6.9). ^{13}C NMR (101 MHz, CD_3OD) δ 146.03, 126.29, 100.12, 73.63, 71.63, 71.22, 69.95, 67.89, 61.60, 51.63, 34.66, 32.84, 30.06, 25.70, 16.66. IR (ATR/ν): 3355.71, 2926.05, 1739.78, 1650.91, 1225.62, 1035.23. ESI-MS *m/z*: calcd. for $C_{29}H_{48}N_6O_{12}Na$ $[M+Na]^+$, 695.3; found 695.3.



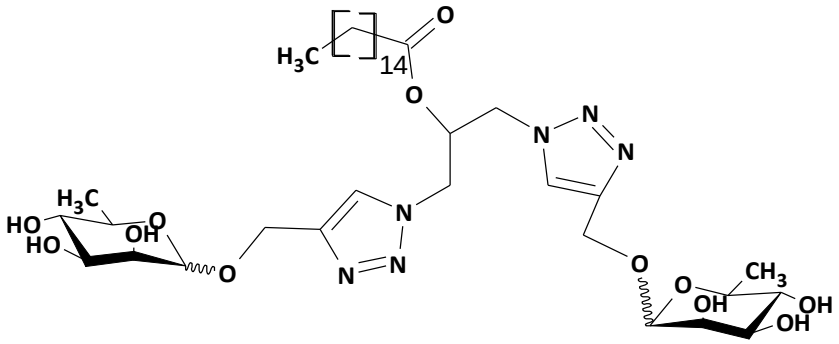
1-(4-(D- Mannopyranosyl)-1H-1,2,3-triazol-1-yl)-3-(4-(D- Mannopyranosyl)-1H-1,2,3-triazol-1-yl)propan-2-yl tetradecanoate (**16K**) was obtained as a white solid, Yield = 47%, M_p(°C)= 64.5-65.2. ^{1}H NMR (400 MHz, CD_3OD) δ 8.07 (2 H, s), 5.68 (1 H, dt, *J*= 11.2, 3.7), 4.81 – 4.57 (4 H, m), 3.88 – 3.49 (12 H, m), 2.22 (2 H, t, *J* =7.4), 1.43 (2 H, dt, *J* =14.7, 7.3), 1.30 (20 H, d, *J* =14.3), 0.89 (3 H, t, *J*= 6.8). ^{13}C NMR (101 MHz, CD_3OD) δ 172.52, 127.73, 126.70, 99.33, 73.57, 71.09, 70.58, 67.19, 61.56, 59.18, 50.23, 48.22, 48.01, 33.23, 31.65, 29.40, 29.38, 29.35, 29.17, 29.06, 28.93, 28.61, 24.28, 22.31, 13.03. IR (ATR/ν): 3330.46, 2925.48, 1739.48. HRMS (ESI) m/z calcd. for $C_{37}H_{65}N_6O_{14}$ $[M+H]^+$ 817.4528, found 817.4486.



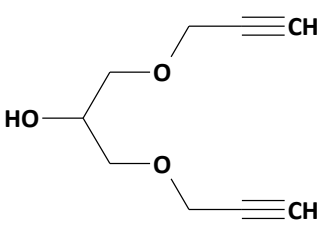
1-(4-(D- Glucopyranosyl)-1H-1,2,3-triazol-1-yl)-3-(4-(D- Glucopyranosyl)-1H-1,2,3-triazol-1-yl)propan-2-yl hexadecanoate (**17j**), was obtained as a yellow solid, Yield = 44%, M_p(°C)= 171.1-189.9. ^{1}H NMR (400 MHz, CD_3OD) δ 8.11 – 8.06 (2 H, s), 5.68 (1 H, dt, *J* =11.2, 3.7), 4.89 (2 H, dd, *J* =9.1, 3.6), 4.71 – 4.63 (4 H, m), 3.94 – 3.19 (12 H, m), 2.25 (2 H, t, *J*= 7.4), 1.45 (2 H, dt, *J*= 14.6, 7.4), 1.38 – 1.12 (24 H, m), 0.90 (3 H, t, *J*= 6.9). ^{13}C NMR (101 MHz, D_2O) δ 172.93, 143.79, 125.68, 97.75, 73.16, 71.95, 71.20, 69.41, 60.60, 31.94, 29.94, 29.74, 29.41, 29.04, 24.54, 22.61, 13.86. IR (ATR/ν): 3346.85, 2922.59, 1741.41. HRMS (ESI) m/z calcd. for C_{37} H_{65} N_6 O_{14} $[M+H]^+$ 817.4528, found 817.4553.



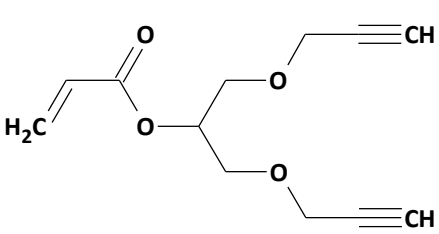
1-(4-(D- Mannopyranosyl)-1H-1,2,3-triazol-1-yl)-3-(4-(D- Mannopyranosyl)-1H-1,2,3-triazol-1-yl)propan-2-yl hexadecanoate (**17k**), was obtained as a yellow solid, M_p(°C)= 156.4-157.9. Yield = 42%, ^{1}H NMR (400 MHz, CD_3OD) δ 8.08 (2 H, s), 5.69 (1 H, m), 4.65 (4 H, dd, *J* =13.2, 5.5), 3.96 – 3.48 (12 H, m), 2.26 – 2.14 (2 H, m), 1.48 – 1.38 (2 H, m), 1.36 – 1.08 (24 H, m), 0.89 (3 H, t, *J* =6.8). ^{13}C NMR (101 MHz, CD_3OD) δ 172.32, 172.29, 99.30, 73.56, 71.09, 70.57, 69.71, 67.18, 61.54, 59.24, 50.28, 33.25, 31.66, 29.41, 29.37, 29.20, 29.07, 28.96, 28.63, 24.30, 22.33, 13.08. IR (ATR/ν): 3360.35, 2921.63, 1741.41. HRMS (ESI) m/z calcd. for C_{37} H_{65} N_6 O_{14} $[M+H]^+$ 789.4241, found 789.4240.



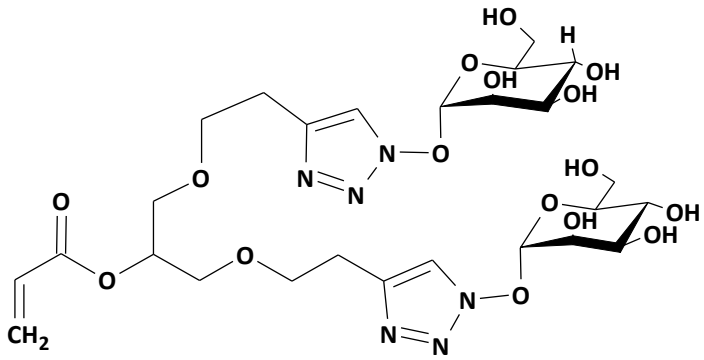
1-(4-(D- Fucopyranosyl)-1H-1,2,3-triazol-1-yl)-3-(4-(D- Fucopyranosyl)-1H-1,2,3-triazol-1-yl)propan-2-yl hexadecanoate (**17l**), was obtained as a yellow solid, Yield = 58%, M_p(°C)= 50.8-51.9. ^{1}H NMR (400 MHz, CD_3OD) δ 8.07 (2 H, s), 5.68 (1 H, dd, *J* =7.4, 3.7), 4.77 (2 H, d, *J*= 3.3), 4.71 – 4.58 (4 H, m), 4.00 – 3.59 (12 H, m), 2.26 – 2.17 (2 H, m), 1.43 (2 H, dt, *J* 14.9, 7.4), 1.39 – 1.24 (24 H, m), 0.94 – 0.83 (3 H, m). ^{13}C NMR (101 MHz, CD_3OD) δ 172.26, 144.56, 124.83, 98.65, 72.18, 70.17, 68.50, 66.43, 60.13, 50.18, 33.38, 33.23, 31.65, 29.39, 29.34, 29.30, 29.25, 29.19, 29.14, 29.05, 29.04, 28.97, 28.94, 28.75, 28.64, 24.60, 24.27, 22.31, 15.24, 13.02. IR (ATR/ν): 3360.35, 2922.59, 1741.41. HRMS (ESI) m/z calcd. for C_{37} H_{65} N_6 O_{12} $[M+H]^+$ 785.4707, found 785.4655.



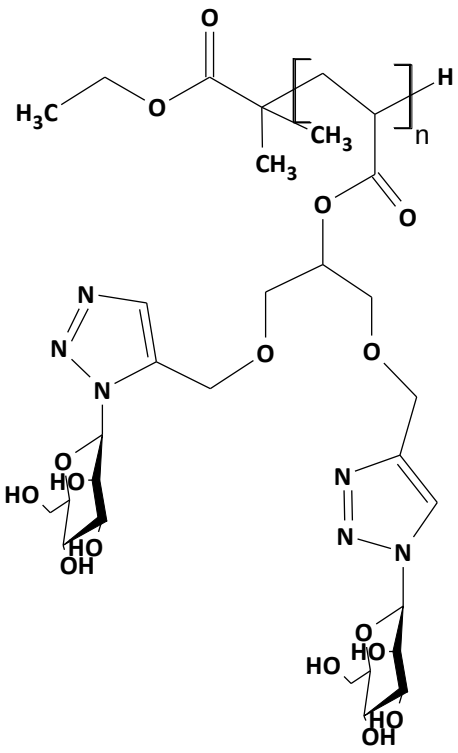
1,3-Bis(prop-2-yn-1-yloxy)propan-2-ol (25) was obtained as an yellow oil. Yield = 18%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.15 (1 H, q, *J* =2.5), 3.65 – 3.50 (8 H, m), 2.48 – 2.45 (2 H, m). ^{13}C NMR (101 MHz, $CDCl_3$) δ 79.14, 75.20, 70.60, 70.07, 58.61. IR (ATR/ν): 3411.46; 3291.89, 2921.63, 2116.49. ESI-MS *m/z*: calcd. for $C_9H_{12}O_3Na$ $[M+Na]^+$, 191.1; found 191.0.



