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# DISSERTATION

## CARBOHYDRATE DERIVED AZIDES FOR PHARMACEUTICAL TECHNICAL APPLICATIONS

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## 1. Summary

Based on chemical and enzyme catalyzed procedures, new carbohydrate derived azides were synthesized. These azides are expected to have a wide range of pharmaceutical and technical applications. Starting from cellobiose, a chemical synthetic pathway has been developed, based on a conventional protection-deprotection scheme to synthesize 6'-azido-6'-deoxy-cellobiose (in which the azido group is located on the non-reducing glucose unit). In a similar way 6-azido-6-deoxy-cellobiose was also synthesized. Both cellobiose derivatives can act as precursors for enzyme-catalyzed syntheses of new aminopolysaccharides or chemical polymerization into biomimetic polymers. The two azido derivatives of cellobiose were enzymatically oxidized under catalysis by the cellobiose dehydrogenase to the corresponding lactones. These lactones can function as monomers for the synthesis of new hydrogels. 6-Azido-6-deoxy-D-glucose was also obtained from glucose by a conventional protocol in 10 steps. This azido sugar can be used as a monomer in the synthesis of 6-amino-6-deoxycellulose. Furthermore we have shown that 6-azido-6-deoxy-cellobiose can also be formed by reversal phosphorylase reaction, starting from glucose 1-phosphate and 6-azido-6-deoxy-D-glucose. While the forward phosphorylase reaction using 6-azido-6-deoxy-cellobiose as starting material and inorganic phosphate as buffer resulted in 6-azido-6-deoxy-D-glucose as expected. That means the azido group on C-6 of glucose as well as of cellobiose is tolerated by the enzyme cellobiose phosphorylase. In our hands 6'-azido-6'-deoxy-cellobiose did not function as a substrate for cellobiose phosphorylase. Further investigation may be required to confirm our observation. In alternative pathway we have synthesized 3-azido-3-deoxy-D-glucose as well as  $\beta$ -D-allose starting from 1,2:5,6-di-O-isopropylidene- $\alpha$ -D-glucopyranose. Our purpose of getting these key intermediates was on one hand to be used as precursors in oligosaccharides synthesis, on the other hand to test them as substrates for the reversal cellobiose phosphorylase reaction. In the enzyme-catalyzed synthesis we have used the enzyme cellobiose phosphorylase extracted from *Clostridium thermocellum*. In the reverse reaction, CBP catalyzes the production of heterodisaccharides from various derivatives of glucose. For this purpose we have used  $\beta$ -D-allose, 6-azido-6-deoxy-D-glucose and

3-azido-3-deoxy-D-glucose as different substrates for the action of CBP. The only heterodisaccharide formed was that from 6-azido-6-deoxy-D- glucose, which means substitution of hydroxyl group by azido group in the six position of glucose seems to be tolerated by cellobiose phosphorylase, whereas the 3-azido analogue did not react. This observation showed that the role of the hydroxyl group or its azide derivative at C-6 is less critical than that of the hydroxyl at C-3. The absence of heterodisaccharide in case of  $\beta$ -D-allose showed that the equatorial configuration on C-3 is necessary for the binding on the active site of the enzyme.

Another useful application of 6-azido-6-deoxy-D-glucose remains in its ability to be coupled in 1,3-dipolar cycloaddition reaction to an alkyne. We have coupled 6-azido-6-deoxy-D-glucose to ethylpropiolate to get a stable triazole. This triazole can be used as a part of a diagnostic system for testing of anti-carbohydrate IgE antibodies. In which the alkyne-azide coupled system act as a linking arm between the glycopeptide of interest and a micro titre plate to which the other end of triazole is immobilised.



## Zusammenfassung

Basierend auf chemischen- und Enzym-katalysierten Verfahren wurden neue Azidozucker synthetisiert. Ausgehend von Cellobiose ist ein synthetisch- chemischer Weg auf der Grundlage eines konventionellen Schemas entwickelt worden, um eine 6'-Azido-6'-deoxy-cellobiose (in welcher sich die Azido-Gruppe an der nicht reduzierenden Glukose-Einheit befindet) zu synthetisieren. Auf ähnlichem Wege wurde auch 6-Azido-6-deoxy-cellobiose synthetisiert. Beide Cellobiose-Derivate können als Vorläufer für Enzym-katalysierte Synthesen von neuen Aminopolysacchariden oder für die chemische Polymerisation in biomimetische Polymere fungieren. Die beiden Azido-Derivate der Cellobiose wurden unter Katalyse durch Cellobiose Dehydrogenase in die korrespondierenden Lactone enzymatisch oxidiert. Diese Lactone können als Monomere für die Synthese neuer Hydrogele fungieren. 6-Azido-6-deoxy-D-glucose wurde ebenso in einem konventionellen 10-Schritte-Protokoll erhalten. Dieser Azido-Zucker kann als Monomer bei der Synthese von 6-Amino-6-Deoxycellulose verwendet werden. Darüber hinaus haben wir gezeigt, dass die 6-Azido-6-deoxy-cellobiose auch durch die Rückreaktion der Phosphorylase ausgehend von Glucose 1-Phosphat und der 6-Azido-6-deoxy-D-glucose erzeugt werden kann, während die Hinreaktion der Phosphorylase unter Benützung 6-Azido-6-deoxy-cellobiose als Substrate und des anorganischen Phosphats als Buffer wie erwartet in einer 6-Azido-6-deoxy-D-glucose resultierte. Dies bedeutet, dass die Azido-Gruppe in der C-6 der Glucose als auch der Cellobiose durch die Cellobiose Phosphorylase toleriert wird. Bei unseren Untersuchungen erwies sich 6'-Azido-6'-deoxy-cellobiose nicht als Substrat für die Spaltung durch Cellobiose Phosphorylase. Auf alternativem Wege haben wir sowohl 3-Azido-3-deoxy-D-glucose als auch  $\beta$ -D-Allose ausgehend von 1,2:5,6-Di-O-isopropyliden- $\alpha$ -D-glucofuranose synthetisiert. Unser Ziel bei der Herstellung dieser Schlüsselsubstanzen war einerseits sie als Vorläufer in der Oligosaccharid Synthese zu verwenden und andererseits sie als Substrate bei der Rückreaktion der Cellobiose Phosphorylase zu testen. Bei der Enzym-katalysierten Synthese haben wir die aus *Clostridium thermocellum* extrahierte Cellobiose Phosphorylase(CBP) verwendet. Bei der Rückreaktion katalysiert die CBP die Produktion von Heterodisacchariden aus verschiedenen Glucose-Derivaten. Für diesen Zweck haben wir  $\beta$ -D-Allose, 6-Azido-6-deoxy-D-glucose und 3-Azido-3-deoxy-D-glucose als unterschiedliche Substrate

für die CBP verwendet. Das einzige gebildete Heterodisaccharid entstand aus der 6-Azido-6-deoxy-D-glucose. Das bedeutet, dass die Substitution der Hydroxyl-Gruppe durch die Azido-Gruppe in der sechsten Position der Glucose von der Cellobiose Phosphorylase toleriert wird, während das 3-Azido-Analoge nicht reagierte. Dieses Ergebnis zeigt, dass die Rolle der Hydroxyl-Gruppe oder des Azids am C-6 weniger kritisch ist als das Hydroxyl am C-3. Das Fehlen von Heterodisacchariden im Falle der  $\beta$ -D-Allose zeigt, dass die äquatoriale Konfiguration am C-3 für die Bindung am aktiven Zentrum des Enzyms erforderlich ist.

Eine andere nützliche Anwendung der 6-Azido-6-deoxy-D-glucose liegt in ihrer Fähigkeit in einer 1,3-dipolaren Cycloadditionsreaktion mit einem Alkin gekoppelt zu werden. Wir erhielten ein stabiles Triazol durch die Kopplung von 6-Azido-6-deoxy-D-glucose mit Ethylpropiolat. Dieses Triazol kann als Teil eines diagnostischen Systems zur Bestimmung von Anti-Carbohydrat-IgE-Antikörpern verwendet werden, wobei das Alkin-Azid-Kopplungssystem als Bindungsarm zwischen dem zu untersuchenden Glycopeptid und der Mikrotiter-Platte fungiert, an der das andere Ende des Triazols immobilisiert wird.

Es wird erwartet, dass die im Rahmen der vorliegenden Arbeit synthetisierten Azidozucker einen vielfältigen pharmazeutisch-technischen Anwendungsbereich finden werden.

## 2. Introduction

Carbohydrate derived azides are important synthetic intermediates. In this work new approaches have been explored to design new azido sugars with unique structures for pharmaceutical and technical applications. These applications include the synthesis of new aminopolysaccharides as pharmaceutical excipients, hydrogels and new stable carbohydrate derived 1,2,3-triazoles for diagnostic purposes.

### 2.1 *Pharmaceutical applications of $\beta$ -glycans*

#### 2.1.1 Cellulose and its derivatives

Cellulose is the most abundant biopolymer on earth, the molecular and supramolecular structure of this macromolecule allows a lot of important applications.

The cellulose formed by plants is the main source of this biopolymer but it is accompanied by many copolymers of biogenic origin, e.g. lignins, hemicelluloses, proteins. These impurities make the production of pure cellulose costly.

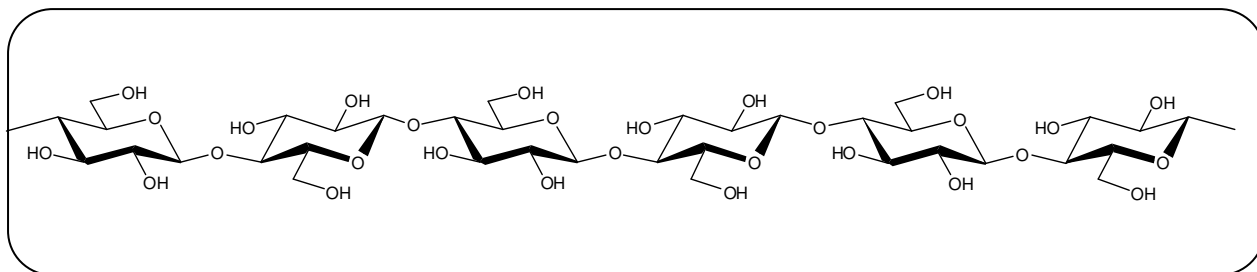


Figure 1. Cellulose

Cellulose is a polydisperse, linear homopolymer consisting of regio- and enantioselective  $\beta$ -1,4-glycosidic linked D-glucose units. It contains three reactive

hydroxyl groups at the C-2, C-3, and C-6 positions, which are, in general, accessible to typical conversions of primary and secondary alcoholic groups. Hydrogen bonding between these hydroxyl groups leads to various supramolecular, semicrystalline cellulose structures; thus, crystallinity and H-bonding associations have a strong influence on its ultimate chemical reactivity and solubility characteristics. As a consequence of its inherent polyfunctional structure, naked cellulose is insoluble in water as well as common organic solvents. New cellulose derivatives with supramolecular architectures are of increasing interest due to their various applications, such as in ultra-thin coatings, host-guest systems, biosensors, liquid crystalline polymers, and biomaterials. Regioselective functionalization of cellulose, i.e. the introduction of either a substituent or more than one substituent onto the cellulose chain at specific hydroxyl group, has attracted ever-increasing attention because of its potential to prepare precisely modified cellulose materials possessing new properties differing from those derived from simple statistical substitution<sup>1</sup>. The distribution of substituents in cellulose derivatives is considered to be one of the most influential factors determining their physical properties, such as, solubility, crystallization, gel formation, liquid crystal formation, and also resistance to enzymic degradation<sup>2</sup>.

#### MICROCRYSTALLINE CELLULOSE (Avicel®)

Avicel® is manufactured by the controlled hydrolysis of  $\alpha$ -cellulose, obtained as a pulp from fibrous plant materials, with dilute mineral acid solutions.

Following hydrolysis, the hydrocellulose is purified by filtration and the aqueous slurry is spray dried to form dry, porous particles of a broad size distribution ( Table1).

#### **Function,**

Tablet and Capsule diluent; tablet disintegrant; suspending and/or viscosity increasing agent.

**Molecular wt. and Formula** , (36,000 approx.)  $(C_6H_{10}O_5)_n$  n = 220.

White, odourless, tasteless, crystalline powder composed of porous particles.

Available in different size grades with different properties i.e 101 and 102

Type	Particle size in $\mu\text{m}$
PH- 101	50
PH- 102	100
PH- 103	50
PH- 104	20

Table 1



Figure 2 Microcrystalline cellulose

### Application in pharmaceutical formulation or technology use;

use	concentration %
Tablet binder / diluent ( wet granulation)	5-20
Tablet binder / diluent ( direct compression)	5-20
Tablet disintegrant	5-15
Tablet glidant	5-15
Anti-adherent	5-20
Adsorbent	--
Capsule diluent	10-30

### POWDERED CELLULOSE (Elcema<sup>®</sup>)

Is naturally occurring cellulose and is manufactured by mechanical processing of  $\alpha$ -cellulose obtained as a pulp from fibrous plant materials, and is commercially available in different types as shown in Table 2. Powdered cellulose occurs as a

white, odourless, tasteless, powder of various finenesses, ranging from a free-flowing, dense powder to a coarse, fluffy, non-flowing material.

**Function**, Tablet and Capsule diluent; sorbent and suspending agent.

**Molecular wt. and Formula**, (243,000 approx.)  $(C_6H_{10}O_5)_n$   $n = 1500$

Type	Particle size in $\mu\text{m}$
Elcema Grade P050	1-50
P 100	1-100
P 150	1-150
P 250	1-250

**Application in pharmaceutical formulation or technology use;**

use	concentration %
Binder	5-20
Filler	--
Disintegrant	5-15

## HYDROXYETHYL CELLULOSE

A purified form of cellulose is reacted with sodium hydroxide to produce a swollen alkali cellulose. This alkali-treated cellulose is chemically more reactive than untreated cellulose. By reacting the alkali cellulose with ethylene oxide, a series of hydroxyethyl cellulose ethers is produced.

### **Function,**

Suspending and/ or viscosity-increasing agent

Others: Binder; film-former; thickener

### **Applications in pharmaceutical formulation or technology use;**

Hydroxyethyl cellulose is an effective film former, binder, thickener, stabilizer and dispersant in shampoos, hair sprays, neutralizers, creams and lotions.

### **HYDROXYPROPYLCELLULOSE**

Hydroxypropylcellulose is manufactured by reacting alkali cellulose with propylene oxide at elevated temperatures and pressures. The propylene oxide can be substituted on the cellulose through an ether linkage at the three reactive hydroxyls present on each anhydroglucose monomer unit of cellulose chain.

### **Function,**

Suspending and/or viscosity-increasing agent; coating agent

Others: Emulsifier; film former; protective colloid; stabilizer; suspending agent; thickener; binder; and granulating agent.

**Molecular wt. and Formula, (50,000 – 1,250,000)  $(C_{15}H_{28}O_8)_n$**

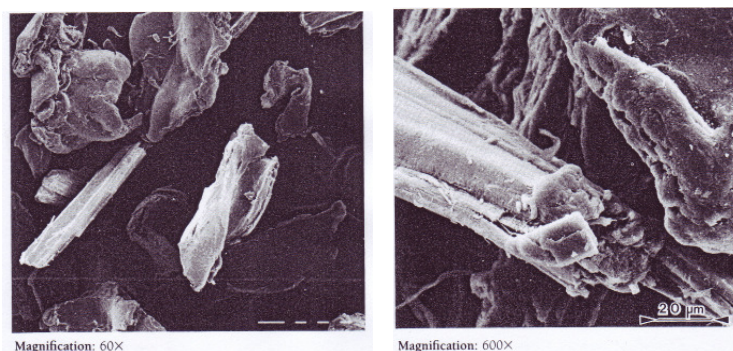


Figure 3 hydroxypropylcellulose

## **Applications in pharmaceutical formulation or technology use;**

Granulating agent (binder), Film coating, thickener, microencapsulation.

These properties depend on the molecular weight.

## Hydroxypropyl Methylcellulose

### **Method of Manufacture;**

Cellulose fibers, obtained from cotton linters or wood pulp, are treated with sodium hydroxide solution. The alkali cellulose thus obtained is in turn treated with methyl chloride and propylene oxide to produce methylhydroxypropyl ethers of cellulose. The fibrous reaction product is then purified and ground to a fine, uniform powder or granules.

### **Function,**

Suspending and/or viscosity-increasing agent;  
tablet binder; coating agent; film former  
and emulsion stabilizer.



Figure 4

Hydroxypropylmethylcellulose

### **Molecular wt. and Formula;**

Approx. 86,000       $C_8H_{15}O_6 - (C_{10}H_{18}O_6)_n - C_8H_{15}O_5$

### **Application in pharmaceutical formulation or technology use**

Film-former in tablet film coating, binder in tablet granulations, thickening agent added to vehicles for eye drops and artificial tear solutions, emulsifier, suspending agent and stabilizer in gels and ointments.



## 2.2 Chitosan

Chitosan has been considered for pharmaceutical formulation and drug delivery applications in which attention has been focused on its absorption-enhancing, controlled-release and bioadhesive properties. Synthesized from a naturally occurring source, this polymer has been shown to be both biocompatible and biodegradable. Chitosan is a linear copolymer of  $\beta(1-4)$  linked 2-acetamido-2-deoxy- $\beta$ -D-glucopyranose and 2-amino-2-deoxy- $\beta$ -D-glucopyranose.

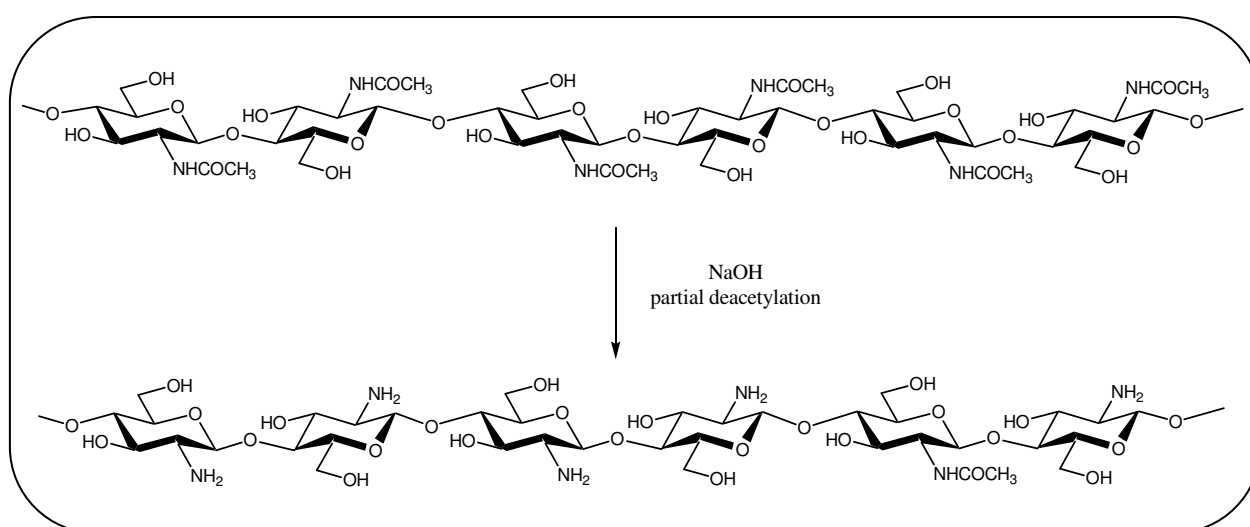


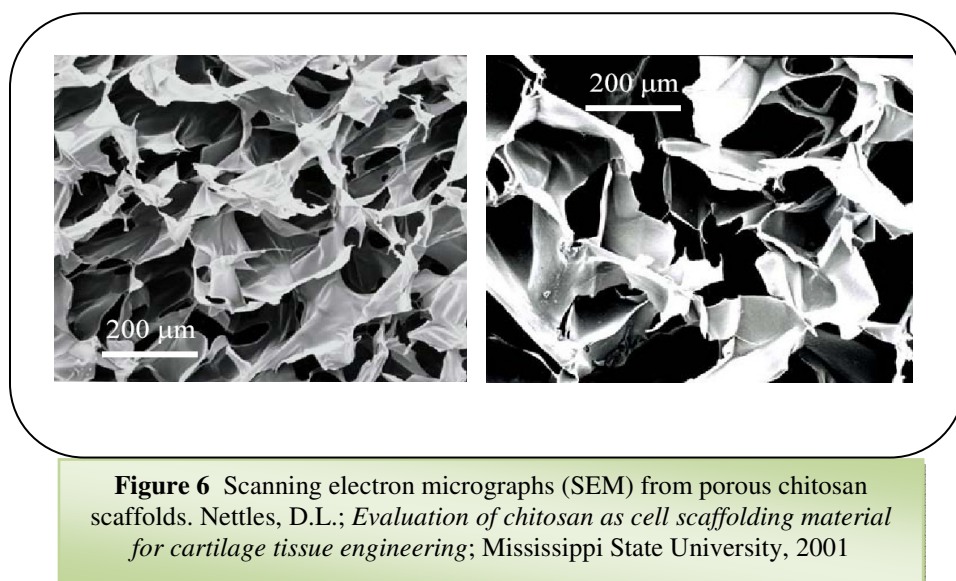
Figure 5 Chitosan

It is easily obtained by deacetylation of chitin (Fig.5), a polysaccharide widely distributed in nature (e.g. shells of crustaceans, insects and certain fungi). The degree of deacetylation affects the solubility of chitosan in aqueous solutions, the polymer is soluble in acidic solutions and slightly soluble in weakly alkaline solutions.

Chitosan lacks irritant or allergic effects and is biocompatible with both healthy and infected human skin. The intriguing properties of chitosan have been known for many years and the polymer has been used in the field of agriculture, industry and medicine. In agriculture, chitosan has been described as a plant antivirus, an additive in liquid multicomponent fertilizers and it has also been investigated as a metal-recovering agent in agriculture and industry. Chitosan has been noted for its

application as a film-forming agent in cosmetics, a dye-binder for textiles, a strengthening additive in paper and a hypolipidic material in diets. It has been used extensively as a biomaterial, owing to its immunostimulatory activities, anticoagulant properties, antibacterial and antifungal action and for its action as a promoter of wound healing in the field of surgery.

In addition chitosan has a variety of promising pharmaceutical uses and is presently considered as a novel carrier material in drug delivery systems.



## Pharmaceutical Applications Of Chitosan

### Oral drug delivery

The bioavailability of drugs has been improved by the use of mucoadhesive dosage forms. By prolonging the residence time of drug carriers at the absorption site, sustained release and improved bioavailability of drugs can be achieved.

Chitosan has better mucoadhesive properties compared with hydroxypropylcellulose and carboxymethylcellulose<sup>3</sup>. Among chitosans of various ranges of molecular-weight, better mucoadhesion was

observed for higher-molecular weight (approximately 1400 kDa) compared to lower-molecular weight chitosans ( 500 to 800 kDa).

### **Parenteral drug delivery**

In controlled-release technology, biodegradable polymeric carriers offer potential advantages for the prolonged release of low-molecular weight compounds to macromolecular drugs. The susceptibility of chitosan to lysozyme makes it biodegradable and an ideal drug carrier. Molecules such as bovine serum albumin, diphtheria toxoid(DT)<sup>4</sup> and bisphosphonates<sup>5</sup> have been successfully incorporated into chitosan microspheres.

The use of chitosan in injectable preparations has received recent attention. Pharmacokinetic and tissue-distribution studies were performed in mice using fluorescent glycol-chitosan and N-succinyl-chitosan. Both chitosan derivatives demonstrated good retention in the blood circulation and a slight accumulation in tissues, suggesting that chitosan is an effective carrier for drugs that are excreted rapidly<sup>6</sup>.

### **Ocular drug delivery**

The poor bioavailability of topically applied ophthalmic drugs implies a necessity for frequent instillation to achieve a therapeutic effect. This inconvenience could be overcome by a prolonged release of the drug in the corneal area. Use of chitosan-based colloidal suspensions in *in vivo* studies showed a significant increase in ocular drug bioavailability<sup>7</sup>.

## Gene delivery

The development of new carrier systems for gene delivery represents an enabling technology for treating genetic disorders. However, a critical barrier to successful gene therapy remains the formulation of an efficient and safe delivery vehicle. Non-viral delivery systems have been increasingly proposed as alternatives to viral vectors owing to their safety, stability and ability to be produced in large quantities. Some approaches employ DNA complexes containing lipid, protein, peptide or polymeric carriers, as well as ligands capable of targeting DNA complexes to cell-surface receptors on the target cells and ligands for directing the intracellular trafficking of DNA to the nucleus. Promising results were reported in the formation of complexes between chitosan and DNA<sup>8</sup>. Although chitosan increases transformation efficiency, the addition of appropriate ligands to the DNA-chitosan complex seems to achieve a more efficient gene delivery via receptor mediated endocytosis. Furthermore, incubation of cells with chitosan demonstrated low cytotoxic activity<sup>9</sup>. These results suggest that chitosan has comparable efficacy without the associated toxicity of other synthetic vectors and can, therefore, be an effective gene-delivery vehicle *in vivo*.

## Nasal drug delivery

There is a growing interest in the development of nasal delivery systems for many drugs, including peptides and proteins. To increase the bioavailability of these compounds, various absorption enhancers have been investigated, including surfactants, bile salts and cyclodextrins. However, most of these are associated with side effects, such as irreversible changes in the nasal mucosa. Several studies have reported the use of chitosan as a safe nasal-delivery system for proteins<sup>10</sup>. Illum et al. have shown that glutamate chitosan can enhance the transport of insulin across the nasal mucosa of sheep and rats. The efficacy of chitosan as a nasal-absorption enhancer was confirmed using salmon calcitonin in a rat model<sup>11</sup>.

## **Chitosan with antitumor activity**

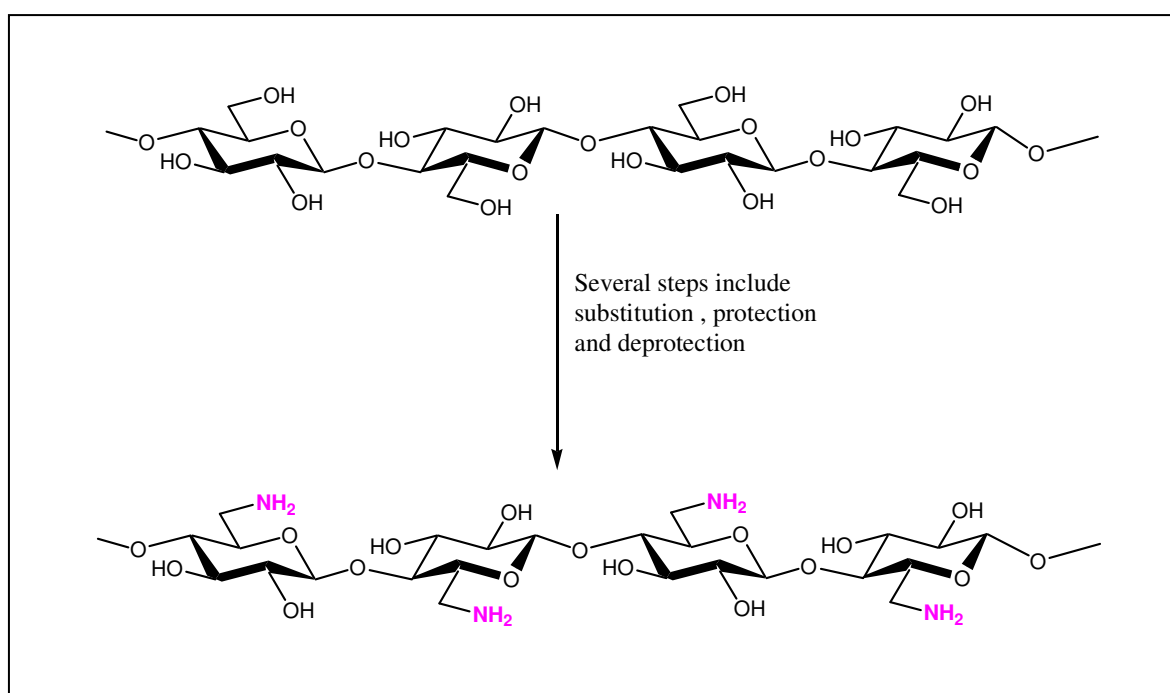
Modified chitosans were reported to display a growth inhibitory effect on tumor cells<sup>12</sup>. This property was employed by Ouchi et al.<sup>13</sup> by conjugating chitosan or chitosaminooligosaccharide (COS) to 5-flurouracil (5FU) in order to provide a macromolecular system with strong antitumor activities and reduced side effects. Indeed the strong antitumor activity exhibited by 5FU is accompanied by undesirable side effects. In vivo studies demonstrated that chitosan-5FU conjugate exhibited a strong survival effect against lymphocytic leukemia in mice. Furthermore, chitosan-5FU and COS-5FU conjugates showed remarkable growth-inhibitory effects on Met-A fibro sarcoma and MH-134Y hepatoma. Both conjugates displayed no acute toxicity, even in high dose ranges. Therefore, chitosan-5FU and COS-5FU are expected to act clinically as macromolecular prodrugs of 5FU.

## 2.3 Aminopolysaccharides

Aminopolysaccharides have attracted interest because of the unique properties of chitosan which are generally different from those of normal polysaccharides such as cellulose<sup>14</sup>.

There are two possible strategies, by which amino derivatives of cellulose can be synthesized;

### 1. Modification of the cellulose polymer itself by regioselective introduction of the amino group.



**Figure 7.** Regioselective introduction of amino groups into cellulose backbone as a synthetic strategy of aminopolysaccharides

Numerous attempts have been made to synthesize aminodeoxycellulose by replacing hydroxyl groups by amino groups in order to change the nature of cellulose. In 1926 Karrer<sup>15</sup> prepared the first aminodeoxycellulose by the reaction of cellulose tosylate with ammonia with degree of substitution of 0.1 (DS 0.1).

Later Sakurada<sup>16</sup> and Scherer and Field<sup>17</sup> synthesized aminodeoxycellulose with DS of 0.5 and 1.0, respectively.

Attempts have also been made to synthesize 6-amino-6-deoxycellulose selectively by using protecting groups. A common pathway is to prepare 6-*O*-tritylcellulose and protect C-2 and C-3, e.g., by acetyl or phenylcarbamoyl groups. After removing the trityl group, the tosyl group is introduced into C-6. The tosyl group is used as a leaving group to perform an S<sub>N</sub> reaction. Haskins and Weinstein<sup>18</sup> were the first who introduced the phthalimido group into C-6 with a DS of 0.25 by previously protecting the C-2 and C-3 positions with acetyl groups. However, they failed to remove the phthalic acid residues. In 1973, Usov et al.<sup>19</sup> prepared 6-amino-6-deoxy-2,3-di-*O*-phenylcarbamoylcellulose with a DS of 0.89 at the 6-position. Using the same procedure, Teshirogi et al.<sup>20</sup> obtained a 6-amino-6-deoxycellulose derivative with a DS of 0.90.

Kern et al.<sup>21</sup> synthesized 6-deoxy-6-pyridinium-2,3-di-*O*-methylcellulose with a DS of 0.8 by the reaction of 6-*O*-tresyl-2,3-di-*O*-methylcellulose with pyridine in five steps. Heinze et al.<sup>22</sup> reported on the synthesis of 6-amino-6-deoxycellulose derivatives with DS of 0.4–0.6 without using protecting groups. These derivatives were obtained by the reaction of cellulose tosylates with (+)-*R*-, (-)-*S*- or racemic amines, e.g., 1-phenylethylamine in DMF–water.

6-Azido-6-deoxycellulose and 6-deoxy-6-halocellulose, which are excellent intermediates for the preparation of 6-amino-6-deoxycellulose, have also been synthesized. Clode and Horton<sup>23</sup> reported on a method for the introduction of azide into C-6 of cellulose. Two levels of DS (0.25 and 0.68) were achieved after having used the above-mentioned activating and protecting group strategy. Saad and Furuhashi<sup>24</sup> obtained 6-chloro-6-deoxycellulose (DS 0.87) and 6-bromo-6-deoxycellulose (DS 0.92). 6-Deoxy-6-iodocellulose derivatives with a DS in the range of 0.46–0.94 were prepared by nucleophilic exchange of cellulose tosylate with NaI in acetylacetone.<sup>25</sup> However, these 6-deoxycellulose intermediates were not used to introduce the primary amino group into C-6.

Only two groups have reported the synthesis of 6-amino-6-deoxy-cellulose with a DS of 0.9 and 1.0. In the first case Teshirogi et al.<sup>26</sup> prepared 6-amino-6-deoxy-cellulose using a 6-*O*-tosylcellulose derivative having protecting groups at C-2 and C-3 as an intermediate. In the second case Liu and Bauman<sup>27</sup> described the





Kadokawa et al<sup>15</sup>. reported the spontaneous polymerisation of 6-amino-6-deoxy-D-glucose and 2,6-diamino-2,6-dideoxy-D-glucose to the corresponding aminopolysaccharides respectively.

### 3. Hydrogels

Hydrogels are the same as aquagels, they are three dimensional network materials which do not dissolve in water but able to swell rapidly and retain large volumes of water in their swollen structures. Hydrogels are usually made of hydrophilic polymer molecules, which are crosslinked, either by chemical bonds or other cohesion forces such as ionic interaction, hydrogen bonding, or hydrophobic interaction. Hydrogels are elastic solids in the sense that there exists a remembered reference configuration to which the system returns even after being deformed for a very long time<sup>28</sup>.

A hydrogel swells for the same reason that an analogous linear polymer dissolves in water to form an ordinary polymer solution<sup>29</sup>. If a hydrogel, for any reason, dissolves in an aqueous solvent, then the gel has become a hydrosol, which is a dispersion of colloidal particles in water. From a general physicochemical point of view, a hydrogel is simply an aqueous solution. Many polymers can undergo a reversible transformation between hydrogel and hydrosol. Chemical crosslinking of dispersed particles in hydrosols will result in an irreversible hydrogel. It is noted that a gel is an infinitely large macromolecule, or a super macromolecule, which forms a network extending from one end to the other and occupying the whole reaction vessel. The polymer networks of small particles with diameters smaller than 1  $\mu\text{m}$  (typically in the range of 100 nm) are called microgels. Microgels have crosslinked networks just like the super macromolecules. Microgels, however, dissolve in water like linear or branched macromolecules due to their molecular nature.

The term hydrogel implies that the material is already swollen in water. The dried hydrogel is called a xerogel or dry gel. During the drying process water evaporates from the gel and the surface tension causes collapse of the gel body. Thus the gel shrinks to only a small fraction of its swollen size. If water is removed without disturbing the polymer network, either by lyophilization or by extraction without organic solvents, then the remaining material is extremely light with porosity as high as 98%. Such a dehydrated hydrogel is called an aerogel or sponge. Aerogels are known to have lower thermal conductivities than all other thermal insulants at ambient conditions. Not all dried hydrogels maintain the ability to swell in water.

Ceramics prepared by the sol-gel process are also called xerogels. Xerogels such as silica gel will not swell again. To indicate the swelling ability of the dry material, a term 'xerogellant' was proposed<sup>30</sup>. There is no lower limit defining how much water a material has to absorb to be called a hydrogel. In general, however hydrogels are expected to absorb at least 10-20 percent of their own weight in water. If a dried hydrogel imbibes at least 20 times its own weight of aqueous fluid while retaining its original shape, it is also called a super absorbent. Due to their high water content, hydrogels usually have low mechanical strength.

Hydrogels have been a topic of extensive research because of their unique bulk and surface properties. Although the research on hydrogels is more than three decades old, the research interest in hydrogels is still growing.

Since the first report on the biomedical use of poly (2-hydroxyethylmethacrylate) hydrogel, hydrogels with various properties have been prepared<sup>31</sup>. Hydrogels can be made to respond (i.e., either shrink or expand) to changes in environmental conditions and the extent of the response can be controlled. Sometimes the volume change in response to the alteration in environmental conditions is so drastic that the phenomenon is called volume collapse or phase transition. The environmental conditions include pH, temperature, electric field, ionic strength, salt type, solvent, external stress, light, and combinations of these. It is these unique properties that have made hydrogels find numerous applications in pharmaceutical, agricultural, biomedical, and consumer-oriented fields.

- Hydrogel as a Biomaterial

Biomaterials are any type of material, designed to restore, augment, or replace the natural functions of the living tissues or organs in the body. Simply speaking, biomaterials are those, which become a part of the body either temporarily or permanently. Biomaterials are used not only for prosthetic application but also for diagnostic and therapeutic applications<sup>32</sup>. Biomaterials should perform within appropriate host response in a specific application without toxic, inflammatory, carcinogenic, and immunogenic responses. An appropriate host response ranges

from inertness and no interaction to one of positive interaction. In general, the body's reaction to implants is to extrude them from the body or form sheath-like capsules around the implants if they cannot be removed.

The injury created by the implantation procedure usually results in inflammation which can be defined as the local reaction of vascularized tissue to injury. The implanted biosensors can maintain the required sensitivity only for a few days mainly due to inflammation and immune reactions, which decrease the sensor's sensitivity.

Success in the application of biomaterials relies heavily on the biocompatibility of biomaterials. Biocompatibility is the appropriate biological performance, both local and systemic, of a given polymer in a specific application. A clear, specific, and absolute definition of biocompatibility does not exist at this time. Numerous interdisciplinary factors must be used to describe the biocompatibility of a given polymer in a given application for a given duration. The importance of biocompatibility cannot be overemphasized. The recent highly publicized controversy on silicone gel-filled breast implants is a case in point. The typical tissue reaction around the implanted biomaterial is the formation of a thin fibrous capsule similar to scar tissue. The fibrous capsule often contracts and causes pain and deformity. The formation of a fibrous membrane capsule around the implant is an attempt by the body to extrude the implant. Some silicone implants were coated with polyurethane foam featuring micropillars, which were designed to disrupt the fibrous capsule architecture and prevent the formation of scar tissue. The polyurethane foam, however, was slowly degraded in the body. Furthermore, the silicone shell of the implant ruptured in many cases releasing silicone gel into the body. Such leakage was blamed for harmful immune reactions and for causing cancer. The issue of biocompatibility becomes even more important if biomaterials are in contact with blood. Interactions between blood and biomaterials may result in thrombus formation, destruction or sensitisation of cellular elements in blood, infection, and adverse immune responses.

Cumulative evidence shows that hydrogels are highly biocompatible. Hydrogels possess a few unique properties that make them biocompatible. First, hydrogels have low interfacial tension with surrounding biological fluids and tissues and that minimizes the driving force for protein adsorption and cell adhesion. Because of its high water content, the hydrogel surface is called a superhydrophilic diffuse surface. The superhydrophilic diffuse surface is known to be highly

biocompatible. This is due to the fact that the hydrogel surface makes the actual interface more vague. Second, hydrogels simulate some hydrodynamic properties of natural biological gels, cells, and tissues in many ways. The high mobility of polymer chains at the hydrogel surface contributes to the prevention of protein adsorption and cell adhesion. This is mainly due to the steric repulsion resulting from “entropic” and “mixing” interactions between the polymer chains and proteins or cell membranes. This phenomenon is the same as well-known steric stabilisation of protein molecules and colloidal particles. Third, the soft, rubbery nature of hydrogels minimizes mechanical and frictional irritation to the surrounding tissue. Low friction surfaces cause no pain and no damage to mucous membranes, or mural thrombus formation.

A major disadvantage of using hydrogels is that they have poor mechanical strength and toughness after swelling. This disadvantage can be overcome by grafting a hydrogel with good mechanical properties onto the biomaterial. The grafting of hydrogels onto biomaterial surfaces changes only the surface properties while the bulk properties remain unchanged.

Hydrogels can be grafted onto biomaterials by physical adsorption, physical entrapment, graft coupling, and polymerisation.

The principal markets for biomaterials are in the areas of cardiovascular implants, orthopedic implants, intravascular and urinary tract catheters, soft tissue replacements, intraocular lenses, wound dressings, biosensors, and controlled release devices. All of these biomaterials will improve their biocompatibility through coating with hydrogels

- Biodegradable Hydrogels

Biodegradable polymeric systems have been used frequently in the development of advanced drug delivery systems. The use of biodegradable polymeric systems in controlled release drug delivery is desirable, since the dosage forms will be degraded and eliminated from the body. This will avoid removal of the device from the body by surgery or other means when the device is no longer needed. Biodegradable polymeric systems also provide flexibility in the design of delivery systems for large molecular weight drugs, such as peptides and proteins, which are not suitable for

diffusion controlled release through non-degradable polymeric matrices. Our impression of biodegradable polymeric systems has changed significantly in recent years. When the rate of drug release was mainly controlled by diffusion through the polymer matrix, the degradation of the polymer was considered to be a less well-defined and unnecessary variable. Currently, the degradation of polymer is regarded as highly desirable and is frequently used to control the drug release rate.

Most of the research on biodegradable drug delivery systems has employed water-insoluble polymers such as poly (glycolic acid) or poly (lactic acid). Not much work has been done, however, on biodegradable hydrogel systems. Because of the unique properties of hydrogels, the biodegradable hydrogels are expected to find wide applications in the improvement of existing dosage forms and the development of new and better drug delivery systems<sup>33</sup>.

In our hands, polymeric products were obtained from 6'-azido-6'-deoxy-cellobiose or its derivatives on several occasions. However, so far, attempts to develop suitable conditions for the controlled preparation of such polymers have been unsuccessful.

## 4. Immobilised glycans for testing of anti-carbohydrate IgE

Protein-linked carbohydrates from plants and insects have different structures from those in mammals, thus there is potential for these to be recognised as “foreign“. Indeed antibodies raised against plant and insect glycoproteins are often directed against the carbohydrate portion of the glycoprotein. One more biomedical significance is the finding that patients with allergies towards plant pollens, plant foods and insect venoms have antibodies (so-called IgE ) recognising carbohydrate epitopes on N-linked oligosaccharides. IgE is of particular importance to allergy since, in conjunction with appropriate allergen, cross-linking of IgE receptors leads to the release of histamine which causes many of the well-known effects of allergic reactions, in the worst case anaphylactic shock. The correct identification of the proteins which cause the allergic reactions is a major part of modern allergy research and diagnosis. However, since the same carbohydrate structure can be present on many proteins, cross-reactions are possible- some of which are clinically significant, others only complicating the identification of the relevant allergen. Often recombinant proteins are used in the testing of patients' sera and in immunotherapies. For the detailed analysis of IgE binding to carbohydrate determinants, practical tools are required that permit the coupling in high yields, to carriers such as micro-titre plates, of small or trace amounts of glycopeptides isolated and purified from natural sources.

### Conventional coupling procedures

Conventional coupling to supports of peptides has been performed by means of reactions of the amino-terminal amino group to form Schiff bases with an aldehyde function or amide linkages with a carboxylic ester bound to the support. In the reductive amination procedure, the initially formed Schiff base is reduced, by the action of borohydride, to afford the more stable secondary amine. In amide formation, activation of the carboxylic acid can be achieved by means of an ester, activated ester such as a hydroxysuccinimide ester or thioester, or lactone. In principle, these conventional coupling procedures are well suited if the peptide antigen to be coupled is freely available in large amounts. Since the number of binding sites on the micro-

titre plate is naturally limited, antigen must be applied in excess and of necessity, a significant portion of it will be wasted in the course of the coupling procedure.

Following their release by hydrolytic procedures from allergenic glycoproteins, the glycopeptides of interest are part of complex mixtures and may be present in minute amounts. In most cases, extensive column-chromatographic purification procedures will be required to obtain pure substances for investigations of the molecular details of allergenicity. It is therefore essential to develop coupling procedures that avoid wastage of the glycopeptides and permit quantitative or near quantitative coupling of the materials to the required carriers.

### **A quantitative reaction is required for the coupling step**

Conventional coupling procedures require an excess of the antigen to be coupled. However, recent work by Sharpless<sup>34</sup> and his associates has demonstrated that certain 1,3-dipolar cycloaddition reactions proceed quantitatively with 1:1 stoichiometry, avoiding the need for excess of one of the reactants. This type of efficient reactivity has been termed “click-chemistry”. An example is the dipolar cycloaddition of alkynes with alkyl and aryl azides to give 1,2,3-triazoles. This reaction has been employed for coupling of antigens to carriers.

### **Carbohydrate-derived azides as linking arms**

Within this work an organic-chemical protocol has been developed, based on a conventional protection-deprotection scheme, by which 6-azido-6-deoxy-glucose as carbohydrate-derived azide can be obtained in ten steps starting from glucose.

For the purposes of allergen testing the azido sugar can be oxidized to the corresponding lactone, this azido lactone can be used in excess, so as to quantitatively derivatize the glycopeptides of interest. Secondly to couple the azido-glycopeptides, by 1,3-dipolar cycloaddition, to micro-titre plates derivatized with alkyne substituents.



The use of carbohydrate-derived azides as linking arms offers several advantages, as follows.

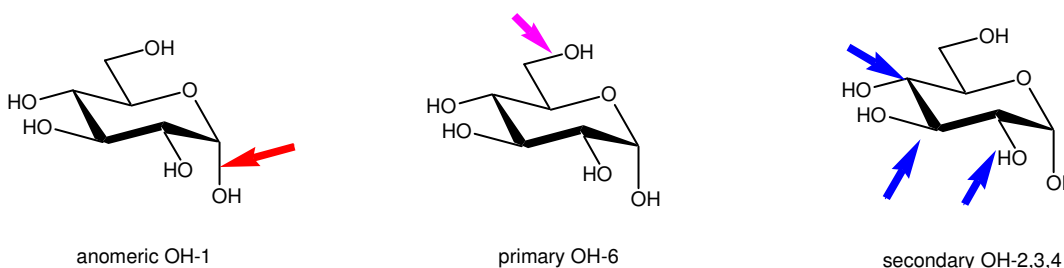
- The aldehyde function of sugars can be oxidized to afford carboxylic acids which may be transformed into esters or lactones
- Azido sugar derivatives are intrinsically more stable than low-molecular weight azides such as azidoacetic acid
- Azido sugar derivatives are easily prepared in practical amounts by conventional carbohydrate chemistry.

## 5. Carbohydrate Syntheses

### 5.1 Chemistry discussion

Many complications in the carbohydrate chemistry arise only because of the fact that there are many hydroxyl groups on the sugar molecule. The presence of three types of hydroxyl groups namely

- the anomeric hydroxyl group, at C-1 in the case of D-glucose
- the primary hydroxyl group at C-6
- the secondary hydroxyl groups which in D-glucopyranose are at C-2, 3, and 4.



make the 'free sugars' very polar in nature, which means they are very soluble in polar solvents, especially those that are capable of H-bonding to these free hydroxyls (e.g. water) but completely insoluble in the standard, more non-polar solvents used for performing organic reactions, product purification and manipulation ( e.g. dichloromethane, ether, hexane, toluene etc.).

Another complication arises not only from double character of the anomeric hydroxyl which reacts in two ways – as an alcohol or as an aldehyde, but also the process of mutarotation allows equilibration of  $\alpha$ - and  $\beta$ -hydroxyls at the anomeric position, that is, the equilibration of the  $\alpha$ -anomer with the  $\beta$ -anomer. Therefore when considering

reactivity of the anomeric center as a hydroxyl group we have to bear in mind potential competing inter-conversion between these  $\alpha$  and  $\beta$ -forms.

These complications make a regioselective reaction difficult without using protecting groups.

### **Protecting groups**

Protecting groups should satisfy several important criteria. First, they should be formed in good yield, they should be stable to subsequent reaction conditions, and finally, they should be readily removed under appropriate conditions.

The most commonly used hydroxyl protecting groups are ether and esters protecting groups.

Sugar hydroxyls react readily as alcohols with activated carboxylic acid derivatives such as acid anhydrides, or acid chlorides under appropriate conditions to form the corresponding esters. These esters are stable due to the non-nucleophilic characters and can bear a wide range of reaction conditions. In addition esters are much less polar than the corresponding alcohols. As a result esters are frequently used as protecting groups.

**Acetates** are the most commonly used esters. They can be easily prepared by stirring the alcohol in a mixture of acetic anhydride and a base. This base not only serves to mop up the equivalent of acetic acid that is produced during the reaction but it also catalyses the reaction itself. This is necessary since the uncatalysed reaction of alcohols with acetic anhydride is very slow at room temperature.

Acetylation of free sugars can be done in one of three ways, each way has different stereochemical consequences for the anomeric position.

Acetylation with acetic anhydride and anhydrous pyridine, often used in equal proportions as the reaction solvent, at room temperature produces the fully acetylated products as a mixture of  $\alpha/\beta$  anomers, which correlate directly to the composition of the anomeric mixture in the free sugar starting material. This result is due to the fact that under these reaction conditions the acetylation of the free hydroxyl at the anomeric centre is faster than the competing process of mutarotation.

Another way of acetylation is to use acetic anhydride and sodium acetate at 100 °C in this case the fully acetylated products are usually the  $\beta$  acetates. This selective formation of the  $\beta$  product is due to the fact that at elevated temperatures the mutarotation, which leads to  $\alpha/\beta$  equilibration, is much faster than the actual acetylation reaction itself. Since the equatorial  $\beta$  hydroxyl is more nucleophilic than the axial  $\alpha$  counterpart, the  $\beta$  component reacts in preference.

Acetylation can also be done using acetic acid and Lewis acid catalysis such as zinc chloride. Since the  $\alpha$ -anomer is thermodynamically favoured by the anomeric effect, the predominant reaction product is the  $\alpha$ -acetate.

**Benzoyl esters** (ROBz) are also very commonly used ester protecting groups and are readily formed by treatment of the alcohol with benzoyl chloride in the presence of an amine base, such as pyridine.

Acetate and benzoyl ester protecting groups are readily removed by treatment with a suitable nucleophile, most commonly methoxide, in a transesterification reaction. Selective anomeric deacetylation is also possible under mild conditions using copper(II) acetate dihydrate in methanol/ water( 9:1).

**Sulfonate esters** play also an important role as hydroxyl protecting groups. They are also good leaving groups for nucleophilic substitution reactions. The widely used sulfonate ester is the toluenesulfonate (tosylate) which is more stable than other sulfonate esters. Tosylate esters of the primary hydroxyl group may be selectively formed using tosyl chloride in dried pyridine.

## Ether protecting groups

**Benzyl ethers** (ROBn) are commonly used as alcohol protecting groups. Benzyl ethers can be easily prepared by reacting the alcohol with benzyl halides, in the presence of a strong base such as sodium hydride. Whereas ester protecting groups are base-labile, ethers are base-stable.

**Tryl ethers**, triphenylmethyl(trityl) groups as a result of their bulky nature, they are useful for selective protection of the primary hydroxyl group to produce trityl ethers. The trityl ethers can be cleaved under mildly acidic conditions to regenerate the primary hydroxyl group. Trityl ethers are base-stable.

## Acetals

Cyclic acetals are often used in the carbohydrate chemistry as alcohol protecting groups. The benzylidene acetal protecting group is very selective for reaction with the 4- and 6-hydroxyls of carbohydrates, to form either *cis*- or *trans*-fused ring systems. The formation of cyclic benzylidene acetals can be achieved by the reaction of benzaldehyde with a diol. Since acetal formation is reversible, all the cyclic acetals can be easily hydrolysed by treatment with aqueous acid. Therefore the extraction of cyclic benzylidene acetals has to be done carefully in alkaline solution. Acetals are generally base-stable.

**Acetonide** protection groups are cyclic acetals and result from the condensation of two hydroxyl groups of a molecule with acetone. The reaction conditions involve treatment of the diol either with acetone itself, or sometimes the dimethyl acetal of acetone under acidic conditions. The formed cyclic acetonides are generally 5-ring instead of 6-ring, which means the two hydroxyl groups must be *cis* to one another. Where there is possibility of reaction with multiple hydroxyl groups, this usefully allows selective access to particular hydroxyl groups of a carbohydrate by selective

protection of the *cis* hydroxyl pairs. The use of acetonide protection in case of D-glucose makes it possible for selective substitution on the 3-hydroxyl group where as the other hydroxyls are acetonide protected.

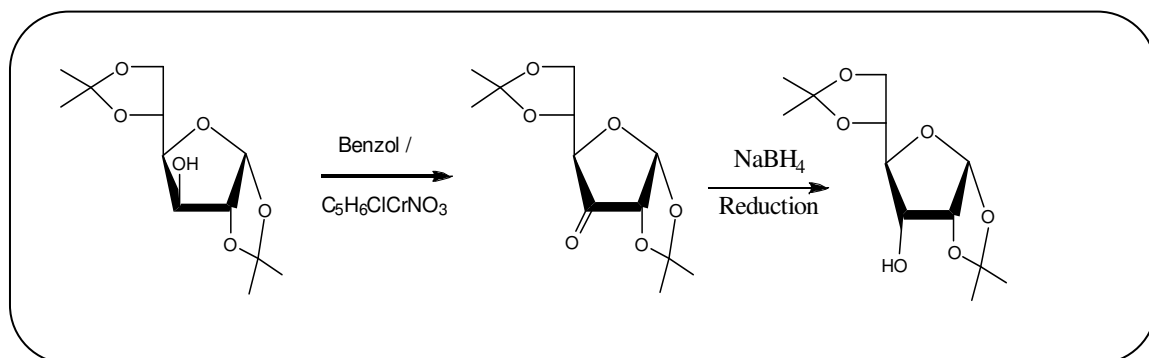
### Nucleophilic substitution reactions

Nucleophilic substitution of sugar hydroxyls takes place if hydroxyls are activated by good leaving groups. The treatment of the sugar with tosyl chloride or mesyl chloride in the presence of anhydrous pyridine leads to the formation of the corresponding tosylate or mesylate respectively. These groups are good leaving groups and allow a nucleophilic substitution reaction by an  $S_N2$  mechanism.

**The azido** group can be easily introduced into a carbohydrate via nucleophilic displacement of a leaving group such as mesylate or tosylate. These displacement reactions are  $S_N2$  type. Subsequent reduction of the azide to an amine allows access to amino sugars.

### Oxidation reactions

Oxidation reactions play an important role in the formation of epimeric sugars. For example the oxidation of diacetone glucose with pyridinium chlorochromate ( $C_5H_6ClCrNO_3$ ) or with dimethylsulfoxide leads to the formation of a ketone.

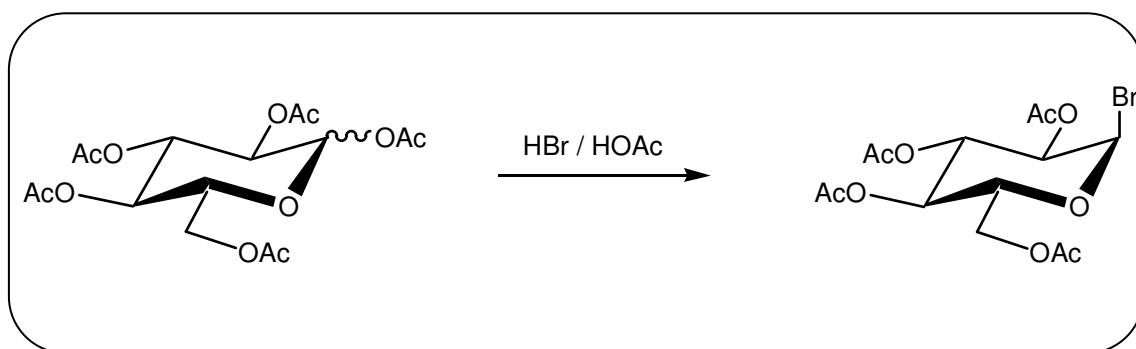


This ketone can undergo the usual range of reactions. For example the treatment of the ketone with a reducing agent such as  $\text{NaBH}_4$  leads to the formation of the corresponding alcohol again with the inversion of the configuration. In case of diacetone glucose, the epimer diacetone allose will be formed.