1,3-Bis(prop-2-yn-1-yloxy)propan-2-yl prop-2-enoate (**27**) was obtained as an yellow oil, Yield = 95%. ^{1}H NMR (400 MHz, CD_3OD) δ 6.47 – 6.34 (1 H, m), 6.22 – 6.11 (1 H, m), 5.89 (1 H, d, *J* 10.4), 5.22 – 5.14 (1 H, m), 4.17 (4 H, dd, *J* =7.8, 2.3), 3.77 – 3.62 (4 H, m), 2.82 (2 H, dt, *J* =8.8, 2.4).^{13}C NMR (101 MHz, CD_3OD) δ 165.64, 130.42, 128.07, 78.87, 74.71, 71.45, 67.94, 57.86, 48.23, 48.02, 47.81, 47.59, 47.38. IR (ATR/ν): HRMS (ESI) m/z calcd. for C_{12} H_{15} O_4 $[M+H]^+$ 223.0962, found 223.0965.



D-Mannose glycomonomer (**28k**), was obtained as am yellow sticky oil. Yield = 24%. ^{1}H NMR (400 MHz, CD_3OD) δ 8.18 – 8.08 (2 H, m), 6.38 (1 H, dd, *J*=17.3, 1.5), 6.14 (1 H, dd, *J*=17.3, 10.4), 6.01 (1 H, dd, *J*=9.9, 1.6), 5.89 (1 H, dd, *J*=10.4, 1.5), 5.21 – 5.12 (1 H, m), 4.65 (4 H, dt, *J*=29.8, 7.8), 3.83 – 3.25 (mannose residues + 4H). ^{13}C NMR (101 MHz, CD_3OD) δ 165.65, 144.53, 130.68, 128.01, 123.69, 86.95, 77.17, 71.53, 71.13, 68.66, 68.43, 67.79, 67.15, 63.60, 61.09. IR (ATR/ν): 3339.14, 2928.38, 2115.53, 2070.21, 1716.34. HRMS (ESI) m/z calcd. for $C_{24}H_{35}N_6O_{14}$ $[M+H]^+$ 631.2227, found 631.2217.



Mannose polymer (**31k**) was obtained as a 182hite solid. ^{1}H NMR (400 MHz, D_2O) 8.11 (broad band), 6.00 (broad band), 4.02 (broad band), 3.68 (broad band), 3.19 (broad band), 1.94 – 1.49 (broad band).IR (ATR/ν): 3337.21, 2933.20, 1719.23.

MALDI-ToF m/z calc for DP=2 $C_{54}H_{84}N_{12}Na_1O_{30}$ $[M+Na]^+$ calcd. 1403.5, found 1403.5; DP=3 $C_{78}H_{120}N_{18}Na_1O_{44}$ $[M+Na]^+$ calcd. 2035.8, found 2035.8; DP=4 $C_{102}H_{156}N_{24}Na_1O_{58}$ $[M+Na]^+$ calcd. 2668.0, found 2667.9; DP=5 $C_{126}H_{192}N_{30}Na_1O_{72}$ $[M+Na]^+$ calcd. 3300.2, found 3300.4

4.4.- RESULTS AND DISCUSION.

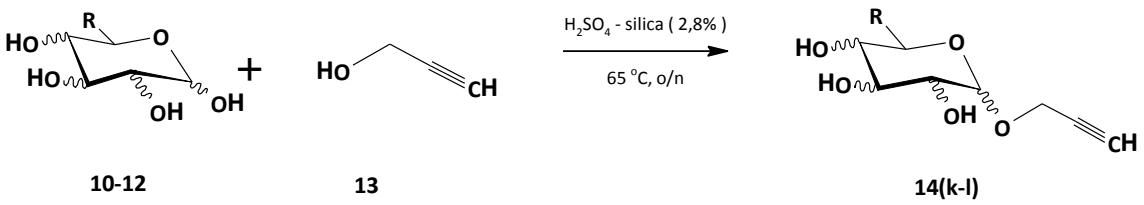
4.4.1.- MOLECULES AS POSSIBLE SUGAR ARRAYS FOR MICROLITER PLATES.

As we described in the introduction part the attachment of sugar azides to hydrocarbon chains through 1,3-dipolar cycloadditions has been already described. These molecules were linked to microliter plates, resulting in an array of alkane chains bearing one sugar moiety[21].

Based in this idea, we designed the synthesis of new compounds having alkyl chains bearing two sugar moieties per chain in the head. Our hypothesis was that these molecules could be used to prepare microarray plates useful for the study of the interaction mechanisms present on the cell-interaction processes.

4.4.1.1.- Synthesis of Alkyne Functionalized Sugars.

Scheme 4.8 shows the one-pot Fischer glycosylation reaction used for the synthesis of alkyne functional sugars. Mannose, glucose and fucose free sugars were reacted with propargyl alcohol at 65ºC using silica-H_2SO_4 as catalyst.



10-12 **13** **14(k-l)**

Scheme 4.8: Synthesis of alkyne functionalised sugar using H_2SO_4-silica catalysis.

The reaction crudes were purified using a column chromatography, which allowed the elimination of the excess of the propargyl alcohol and of the unreacted free sugar. The reaction yields ranged 45 to 60% of pure compounds, depending on the starting sugar. These yields correspond to an anomeric mixture of both α and ß propargyl glycosides in a thermodynamic equilibrium. Both isomers have been already separated for a detailed characterization. This approach implies the per-O-acetylation of the alcohol

groups previous the isomer separation[33]. The assignation of the NMR signals to each isomer was then performed[33]. Considering this available information, we could set the identification of both isomers through ^{1}H NMR.

4.4.1.2.- CuACC Reaction.

In order to prepare sugar functionalized molecules from our diazide derivatives (**1**) we decided to synthesize compounds from the C8 (**1b**) and C18 (**1d**) diazido derivatives and various alkinyl glycosides (**14j-14l**) prepared using the Fischer glycosilation reaction[41]. The desired products were prepared using the CuACC reaction. This reaction was carried out using $CuSO_4 \cdot 5H_2O$/NaASc system as catalyst and MeOH: H_2O as solvent (Scheme 4.9).

Table 4.2 shows the three different C8 derivate prepared. Each of these solid compounds bear two sugar moieties and are stable compounds. The yields ranged 40 to 55%, after column purification.

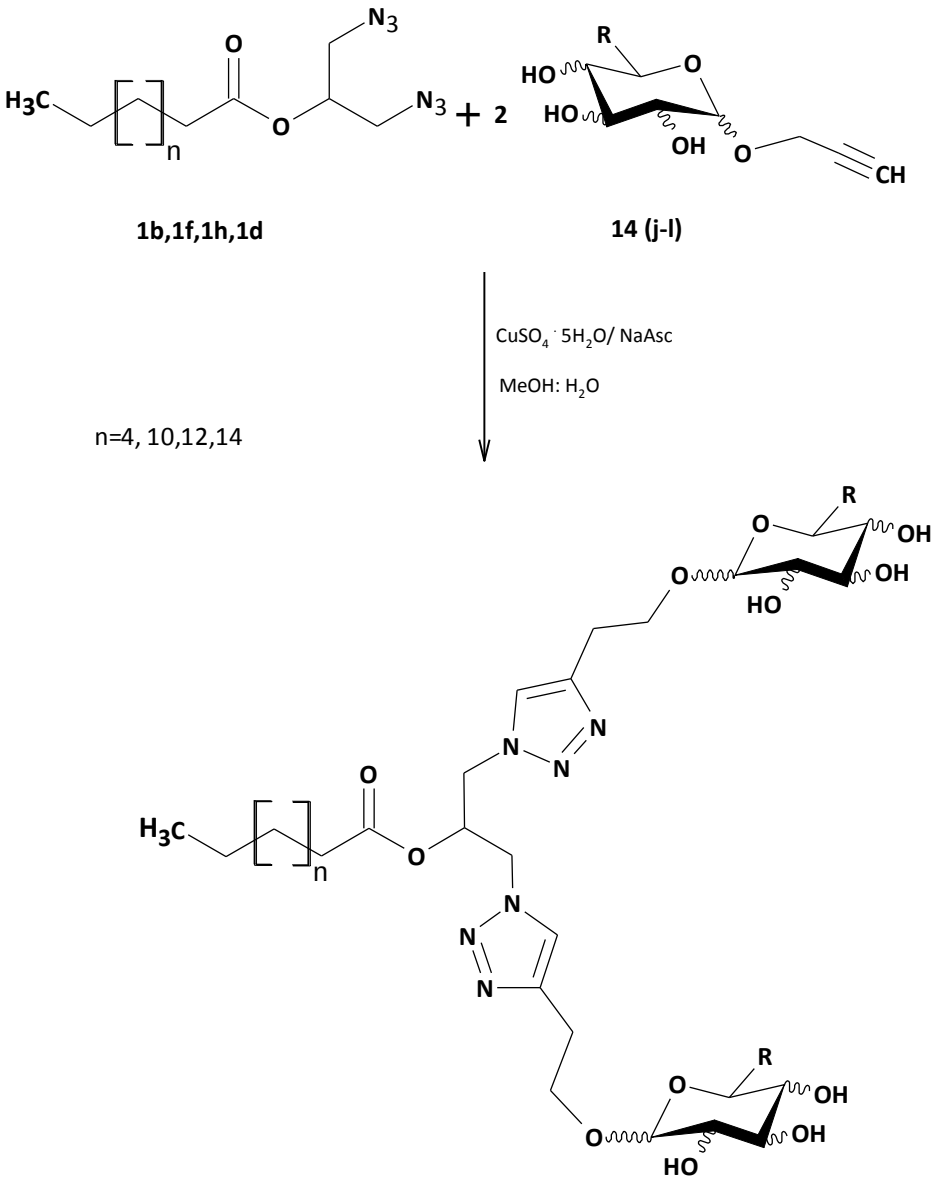
Table 4.2: *Yields corresponding to the* **a**lkane derivates bearing two sugar moieties**.**

	Alkane Chain			
Propargyl Sugar	**15 Yield (%)**	**18 Yield (%)**	**16 Yield (%)**	**17 Yield (%)**
J	55.3	*	ns	44.2
K	40.9	*	47.4	42.4
L	54.5	*	ns	57.7

ns= no synthesized. * cannot be purified

The same reactions were performed with C18 derivatives using the same conditions. However, although the ^{1}H NMR of the crude reaction clearly showed the signal corresponding to the triazol ring, we were not able to obtain the pure products. All the attempts to purify the product using column chromatography were unsuccessful, observing that the desired compound decomposed into the column.