### Reactions of the anomeric centre

The anomeric hydroxyl group can act as a nucleophile in simple reactions such as acetylation. Conversion of the hydroxyl group at the anomeric position into acetate is particularly useful since acetates can readily act as leaving groups under the appropriate reaction conditions, and, therefore, many other anomeric substituents may be introduced by nucleophilic substitution reactions.

**Glycosyl halides**, such as bromides, are easily prepared by reacting of sugar acetates with a solution of  $\text{HBr}$  in acetic acid.



Glycosyl chlorides can be formed analogously, whereas iodides are rather too reactive, and due to their high instability have not been used to a great extent in synthesis. Glycosyl bromides are the most commonly used halides. They can undergo an extremely useful and diverse array of reactions. For example, they can react with thiolate anions to form thioglycosides, they can also react with azides to form azidoglycosides, which can be reduced to the corresponding glycosyl amine.

The reductive elimination of peracetylated glycosyl bromides leads to the formation of glycols.

### **Oxidation**

The anomeric center can undergo oxidation reactions to the carboxylic acid. This can be done selectively using bromine in water. The product can either be isolated as the acid salt, or, if the reaction is performed under mildly acidic conditions, then, as the corresponding lactone.

### **Reduction**

Treatment of sugars with good reducing agents, such as sodium borohydride produces the open chain alditol in good yield. These reduction reactions proceed via reduction of the small amount of open chain aldehyde in solution with, once again, the corresponding shift in equilibrium.

### **Chemical disaccharide formation**

Chemical disaccharide formation is known as glycosylation reaction in which a glycosidic bond is formed by the displacement of a leaving group at the anomeric position of one sugar, termed the glycosyl donor, with the free hydroxyl group of another, itself termed the glycosyl acceptor. The bond formed between the donor and acceptor is called glycosidic bond.

### **Glycosyl acceptors**

Due to the nucleophilicity of different hydroxyls around a sugar ring, a regioselective glycosylation reaction is not possible without selective protection of the hydroxyl groups, and a mixture of glycosylation products is to be expected. This problem can be overcome by selective protection of the glycosyl acceptor. Hence most glycosyl



acceptors are monosaccharides that only have a single free hydroxyl group. The anomeric position of a glycosyl acceptor should be suitably protected or differentially functionalised from the anomeric position of the glycosyl donor to hinder a self reaction and/or polymerization.

### **Glycosyl donors**

A glycosyl donor is the other reactant of a glycosylation reaction, in which the leaving group at the anomeric position is displaced by the free hydroxyl group of the glycosyl acceptor. The activation of the anomeric leaving group is necessary in order to achieve this displacement. On activation not only the substituent at the anomeric center of the glycosyl acceptor must remain unaffected but also any other existing glycosidic linkages, be they in the donor or acceptor.

### **Glycosyl bromides**

Glycosyl bromides are widely used as glycosyl donors. They were the first glycosyl donors used for disaccharide formation by Königs and Knorr. They can be activated by the addition of halophiles such as silver salts e.g. soluble silver triflate or perchlorate (AgOTf or AgClO<sub>4</sub>), or such soluble mercury salts (e.g. Hg (CN)<sub>2</sub> or HgBr<sub>2</sub>) or. Glycosyl bromides are easily formed by treatment of a sugar acetate with a solution of HBr in acetic acid.

### **Glycosyl fluorides**

Glycosyl fluorides are much more stable than the corresponding bromides and chlorides. They can be readily synthesized from the corresponding thioglycosides by treatment with diethylaminosulfur trifluoride (DAST, Et<sub>2</sub>NSF<sub>3</sub>).

## Thioglycosides

Thioglycosides are most commonly used as glycosyl donors. They are readily prepared from the corresponding anomeric acetates by treatment with a thiol and a Lewis acid, such as boron trifluoride etherate ( $\text{BF}_3 \cdot \text{Et}_2\text{O}$ ). Thioglycosides are more stable than glycosyl bromides, and will not react until they are activated under particular reaction conditions for example using activating groups such as N,N-dialkylsulfonamide and trifluoromethanesulfonic anhydride ( $\text{Tf}_2\text{O}$ )<sup>35</sup>.

## 5.1.1 Synthesis of 6'-azido-6'-deoxy-cellobiose

(Scheme 1)

The use of cellobiosyl fluorides as precursors for the in vitro enzymatic synthesis of cellulose has given cellobiose derivatives more attention to be used as substrates for the same catalytic reaction. Important cellobiose derivatives are those which carry azido groups. The azido group serves for introduction of nitrogen, functions as a protecting group for nitrogen, and, being electrically neutral, is frequently compatible with enzyme-catalyzed reactions.

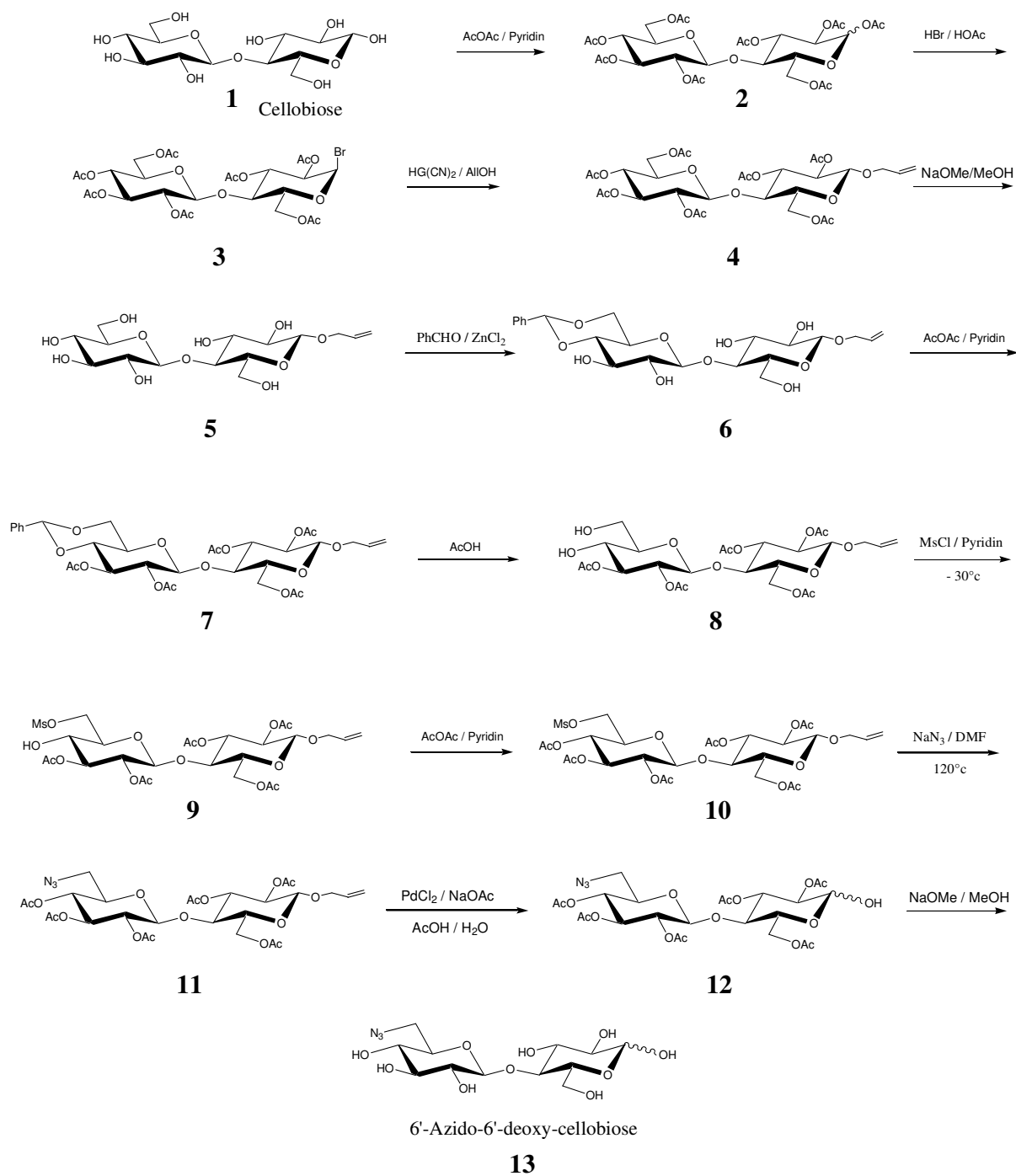
There are theoretically 6 positions on the cellobiose molecule where we can introduce an azido group, namely C-2, C-3 and C-6 of the reducing glucose unit and analogous positions of the non-reducing glucose unit. Within this work we have synthesized two cellobiose azido derivatives, in which the azido function is located at the C-6 position of the reducing or the non-reducing sugar unit(C-6').

6'-Azido-6'-deoxy-cellobiose was synthesized from cellobiose in twelve steps. Briefly, cellobiose was first completely acetylated with acetic anhydride in pyridine to form the octaacetate derivative of cellobiose. Then the anomeric position was activated by the formation of the corresponding glycosyl bromide by treatment of the cellobiose octaacetate with a solution of HBr in acetic acid, to form exclusively the thermodynamically favored  $\alpha$ -anomer<sup>36</sup>. Recrystallisation from a mixture of tetrahydrofuran and diisopropylether has offered the formed glycosyl bromide extra stability. The crystalline glycosyl bromide is then treated with allyl alcohol under catalysis by Hg (CN)<sub>2</sub> in dried dichloromethane. The formed allyl- $\beta$ -D-cellobiose derivative was then deacetylated with sodium methoxide in methanol (Zemplén deacetylation) to give the free allyl- $\beta$ -D-cellobiose. A regioselective protection of the 4',6'-hydroxyls was carried out using benzaldehyde under the catalytic action of zinc chloride. The formed 4',6'-benzylidenated  $\beta$ -allyl glycoside was directly acetylated. Heating at reflux in 60% acetic acid resulted in the deprotection of 4' and 6' positions. The formed  $\beta$ -allyl glycoside of cellobiose contains only 2 free hydroxyls at C-4' and

C-6'. Selective 6'-O-mesylation followed by per-O-acetylation afforded the 6'-O-mesyated derivative of cellobiose glycoside.

Treatment of the mesylate derivative with sodium azide in dimethyl formamide at 120°C has introduced the azido group in the six position of the non-reducing sugar unit. Finally the anomeric allyl group was removed by the action of palladium chloride and sodium acetate in acetic acid-water mixture. Further deacetylation with methoxide in methanol afforded the 6'-azido-6'-deoxy-cellobiose.

## Synthesis of 6'-azido-6'-deoxy-cellobiose



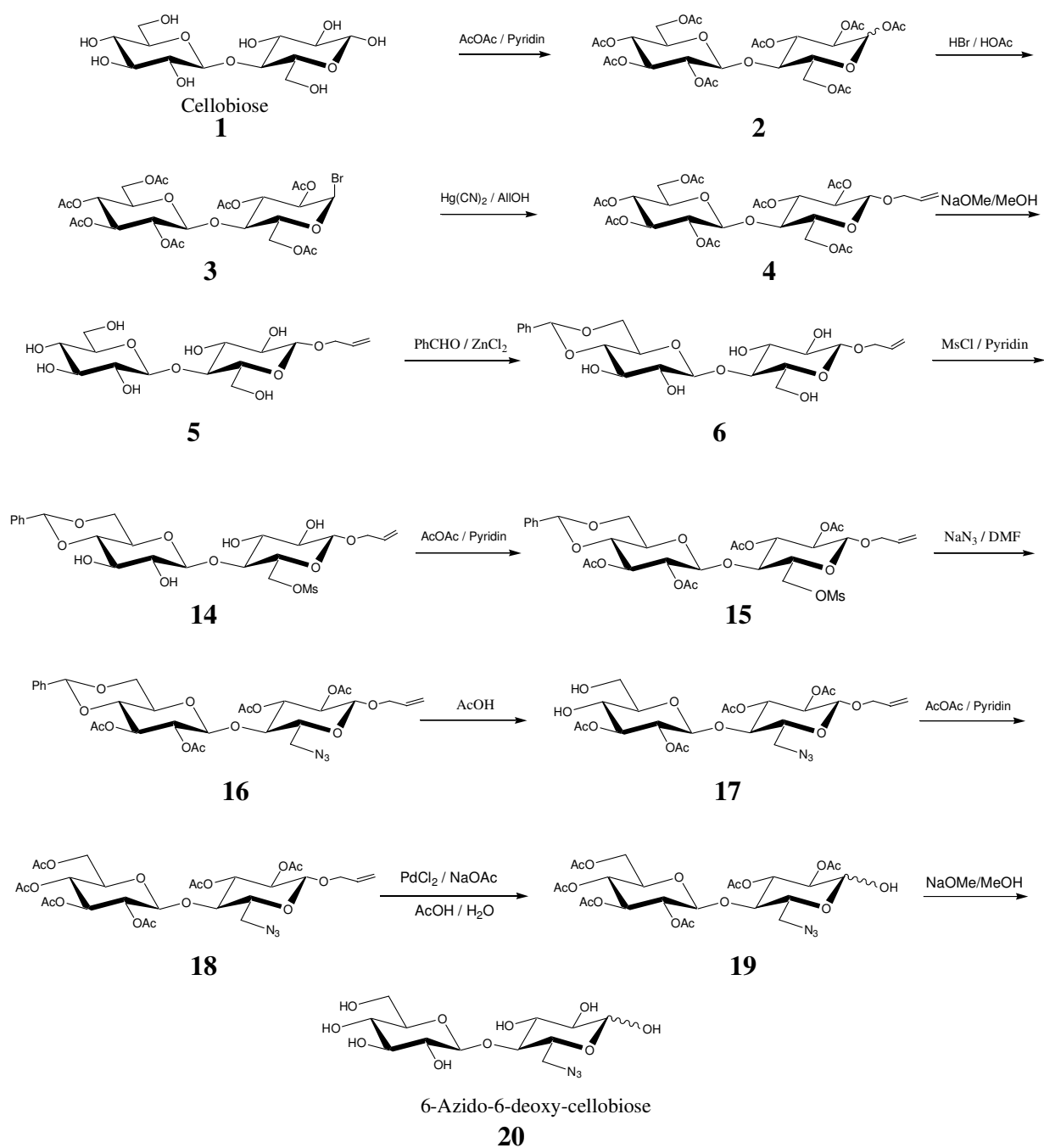
Scheme 1

## 5.1.2 Synthesis of 6-azido-6-deoxy-cellobiose<sup>37</sup>

(Scheme2)

In a similar protocol 6-azido-6-deoxy-cellobiose was also synthesized from cellobiose in twelve steps. First, the cellobiose was peracetylated with acetic anhydride in pyridine, followed by treatment with HBr in acetic acid that converted the anomeric acetate to the corresponding glycosyl bromide. The anomeric position was then protected through the formation of allyl glycoside<sup>38</sup> on treatment of the glycosyl bromide with allyl alcohol. Deacetylation of the allyl glycoside with methoxide in methanol and regioselective protection of the 4',6'-hydroxyl groups by the action of benzaldehyde, catalyzed by zinc chloride, the 4',6'-benzylidenated  $\beta$ -allyl glycoside of cellobiose was obtained, containing only one free primary hydroxyl function at C-6. Selective 6-O-mesylation followed by per-O-acetylation and treatment with sodium azide in dimethyl formamide at 100°C afforded the 6-azido-6-deoxy-cellobiose glycoside, in which the azido group was introduced into the C-6 of the reducing sugar unit. Deprotection of the benzylidene group was achieved by reflux with 60% acetic acid, followed by ester protection (with acetic anhydride/ pyridine). The anomeric allyl group was then removed by palladium chloride and sodium acetate in acetic acid-water. Finally, deacetylation with sodium methoxide in methanol afforded 6-azido-6-deoxy-cellobiose.

## Synthesis of 6-azido-6-deoxy-cellobiose



Scheme 2

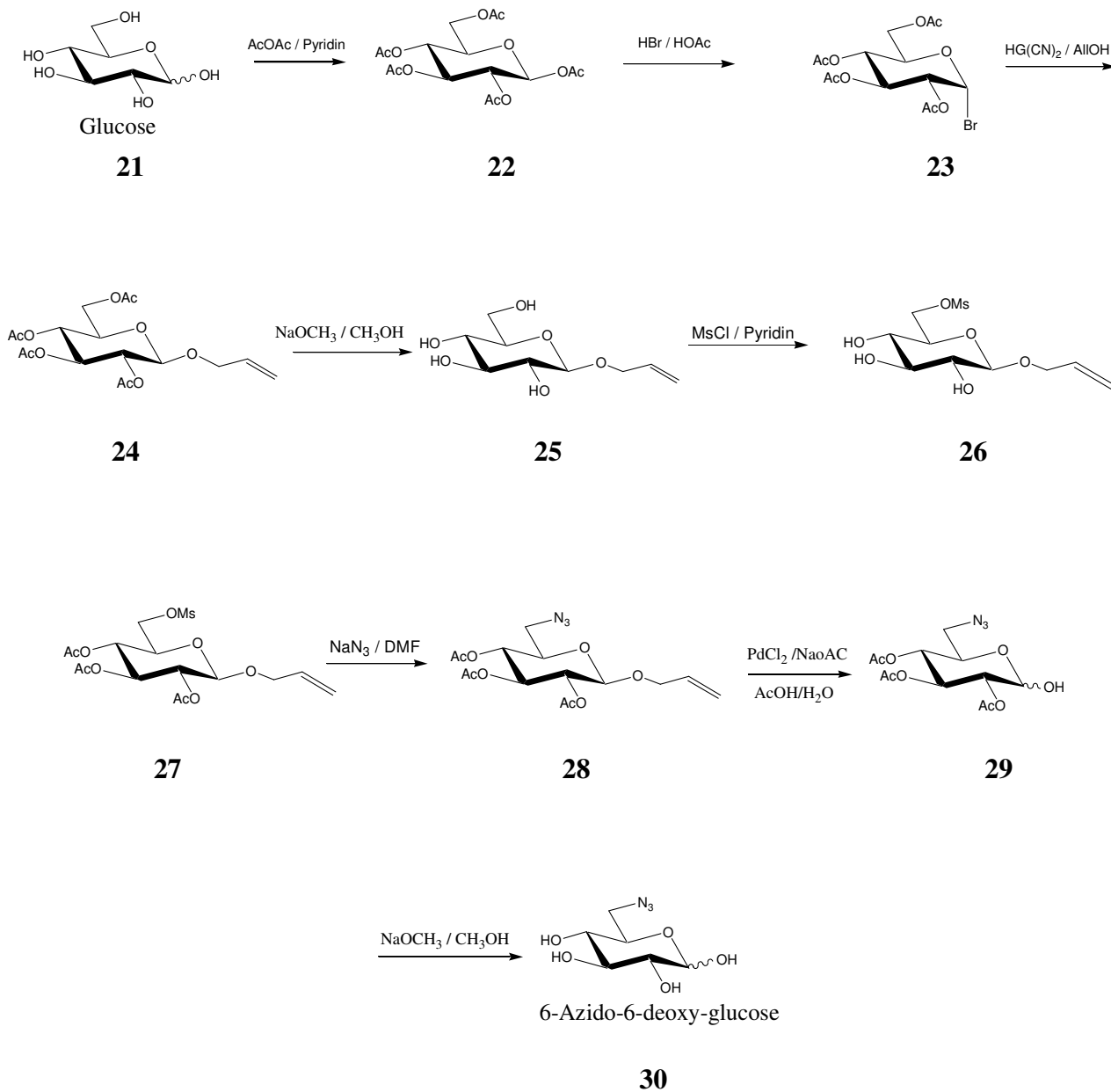
### 5.1.3 Synthesis of 6-azido-6-deoxy-D-glucose

(Scheme 3)

6-Azido-6-deoxy-D-glucose is obtained from glucose by a conventional protocol in 10 steps<sup>39</sup>. D-glucose was peracetylated by acetic anhydride in pyridine. The anomeric acetate was then activated through the formation of the corresponding bromide glycoside. This was done by reacting the glucose pentaacetate with HBr in acetic acid. The formed glycosyl bromide was then recrystallized from diisopropyl ether. The white crystals of the  $\alpha$ -bromo glycoside were stable over several months. Protection of the anomeric position was easily done by reacting the glycosyl bromide with allyl alcohol under catalysis by mercuric cyanide in dichloromethane. After deacetylation with methoxide in methanol, the formed free allyl glycoside, containing one primary hydroxyl at C-6, was selectively O-mesylated. The temperature was kept at  $-30^{\circ}$  using a dry ice bath in order to minimize any side products on the other hydroxyls. Per-O-acetylation was performed to protect the hydroxyls on C-2, C-3 and C-4. The mesylate derivative was then refluxed for 2 hours with sodium azide in dimethyl formamide to give the corresponding azido derivative. Deprotection of the allyl group was carried out using palladium chloride and sodium acetate in acetic acid-water mixture. Finally, deacetylation afforded the 6-azido-6-deoxy-D-glucose.



## Synthesis of 6-azido-6-deoxy-D-glucose



Scheme 3

## 5.1.4 Synthesis of 3-azido-3-deoxy-D-glucose

(Scheme 4)

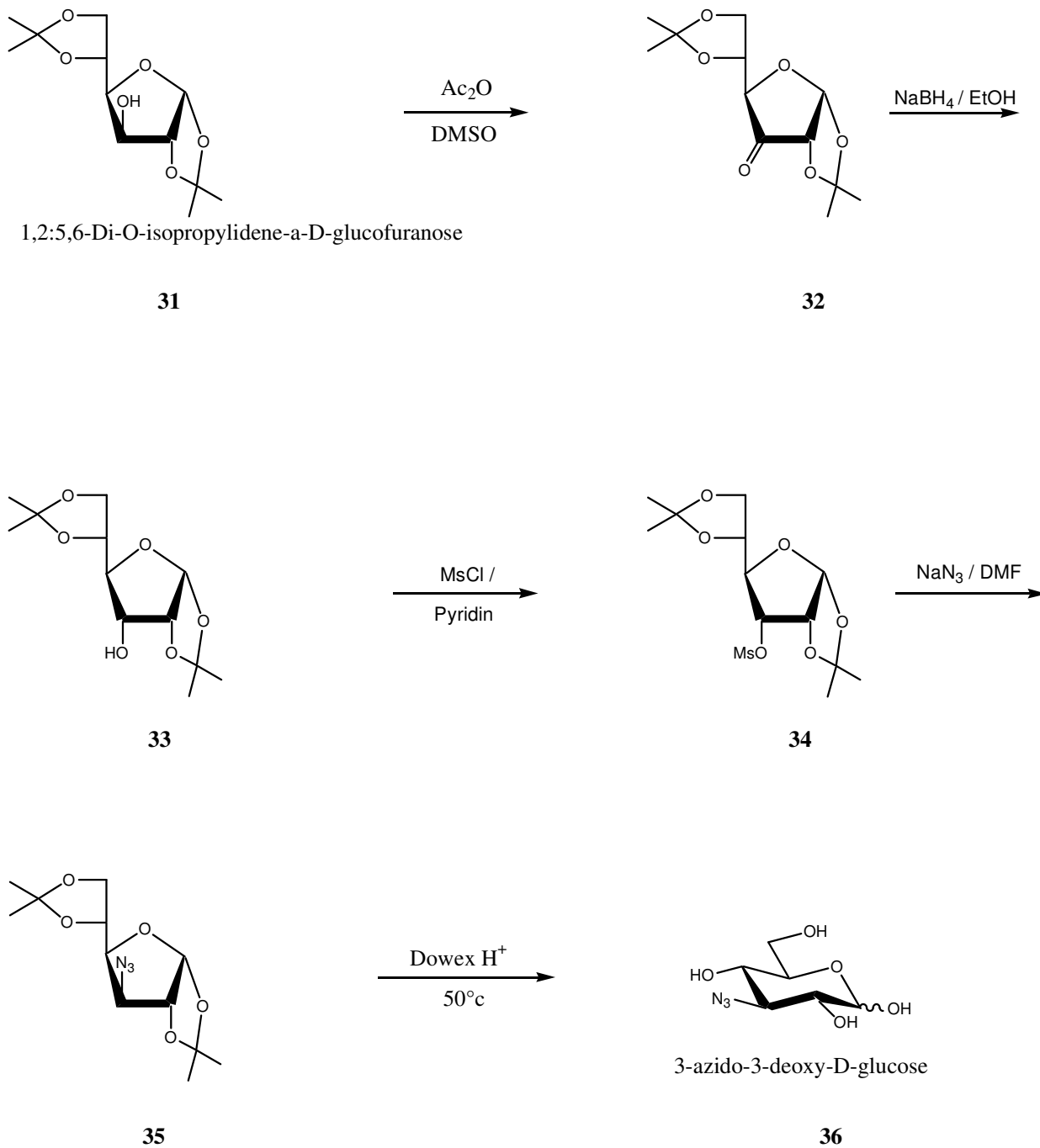
3-Azido-3-deoxy-D-glucose was synthesized from the commercially available 1,2:5,6-di-O-isopropylidene- $\alpha$ -D- glucofuranose in 5 steps. In the first step the protected  $\alpha$ -D-glucofuranose was oxidized with acetic anhydride in dimethyl sulfoxide at 70°C to give the hexofuranose analogue. The hexofuranose derivative was then reduced with sodium borohydride in ethanol as a solvent to give the allofuranose derivative. The allofuranose derivative was then O-mesylated in the free hydroxyl group at C-3 to give the 3-O-mesylate derivative. The mesyl group was then substituted with azido, through the reaction with sodium azide in DMF. Under gently heating the protecting groups were removed with Dowex<sup>®</sup> H<sup>+</sup> to give the 3-azido-3-deoxy-D-glucose.

## 5.1.5 Synthesis of $\beta$ -D-allose

(Scheme 5)

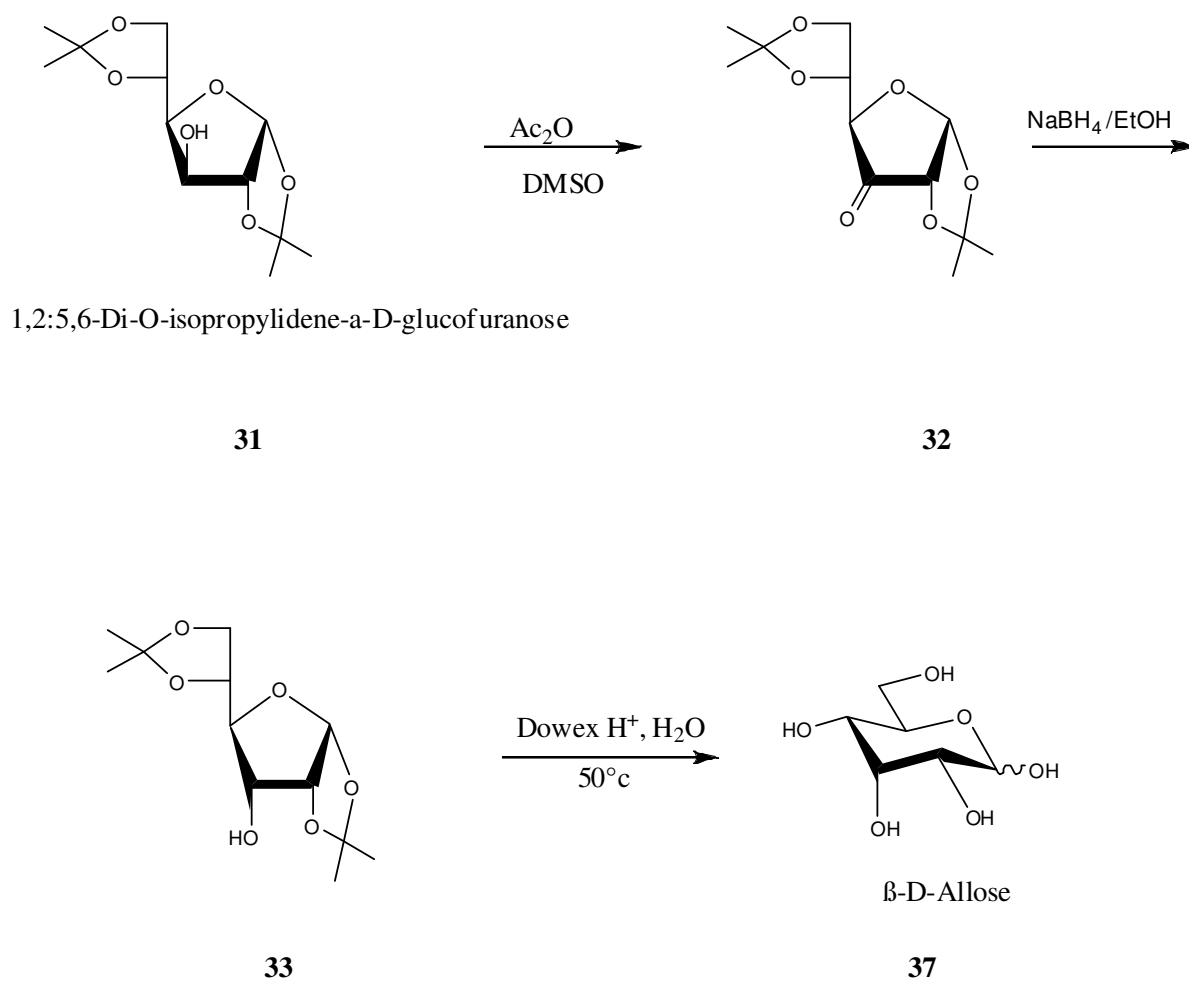
The synthesis of  $\beta$ -D-allose was simply achieved by the action of Dowex<sup>®</sup> H<sup>+</sup> resin on allofuranose (compound **33**) in water under gentle heating. In this reaction the two isopropylidene protecting groups were removed<sup>40</sup>.

## Synthesis of 3-azido-3-deoxy-D-glucose



Scheme 4

## Synthesis of $\beta$ -D-allose



Scheme 5

## 5.2 *Enzyme-Catalyzed Syntheses*

### 5.2.1 Carbohydrate Processing Enzymes

The chemical formation of a particular glycosidic bond involves the consideration of three factors:

- Reactivity
- Regioselectivity
- Stereoselectivity

Despite the development of a number of chemical techniques that allow partial control of the configuration at the anomeric center, these methods are rarely general.

As a result, mixtures of products are often formed that require laborious purification. The formation of side products with the "wrong" stereochemistry also lowers the yields of the desired products. Enzymes are powerful catalysts that are able to overcome some of these problems. They contain a region which is called the active site, that control the reaction they catalyse, this active site can be considered as made of two parts: the catalytic site, which increases the rate of the reaction, and the binding site or sites which hold(s) the reactants in place and determines the selectivity of the reaction.

The mechanistic action of carbohydrate processing enzymes can be understood in the terms of the problems of glycosidic bond formation as follows.

1. **Reactivity** – just for chemical methods of forming glycosidic bonds, enzymatic mechanisms also rely on the loss of a good leaving group from the anomeric centre of the glycosyl donor. This bond breaking is typically assisted by the involvement of the lone pair on the ring oxygen atom and leads to the

formation of a glycosyl cation intermediate. The transition state that leads to the formation of this reactive intermediate is stabilised by the catalytic site of the enzyme. As the formation of this transition state is the lowest step, lowering the associated energetic barrier causes an increase in the rate of reaction. The shape of the binding site of the enzyme now holds this reactive species ready for attack by a nucleophile such as the hydroxyl group of another sugar, the glycosyl acceptor.

2. **Regioselectivity** - because the reactants fit perfectly into the binding sites of the enzyme in only one particular orientation, the shape of the enzyme determines which groups will react. Both the glycosyl acceptor and glycosyl donor are held tightly in the enzyme's binding site. Their relative orientations allow the reaction of only one of the hydroxyl groups of the acceptor. The other hydroxyl groups of the glycosyl acceptor are buried in the folds of the binding site; they are therefore temporarily protected and so do not react. Furthermore, the reactive anomeric carbon atom in the glycosyl donor is close enough to only one particular hydroxyl group to react.
3. **Stereoselectivity** – The enzyme also binds the reactive glycosyl donor in such a way that it can only be attacked from one side: either above or below. The result is that the formation of only one stereochemical configuration is possible. Which configuration is formed therefore depends on the shape of the enzyme active site. Different enzymes have evolved with differently shaped active sites; some allow only the formation of  $\alpha$  products whilst some will catalyze formation of only the  $\beta$ -glycosides.

### 5.2.1.1 Cellulase enzyme system

All cellulases hydrolyze<sup>41</sup> the  $\beta$ -1, 4-glucosidic bond between glucosyl moieties by a general acid catalysis requiring a proton donor and a nucleophile/base. The hydrolysis products can either result in the inversion or retention (double replacement mechanism) of the anomeric configuration of carbon-1 at the reducing end<sup>42</sup>. The enzymes have a complex molecular architecture comprising discrete modules: the catalytic domains are joined to non-catalytic modules, called carbohydrate binding modules (CBM), by linker regions (commonly proline-threonine-serine- or PTS-boxes).

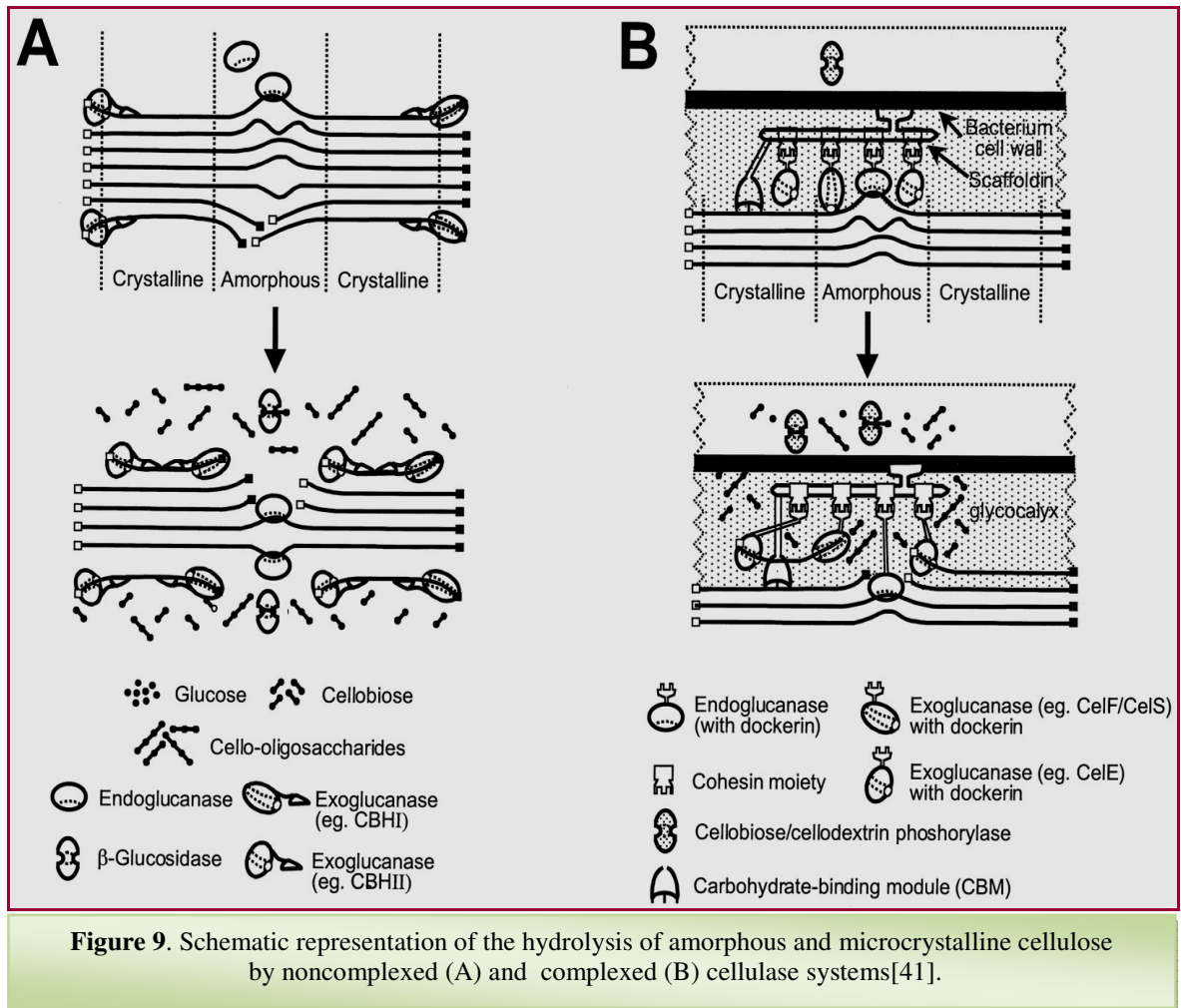
Cellulases differ not only in the action mode (endo or exo), but also in the way they bind to the crystalline surface of the substrate. The crystalline material is hydrolyzed by a number of simultaneously present, interacting enzymes, or alternatively by a multienzyme complex. Only by cooperation with non-catalytic specific binding modules (the carbohydrate binding proteins or modules), the enzymes are able to disrupt the crystal surface at the solid-liquid interphase, to make single cellulose fibers accessible for hydrolysis.

Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure.  $\beta$ -Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose. Cellulases are distinguished from other glycoside hydrolases by their ability to hydrolyze  $\beta$ -1, 4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the  $\beta$ -1, 4-glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base.

A general feature of most cellulases is a modular structure often including both catalytic and carbohydrate-binding modules (CBMs) as shown in Figure 9. The CBM effects binding to the cellulose surface, presumably to facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the substrate, insoluble

cellulose. The presence of CBMs is particularly important for the initiation and processivity of exoglucanases<sup>43</sup>.

In Figure 9 the solid squares represent reducing ends and the open squares represent nonreducing ends. Amorphous and crystalline regions are indicated.

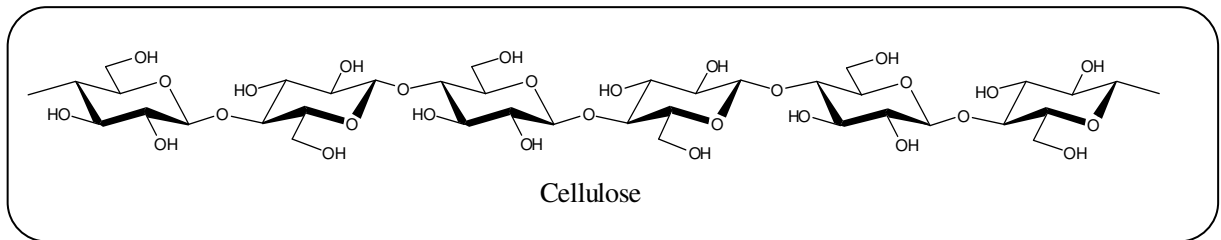


Cellulase systems are not merely an agglomeration of enzymes representing the three enzyme groups (endoglucanases, exoglucanases, and  $\beta$ -glucosidases, with or without CBMs), but rather act in a coordinated manner to efficiently hydrolyze cellulose. Microorganisms have developed different approaches to effectively hydrolyze cellulose, naturally occurring in insoluble particles or imbedded within hemicellulose and lignin polymers.

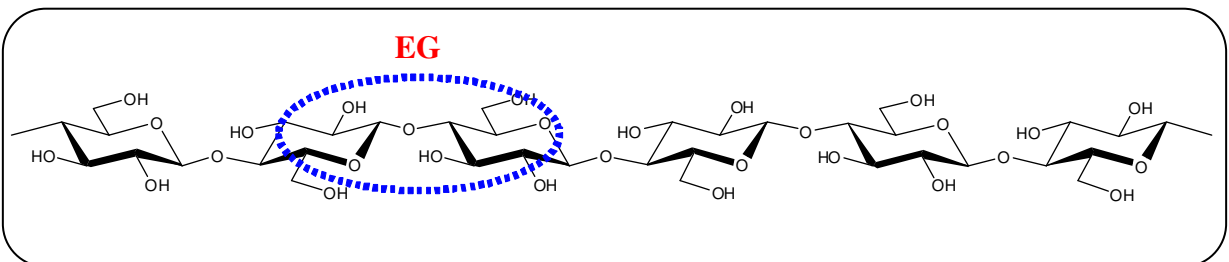


## A schematic representation of cellulase action

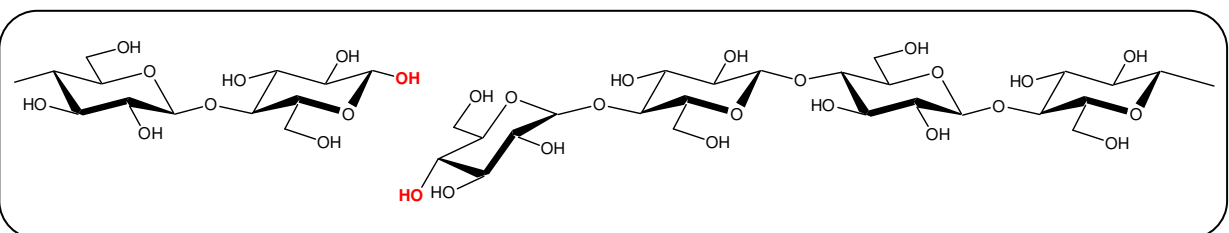
The catalytic activity of the cellulase enzyme system can be summarized in the following steps



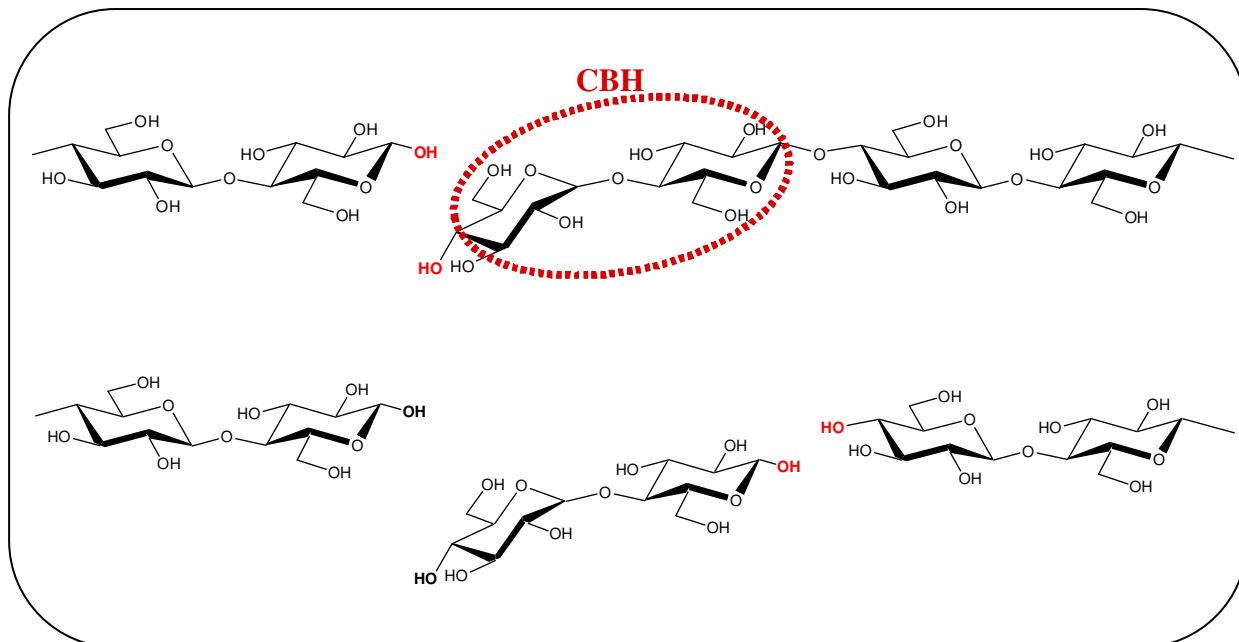
Endoglucanase EG binds randomly to the surface of the cellulose microfibril. The catalytic action of EG breaks a glycosyl bond within a glucan chain.



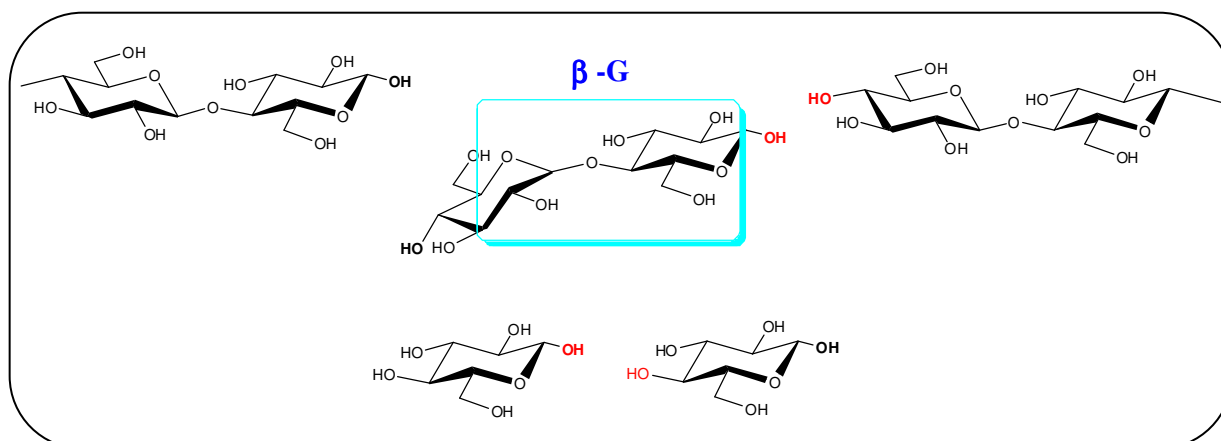
EG leaves the microfibril surface, the nick in the glucan chain exposes a reducing and nonreducing end.



Cellobiosylhydrolase CBH (which is an exoglucanase) can act only on the free nonreducing end of a glucan chain. The catalytic action of CBH cleaves a cellobiose unit from the nonreducing chain end.



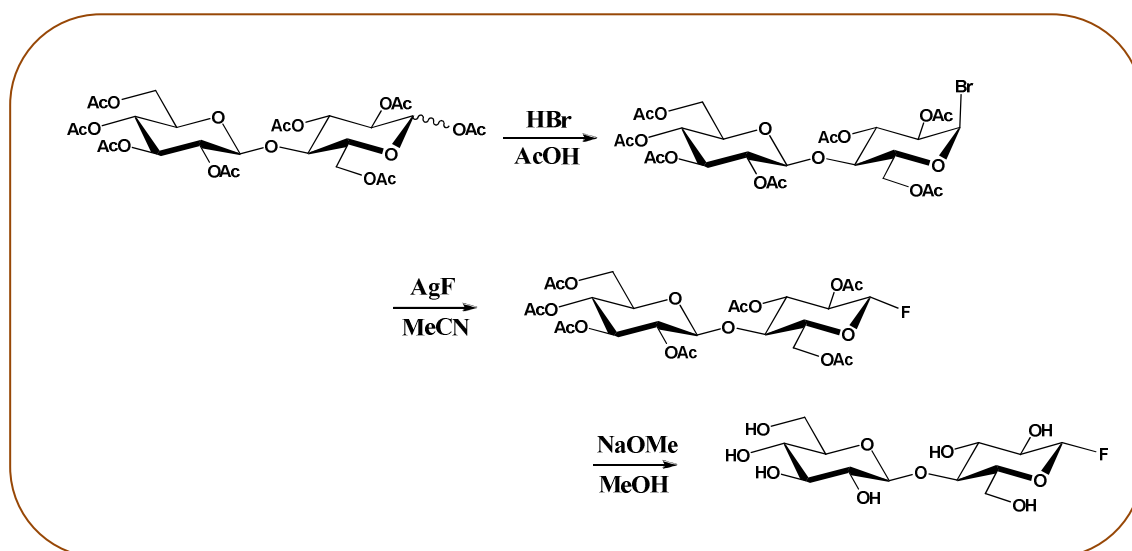
Cellobiose is released into solution, where it is split into glucose monomers by  $\beta$ -glucosidase  $\beta$ -G or by cellobiose phosphorylase (see page 61).



CBH moves to the newly created free nonreducing end and continues to cleave cellobiose units from the glucan chain. EG continuously nicks glucan chains but releases little soluble reducing sugar. CBH is catalytically active only at glucan chain ends. Thus, EG created sites at which CBH may act. The result is synergistic degradation of cellulose.

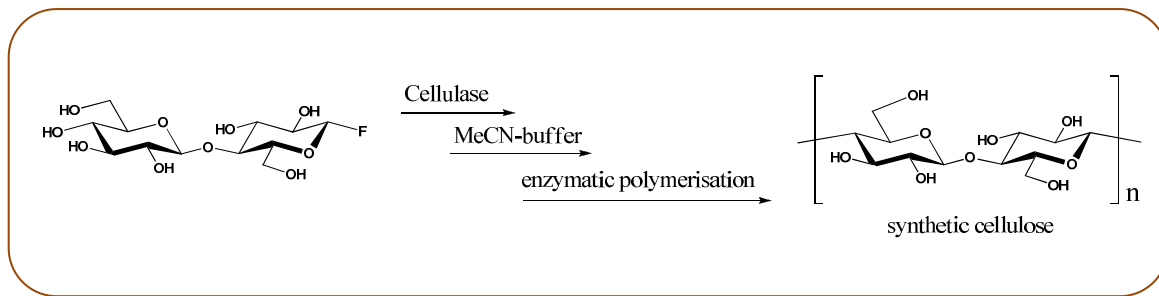
## Reversal of cellulase action

The use of cellulase enzyme as a catalyst for the synthesis of complex polysaccharides has played an important role in polymer synthesis. One example is the first successful in vitro synthesis of cellulose via a nonbiosynthetic pathway by utilizing cellulase as a catalyst, where the enzymatic synthesis of cellulose can be achieved via polycondensation of  $\beta$ -cellobiosyl fluoride monomer<sup>44,45,46</sup> (Figure 10). Thereby it was postulated that a cellobiose unit would be preferable as a substrate because it can be recognized by the binding site of the enzyme more readily than a glucose derivative. For this transglycosylation reaction  $\beta$ -cellobiosyl fluoride was chosen as activated glycosyl donor.



**Figure 10:** Synthetic route of  $\beta$ -cellobiosyl fluoride monomer from cellobiose octaacetate [46]

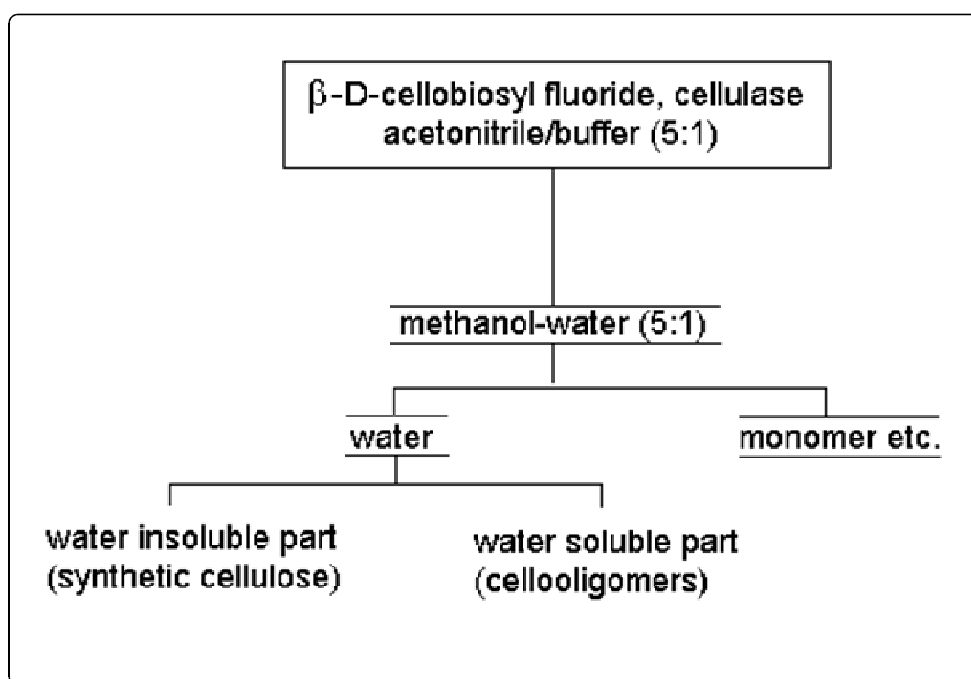
The cellulase promotes the transglycosylation of the cellobiose moiety toward the 4' hydroxy group of another monomer, eliminating hydrogen fluoride. The polymerization has to be carried out in an aqueous organic solvent system e.g. acetonitrile and acetate buffer in order to make the desired polycondensation predominant over the competitive hydrolysis reaction. The configuration of the C1 fluorine atom of the starting material has been designed in order to form a reactive intermediate leading to a  $\beta$  (1 $\rightarrow$ 4) product via "double displacement mechanism" at the active site of the enzyme.



**Figure 11:** The enzymatic polymerisation of  $\beta$ -cellobiosyl fluoride monomer catalysed by cellulase in acetonitrile-buffer [46]

The experimental procedure for the enzymatic polymerisation is as follows. In a test tube a solution of *Trichoderma viride* is added to a solution of  $\beta$ -cellobiosyl fluoride monomer in a mixed solvent of acetonitrile/acetate buffer. The addition of acetonitrile is essential for the cellulase catalyst to promote the reverse reaction of the hydrolytic decomposition<sup>47</sup>. The polymerisation perfectly controls the regio- and stereochemistry of the reaction, giving rise to the synthetic cellulose (degree of polymerisation < 22). Alternatively, under the reaction conditions of a higher substrate concentration or higher acetonitrile concentration, water-soluble cello-oligomers (degree of polymerisation < 8) are produced predominantly. After inactivating the enzyme, the reaction mixture is purified, yielding a water-insoluble white powdery material, the synthetic cellulose and a mixture of cello-oligomers.

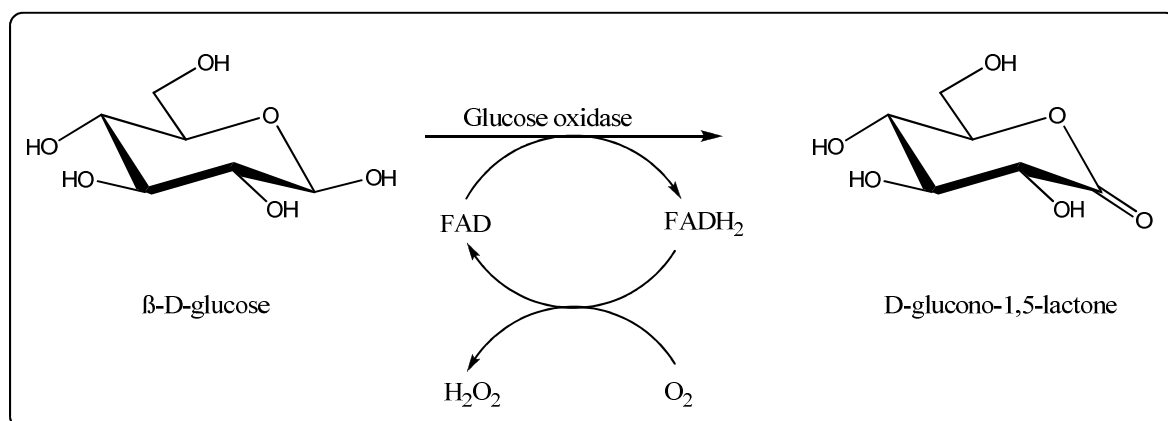
Figure 12 shows the flow chart for the preparation of synthetic cellulose (the water-insoluble part) as well as cello-oligomers (the water-soluble part).