The reaction was repeated with the different sugars, to rule out an effect on the stability of the sugar. In all the cases, the triazol ring signal clearly appears in the crude ^{1}H NMR spectra. However, the purification of the compounds either through column chromatography or crystallization with various solvents was unsuccessful. The products seem to decompose in all our attempts.



Scheme 4.9: CuACC reaction.

At this point, we decided to work with alkane chains smaller than C18. Our experience showed that this chain length seems to present disadvantages with some reactions. Consequently, we decided to prepare the C16 derivatives starting with **1h.** We expected that the alkyl sugar derivatives of palmitic acid will present many interesting properties. The reaction was carried out as described in Materials and Methods section using the same conditions than C8 derivative. However, lower yields were reached for mannose and glucose derivatives using C16 than C8. Yields were similar for both acids using fucose derivative (Table 4.2). The reaction between compound **1f** derivative (C14) and mannose derivative (**14k**) was also performed in

order to study if alkane chain can affect the yield. Nevertheless, the resulting yield did not allow the establishment of a clear relationship between chain length and final yield.

4.4.1.3.- Protein Interaction Array Test.

The interactions between the alkane sugars and C-lectin glycoproteins were measured using surface plasmon resosnance spectroscopy (SPR) in a high-troughput multichannel mode, with a GLC chip.

We started working only with the **15k** derivative, which was expected to present the higher affinity. Hence, we prepared 6 different concentrations of the corresponding disugar derivative.
The chip was first activated with SNHS-EDAC. Then, 5 different human lectins (LANGERIN, DC-SIGNR, DC-SIGN, MR2 and MGL1), from different organs, were added in an acetate buffer at pH 5. One channel was only kept with the buffer acetate as a control.
The buffer solution was flowed over the chip alone before (90 s) and after (650 s) the injection of the analyte (900 s).

Over the ligands were ran the samples, and after them the deactivator. No response on the SPR sensograms was showed from non-of them at the higher concentration (100 μM solution). Consequently, our molecules did not show any binding capability over C-ligands.

4.4.2.- POLYMER SYNTHESIS.

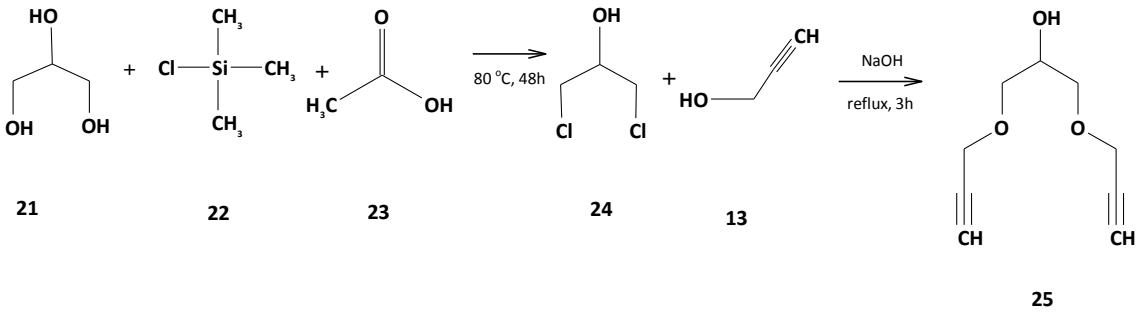
Considering that a polymer bearing one sugar moiety per chain showed an interesting effect in the gps120 DC-SIGN interaction, acting as competitors for gp120 to interact with DC-SIGN[33], we decided design a synthetic pathway from glycerol to prepare a polymer derivate bearing two sugars per chain.
We decided to perform a new synthetic route capable to synthesise similar polymer structures to the described above, doubling the sugar moieties compared with the previously polymer molecules. This approach used glycerol as starting material and

acrylic acid as carboxylic acid. Our aim was synthesise acrylate monomers with equivalent structures the ones prepared in the previous section.

We decided to invert the functionalization of the compounds to avoid any step using a dangerous azide molecule. Hence, instead of working with propargyl sugars and alkane chains with a high content of azide groups, we planned to work with sugar azides, which are more stable and less dangerous.

4.4.2.1.- Preparation of Acrylates.

Starting from glycerol we synthesize the 1,3-dichloropropan-2-ol (**24**) using the procedure previously described in our group[95]. Once this product was obtained, 1,3-bis(prop-2-yn-1-yloxy)propan-2-ol (**25**) was synthesised using propargyl alcohol as reagent (**13**) in a basic media. (Scheme 4.10).



Scheme 4.10: 1,3-bis(prop-2-yn-1-yloxyl)propan-2-ol (**25**) synthesis from glycerol.

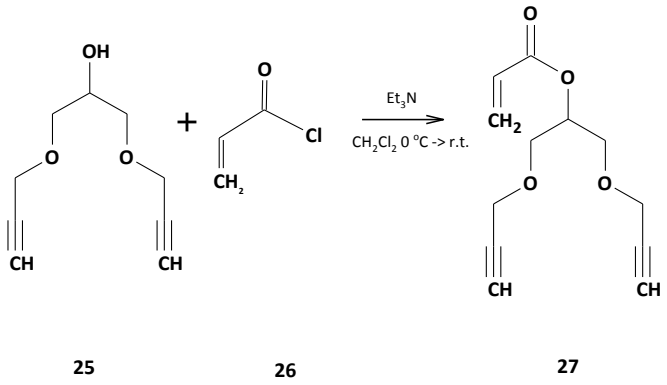
Table 4.3 shows the several attempts performed to improve the low reaction yields. Initially, we decided to use Cs_2CO_3 as a base, entry 3.2, instead of NaOH. We expected that this base could work better in not strictly water systems. However, the crude reaction present a complex mixture of products. Finally, we decided to use the conditions described[93], which led to 18%, Table 4.3 entry 3.4, of the desired product (25) after column purification. This was the best yield reached using these conditions.

Table 4.3: Effect of the base and the ratio reagent: base in the yield synthesis of **25** after column purification

Entry	base	Solvent	19: base	Conversion (%)[a]	25 Yield(%)[b]
3.1	NaOH	H_2O	1.4: 1	20%	-
3.2	Cs_2CO_3	THF : H_2O	1: 4: 4	100%	-
3.3	NaOH	H_2O	1: 1.2	100%	13.34
3.4	NaOH	H_2O	1: 1.2	100%	18

[a]: NMR ^1H calculated. [b]: column purificated

Scheme 4.11 shows the synthesis of the desired acrylate (**27**) starting from the purified 1,3-bis(prop-2-yn-1-yloxy)propan-2-ol (**25**). Acryloyl chloride (**26**) was added to a solution of the alcohol **25** to yield compound **27**, which is a free-radical-curable monomer since the double CC bond present in the acrylic part.



25 **26** **27**

Scheme 4.11: 1,3-bis(prop-2-yn-1-yloxyl)propan-2-yl prop-2-enoate (27) synthesis from 1,3-bis(prop-2-yn-1-yloxyl)propan-2-ol

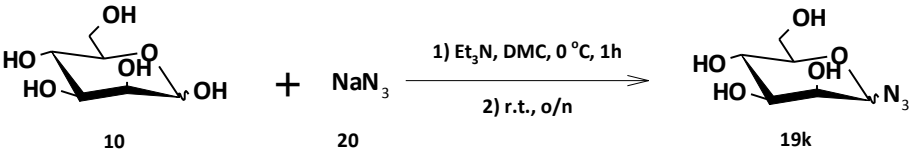
The first attempt to prepare the acrylate ester was performed using a 25:26: Et_3N ratio of 1: 1.7: 1.4. Although acryloyl chloride (**26**) was present on excess the yield was moderate (31.4%). The reaction was again performed increasing the relation of **26** and the base (25:26: Et_3N ratio = 1:2.6:2.25) In that case we achieved to synthesize the desired product (**27**) with a 95% yield, without any further purification.

4.4.2.2.- Synthesis of Azide Functionalized Sugars.

The synthesis of the sugar azides was performed as was previously described in section 3.3.2 c using DMC, which allowed the selective substitution of the anomeric hydroxyl group by the azide without the need of protecting the other hydroxyl groups (Scheme 4.12).

To improve the yield, the reaction was studied following two purification procedures. In the first one, the purification was conducted through a gel silica column. Before the purification, the crude reaction was concentrated and ethanol was added, which precipitated the sodium azide excess. Hence, the sodium azide was easily removed by

filtration. It must be noted, that this process should be repeated at least twice to almost completely remove the sodium azide.



Scheme 4.12: Mannose azide synthetic pathway

Once the solid was removed, the filtrate was concentrated and purified through silica gel column chromatography, (CH_2Cl_2: MeOH / 5: 1). Nevertheless, triethylamine still remains in the mixture after column purification, as it eluted with the product. Consequently, we decided to crystallize the product using MeOH, which results in a final yield of 22.6% (Table 4.4, entry 4.1). which was lower than the described in the bibliography (82-92%)[43, 95, 96].

Table 4.4: Azide mannose **(19k)** synthesis trials**.**

Entry	Mannose	NaN_3	Et_3N	DMC	19k Yield (%)
4.1	1	7.8	10.1	3.2	22.6
4.2	1	11.5	16	3	76.9

Because of the low yield and the difficult to completely remove the triethylamine, we decided to repeat the synthesis using a different process to purify the reaction crude. After the excess of sodium azide was removed adding ethanol into the crude and filtrating the solution, the filtrate was again concentrated under vacuum. The residue was dissolved in water and washed with CH_2Cl_2 three times until triethylamine cannot be detected by TLC in the organic layer. Once the triethylamine was completely removed, the aqueous layer was passed through an Amberlite column previously activated, to remove the extra salts present in the crude mixture. Finally, the aqueous solution was freeze-dried.