**Figure 12:** Flow chart for the preparation of synthetic cellulose [46]

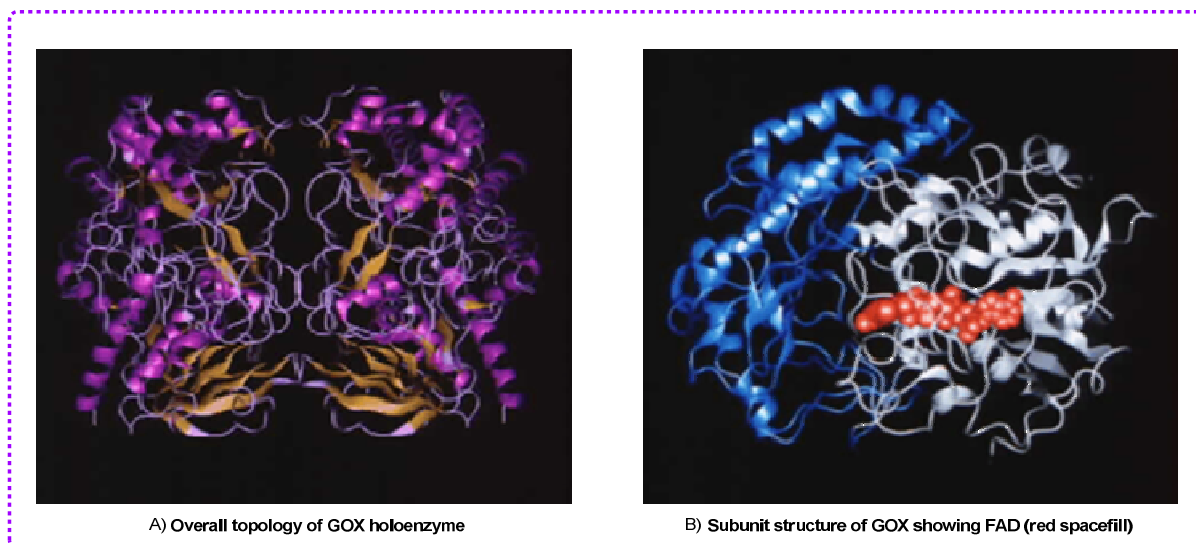
### 5.2.1.2 Glucose Oxidase

Glucose oxidase ( $\beta$ -D-glucose:oxygen 1-oxidoreductase, EC1.1.3.4) catalyses the oxidation of  $\beta$ -D-glucose to D-glucono-1,5-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor<sup>48</sup>.



Glucose oxidase has been used in the food industry for the removal of glucose from powdered eggs and for a source of hydrogen peroxide in food preservation. Moreover, it is also used for quantitative determination of D-glucose in blood, food and fermentation products and enzyme-antibody conjugates for enzyme immunoassays<sup>49</sup>.

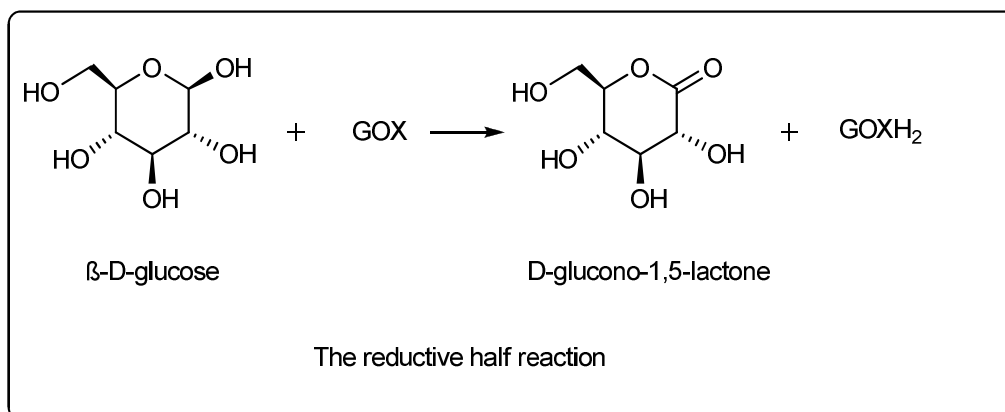
Glucose oxidase is a flavin-containing glycoprotein. The enzyme is a homodimer, composed of two subunits; the monomer folds into two structural domains (Figure13). One of the domains binds FAD noncovalently and the other is involved with substrate binding. The FAD is acting as a redox carrier in catalysis and can be released from the holoprotein after denaturation<sup>50</sup>. FAD functions as a coenzyme owing to its ability to undergo reversible redox reactions.



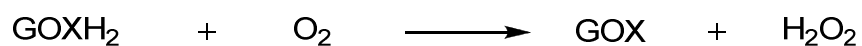
**Figure13:** High resolution crystal structure of GOX: A) overall topology of holoenzyme  
 B) Subunit structure of GOX showing FAD (red space fill)  
*M. Cardosi, Electrochemical Sensors Group, University of Paisley, Paisley, Scotland*

## Overall Reaction Mechanism

The enzymatic oxidation of glucose proceeds in two steps, the reductive half reaction in which two protons and electrons are transferred from  $\beta$ -D-glucose to the FAD. This step of the catalytic cycle leads to the oxidation of  $\beta$ -D-glucose to  $\delta$ -gluconolactone.

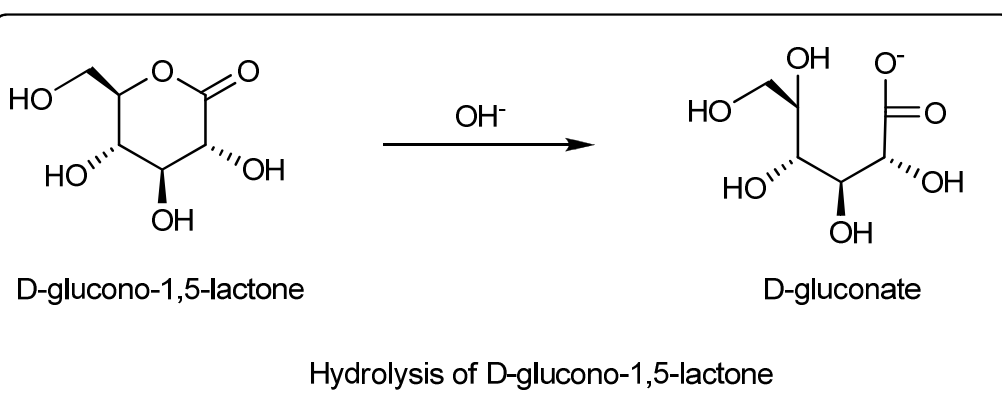


In the second step, the oxidative half reaction, the enzyme is oxidized by molecular oxygen yielding hydrogen peroxide.

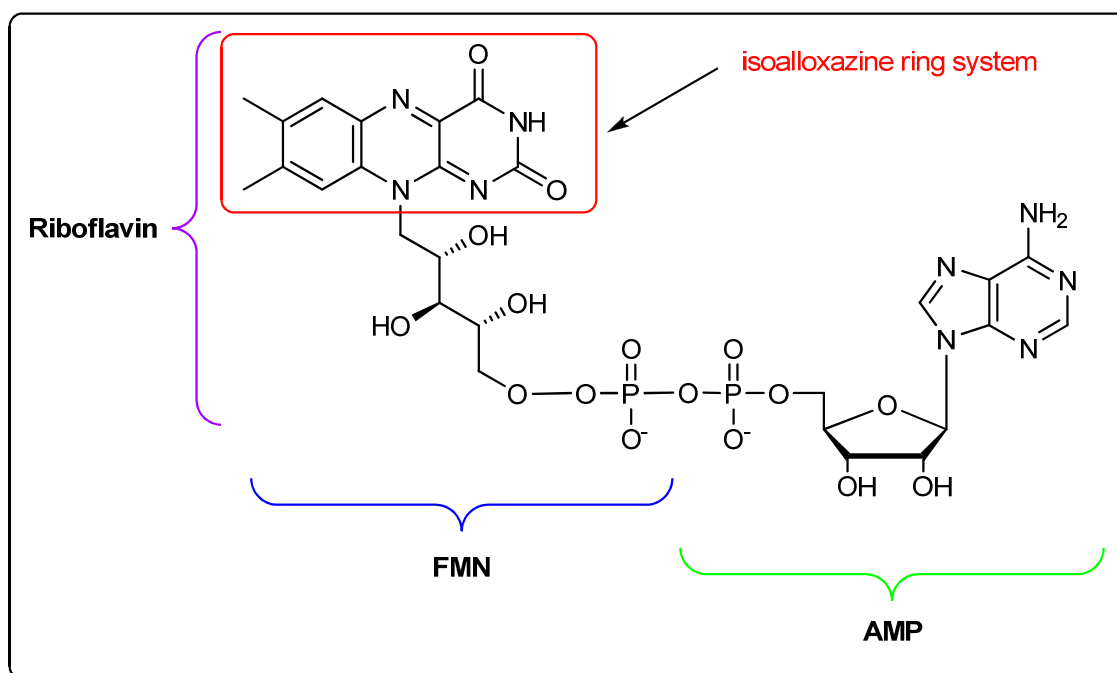


The oxidative half reaction

Finally  $\delta$ -gluconolactone can be hydrolyzed non-enzymatically to gluconic acid. This hydrolysis step is pH dependent.

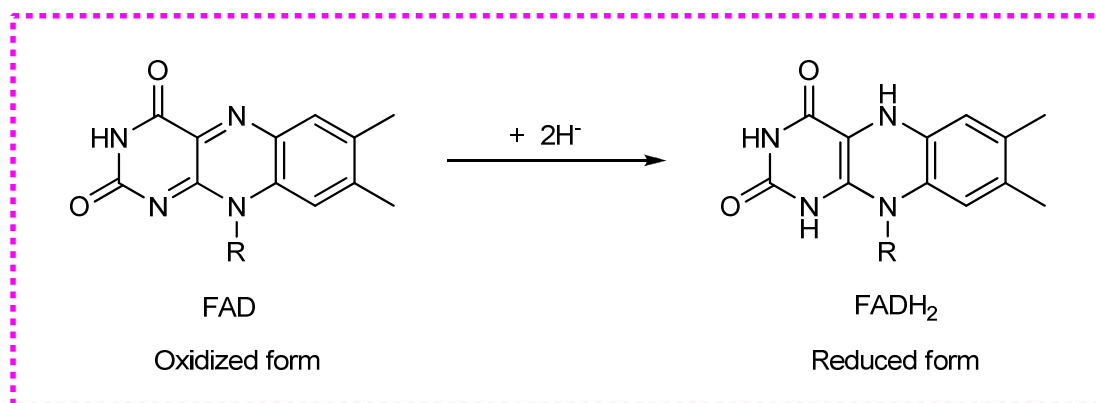


The redox active centre of the FAD coenzyme is the isoalloxazine ring system as shown in Figure14



**Figure14:** Chemical structure of the coenzyme FAD

The reaction sequence below shows the transfer of two electrons and two hydrogen atoms to the isoalloxazine ring system during the process. This electron and hydrogen transfer leads to the reduction of riboflavin to dihydroriboflavin.



Under reaction with molecular oxygen the dihydroriboflavin (FADH<sub>2</sub>) can be reoxidized to the hydroperoxy intermediate. The later gives under loss of hydrogen peroxide the FAD again.

### **Substrate Specificity of glucose oxidase GOX**

Glucose oxidase has affinity toward different sugars such as D-xylose, D-mannose, D-galactose and glucose analogs such as 2-deoxyglucose. The rate of reaction of glucose oxidase with  $\beta$ -D-glucose is very much faster than that with other sugars<sup>51</sup>. It was also reported that  $\alpha$ -hydroxycarbonyl compounds such as dihydroxyacetone, glyceraldehyde, phenacyl alcohol, and furoin are also substrates for glucose oxidase, acting as glucose analogs<sup>52</sup>.

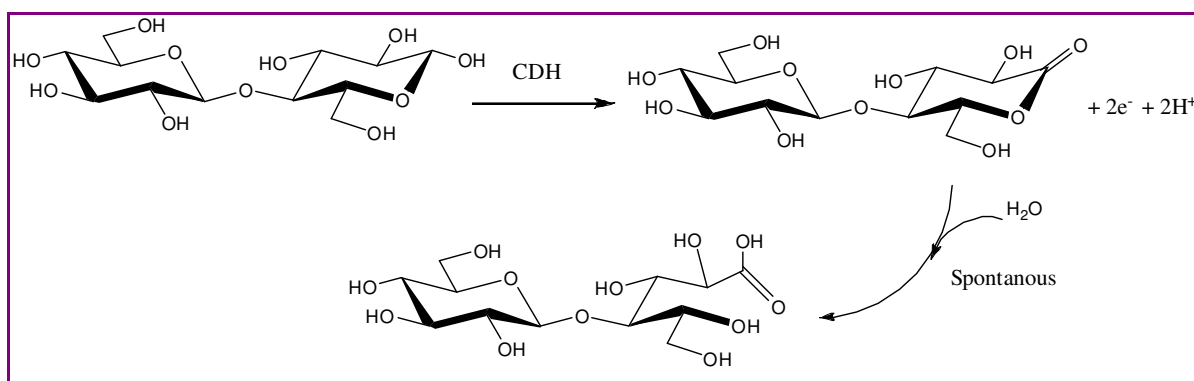
### **6-Azido-6-deoxy-D-glucose as substrate**

Within this work we have synthesized 6-azido-6-deoxy-D-glucose, it was investigated as an analog of glucose to act as a substrate for glucose oxidase. Under the optimum reaction conditions, we could not detect any product formed or any change in the educt. Other investigations may needed to explain either the enzyme-substrate complex is not formed at all, or if the azido group in the six position of the glucose may inhibit the formation of stable intermediate.



### 5.2.1.3 Cellobiose Dehydrogenase

Cellobiose dehydrogenase (CDH) is an extracellular flavocytochrome containing flavin and *b*-type heme, and plays a key role in cellulose degradation by filamentous fungi<sup>53</sup>. In filamentous fungi, cellulose degradation had been thought to proceed via two-step hydrolysis, i.e. cellulose is hydrolyzed to cellobiose by various cellulases and the product is further hydrolyzed to glucose by  $\beta$ -glucosidase. However, recent cytochemical, kinetic, and transcriptional studies have supported another hypothesis concerning the contribution of cellobiose dehydrogenase to the extracellular cellulose metabolism of the white-rot fungus *Phanerochaete chrysosporium*, and the importance of a combination of hydrolytic and oxidative reactions in cellulose-degrading fungi. CDH is the only extracellular flavocytochrome known to be secreted by filamentous fungi during cellulose degradation. This enzyme carries flavin and a *b*-type heme in different domains and the flavin domain catalyzes the dehydrogenation of cellobiose and cello-oligosaccharides to the corresponding  $\delta$ -lactones. Although this enzyme was initially characterized as an oxidase (cellobiose oxidase), its higher affinity for quinones and ferric compounds than for oxygen and the low-spin character of the heme in both the ferric and ferrous states indicate that the electron acceptor of this enzyme is not molecular oxygen. Although many candidates have been proposed for the electron acceptor of CDH, its natural electron acceptor and the physiological function of the oxidative half reaction of this enzyme are still uncertain. CDH displays<sup>54</sup> the properties of a typical dehydrogenase with oxidative and reductive half reactions that can be studied separately. The oxidative half reaction represents an oxidation in the C1 position of a saccharide;

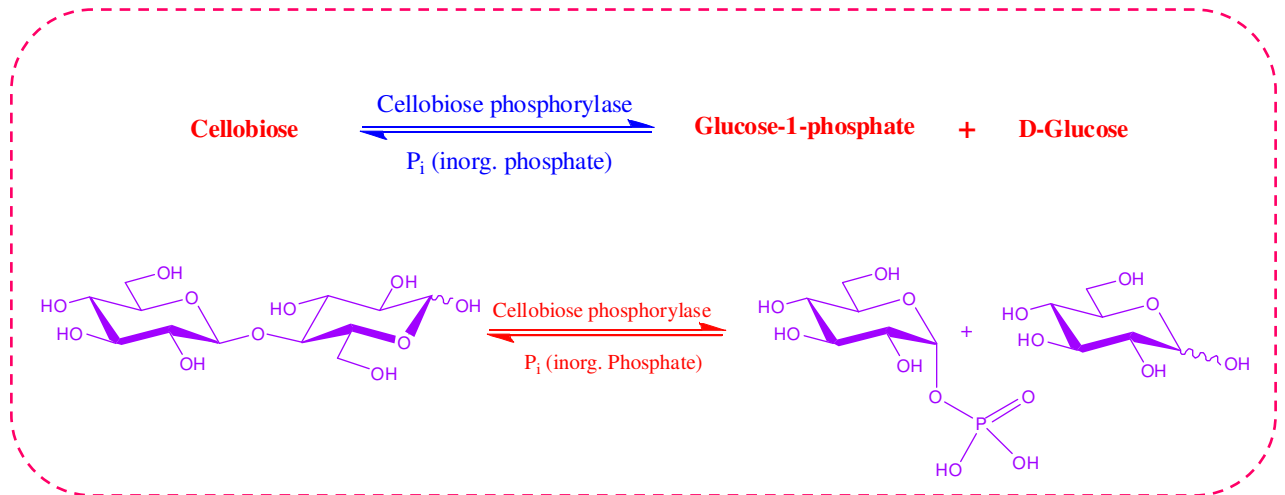


**Figure 15.** The oxidative half reaction of CDH. CDH performs a two-electron oxidation of the C1 of cellobiose to a lactone. The electrons are taken up by the FAD of CDH. The lactone will spontaneously hydrolyze under ring opening to a carboxylic acid [54]

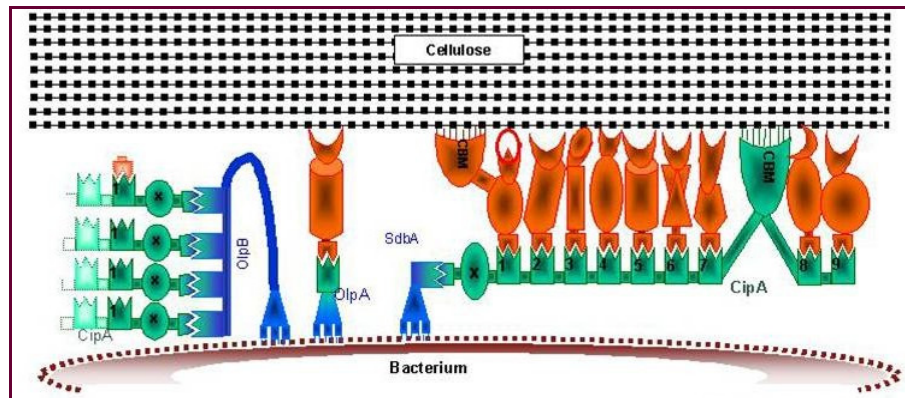
the hemiacetal at this position is converted into a lactone that hydrolyzes spontaneously to a carboxylic acid (cellobionic acid) (Fig. **15**). The two electrons taken up by the enzyme are later transferred further to one two-electron acceptor, or to two one-electron acceptors.

### 5.2.1.4 Cellobiose phosphorylase

Cellobiose phosphorylase (CBP) is one of the phosphorolytic enzymes that catalyze the reversible phosphorolysis of cellobiose to form glucose and  $\alpha$ -D-glucose-1-phosphate, with an inversion of the anomeric configuration.



The cellobiose phosphorylase of *Clostridium thermocellum* is an endocellular enzyme responsible for the reversible phosphorolysis of cellobiose.

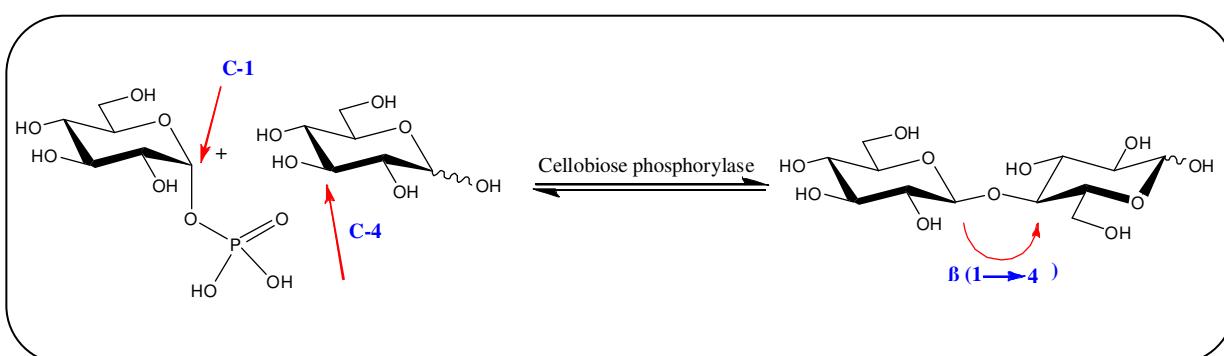


**Figure 16.** A model of *Clostridium thermocellum* cellulosomes and an associated cell-surface anchoring protein

Schwarz WH, *Research Group Microbial Biotechnology*, TU München, Germany(2001)

The physiological role of cellobiose phosphorylase is to convert cellobiose into  $\alpha$ -D-glucose-1-phosphate (G-1-P), which is more efficiently utilized as a carbon source than D-glucose in some microorganisms<sup>55</sup>. It plays an important role in cellulose breakdown<sup>56</sup>.

Cellobiose phosphorylase has been shown to catalyze the synthesis of a number of Glc- $\beta$ - (1 $\rightarrow$ 4) linked disaccharides from glucose-1-phosphate and various glycosyl acceptors. The reversible phosphorolysis reaction requires the acceptor to maintain the configuration of  $\beta$ -D-Glc-P at the C-1 and C4 positions, although the configurations of the C-3 and C-5 positions can also be important. Comparison of the data for 6-deoxy and 2-deoxyglucose showed that the role of the hydroxyl group at C-6 is less critical than that of the hydroxyl group at C-2. Kinetic data suggests that the hydroxyl group at the C-6 plays a role in enzyme-substrate binding<sup>57</sup>.



Synthesis using the reversible reaction of phosphorylases is advantageous over other enzymatic methods, such as the transglycosylation reaction of glycosidases, due to the regioselectivity of the enzyme resulting in only one product and because of the ready availability of donor substrate<sup>54</sup>. Cellobiose phosphorylase can also utilize disaccharides as acceptors to form trisaccharides. The exclusive formation of a  $\beta$  (1 $\rightarrow$ 4) branch on the reducing residue of the (1 $\rightarrow$ 6)-linked disaccharides confirms the specificity of cellobiose phosphorylase and explains why cellobiose phosphorylase did not react with the non-reducing residues of the disaccharides<sup>55</sup>.

### Cellobiose phosphorylase catalysis

Beside the use of organic-chemical protocols based on conventional protection-deprotection schemes, we have also used enzyme-catalyzed synthetic protocols. Firstly the cellobiose phosphorylase enzyme was tested for its ability to use azido sugars as substrates for both synthetic as well as phosphorolytic actions. Cellobiose

phosphorylase (CBP) is one of the phosphorolytic enzymes that catalyze the reversible phosphorolysis of cellobiose to form glucose and  $\alpha$ -D-glucose-1-phosphate, with an inversion of the anomeric configuration.

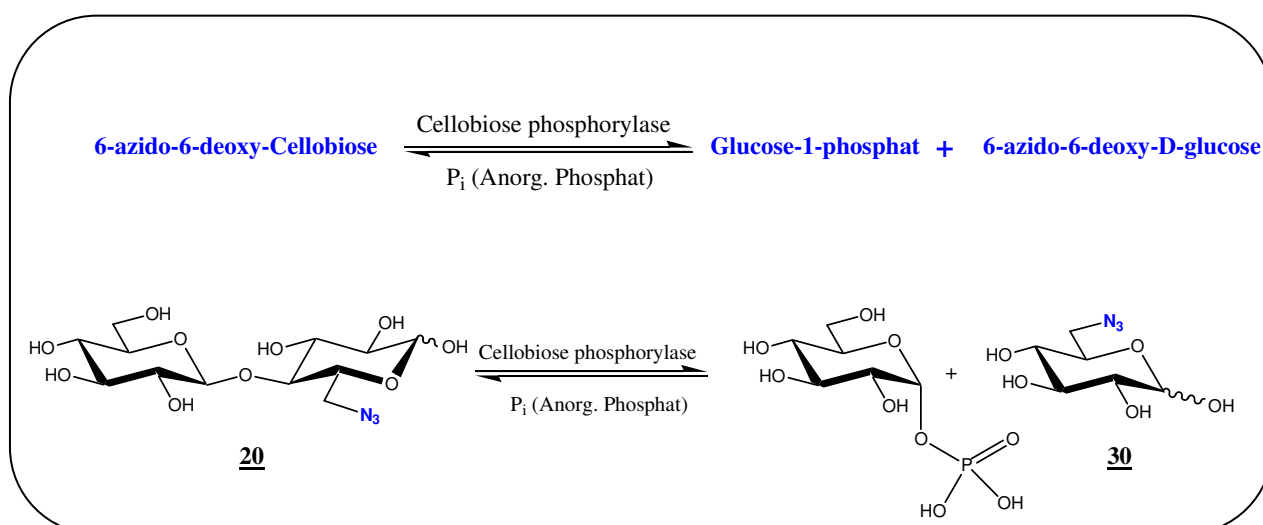
## Phosphorylation reactions

From the cellobiose series we have tested both 6-azido-6-deoxy-cellobiose and 6'-azido-6'-deoxy-cellobiose as substrates for the phosphorolytic reactions. The incubation of each substrate was done at 37 °C with the enzyme extract in phosphate medium as buffer of P<sup>H</sup> 7.0.

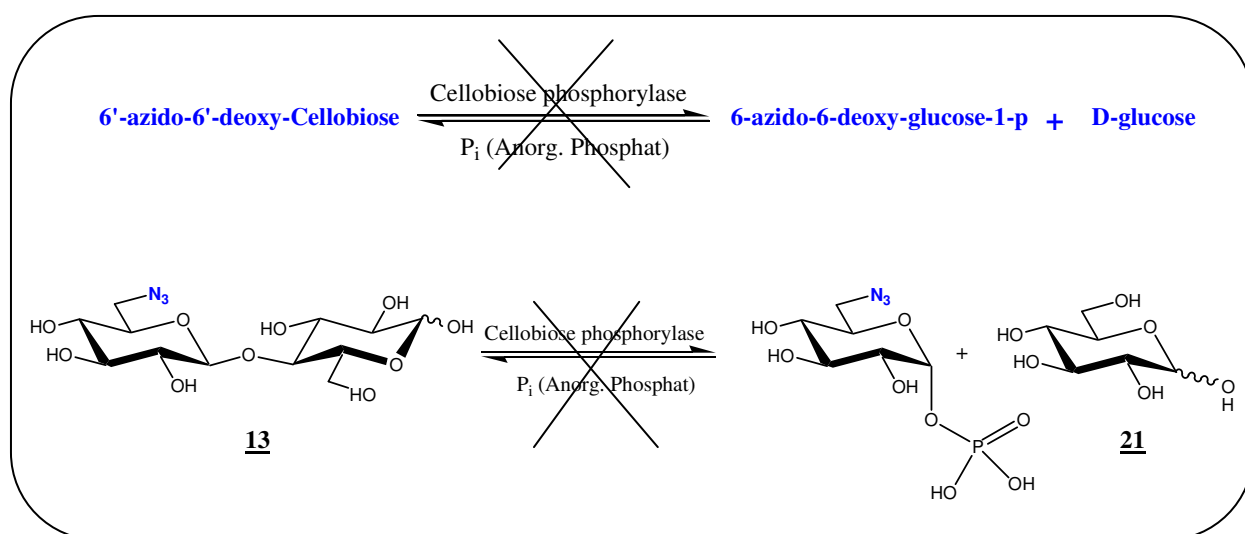
The general equation of the phosphorolytic reaction is as follows.

The enzyme breaks the  $\beta$  (1 $\rightarrow$ 4) glycosidic bond of cellobiose to give glucose 1-phosphate and D-glucose as products.

In the case of 6-azido-6-deoxy-cellobiose, the same  $\beta$  (1 $\rightarrow$ 4) glycosidic bond was broken and has given glucose-1-phosphate and 6-azido-6-deoxy-D-glucose **30** as products. In this example we have shown an alternative synthetic pathway for 6-azido-6-deoxy-glucose using enzymatic route.

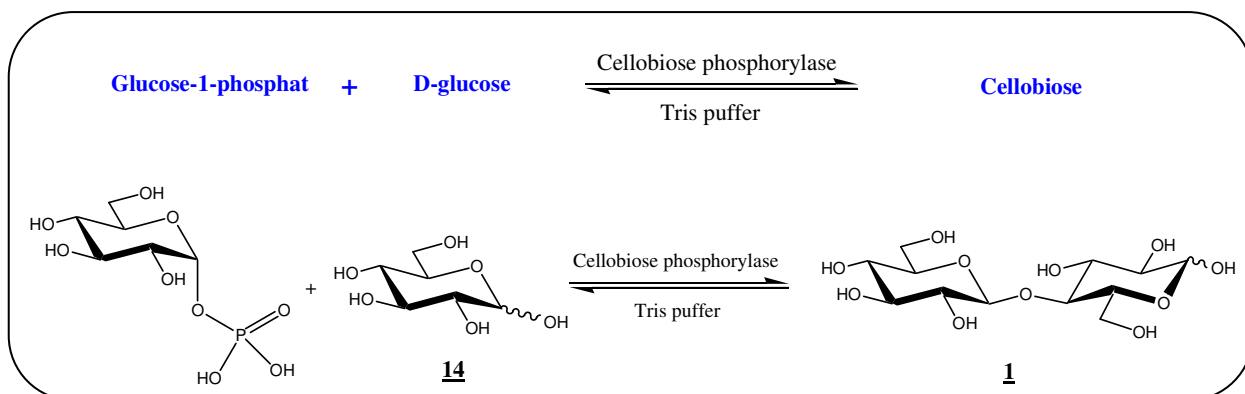


In the case of 6'-azido-6'-deoxy-cellobiose it was expected to get 6-azido-6-deoxy- $\alpha$ -D-glucose-1-phosphate and D-glucose as products. However our investigations did not give any identifiable products. Further investigations may be needed to ascertain if the azido group at C-6 of the non-reducing sugar unit is incompatible with the catalytic action of the enzyme.



## Reversal reaction of cellobiose phosphorylase

In the reverse reaction, CBP catalyzes the production of heterodisaccharides from various derivatives of glucose. The general equation of the reversal reaction of CBP is as follow;

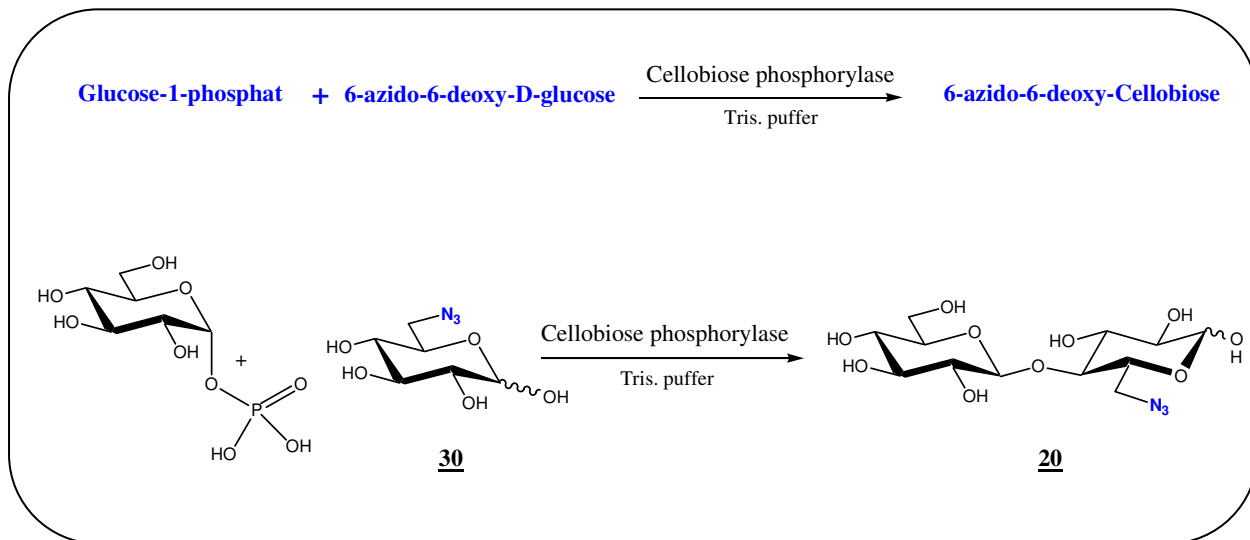


We have investigated the following derivatives.

- I) 6-azido-6-deoxy-D-glucose
- II) 3-azido-3-deoxy-D-glucose
- III)  $\beta$ -D-allose

### I) Enzymatic synthesis of 6-azido-6-deoxy-cellobiose:

6-Azido-6-deoxy-D-glucose **30** was used as substrate in the reversal phosphorylase reaction to synthesize the corresponding disaccharide. The incubation was done at 37°C for 24 h in Tris buffer as medium. The progress of the reaction was detected by TLC. The following equation describes the enzymatic catalysis of disaccharide formation.



The TLC in the **Figure 17** shows the formation of 6-azido-6-deoxy-cellobiose **20** in the reverse reaction, in which glucose-1-phosphate reacted with 6-azido-6-deoxy-D-glucose under the catalytic action of cellobiose phosphorylase in tris buffer, as well as the formation 6-azido-6-deoxy-D-glucose in the forward phosphorylation reaction, in which 6-azido-6-deoxy-cellobiose is phosphorylated in the presence of phosphate buffer to give glucose-1-phosphate and 6-azido-6-deoxy-D-glucose **30**.

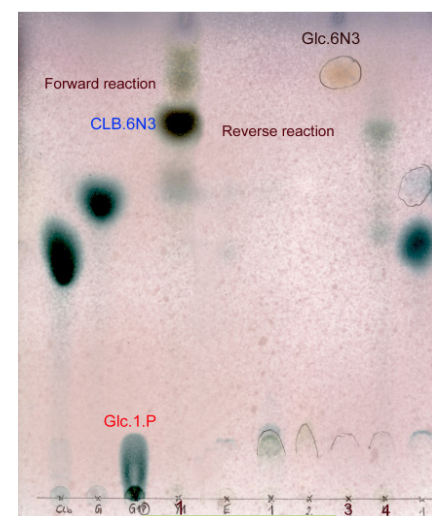
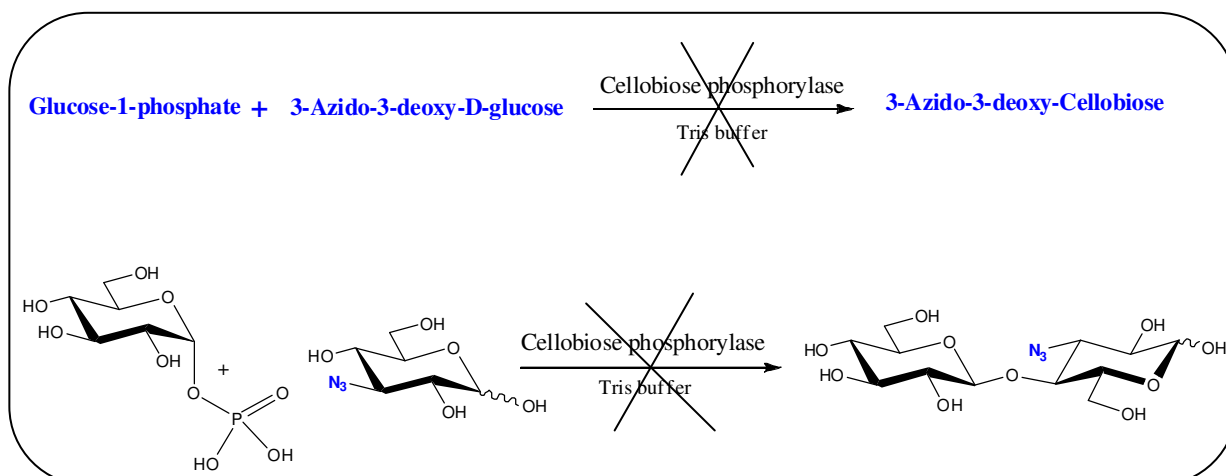


Figure 17

## II) 3-Azido-3-deoxy-cellobiose

Hypothetically the reversed action of cellobiose phosphorylase on 3-azido-3-deoxy-D-glucose could lead to the formation of the corresponding disaccharide according to this equation;

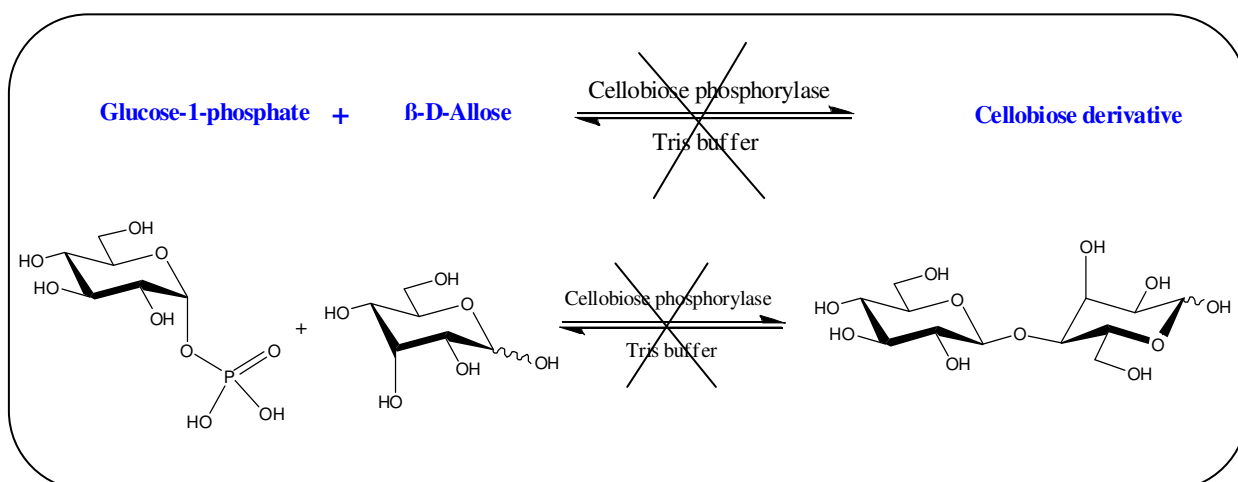




We have incubated glucose-1-phosphate and 3-azido-3-deoxy-D-glucose with the cellobiose phosphorylase in Tris buffer for 24 hours at 37°C. The TLC did not show any noticeable changes. This means the introduction of azido group in the 3 position of glucose is incompatible with the synthetic action of the enzyme.

### III) $\beta$ -D-Allose

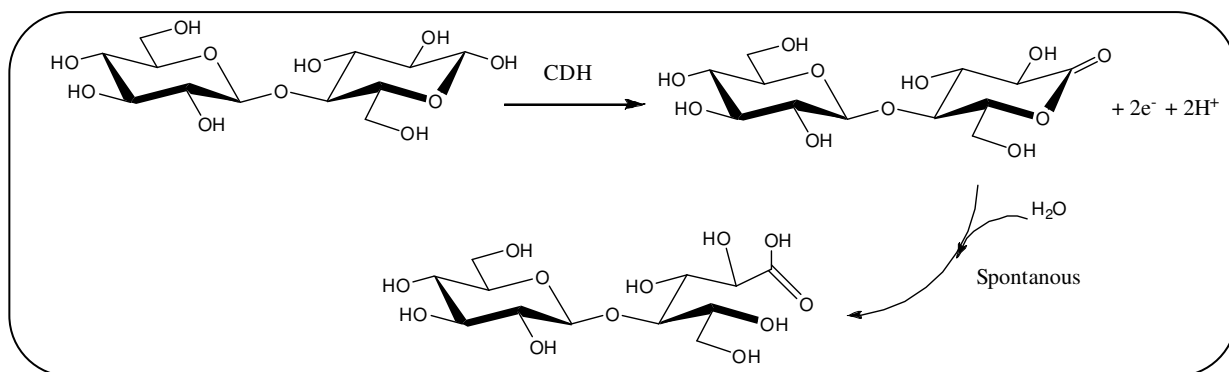
The inverted configuration of only one stereocentre on  $\beta$ -D-allose gives the molecule similarity to that of D-glucose. Hypothetically it could act as a substrate for the cellobiose phosphorylase to yield a cellobiose derivative.



After the incubation of  $\beta$ -D-allose with glucose-1-phosphate in the presence of cellobiose phosphorylase in Tris buffer, the TLC did not reveal any products. That means either the equatorial configuration of the hydroxyl on C-3 is essential for the substrate-enzyme binding, or that the axial orientation of the hydroxyl group inhibits the binding.

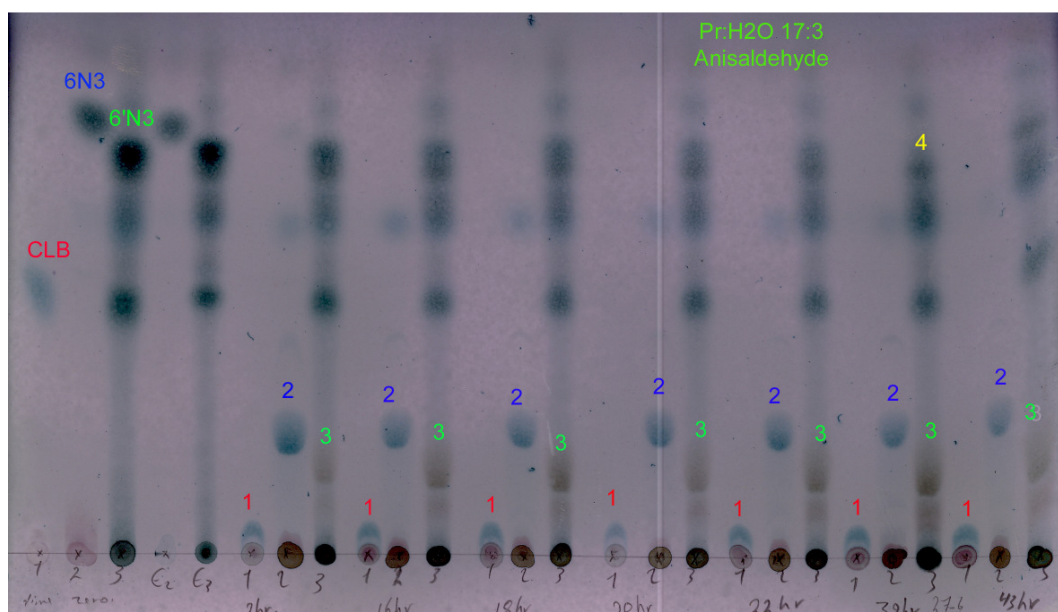
### 5.2.1.5 Cellobiose dehydrogenase catalyzed synthesis

6'-Azido-6'-deoxy-cellobiose **13** and 6-azido-6-deoxy-cellobiose **20** were tested for their ability to act as substrates for cellobiose dehydrogenase CDH. Cellobiose dehydrogenase oxidizes cellobiose to cellobionolactone. The following reaction equation describes this oxidation;



The oxidative half reaction of CDH. CDH performs a two-electron oxidation of the C-1 of cellobiose to a lactone. The electrons are taken up by the FAD of CDH. The lactone will spontaneously hydrolyze under ring opening to a carboxylic acid.

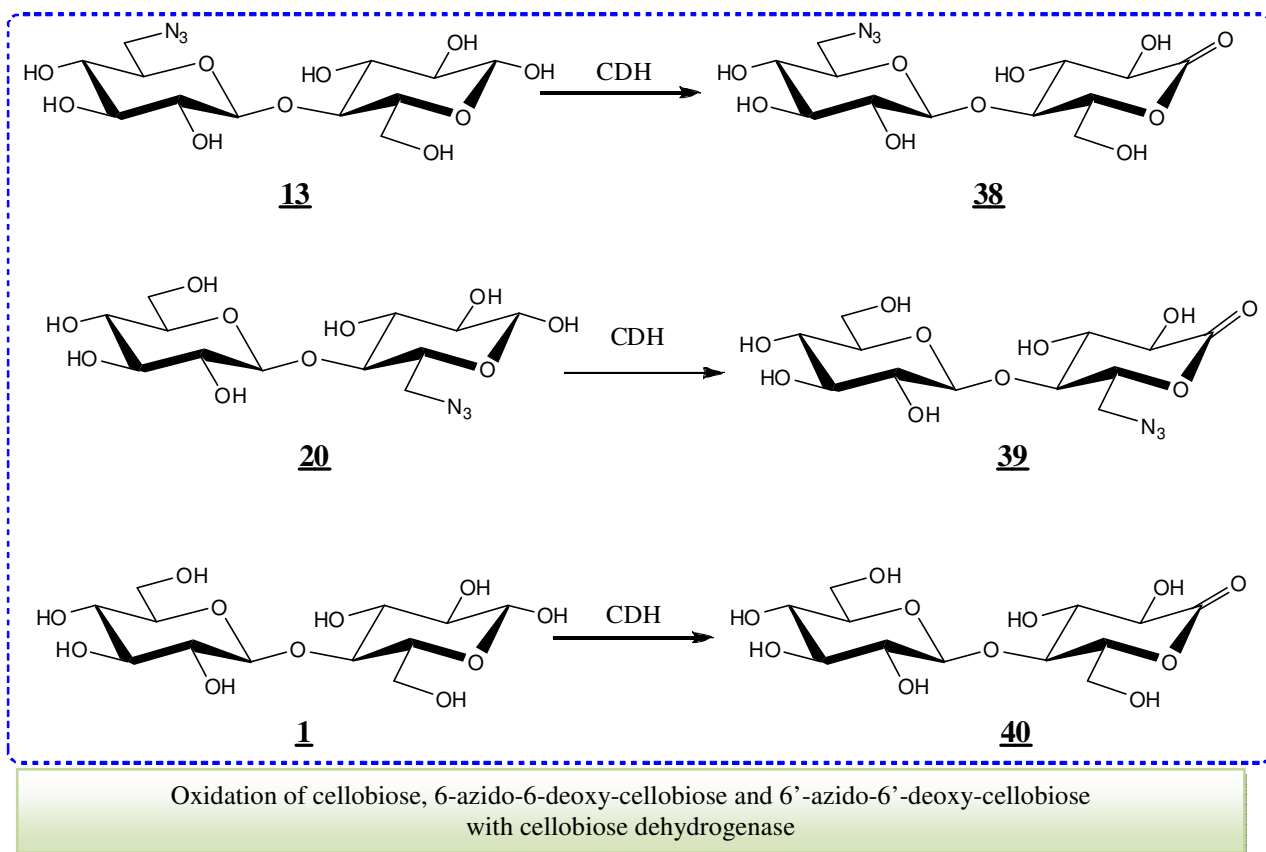
Compounds **13** and **20** as well as cellobiose **1** were incubated with CDH in the presence of potassium hexacyanoferrate  $K_3 [Fe (CN)_6]$  at 30°C. The progress of the reaction was detected using TLC.



**Figure 18.** Oxidation of CLB, CLB-6-N<sub>3</sub> and CLB-6'-N<sub>3</sub> with cellobiose dehydrogenase  
**1** = cellobiose, **2** = cellobiose-6-N<sub>3</sub> and **3** = cellobiose-6'-N<sub>3</sub>

After 2 h the oxidation of cellobiose was completely finished, whereas that for 6'-azido-6'-deoxy-cellobiose and for 6-azido-6-deoxy-cellobiose required longer than 24 hours.

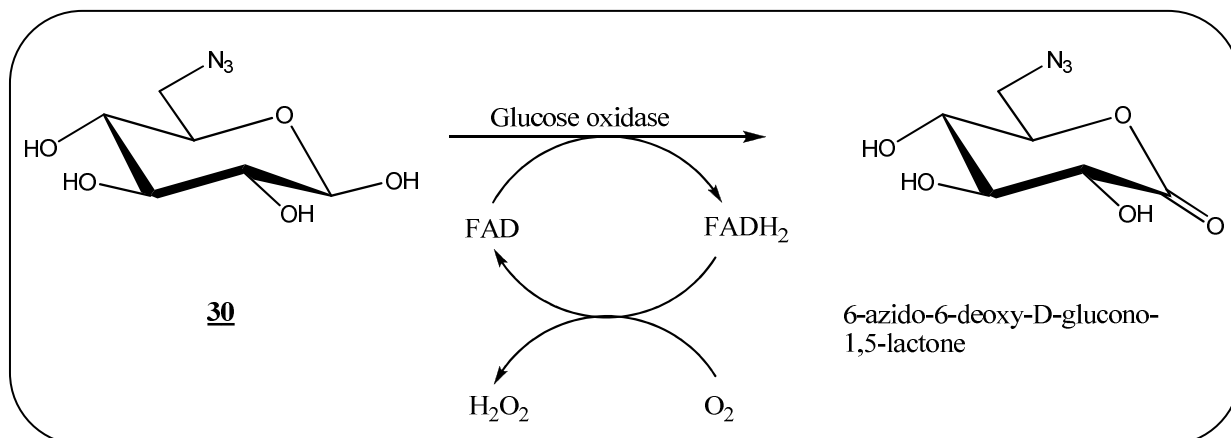
### Scheme of the oxidation reactions



### 5.3. Alkyne-azide coupling; 1,2,3-Triazoles formation

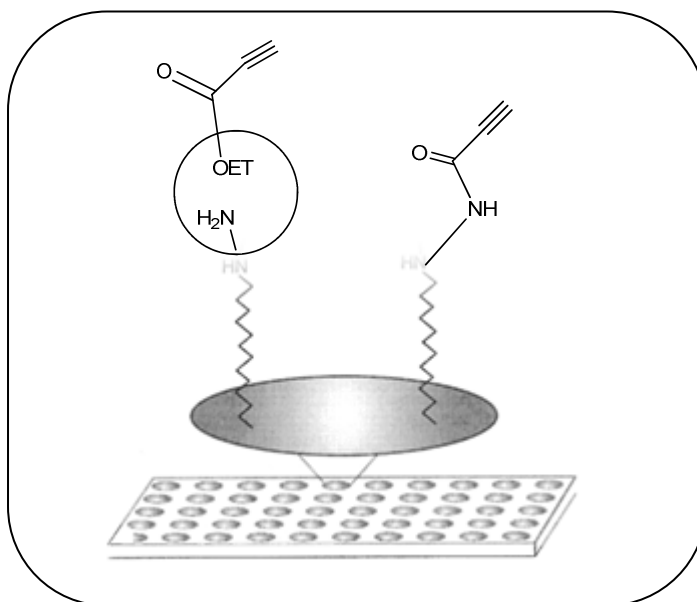
Click chemistry is a newer approach to the synthesis of drug-like molecules that can accelerate the drug discovery process by utilizing a few practical and reliable reactions. Sharpless and coworkers defined what makes a click reaction as one that is wide in scope and easy to perform, uses only readily available reagents, and is insensitive to oxygen and water. In fact, in several instances water is the ideal reaction solvent, providing the best yields and highest rates. Reaction work-up and purification uses benign solvents and avoids chromatography<sup>58</sup>. Of the reactions comprising the click universe, the “perfect” example is the Huisgen 1,3-dipolar cycloaddition of alkynes to azides to form 1,4-disubstituted-1,2,3-triazoles. The copper(I)-catalyzed reaction is mild and very efficient, requiring no protecting groups, and requiring no purification in many cases<sup>59</sup>. The azide and alkyne functional groups are largely inert towards biological molecules and aqueous environments, which allows the use of the Huisgen 1,3-dipolar cycloaddition in target guided synthesis and activity-based protein profiling<sup>60</sup>. The triazole has similarities to the ubiquitous amide moiety found in nature, but unlike amides, is not susceptible to cleavage. Additionally, triazoles are nearly impossible to oxidize or reduce.

In this work our aim was to immobilize glycans for testing of anti-carbohydrate **IgE**. Our strategy based on the formation of a carbohydrate derived azide as a linker for the building of a 1,2,3-triazole. For this purpose we have chosen 6-azido-6-deoxy-D-glucose **30** as a candidate, which can be oxidized to the corresponding carboxylic acid which may be transformed into an ester or lactone.



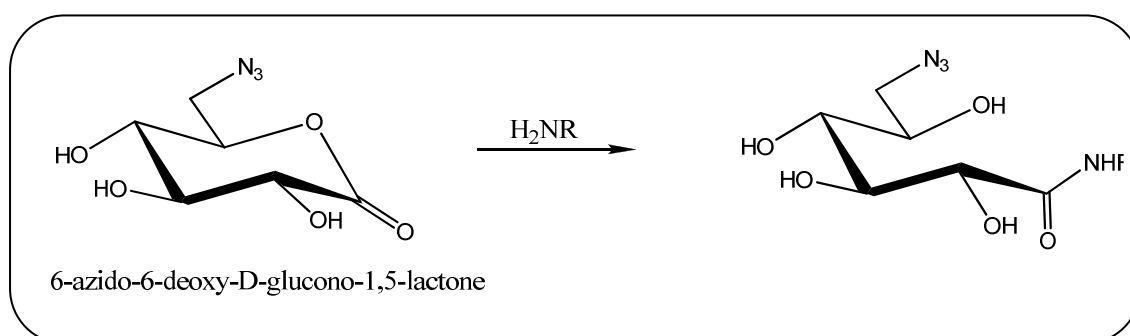
**Figure23** Oxidation of 6-azido-6-deoxy-D-glucose

In the second step the alkyne derivative can bind to a microtitre plate which carries amino groups on the surface, in our work we have used ethyl propiolate as alkyne source which can bind to the microtitre plate through the formation of amide bond as shown in Figure 24.



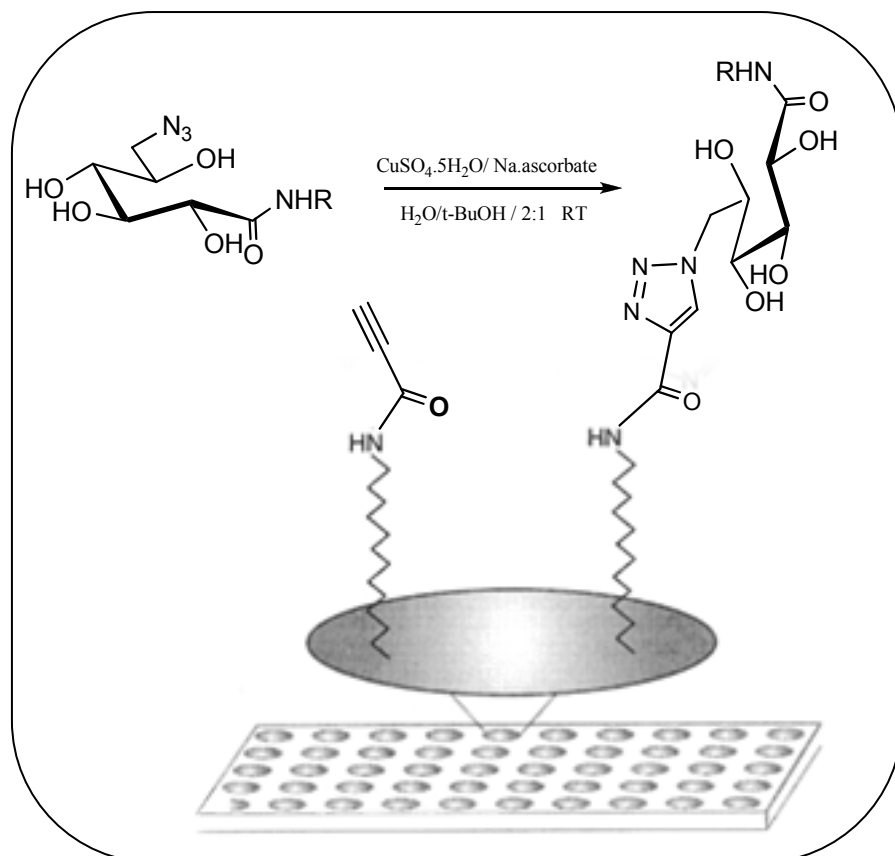
**Figure 24** The binding of the alkyne ester to the micro titre plate

The lactone formed in the first step can react in excess with the glycopeptide of interest as shown in Figure 25.