The residue containing mainly mannose azide was recrystallized from MeOH yielding a completely pure compound. Although the yield was still lower than the described[44, 95, 96] (Table 4.5 entry 5.2),

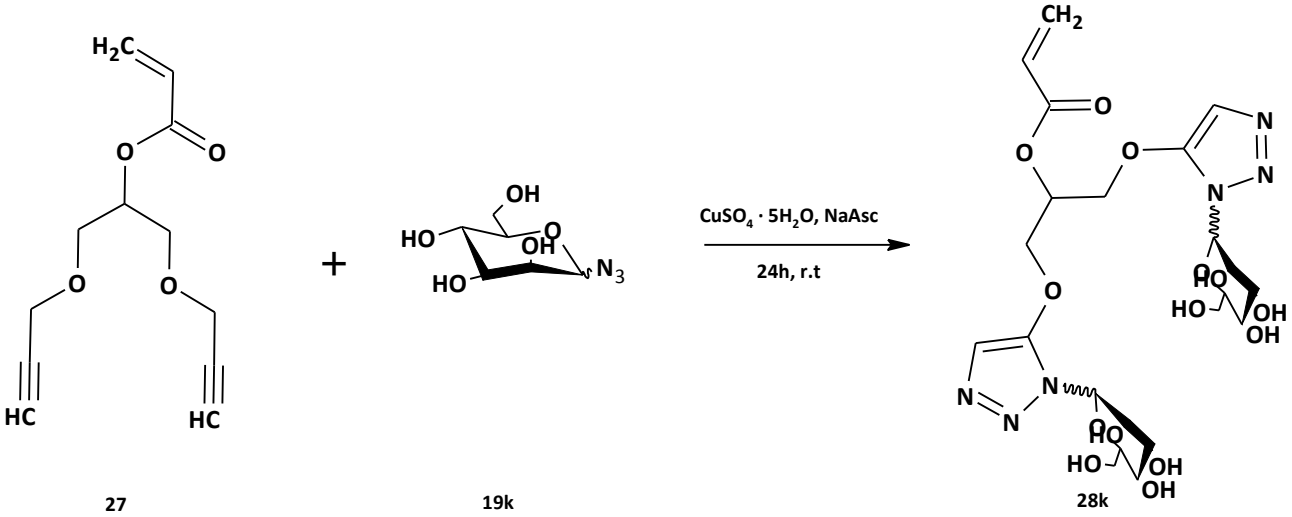
it was higher than the obtained using the silica gel column chromatography, which was not necessary to obtain the final product.

The ^{1}H and ^{13}C spectra of the pure product showed signals corresponding to the literature described products[95, 96].

4.4.2.3.- CuACC for Monomer Functionalization.

Once the two reagents (**27** and **19k**) for the monomer synthesis were prepared and purified, the synthesis of the monomer was performed as shown in Scheme 4.13.

The first synthesis was performed using a MeOH: H_2O mixture as a solvent. The reaction was conducted in air atmosphere, expecting that the O_2 present performed as a polymerization inhibitor. But, the changes The addition of Cu^{2+} induced a drastic modification of the reaction mixture aspect. Indeed, the ^1H NMR spectra of the final product showed the complete polymerization of the starting reagent, (Table 4.5, entry 5.1), as showed the fact that the acrylic signals completely disappeared. Hydroquinone was then selected as a polymerization inhibitor. Entry 5.2 shows that using a 10% of hydroquinone the triazol ring was formed, as showed the fact that a signal near 8 ppm appeared in the ^1H NMR spectrum. The moderate yield reached, 51% by ^1H NMR, prompted us to perform new approached to this synthesis.



27 **19k** **28k**

Scheme 4.13: Monomer synthesis (**28k**) using CuAAC click reaction.

Firstly, a third trial with less hydroquinone quantity was performed (entry 5.3). Although the formation of the triazol ring was observed, acryloyl signals disappeared indicating the polymerization of the initial reagent. Consequently, we decided to maintain the proportion of 10% hydroquinone as polymerization inhibitor in the next trials.

Entry 5.4 shows the yield reached in our attempt to scaled-up the synthesis of **28k.** This low yield conducted as to hypothesise that the H_2O: MeOH might be not was the

same solvent considering the low solubility of **27** in this solvent mixture. Hence, we decided to change the solvent using H_2O:THF (1:1) as a solvent mixture at 50°C[97]. The THF was evaporated under vacuum after 24 h of reaction and the remaining aqueous solution was freeze-dried.

Once freeze-dried, a slightly green solid with a similar aspect to the other previously synthesized sugars was obtained. The triazol ring formation without signs of polymerization could be identified by the signal at 8.14ppm in the ^{1}H NMR spectrum. Hence, the crude mixture was purified through silica gel column. Unfortunately, the yield of the purified product clearly decreased in relation to the yield of the crude product determined by ^{1}H NMR (entry 5.5). The study of other column fractions showed that part of the product polymerized during the purification process.

Entry 5.6 shows the results reached performing a quick column purification trying to avoid large periods of contact between the silica gel and the product. The yield , was clearly improve, although polymerization was still observed during the column purification.

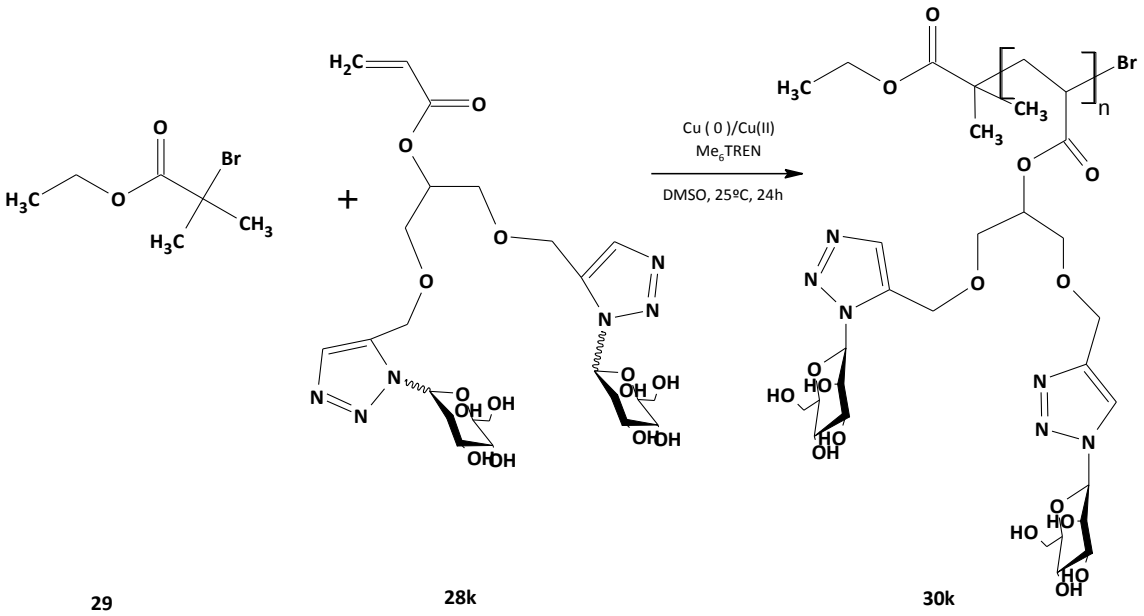
Table 4.5: Effect of reagents ratio, solvent and inhibitor in the synthesis of **28k**

Entry	27: 19k: Cu^{2+}: NaAsc	Solvent	Inhibitor	Conversion[a] (%)	28k Yield[b] (%)	28k Yield[c] (%)
5.1	4.2: 12.9: 1.18: 1	H_2O: MeOH	Oxygen	100	0	-
5.2	5.35: 11: 1.2: 1	H_2O: MeOH	O_2/Hydroquinone (10%)	100	51.0	-
5.3	5.2: 11.5: 1.23: 1	H_2O: MeOH	O_2/Hydroquinone (5%)	100	41.6	-
5.4	7.7: 15.5: 1: 1.2	H_2O: MeOH	O_2/Hydroquinone (10%)	100	31.5	-
5.5	5: 15: 1.2: 1	H_2O: THF	O_2/Hydroquinone (10%)	100	59.5	11.04
5.6	5: 14: 1: 1.3	H_2O: THF	O_2/Hydroquinone (10%)	100	54.0	23.82

[b] ^{1}H NMR calculated, [c] column purified

4.4.2.3.- Polymer Synthesis.

Scheme 4.14 shows the with the polymerization of the glycomonomer D-mannose (**28k**) at room temperature. The process was performed using Cu(0)/Cu(II)/Me_6TREN as catalyst with EBiB (**29**) as initiator and DMSO as solvent.



Scheme 4.14: SET-LRP polymerization of **28k**

The reaction was completed after 24h. The crude mixture was dialysated against water and a ^{1}H NMR was recorded. Spectra did not provide many information, but the triazol ring signal and also the sugar protons were present. Moreover, there were not present the signals corresponding to the acrylate part. Meanwhile, signals have appeared at 1-2 ppm, which indicated the formation of the alkane chain of the polymer backbone and their linkage through the triazol ring of the mannose units.

Figure 4.3 shows the MALDI-ToF analysis performed to characterize the structure of the glycopolymer. Peaks at 1403, 2035, 2668 and 3301 Da (*m*/*z*) cationised with Na^{+} were attributed to dead polymer chains with terminal hydrogen. with 2 to 5 chains added,respectively (Scheme 4.14). The exchange of the bromine for the proton were mainly caused by disproportionation and chain transfer side reaction and thus leading to lose of terminal bromine. It must be note that there appears also repited not identified signals at +34, +58, +106 m/z. Hence, the polymer with terminal bromine was not detected using the MALDI-ToF technique.