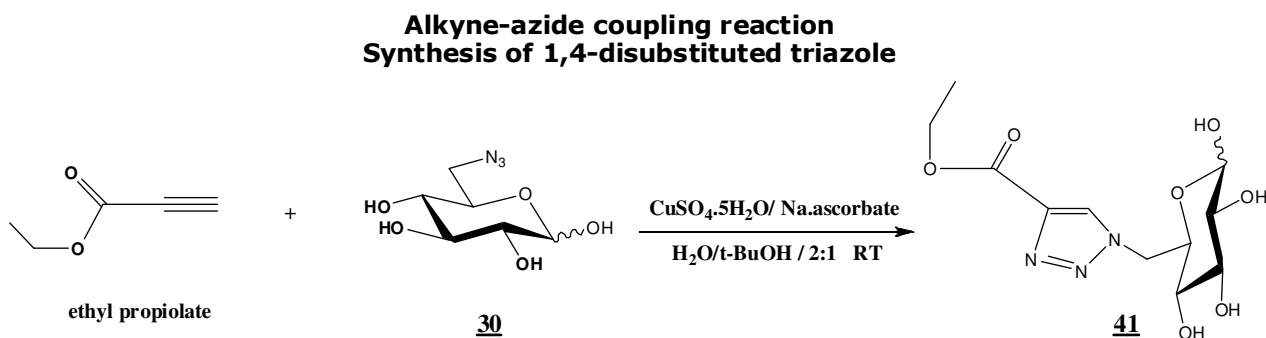
**Figure 25** Reaction of the azidogluconolactone with a glycopeptide of interest

Finally the formed glycopeptide-azidogluconolactone can bind in a click reaction to the microtitre plate through triazole formation as shown in Figure 26.



**Figure 26** Formation of triazole on the microtitre plate

6-Azido-6-deoxy-D-glucose **30** was coupled with ethyl propiolate. The reaction between the azide derivatives and ethyl propiolate was done in the presence of copper (II) sulfate and sodium ascorbate in a ratio of 1:5. The total reaction was carried out in a mixture of 2:1 of water to *tert*-butyl alcohol at room temperature for 8h to afford the 1,4-disubstituted triazole product in a crystalline form.

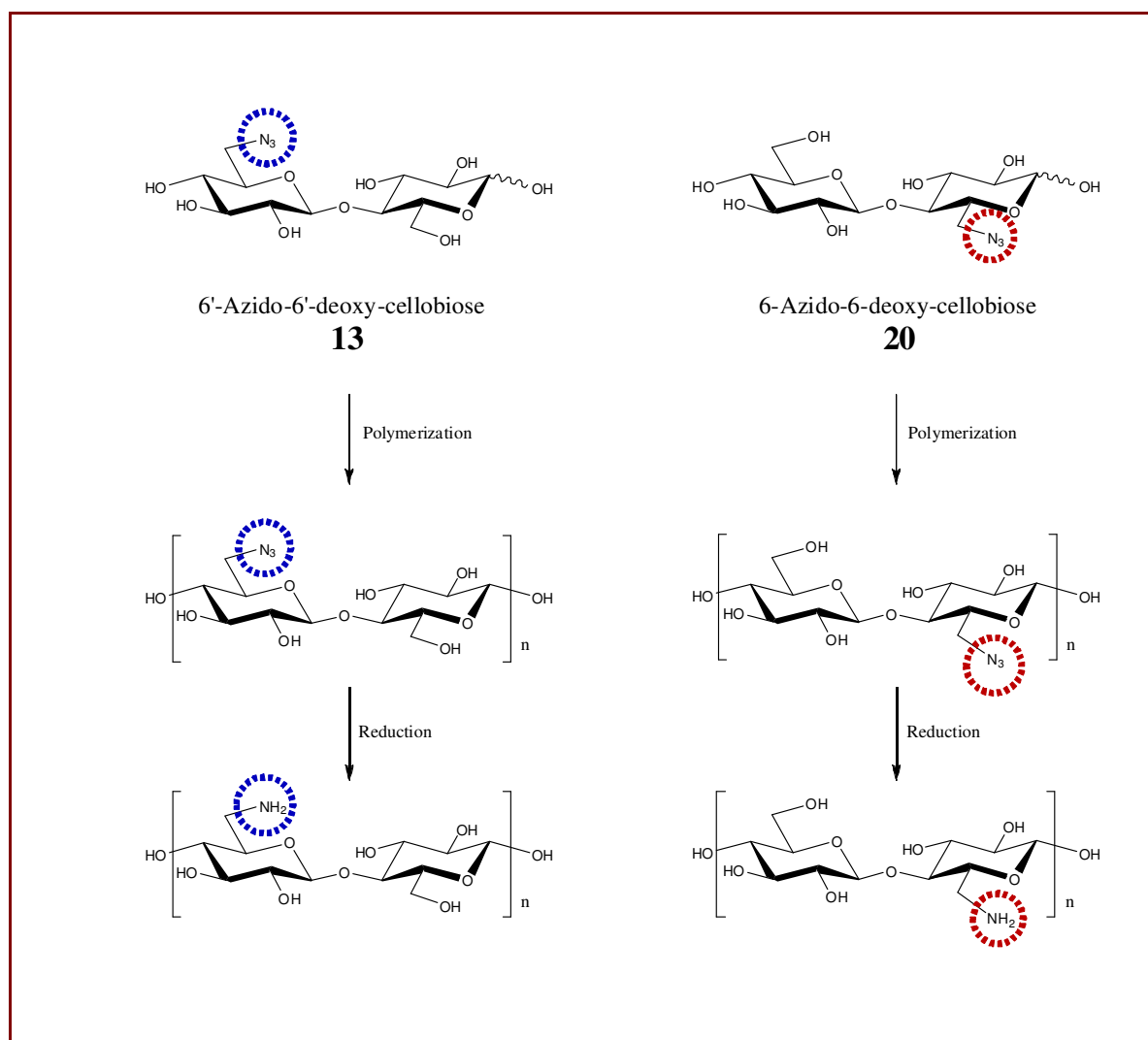


## 5.4 Pharmaceutical and Technical applications

The carbohydrate derived azides synthesized within this work can be used in different applications as follow;

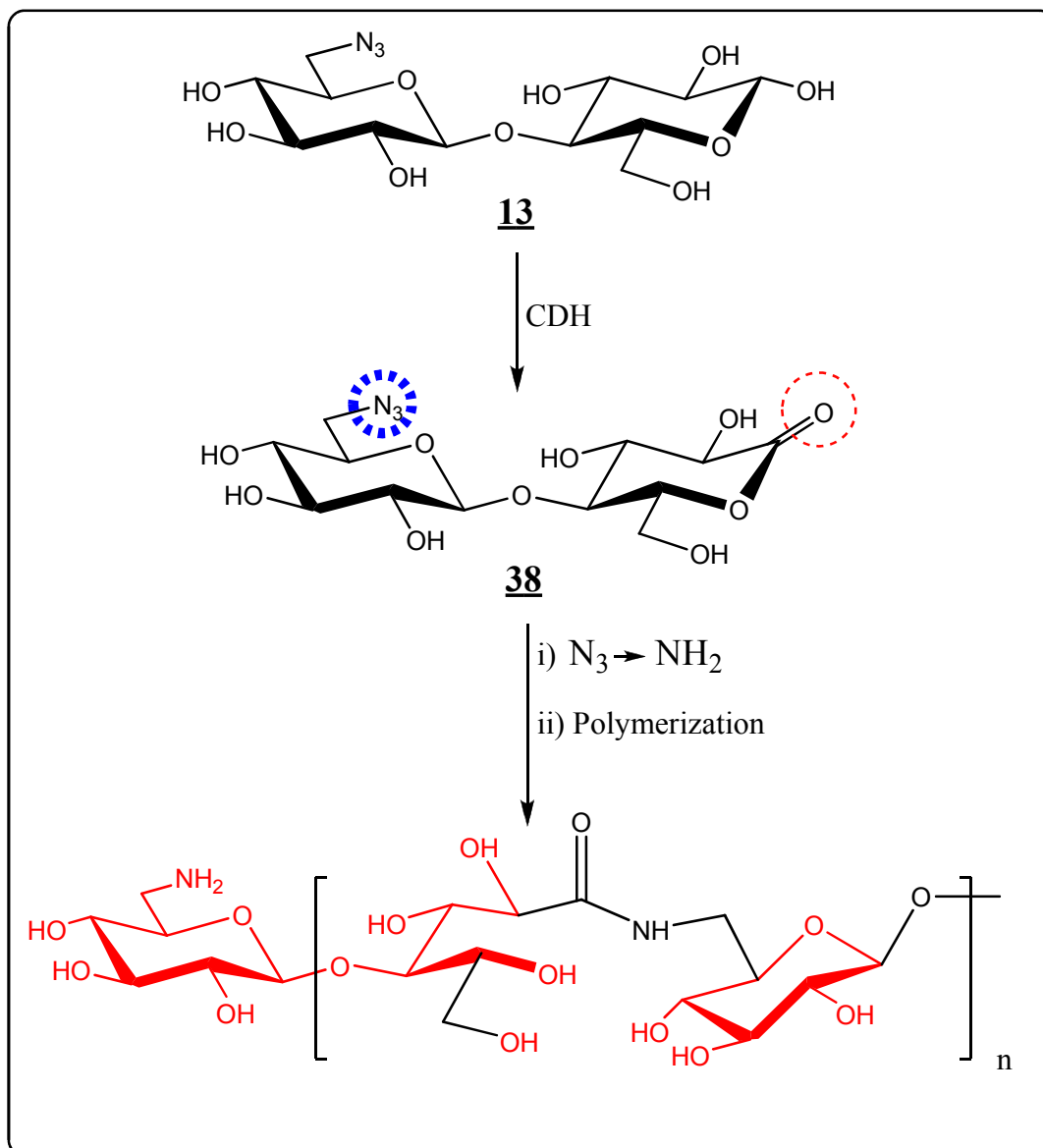
### 5.4.1 Azido-cellobiose derivatives

1. Precursors for the formation of **aminopolysaccharides** with unique structures. These aminopolysaccharides can play an important role as pharmaceutical excipients



Aminopolysaccharides with amino groups on different glucose moieties

- In the case of 6-azido-6-deoxy-D-cellobiose, the resulting polymer carries the azido group on the reducing glucose moiety.
  - While in the case of 6'-azido-6'-deoxy-D-cellobiose the azido group is located on the non-reducing glucose unit.
2. **6'-Azido-6'-deoxy-cellobionolactone 38** can act as a starting material for the preparation of new hydrogels shown in Figure 27.



**Figure 27** shows the polymerization of 6'-azido-6'-deoxy-cellobionolactone to a hydrogel

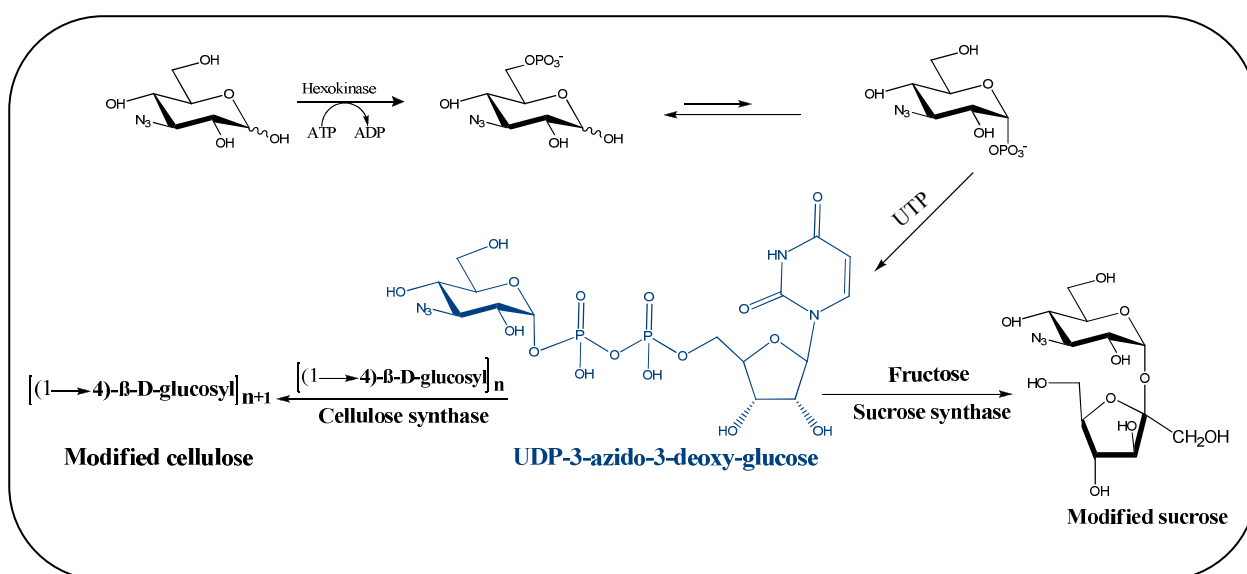
The polymerization reaction leads to a biodegradable hydrogel with unique structure, such biodegradable hydrogels can find wide applications in the improvement of existing dosage forms and the development of new and better drug delivery systems.



## 5.4.2 Azido glucose derivatives

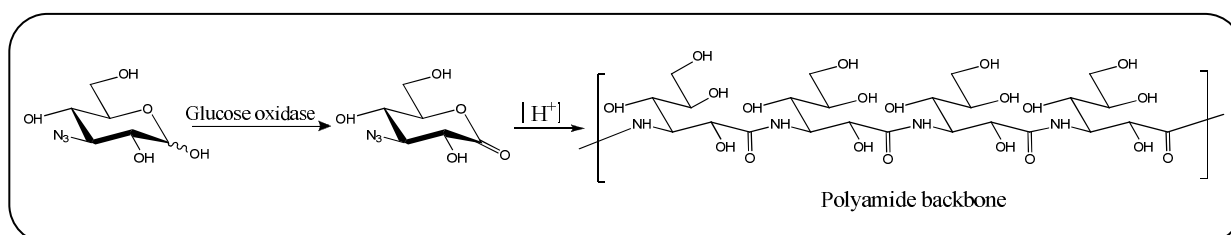
### 3-Azido-3-deoxy-D-glucose

3-Azido-3-deoxy-D-glucose can be transformed under catalysis by hexokinase and ATP into 3-azido-3-deoxy-D-glucose-6-phosphate, the latter can be incorporated as a glucose analog into an UDP sugar molecule to give the UDP-3-azido-3-deoxy-glucose, which could be used as a substrate for diverse synthase enzymes for example sucrose synthase and cellulose synthase. In the first example a synthesis of new sweetener is possible while in the second case the incorporation of 3-amino-3-deoxy-D-glucose in a polysaccharide can be considered.



**Figure 28:** Incorporation of 3-azido-3-deoxy-D-glucose in UDP

Another application for 3-azido-3-deoxy-D-glucose could drive from its ability to be oxidized to the corresponding lactone which is an appropriate precursor for the polymerization of a new polyamide backbone.

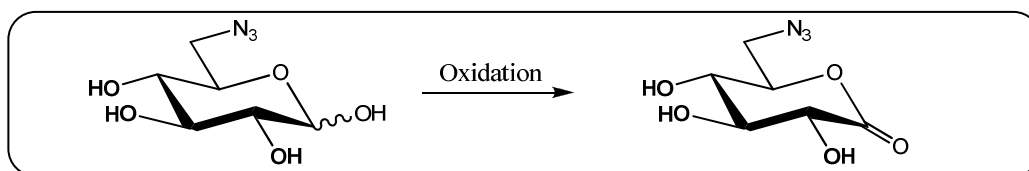


**Figure 29** Hypothetical polyamide backbone from 3-azido-3-deoxy-D-glucose

### 5.4.3 Applications of 1,2,3-Triazoles

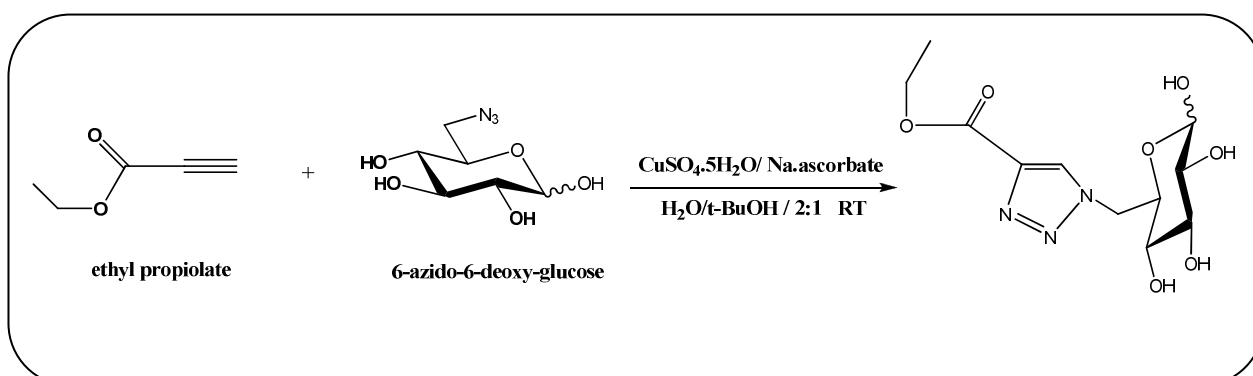
It is reported that 1,2,3-triazoles show biological activities and there are numerous examples in the literature including anti-HIV activity, antimicrobial activity against gram positive bacteria and selective  $\beta$ 3 adrenergic receptor agonism<sup>61</sup>. Moreover 1,2,3-triazoles have found broad use in agrochemicals and industrial applications such as dyes and corrosion inhibitors, photostabilizers and photographic materials<sup>62</sup>.

1,2,3- Triazoles derived from azido sugars offer other advantages owing to the presence of the aldehyde function of glucose that can be oxidized to carboxylic acids.



The later can be transformed into esters or lactones and can bind to glycopeptides through an amide bond. This property offers 1,2,3-triazole the ability to act as a linking arm and can be widely used in diagnostic purposes.

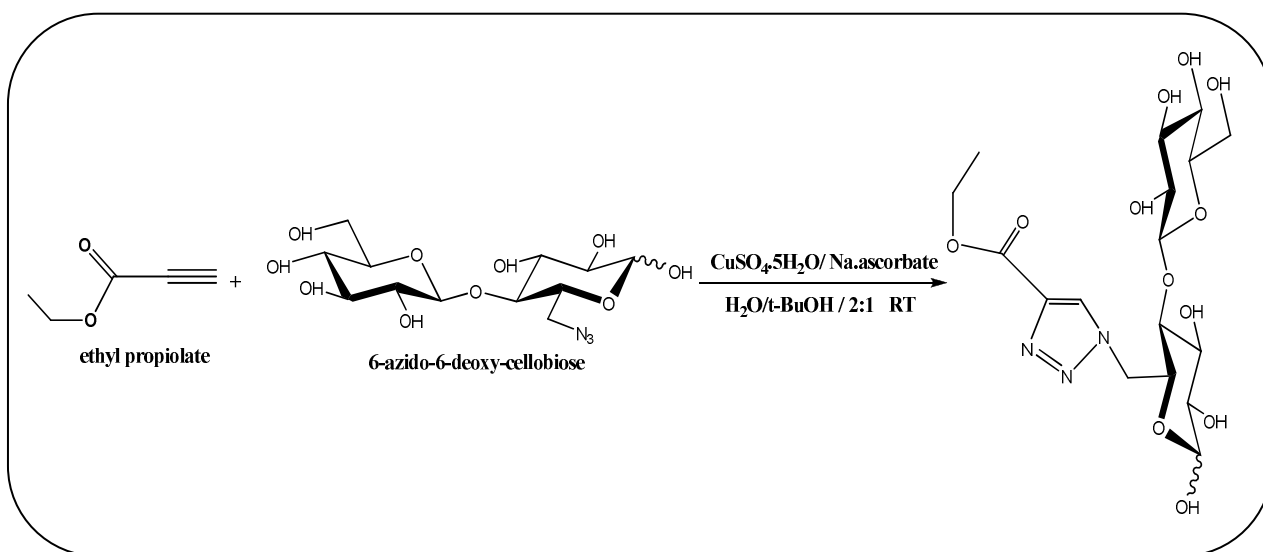
The Figure below show the formation of a 1,2,3-triazole from ethyl propiolate as alkyne and 6-azido-6-deoxy-D-glucose as azido sugar.



**Figure 30** bifunctionalized triazole from ethylpropiolate and 6-azido-6-deoxy-D-glucose

Both the propiolate and sugar moieties contain additional functional groups suitable for coupling to conventional carriers and biological ligands prior to triazole formation.

In a similar way, 6-azido-6-deoxy-cellobiose **20** could potentially be coupled with the same alkyne to afford the 1,4- disubstituted triazole product as shown in Figure **31**.



**Figure 31** Bifunctionalized triazole from ethylpropiolate and 6-azido-6-deoxy-D-cellobiose

Beside the application of these 2 triazoles as linking arms for diagnostic purposes, they could also be tested for biological activity, for example if they show any cytotoxic properties.

## 6. Outlook

The organic-chemical and enzyme-catalyzed syntheses described in this work are a part of a wide plan aims to postulate innovative synthetic strategies for different structures including aminopolysaccharides and hydrogels. Such biomimetic artificial materials can solve many problems accompanied with the naturally occurring polymers such as the presence of copolymers of biogenic origin. Furthermore such materials can be used in pharmaceutical, medical and technical applications.

Concerning azido derivatives of cellobiose, this work can be enriched if other derivatives can be synthesized; e.g. the introduction of azido group in the 2<sup>nd</sup> and 3<sup>rd</sup> positions of both reducing and non-reducing glucose moieties of cellobiose.

As to the enzymatic synthesis using cellobiose phosphorylase, there is a need for cooperation with enzymologists to develop genetically modified mutants of CBP that catalyzes only the reversal synthetic reactions rather than the forward one. In this manner, the large scale synthesis of new sugars will be possible. Our observation that CBP can also catalyze the synthesis of a trisaccharide is considered to be of great importance and needs more verification.

Concerning the triazole synthesis and their applications, screening tests may be needed to verify any biological activity and in case of positive results the formation of these stable triazoles can be expanded, so that new library of different triazoles prepared from all azido sugars gained in this work can be achieved.

For the application of the coupled product in diagnostics some efforts are needed in order to quantify the binding glycopeptide of interest on the microtitre plate. This work is under development.

## 7. Experimental Section

### 7.1. Materials

- Cellobiose (compound 1), 1,2:5,6-Di-O-isopropylidene- $\alpha$ -D-glucofuranose (compound 31), were purchased from GLYCON Biochemicals, Luckenwalde, Germany.
- Acetic anhydride, acetone, acetonitrile, activated charcoal (untreated, granular, 4-8 mesh), allyl alcohol, benzaldehyde, benzene, benzoyl chloride, calcium hydride, chloroform,  $\alpha$ -D-Cellobiose octaacetate 98%, Diazald<sup>®</sup>, dichloromethane, diisopropyl ether, dimethyl sulphoxide, Drierite<sup>®</sup> 6 mesh with and without indicator, ethylacetate, glacial acetic acid, ethanol, hexane, hydrobromic acid solution 33% in acetic acid, isopropanol, magnesium sulfate, magnesium turnings, mercuric cyanide, methanesulfonyl chloride, methanol, molecular sieves 4 Å pellets, N,N-dimethylformamide(DMF), silver fluoride, sodium azide, sodium bicarbonate, sodium borohydride, sodium carbonate, sodium chloride, sodium hydrogen sulfate, palladium(II)chloride, propanol, pyridine, zinc chloride, toluene, were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

Dried pyridine: commercially available pyridine (Sigma-Aldrich) was refluxed over calcium hydride for 4h, and then distilled from calcium hydride with exclusion of moisture.

Dried chloroform: commercially available chloroform was refluxed over P<sub>2</sub>O<sub>5</sub> for 4h and then distilled.

Dried dichloromethane: commercially available dichloromethane was refluxed over calcium hydride for 4h, and then distilled from calcium hydride with exclusion of moisture.

Dried acetonitrile: commercially available acetonitrile was refluxed over calcium hydride for 4h, then distilled from calcium hydride with exclusion of moisture.

Dried methanol: 500 ml commercial methanol (Sigma-Aldrich) reacted with magnesium turnings (~1 g), refluxed for 4h, and then distilled from the Mg-methoxide formed with exclusion of moisture (Drierite® drying tube).

Dried allyl alcohol: 500 ml commercially available allyl alcohol (Aldrich) was dried over ~80 g Drierite® for 48h at room temperature.

Dried dimethylformamide: 500 ml commercially available dimethylformamide (Aldrich) was dried over ~80 g Drierite® for 48h at room temperature.

Dried dimethyl sulphoxide: 500 ml commercially available dimethyl sulphoxide was dried over preheated molecular sieves® 4Å for 48h.

Dowex® mixed bed (AG 501-X8 Resin), and Dowex® 50-H<sup>+</sup> (Ag 50W-X8 Resin): Bio-Rad Laboratories, Richmond, California

Cellobiose phosphorylase: from *Clostridium thermocellum* was extracted in our laboratories as described under methods.

Cellobiose dehydrogenase: was obtained from Dr. Roland Ludwig, Department of Food Science and Technology, BOKU, University of Applied Life Sciences, Muthgasse 18, 1190 Wien.

## 7.2 Methods

- Preparative Column Chromatography was performed on columns of Silica gel 60 (0,040-0,063 mm), supplied by Merck KgaA, Darmstadt, Germany.
- Thin layer chromatography (TLC): Thin layer chromatograms were developed in glass jars on glass-backed, standard 5×10 cm plates of silica gel 60 F<sub>254</sub> supplied by Merck KgaA, Darmstadt, Germany. For visualization, plates were sprayed, using glass vaporizer bottles equipped with a rubber balloon, with a modified anisaldehyde-sulfuric acid spray reagent (anisaldehyde, 0.5 ml; ethanol, 9 ml; conc. H<sub>2</sub>SO<sub>4</sub>, 0.5 ml; glacial acetic acid, 0.1 ml)<sup>63</sup>. To protect the operator from the harmful mist, it is advantageous to use a spraying cabinet in a well-ventilated hood. After spraying, plates were heated for ~3 min on a hotplate equipped with an aluminium block at a setting of ~150°C (not calibrated). Colorations are best observed immediately, as differences in color shades tend to become less prominent with time.

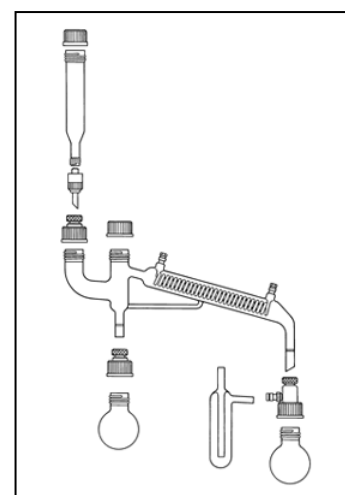
For graphic TLC representation, sprayed plates were scanned with the aid of a canon scanner, and the digital files printed directly or edited using the program Adobe Photoshop 5.0. Edited chromatograms have been prepared to emphasize the differences in colorations rather than to indicate quality of the chromatographic separations or purity of the materials used.

- Preparative layer chromatography (PLC): 20×20 cm PSC-plates of silica gel 60 F<sub>254</sub>, 2 mm, supplied by Merck KgaA, Darmstadt, Germany.
- Melting points were determined on a *Kofler* hot stage and are uncorrected.
- Optical rotations were performed with the aid of a “Perkin Elmer 341” polarimeter.
- Elemental analyses were performed with a “2400 CHN Elemental Analyzer” from Perkin Elmer.
- Infra-red-spectra were performed with a “Perkin Elmer 1600 FT-IR” spectrometer on silica-plates.

- NMR-spectra were performed with NMR-spectrometers from Bruker, an “Avance DPX 250” at 250.13 and 62.90 MHz, an „Avance DRX 400“ at 400.13 and 100.61 MHz, and an „Avance DRX 600“ at 600.13 and 150.92 MHz. The calibration was performed on internal standards of solvents ( $^1\text{H-NMR}$ :  $\text{CHCl}_3$  in  $\text{CDCl}_3$  7.24 ppm,  $\text{H}_2\text{O}$  in  $\text{D}_2\text{O}$  4.79 ppm;  $^{13}\text{C-NMR}$ :  $\text{CDCl}_3$  77.00 ppm). The chemical shift ( $\delta$ ) is given in ppm, the coupling constant  $J$  is given in Hertz (Hz).