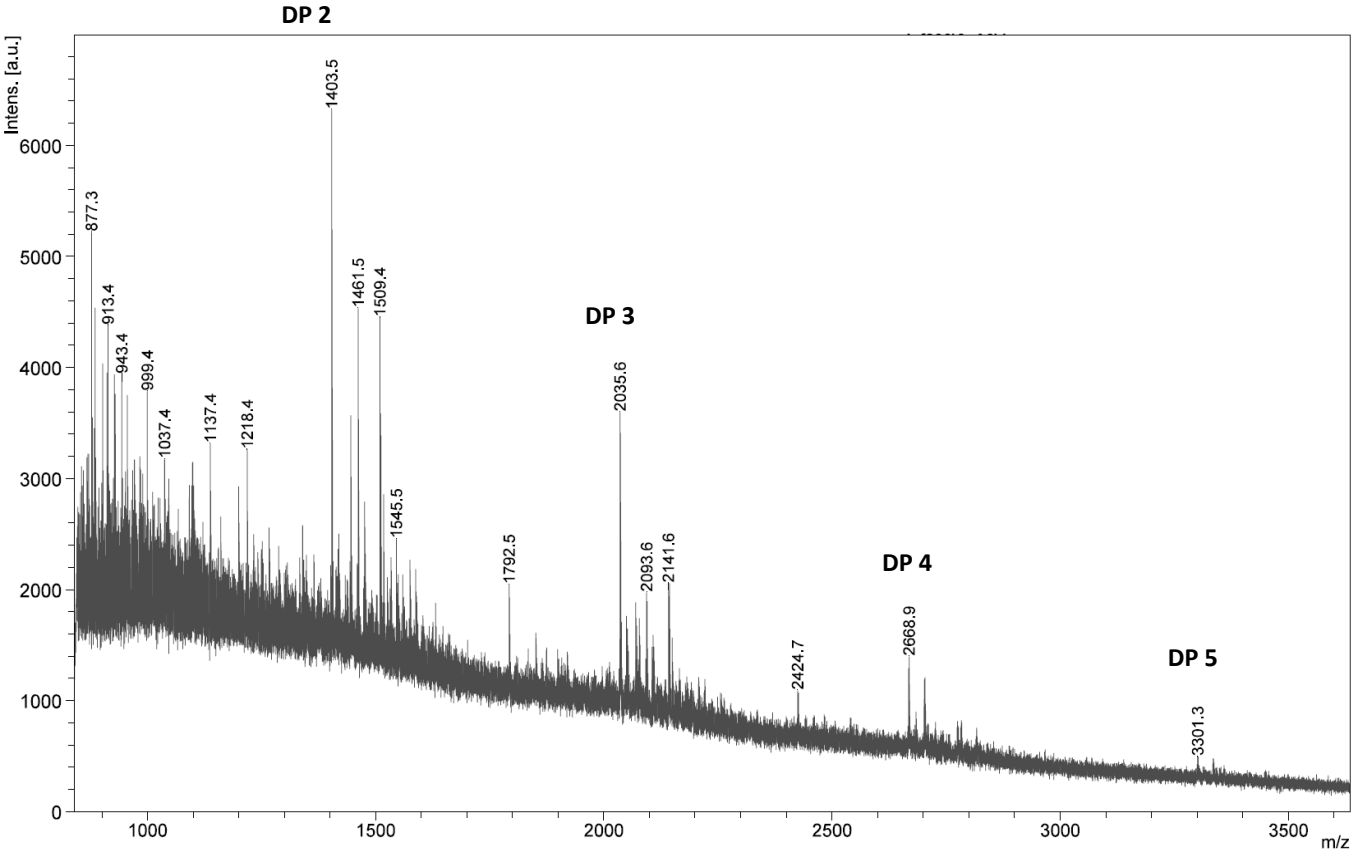
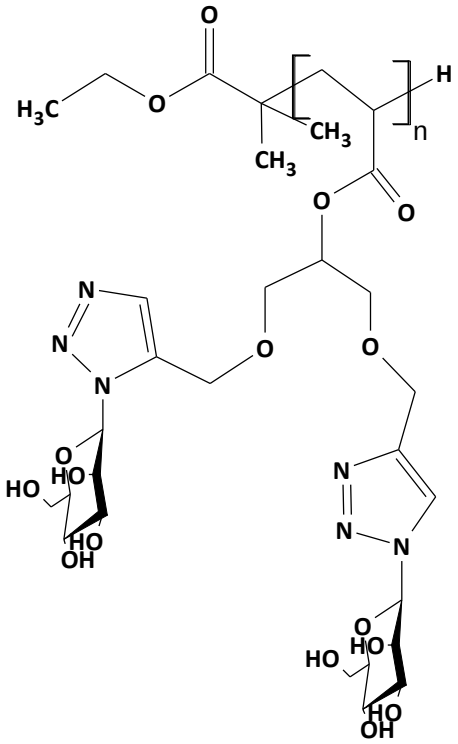


Figure 4.3: MALDI-ToF spectra of the glycopolimer (**31k**) obtained by SET-LRP.



31k

Scheme 4.14: Proposed structure for the dead polymer synthesized (**31k**).

4.5.- CONCLUSIONS.

We have synthesised various alkane linear chains bearing two sugar moieties each one. The synthesis was performed using the CuAAC click reaction between sugar alkynes and diazide fatty esters with various alkyl chain lengths. The sugar alkynes were prepared from free sugar via a one-step Fisher type glycosylation using H_2SO_4-silica catalyst. The diazide esters were prepared from glycerol. These products did not show a direct response with SPR technique, although we could expect other possibilities for them as sugar arrays as it they can present many other applications[98, 99].

We also have achieved the polymerization of a glycomonomer via SET-LRP demonstrating the possibility to synthesize it from glycerol. The glycomonomer was synthesized with two mannose moieties per monomer chain. The monomer was prepared from a sugar azide and a glycerol derivate using the CuAAC click reaction. The final product bears of a twice more sugars per chain than the ones prepared until now, which is expected to improve the efficiency of the cell disruption of this polymers. In fact, the use of linear polymers on effective lectin binding has been demonstrated by several research groups[30-32].Indeed, relatively simple mannose containing glycopolymers can effectively bind human dendritic cell associated lectin (DC-SIGN) and disrupted the interaction of DC-SIGN with the HIV envelope glycoprotein gp120. This activity has been proposed as a new approach for therapeutic applications[100].

4.6.- REFERENCES.

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GENERAL CONCLUSIONS

1.-Different biomass sources have successfully been modified using enzymes, to produce high value added products.

2.-Some of the enzymatic modifications applied to fruit fibres have improved their WHC and SCW. FTIR has been demonstrated a powerful spectroscopic technique to study the modification effects of various enzymes on the fruit fibres.

3.-The use of glycerol as starting material to prepare products with very different applications has been demonstrated.

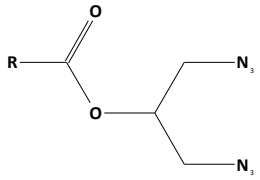
4.-New glycerol derivatives with high industrial interest were synthesized. The synthetic pathway includes the use of CO_2 or CO_2 derivatives making the process more greener.

5.-The study of the unexpected behaviour presented by some of the synthesized products, was demonstrated to be due to the presence of hydrogen bond interactions.

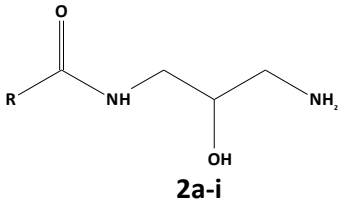
6.-Compounds with different lateral chain lengths were synthesized from glycerol through click chemistry.

7.-Polymers with high expected capacity to be used as DC-SIGN gp120 disruptors have been obtained from glycerol as starting material using an easy and cheap methodology.

ANNEX 1.

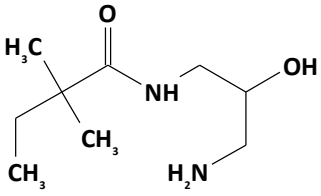


1a-i

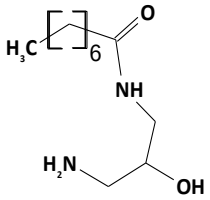


2a-i

a= $CH_3CH_2C(CH_3)_2$; **b**= $CH_3(CH_2)_6$; **c**= $(C_6H_5)_2CH$; **d**= $CH_3(CH_2)_{16}$; **e** =$CH_3(CH_2)_{11}$; **f**= $CH_3(CH_2)_{12}$; **g**=$CH_3(CH_2)_{13}$; **h**= $CH_3(CH_2)_{14}$; **i**=$CH_3(CH_2)_{15}$,.



2a



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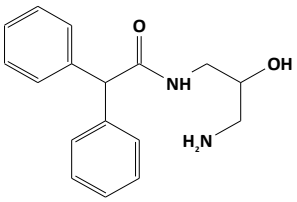

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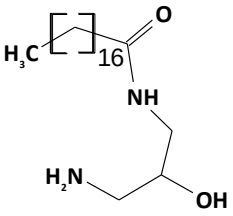
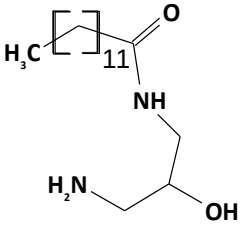


2d



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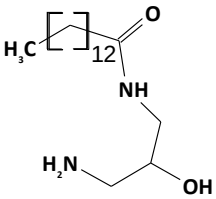

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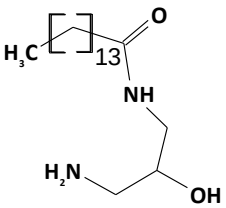

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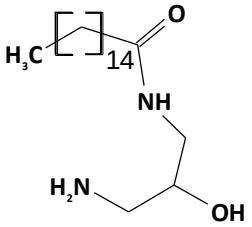
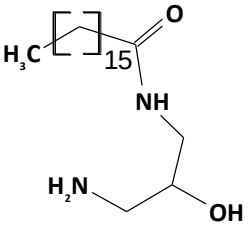


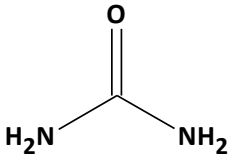
2h



2i

CO_2

3



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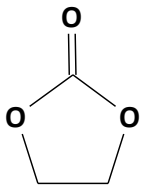

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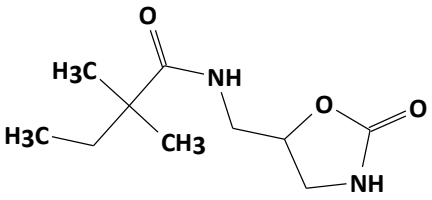
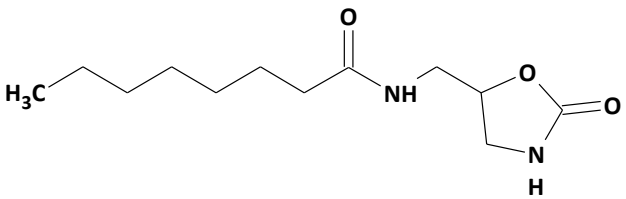
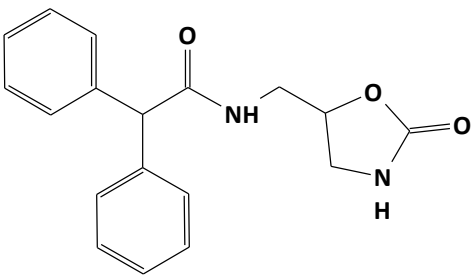


6a



6b



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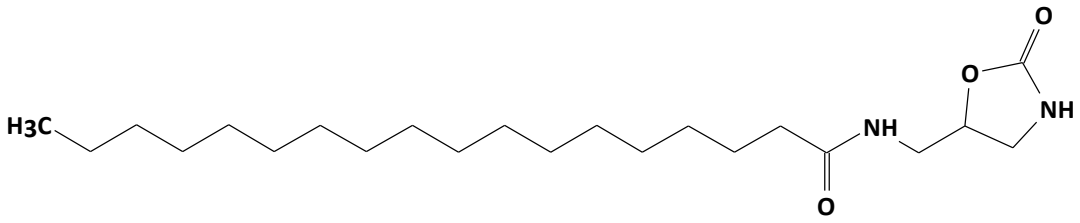

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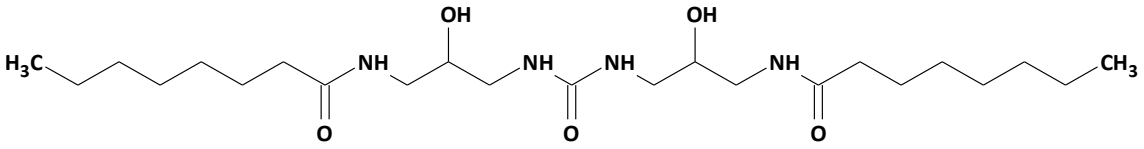
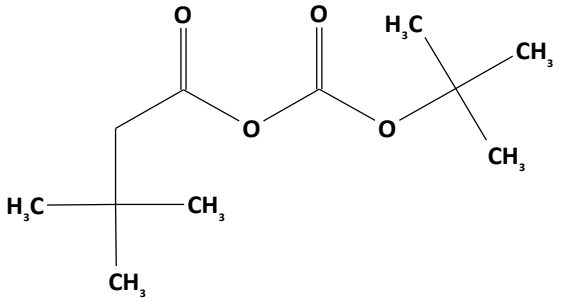


7b



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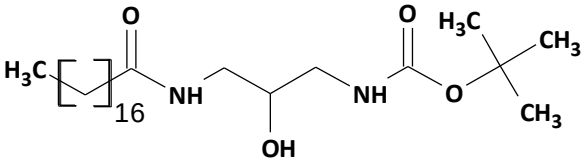

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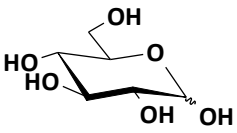

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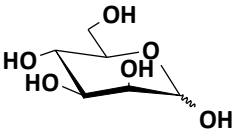

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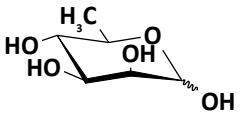

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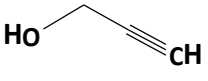

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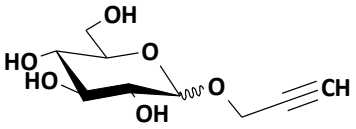

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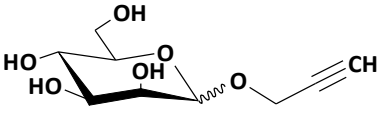

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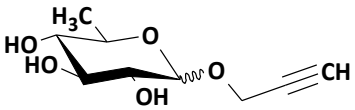

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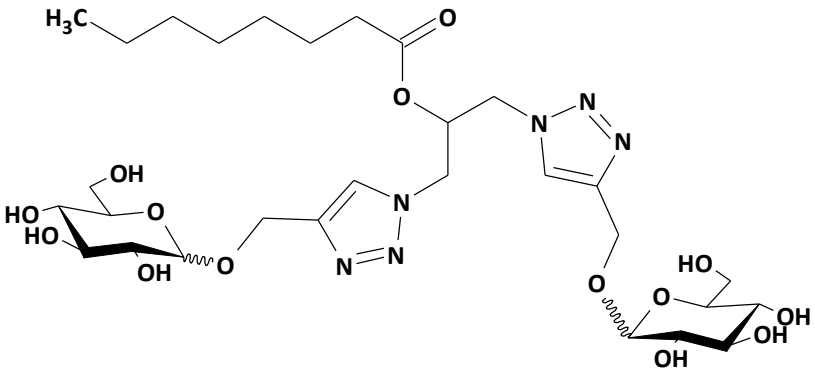

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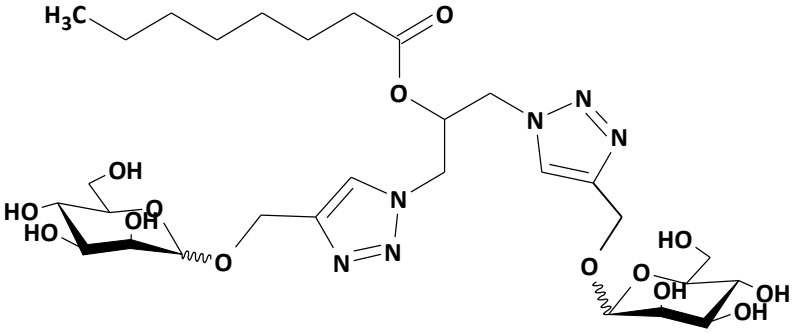

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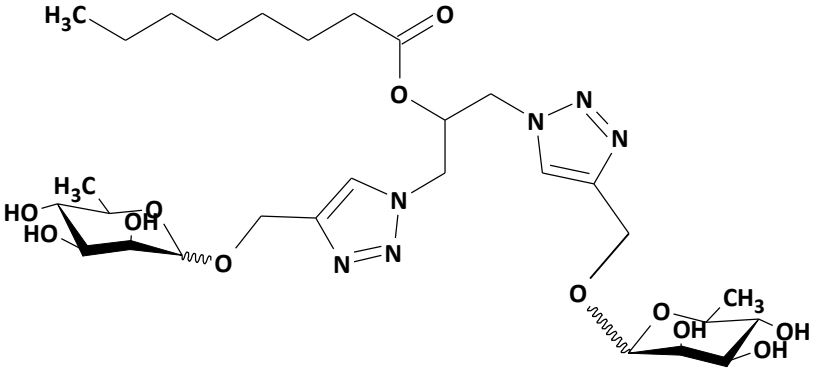

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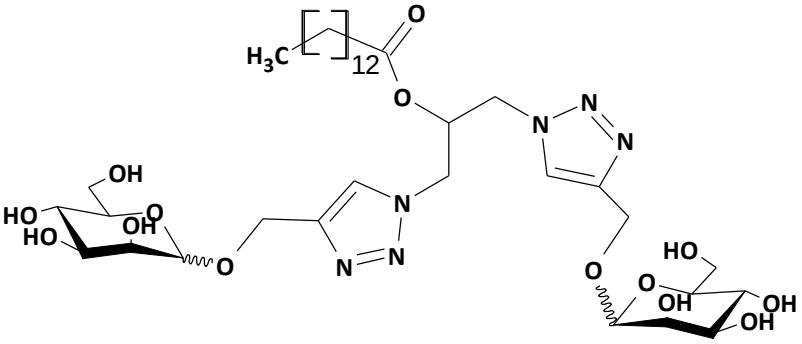

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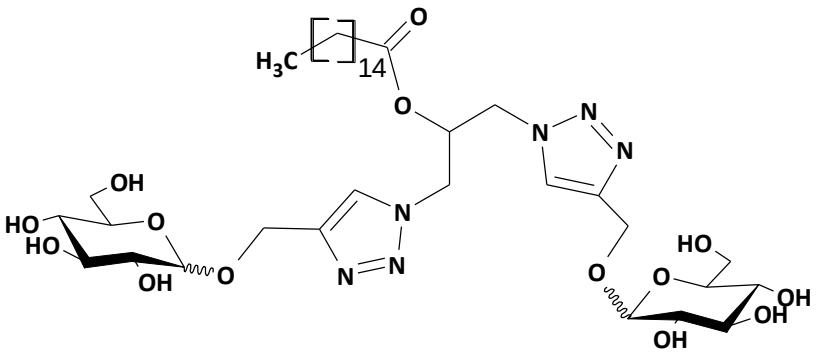

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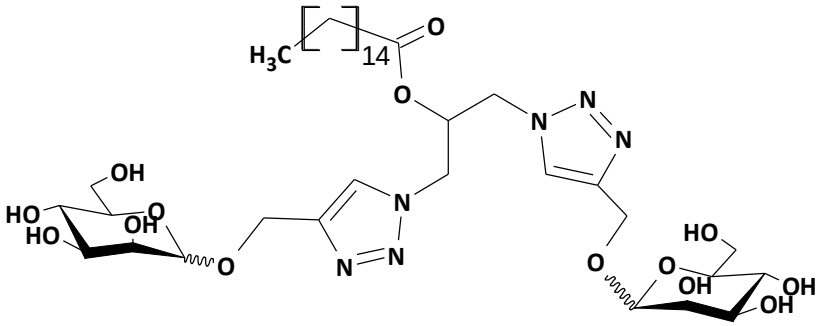

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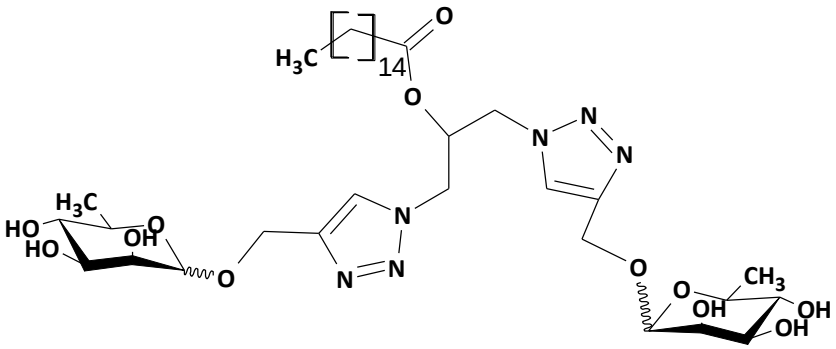

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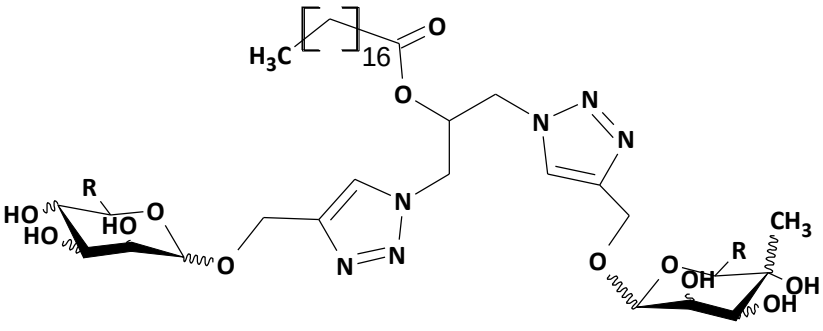

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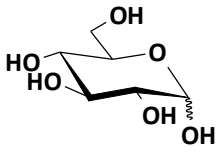

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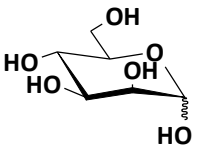

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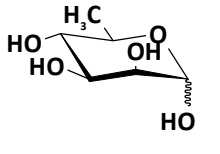

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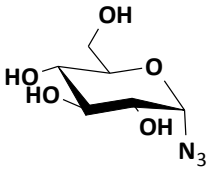

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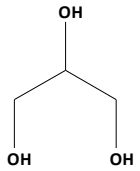

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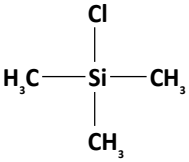

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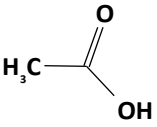

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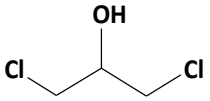

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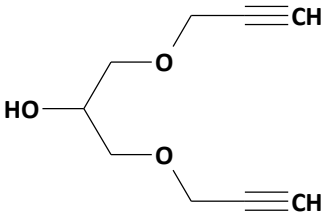

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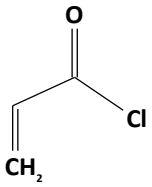

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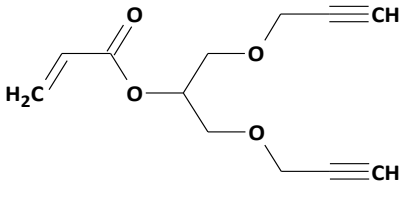

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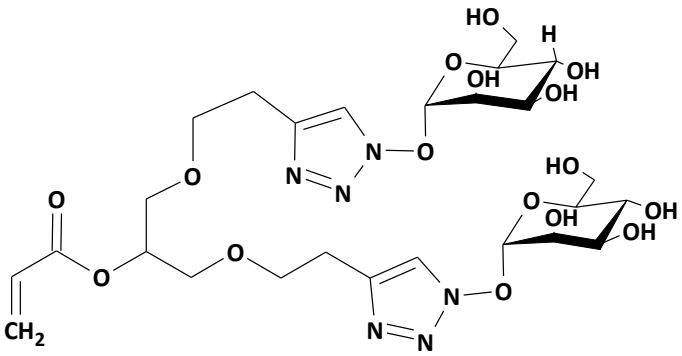

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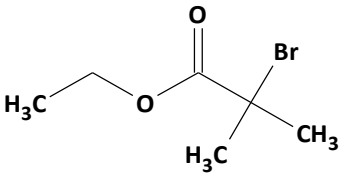

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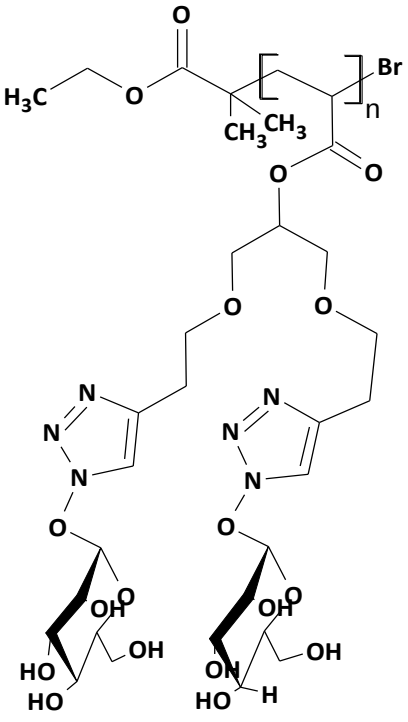

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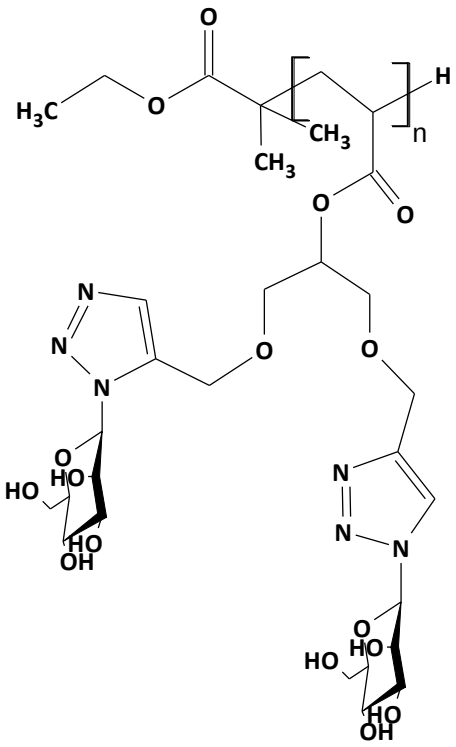
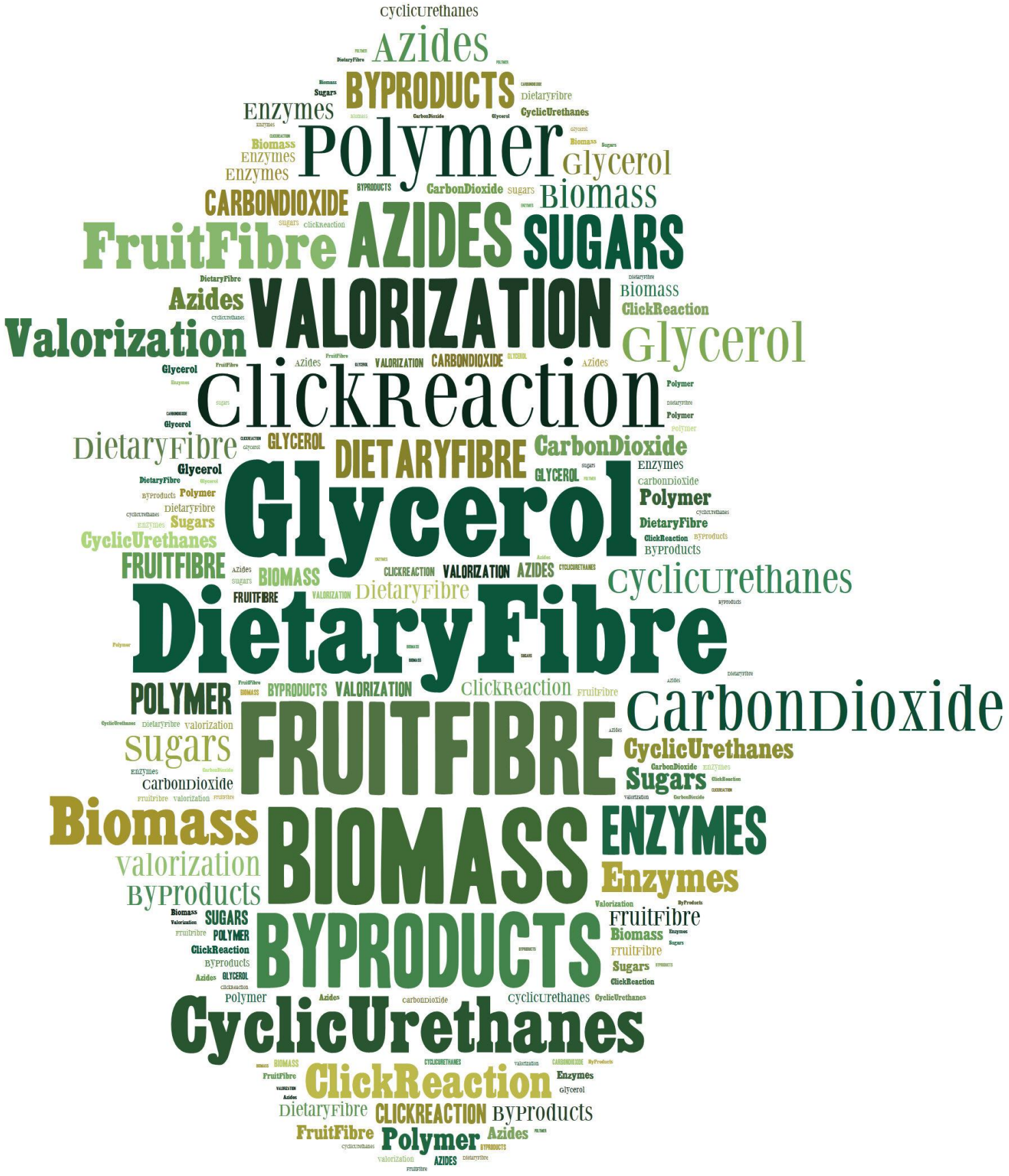


31k

ANNEX 2.

WHC		CITRUS N	CITRUS M	APPLE N	APPLE M	PEACH N
FER						
	45°C 24h	12.3971±0.7476	14.2618±0.3429	8.9064v0.1275	10.1424±0.3456	10.2971±0.1853
	45°C 72h	13.7660±0.5499	15.7302±0.4301	9.7866±0.1374	13.0243±1.0860	11.3672±1.4620
	65°C 24h	12.7310±0.1892	16.1273±0.7667	11.1713±0.2515	12.57030.4426	13.0256±1.0256
	65°C 72h	13.5571±0.6159	14.8874±1.0199	11.4152±0.2997	13.6431±0.8467	11.4395±0.8193
PME A						
	30°C 1/2 h DESTILLED	14.0633±0.7622	13.5778±0.5804	9.8854±0.6445	10.5672±1.5693	11.6677±0.3965
	30°C 2h DESTILLED	11.6533±0.1771	14.1100±1.2141	9.9480v0.1522	11.5143±0.3084	8.4771±1.2824
	50°C 1/2 h DESTILLED	13.4806±0.1993	15.1921±2.0697	10.0551±0.4765	9.7911±0.6909	10.4911±0.4933
	50°C 2 h DESTILLED	14.0839±0.6124	14.1259±0.9608	9.7566±1.2218	10.3958±0.7494	9.4666±1.3830
	30°C 1/2h TAP	12.1501±0.3052	13.9689±0.9412	8.9998±1.0440	9.8849±0.3887	10.7448±1.0669
	30°C 2h TAP	11.5585±0.2670	13.7987±0.4543	9.8047±0.2719	10.5532±0.3364	7.8165±1.1791
	50°C 1/2h TAP	12.1728±0.4237	14.0691±0.7278	10.1506±0.4227	11.1855±1.0239	10.1545±0.4904
	50°C 2h TAP	13.6950±0.4549	14.8952±1.3593	10.3214±0.5841	11.3821±0.7007	10.1165±0.1205
PME B						
	50°C 24h DESTILLED	13.65647±0.2764	15.5670±0.6632	10.6065±1.3774	14.4380±0.175	11.3274±0.3122
	50°C 24h TAP	13.32709±0.0706	15.8292±0.3411	7.9818±1.7001	13.4293±0.2077	12.2852±0.3836
	50°C 60h DESTILLED	13.49982±0.0700	16.6616±1.0233	9.0752±0.3540	15.8687±1.3133	11.9127±0.6441
	50°C 60h TAP		17.6647±0.3734		16.9939±0.9961	
PEC A						
	30°C 1/2h		17.0770±1.552		15.0662±1.8851	
	50°C 1/2h	10.5855±0.5250	17.5519±1.9862	12.2654±0.4559	15.4750±0.4524	11.6496±0.8773
	50°C 1h	10.1464±0.8509		13.0425±1.2700		11.4494±1.3564
	55°C 1/2h	10.3598±0.3621		12.2935±0.1925		11.1528±0.4095
	55°C 1h	11.0564±0.8089		11.0116±1.7572		10.5522±0.2412
PEC B						
	50°C 4h	11.5266±0.3891	13.7193±0.6453	12.6521±0.2361	15.1824±0.5395	11.9949±0.7708
	50°C 8h	10.7118±0.8762		13.1928±1.2444		12.2427±0.5297
	50°C 16h	11.1011±0.2699		14.3774±0.3791		13.9684±1.6143
	55°C 4h	11.2784±0.3792		12.7489±0.6796		11.5179±0.5509
	55°C 8h	12.0358±0.9022		12.6135±0.2169		12.0344±0.3765
	55°C 16h	11.2549±0.2664		14.7351±0.7673		12.7853±1.3412
CEL						
	60°C 2h	9.8985±0.3951		13.3930±0.6961		11.1246±0.4772
	60°C 6h	10.9161±0.0821	15.6855±0.5301	12.7411±0.7264	9.6892±0.0586	12.3371±0.8628
	60°C 18h	11.6749±1.3025		13.3708±0.1855		11.2707±0.9980
	65°C 2h	10.6035±0.6570		11.8824±0.8946		11.2292±0.3740
	65°C 6h	10.5225±0.3630		12.6928±0.3287		12.4891±0.5450
	65°C 18h	11.1378±0.2504		13.4170±0.3725		11.2898±0.3672

	SWC	CITRUS N	CITRUS M	APPLE N	APPLE M	PEACH N
FER						
	45°C 24h	11.8919±0.4015	12.0855±0.1711	6.5847±0.5246	6.6858±0.4611	10.4279±0.1928
	45°C 72h	12.6857±0.5455	12.3850±0.1060	7.0625v0.3018	9.4483±0.2841	8.65940.2407
	65°C 24h	10.8711±0.5172	13.1701±0.4335	7.1792±0.0847	12.4008±0.4813	7.3452±0.0390
	65°C 72h	9.6546±0.3100	10.8342±0.0314	6.7337±0.6311	12.0861±0.1997	7.8985±0.5214
PME A						
	30°C 1/2 h DESTILLED	10.4831±0.1194	12.4762±0.1221	8.7623±0.2199	8.2218±0.6189	6.8793±0.2759
	30°C 2h DESTILLED	13.0707±1.7751	15.1160±0.3147	6.9062±0.1104	8.2678±0.7337	7.3017±0.3630
	50°C 1/2 h DESTILLED	14.4344±0.1946	14.7718±0.6974	8.6193±0.0598	5.5900±1.2776	7.1533±0.6034
	50°C 2 h DESTILLED	11.3373±1.5753	12.0716±0.4985	7.4698±0.5299	8.2902±0.2491	6.2875±0.1874
	30°C 1/2h TAP	11.2732±0.3527	12.1774±0.1909	7.9464±0.6578	8.0371±0.9891	7.51000.6010
	30°C 2h TAP	13.1570±0.5779	13.3601±0.3127	6.4709±0.0792	8.5075±0.1738	6.1685±0.2815
	50°C 1/2h TAP	14.0334±1,2241	11.0234±0.3786	6.7673±0.8052	7.4054±0.3203	6.2251±0.6682
	50°C 2h TAP	12.0231±0.7941	12.4889±0.4578	8.3154±0.1955	7.8854±0.5510	6.7376±0.1546
PME B						
	50°C 24h DESTILLED	11.4449±0.2826	16.8116±1.1328	13.3306±0.3757	10.5986±0.0325	12.7695±0.4978
	50°C 24h TAP	11.6942±0.4116	20.5820±0.7887	7.6728±0.3025	9.4796±0.5839	14.4605±0.4554
	50°C 60h DESTILLED	11.7271±0.3033	17.4021±1.0546	13.7084±0.3258	15.5929±1.3445	13.7847±1.7694
	50°C 60h TAP		20.8737±0.2910		10.5273±0.4799	
PEC A						
	30°C 1/2h		25.3750±1.9906		13.7012±1.4056	
	50°C 1/2h	10.6265±0.5749	21.9690±1.9883	10.2580±0.3957	12.8850±1.1918	11.3451±0.2905
	50°C 1h	8.3345±0.0186		12.3840±0.5185		9.5052±1.8681
	55°C 1/2h	6.5295±0.2967		7.4446±0.3425		6.7594±0.4653
	55°C 1h	9.7356±0.1992		10.0650±0.1746		9.9316±0.6034
PEC B						
	50°C 4h	12.1475±0.8691	13.2335±0.6378	11.5909±0.2981	12.3409±1.7038	12.7624±0.2134
	50°C 8h	12.4170±0.2466		12.8357±0.3294		12.2755±0.4022
	50°C 16h	11.55640.06689		13.3442±0.2921		15.3803±0.8798
	55°C 4h	10.2222±0.7210		11.3121±0.4341		11.4732±0.1911
	55°C 8h	10.4051±0.2730		11.8978±0.4836		13.3963±0.0992
	55°C 16h	6.5942±0.0277		17.0917±0.4226		12.0687±2.1563
CEL						
	60°C 2h	9.2026±0.2865		9.7463±0.2050		11.4257±0.5341
	60°C 6h	9.8273±0.3278	21.2590±1.6168	11.5968±0.9552	4.4780±0.5221	13.1067±0.3220
	60°C 18h	11.1414±0.4983		13.3858±0.3574		12.5389±0.6459
	65°C 2h	9.6047±0.3200		9.9897±0.1315		10.4815±0.2007
	65°C 6h	9.6186±0.2085		11.8081±0.2236		14.6189±0.4927
	65°C 18h	6.0850±0.3805		14.9451±0.1905		13.3108±0.1774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CyclicUrethanes
FRUITFIBRE
BIOMASS
CLICKREACTION
VALORIZATION
AZIDES
cyclicurethanes
DietaryFibre
POLYMER
BYPRODUCTS
VALORIZATION
clickReaction
carbonDioxide
FRUITFIBRE
sugars
CyclicUrethanes
Sugars
carbonDioxide
Biomass
BIOMASS
ENZYMES
valorization
Enzymes
Byproducts
SUGARS
BYPRODUCTS
FruitFibre
POLYMER
Biomass
ClickReaction
FruitFibre
Byproducts
Sugars
Azides
GLYCEROL
ClickReaction
Polymer
cyclicurethanes
CyclicUrethanes
CyclicUrethanes
ClickReaction
Enzymes
Glycerol
DietaryFibre
CLICKREACTION
Byproducts
FruitFibre
Polymer
Azides
valorization
AZIDES