### Synthesis of diazomethane;

5 g of Diazald were suspended in ether, then dropped through dropping funnel into the KOH solution, followed by distillation at  $65^\circ\text{C}$ . The formed diazomethane was collected in a cooled round bottom flask, the solution is yellow Brown. The obtained diazomethane was applied directly.



**Fig. 32** Distillation apparatus for diazomethane

Sigma-Aldrich catalogue 2007

### Extraction of *Cellobiose phosphorylase*;

- Fermentation and harvest of the anaerobic bacterium, *Clostridium thremocellum*, as a source of Cellobiose phosphorylase - *Clostridium thremocellum* NCIMB 10682 was used as CBPase producer and was cultured in a medium containing the following (g/liter): Cellobiose, 2.0g; Yeast extract, 2.0g;  $(\text{NH}_4)$   $\text{SO}_4$ , 1.30g;  $\text{KH}_2\text{PO}_4$ , 1.50g;  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 2.90g;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.0g;  $\text{CaCl}_2$ , 0.15g; Resazurin (0.2%), 1.0ml;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (5%), 25.0 $\mu\text{l}$ ; distilled water, 1l. The microorganism was incubated anerobically at  $60^\circ\text{C}$ . After incubation cells were harvested by centrifugation.
- Sonication of the bacteria – Sonication was performed by ultrasonic probe five times for 20 sec on ice to break down the cells. Cell debris was removed by



centrifugation. The cleared lysate (supernatant) was concentrated with VIVASPIN 20 by centrifugation to a volume of 10ml.

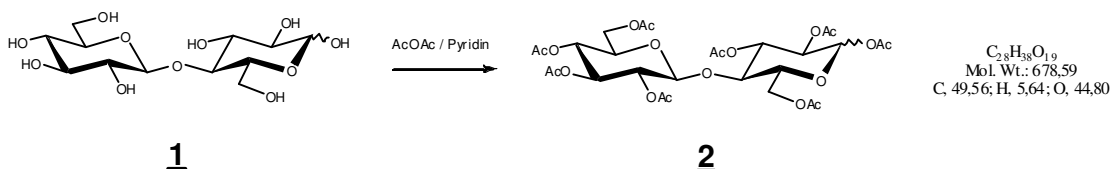
- c) Column chromatographic enrichment and purification of the enzyme CBPase  
– The native CBPase was purified using a column chromatography procedure (DEAE) as reported by Alexander<sup>64</sup>.
- d) Thin layer chromatography (TLC): To ensure the activity of the enzyme CBP, the purified extract was incubated with cellobiose in the presence of phosphate buffer pH 7.0 at 37°C. In order to detect the phosphorolysis of D-cellobiose a TLC-Assay was performed subsequently.

### **7.3 Overview of carbohydrate syntheses. Schemes of syntheses**

- |   |                 |             |
|---|-----------------|-------------|
| <b>1. Synthesis of 6'-azido-6'-deoxy-cellobiose</b> | <b>Scheme 1</b> | see page 44 |
| <b>2. Synthesis of 6-azido-6-deoxy-cellobiose</b>   | <b>Scheme 2</b> | see page 46 |
| <b>3. Synthesis of 6-azido-6-deoxy-D-glucose</b>    | <b>Scheme 3</b> | see page 48 |
| <b>4. Synthesis of 3-azido-3-deoxy-D-glucose</b>    | <b>Scheme 4</b> | see page 50 |
| <b>5. Synthesis of <math>\beta</math>-D-allose</b>  | <b>Scheme 5</b> | see page 51 |

## 7.4 Chemical syntheses description

### 1,2,3,6-Tetra-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl)-D-glucopyranoside **2**



#### Preparation:

In a 500 ml round bottom flask (50g, 0.145 mole) cellobiose **1** was suspended in (150 ml) dry pyridine, 150 ml (1.575 mole) acetic anhydride was dropped from a dropping funnel sealed with a drying tube (Drierite®), into the suspension. The reaction mixture was cooled in ice-water bath, stirred with a magnetic stirrer and allowed to come to room temperature. After stirring over night, the content of the flask was poured onto ice and stirred further for 30 min. The formed product was filtered under vacuum and washed several times with distilled water. The product was stored under vacuum. The mother liquor was evaporated and treated again similar to the previous steps. The 2 products were gathered and dried in a dessicator over Drierite® and gave a total yield 84.38 g 0.124mole (85%) of **2**.

#### Characterisation:

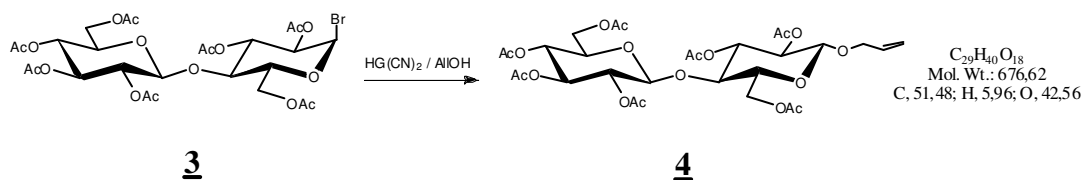
TLC (EE:T 3:2):  $R_F$             educt **1** 0.00  
   product **2** 0.68

**2**: Optical rotation:  $[\alpha]_D^{20} = -12.1^\circ$  (CHCl<sub>3</sub>; 0.5 g/100 ml)

**2**: m.p.: 198-199°C



2',3',4',6'-Tetra-O-acetyl-β-D-glucopyranosyl-(1→4)-allyl 2,3,6-tri-O-acetyl-β-D-glucopyranoside **4**



Preparation: <sup>66</sup>

In a 500 ml round bottom flask, hepta-O-acetyl cellobiosyl bromide (45.0 g 0.064 mole) **3** was dissolved in 100 ml dry  $\text{CH}_2\text{Cl}_2$ , 10 g Drierite® was added. To the magnetically stirred solution 12 g of mercury (II) cyanide was added and the flask sealed with a drying tube (Drierite®). After 36 hours stirring at room temperature, thin layer chromatography (EE: T / 3:2) indicated complete consumption of **3**. The reaction mixture was then diluted with 50 ml of  $\text{CH}_2\text{Cl}_2$  stirred for further 30 min., the reaction mixture was then filtered over Celite®, and the filtrate was evaporated under vacuum to give 42 g (0.062 mole) of **4**. The product was recrystallized from isopropanol to give a yield of 80% (35.0 g) of compound **4**.

Characterisation:

TLC (EE:T/ 3:2):  $R_F$     educt **3** 0.58  
product **4** 0.62

**4**: Optical rotation:  $[\alpha]_D^{20} = -39.43^\circ$  ( $\text{CHCl}_3$ ; 0.05 g/100 ml)

**4**: m.p.: 190-191 °C

**<sup>1</sup>H-NMR** (CDCl<sub>3</sub>, 400 MHz) δ [ppm]:

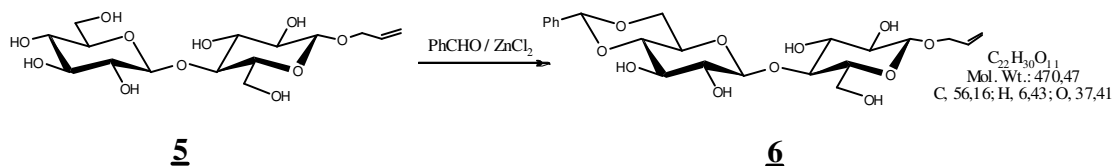
5.33-5.77 (m; 1H; allyl -CH<sub>2</sub>-CH=CH<sub>2</sub>); 5.28-5.19 (m; 2H; allyl =CH<sub>2</sub>); 5.17 (dd; 1H; H3'; J<sub>2',3'</sub>=9.37 Hz); 5.13 (dd; 1H; H3; J<sub>3,4</sub>=9.37 Hz); 5.04 (dd; 1H; H4; J<sub>4,5</sub>=9.54 Hz); 4.94-4.89 (m; 2H; H2', H2); 4.53-4.49 (m; 3H; H1', H1, H6a; J<sub>5,6a</sub>=7.89 Hz); 4.36 (dd; 1H; H6a'; J<sub>5',6a'</sub>=4.44 Hz; J<sub>6a',6b'</sub>=12.42 Hz); 4.12-4.02 (m; 3H; allyl -O-CH<sub>2</sub>-, H6b', H6b); 3.78 (dd; 1H; H4'; J<sub>3',4'</sub>=9.37 Hz; J<sub>4',5'</sub>=9.62); 3.65 (m; 1H; H5); 3.57 (m; 1H; H5'); 2.20-1.95 (7 s; 21H; -COCH<sub>3</sub>).

**<sup>13</sup>C-NMR** (CDCl<sub>3</sub>, 100 MHz) δ [ppm]:

170.86, 170.68, 170.59, 170.19, 169.97, 169.68, 169.41 (7C, -COCH<sub>3</sub>); 133.72 (allyl -CH=CH<sub>2</sub>); 118.02 (allyl=CH<sub>2</sub>); 101.16 (C1); 99.77 (C1'); 73.33 (C3'); 73.07 (C3); 72.93 (C5'); 72.36 (C5); 72.02 (C2); 71.95 (C2'); 70.41 (allyl -O-CH<sub>2</sub>-); 62.26 (C6); 61.96 (C6'); 21.23-20.91 (7C, -COCH<sub>3</sub>).



4',6'-O-Benzylidene- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-allyl  $\beta$ -D-glucopyranoside **6**



Preparation: <sup>67</sup>

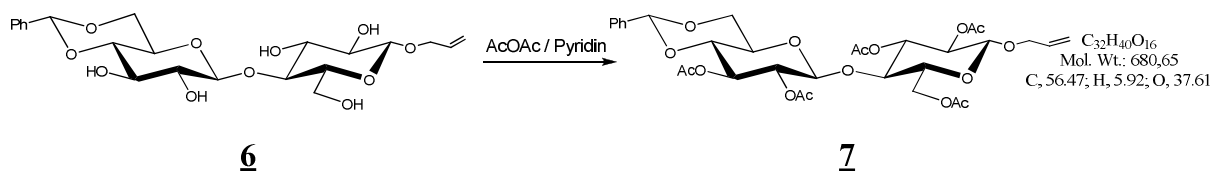
In a round bottom flask, 50 ml of freshly distilled benzaldehyde and 7.3 g of preheated zinc chloride were added to (8.0 g, 0.021 mole) of compound **5**. The flask was tightly stoppered and shaken on a mechanical shaker at 200 rpm for 36 hours. After TLC indicated the complete consumption of the educt, the product was extracted as follows.

- In a 600 ml beaker, a saturated solution of  $NaHCO_3$  (200 ml) and hexane (100 ml) were added
- The solution was vigorously stirred with a mechanical stirrer
- The reaction mixture was pipetted into the stirred solution
- The hexane phase was discarded after 20 min. stirring and a fresh 100 ml of hexane was added; this step was repeated 4 times.
- The precipitated product was then filtered under vacuum and washed twice with hexane, then several times with distilled water.
- Finally the product was stored in a desiccator and dried under vacuum to give 9.48 g (0.020 mole) of **6** with a yield of 96%.





2',3'-Di-O-acetyl-4',6'-O-benzylidene- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-allyl 2,3,6-tri-O-acetyl- $\beta$ -D-glucopyranoside 7



Preparation:

Compound 6 (9.0 g, 0.019 mole) was suspended in 30 ml of dry pyridine, after the addition of 30 ml 0.315 mole of acetic anhydride the reaction mixture was stirred in an ice-water bath overnight. After TLC had indicated the complete consumption of the educt, the whole content of the flask was poured onto ice water. The formed product was then filtered under vacuum and washed several times with distilled water. The product was dried in a desiccator and was recrystallized from isopropanol to afford 12.3 g (0.018 mole) of 7 with a yield of 95%.

Characterisation:

TLC (ethyl acetate:isopropanol / 10:1):  $R_F$  educt 6 0.40  
product 7 0.75

7: Optical rotation:  $[\alpha]_D^{20} = -50.03^\circ$  ( $\text{CHCl}_3$ ; 0.5 g/100 ml)

7:m.p.: 228-230  $^\circ\text{C}$

**IR:**  $\nu$  [ $\text{cm}^{-1}$ ] 2876 (w), 1300 (s) =CH<sub>2</sub> valency and deformation oscillation, 1372 (O)COCH<sub>3</sub> valency oscillation.

**<sup>1</sup>H-NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  [ppm]:

7.40-7.38 (m; 2H; aromatic protons); 7.34-7.32 (m; 3H; aromatic protons); 5.76-5.87 (m; 1H; allyl -CH<sub>2</sub>-CH=CH<sub>2</sub>); 5.45 (s; 1H; -CHPh); 5.23 (dd; 2H; allyl=CH<sub>2</sub>, H<sub>3'</sub>; J<sub>3',4'</sub>=9.45 Hz); 5.15 (dd; 2H; allyl=CH<sub>2</sub>, H<sub>3</sub>; J<sub>3,4</sub>=9.45 Hz); 4.95 (dd; 1H; H<sub>2'</sub>; J<sub>2',3'</sub>=8.29 Hz); 4.89 (dd; 1H; H<sub>2</sub>; J<sub>2,3</sub>=8.29 Hz); 4.57 (d; 1H; H<sub>1'</sub>; J<sub>1',2'</sub>=7.79 Hz); 4.49 (d; 1H; H<sub>1</sub>; J<sub>1,2</sub>=8.12 Hz); 4.47 (dd; 1H; H<sub>6a</sub>; J<sub>6a,6b</sub>=10.12 Hz); 4.33 (dd; 1H; H<sub>6a'</sub>; J<sub>5',6a'</sub>=4.98 Hz; J<sub>6a',6b'</sub>=9.45 Hz); 4.30-4.02 (m; 2H; allyl-O-CH<sub>2</sub>); 4.08-4.02 (m; 2H; allyl -O-CH<sub>2</sub>, H<sub>6b</sub>); 3.78 (dd; 1H; H<sub>4</sub>; J<sub>4,5</sub>=8.46 Hz); 3.70 (dd; 1H; H<sub>6b'</sub>; J<sub>5',6b'</sub>=10.12 Hz); 3.78 (dd; 1H; H<sub>4'</sub>; J<sub>4',5'</sub>=9.62 Hz); 3.55 (ddd; 1H; H<sub>5</sub>); 3.45 (ddd; 1H; H<sub>5'</sub>); 2.10 (s; 3H; -COCH<sub>3</sub>); 2.03 (s; 3H; -COCH<sub>3</sub>); 2.01 (s; 3H; -COCH<sub>3</sub>); 1.99 (s; 6H; -COCH<sub>3</sub>).

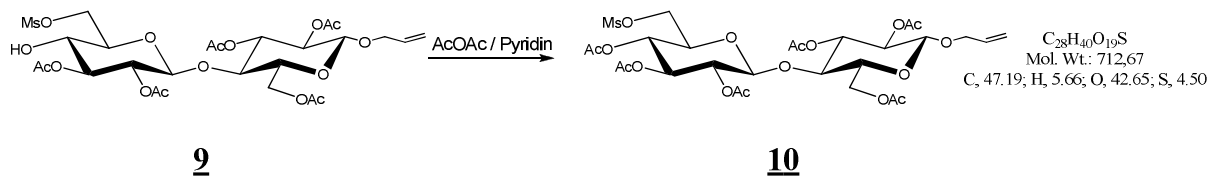
**<sup>13</sup>C-NMR** (CDCl<sub>3</sub>, 100 MHz)  $\delta$  [ppm]:

171.75, 171.53, 171.01, 170.96, 170.79 (5C, -COCH<sub>3</sub>); 137.99 (1 quaternary aromatic carbon atom); 134.75 (allyl -CH=CH<sub>2</sub>); 130.63, 130.40, 129.68, (5 tertiary aromatic carbon atoms); 127.51 (-CHPh); 119.04 (allyl =CH<sub>2</sub>); 102.96 (C<sub>1'</sub>); 100.67 (C<sub>1</sub>), 77.87 (C<sub>4'</sub>); 77.21 (C<sub>4</sub>); 74.86 (C<sub>3</sub>); 74.00 (C<sub>5</sub>); 73.93 (C<sub>2</sub>); 73.34 (C<sub>3'</sub>); 73.16 (C<sub>2'</sub>); 73.12 (C<sub>6</sub>); 70.70 (C<sub>6'</sub>); 67.76 (C<sub>5'</sub>); 63.31 (allyl -O-CH<sub>2</sub>-); 22.38-21.91 (5C; -COCH<sub>3</sub>).





2',3',4'-Tri-O-acetyl-6'-O-methylsulfonyl-β-D-glucopyranosyl-(1→4)-allyl 2,3,6-tri-O-acetyl-β-D-glucopyranoside **10**



Preparation:

To product **9** (4.85 g 7.23 mmole), 30 ml of dry pyridine and 30 ml of acetic anhydride were added. The reaction mixture was stirred in an ice-water bath over night. After TLC has indicated the complete consumption of the educt, the reaction mixture poured onto ice, stirred, filtered under vacuum and washed several times with distilled water. The product was then recrystallized from 96% ethanol to give 4.80 g (6.7 mmole) of pure crystals of **10** with a yield of 92%.

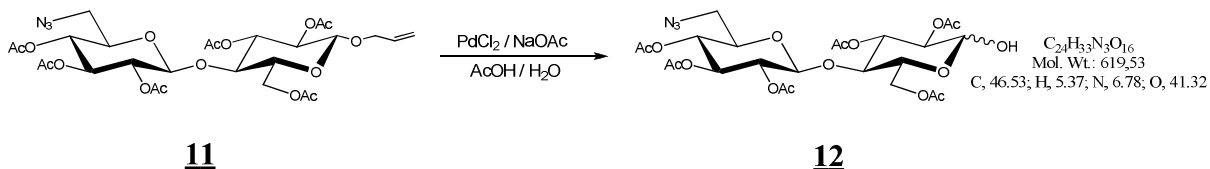
Characterisation:

TLC (EE: T / 3:2):  $R_F$  educt **9** 0.41  
product **10** 0.65

**10**: m.p.: 204°- 206°C



2',3',4'-Tri-O-acetyl-6'-azido-6'-deoxy- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-2,3,6-tri-O-acetyl- $\beta$ -D-glucopyranoside **12**



Preparation:<sup>68</sup>

In a round bottomed flask 750 mg (1.14 mmole) of compound **11**, was dissolved in 60 ml of 88% acetic acid. To this solution, 350 mg of sodium acetate and 350 mg of PdCl<sub>2</sub> were added. The reaction mixture was stirred for 48 hours, after that TLC had shown the complete consumption of **11**. The reaction mixture was neutralized with saturated solution of NaHCO<sub>3</sub>, 50 ml chloroform added and the mixture filtered over Celite®, chloroform phase separated in a separatory funnel, dried over anhydrous MgSO<sub>4</sub>, filtered and evaporated under vacuum to afford 680 mg (1.1 mmole) of **12** with a yield of 96%. Column chromatography (EE: T/ 3:2) afforded 570 mg (0.92 mmole) pure crystals of **12** (80% total yield).

Characterization:

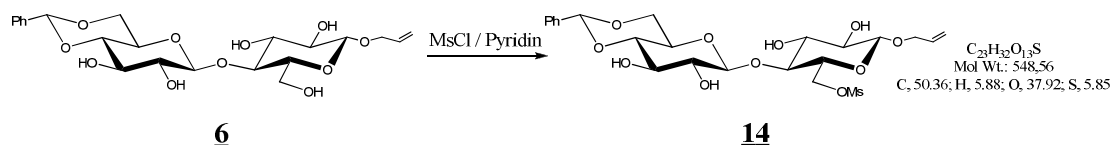
TLC (EE: T / 3:2): R<sub>F</sub>      educt **11** 0.65  
   product **12** 0.24

**12**: m.p.: 170 °-172 °C





4',6'-O-Benzylidene- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-allyl 6-O-methylsulfonyl- $\beta$ -D-glucopyranoside **14**



Preparation:

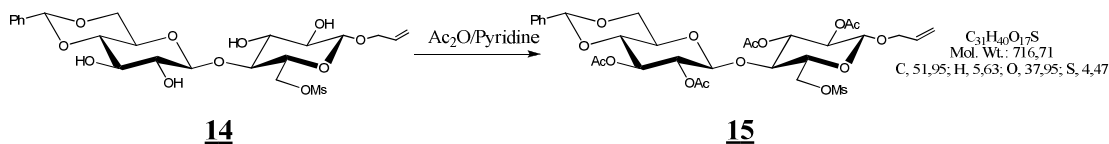
In a 250 ml round bottom flask 8.0 g (0.017 mole) of compound **6** was dissolved in 75 ml dry pyridine. To the magnetically stirred solution, 1.5 ml (0.0187 mole) of methanesulfonyl chloride diluted with 10 ml of dry  $\text{CH}_2\text{Cl}_2$  was dropped through a dropping funnel over 4h. The reaction was cooled at  $-30^\circ\text{C}$  using dry ice. After TLC has indicated the complete consumption of the starting material the reaction mixture was poured onto ice, stirred for a further 30 min, then extracted with chloroform. The chloroform phase was washed once with  $\text{NaHSO}_4$  then with  $\text{NaHCO}_3$ , dried over anhydrous  $\text{Mg}_2\text{SO}_4$ , filtered and finally evaporated under vacuum to give 8.50 g (0.015 mole) of the syrupy product of **9** ( yield of 91%) which is directly applied for the next step without any purification.

Characterisation:

TLC (EE: ISO / 10:1):

$R_F$  educt **6** 0.36  
product **14** 0.51

2',3'-Di-O-acetyl-4',6'-O-benzylidene-β-D-glucopyranosyl-(1→4)-allyl 2,3-di-O-acetyl-6-O-methylsulfonyl-β-D-glucopyranoside **15**



Preparation:

Compound **14** (8.5 g 0.015 mole) was suspended in 70 ml of dry pyridine, and acetic anhydride (70 ml) was added to the magnetically stirred solution under cooling in an ice-water bath. The vessel was sealed with a drying tube (Drierite®), allowed to come to room temperature, and stirring continued over night. TLC indicated the end of the reaction. The content of the flask was then poured onto ice water and extracted with chloroform and the chloroform phase washed with saturated solutions of sodium hydrogen sulfate and sodium hydrogen carbonate, respectively. The organic phase was dried over anhydrous magnesium sulfate, filtered and evaporated under vacuum to give 10.50 g (0.015 mole) of compound **15** in 98% yield.

Characterisation:

TLC (EE:T / 3:2):  $R_F$

educt **14** 0.28

product **15** 0.76

**82**: m.p.: 192-194 °C

**IR:**  $\nu$  [ $\text{cm}^{-1}$ ] 2876 (w), 1300 (s) =CH<sub>2</sub> valency and deformation oscillation, 1372 (O)COCH<sub>3</sub> valency oscillation, 1740(s) -C=O valency oscillation, 1360 (s), 1178 (s) – O-SO<sub>2</sub> oscillation.

**<sup>1</sup>H-NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  [ppm]:

7.41-7.38 (m; 2H; aromatic protons); 7.35-7.32 (m; 3H; aromatic protons); 5.84-5.77 (m; 1H; allyl –CH<sub>2</sub>-CH=CH<sub>2</sub>); 5.45 (s; 1H; -CHPh); 5.25 (dd; 1H; H3'; J<sub>3',4'</sub>=9.71 Hz); 5.25-5.19 (dd; 2H; allyl=CH<sub>2</sub>); 5.19 (m; 2H; allyl=CH<sub>2</sub>, H3); 4.93 (dd; 1H; H2'; J<sub>2',3'</sub>=7.92 Hz); 4.87 (dd; 1H; H2; J<sub>2,3</sub>=8.01 Hz); 4.66 (d; 1H; H1'; J<sub>1',2'</sub>=7.74 Hz), 4.53 (d; 1H; H1; J<sub>1,2</sub>=7.91 Hz); 4.53 (dd; 1H; H6a; J<sub>5,6a</sub>=1.89 Hz); 4.35-4.25 (m; 3H; allyl=CH<sub>2</sub>, H6a', H6b'); 4.10-4.05 (1H; allyl=CH<sub>2</sub>); 3.82 (dd; 1H; H4; J<sub>4,5</sub>=9.26 Hz); 3.70 (dd; 1H; H6b; J<sub>6a,6b</sub>=10.36 Hz); 3.65 (dd; 1H; H4'; J<sub>4',5'</sub>=10.63 Hz); 3.58 (m; 1H; H5); 3.47 (m; 1H; H5'); 3.07 (s; 3H; -OSO<sub>2</sub>-CH<sub>3</sub>); 2.08-1.99 (4 s; 12H; ; -COCH<sub>3</sub>).

**<sup>13</sup>C-NMR** (CDCl<sub>3</sub>, 100 MHz)  $\delta$  [ppm]:

171.41, 171.02, 170.95, 170.82 (4C, -COCH<sub>3</sub>); 137.99 (1 quaternary aromatic carbon atom); 134.53 (allyl –CH=CH<sub>2</sub>); 130.63, 129.68, 127.54, (5 tertiary aromatic carbon atoms); 119.27 (allyl =CH<sub>2</sub>); 103.21 (-CHPh); 103.00 (C1'); 100.79 (C1), 79.38 (C4'); 77.38 (C4); 77.21 (C3); 74.46 (C2'); 74.06 (C5); 73.93 (C3'); 73.84 (C2); 73.23 (allyl –O-CH<sub>2</sub>-); 72.94 (C6'); 67.77 (C6); 67.46 (C5'); 39.401 (1C; -OSO<sub>2</sub>-CH<sub>3</sub>); 22.34-22.05 (4C; -COCH<sub>3</sub>).



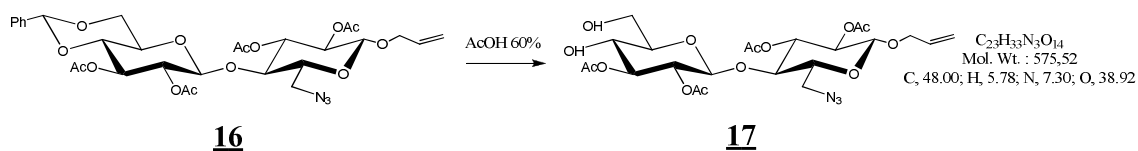
**<sup>1</sup>H-NMR** (CDCl<sub>3</sub>, 400 MHz) δ [ppm]:

7.42-7.38 (m; 2H; aromatic protons); 7.35-7.32 (m; 3H; aromatic protons); 5.86-5.79 (m; 1H; allyl -CH<sub>2</sub>-CH=CH<sub>2</sub>); 5.45 (s; 1H; -CHPh); 5.23 (dd; 1H; H3'; J<sub>3',4'</sub>=9.54 Hz); 5.25-5.16 (dd; 1H; allyl=CH<sub>2</sub>); 5.15 (m; 2H; allyl=CH<sub>2</sub>, H3; J<sub>3,4</sub>=9.24 Hz); 4.91 (dd; 1H; H2'; J<sub>2',3'</sub>=9.54 Hz); 4.89 (dd; 1H; H2; J<sub>2,3</sub>=9.32 Hz); 4.58 (d; 1H; H1'; J<sub>1',2'</sub>=7.80 Hz); 4.54 (d; 1H; H1; J<sub>1,2</sub>=7.94 Hz); 4.32 (dd; 1H; H6a'; J<sub>5',6a'</sub>=5.20 Hz; J<sub>6a',6b'</sub>=10.4 Hz); 4.30-4.06 (2H; allyl-CH<sub>2</sub>); 3.76 (dd; 1H; H4; J<sub>4,5</sub>=9.32 Hz); 3.70 (dd; 1H; H6b'; J<sub>5',6b'</sub>=10.33 Hz; J<sub>6a',6b'</sub>=10.33 Hz); 3.64 (dd; 1H; H4'; J<sub>4',5'</sub>=9.61 Hz); 3.48-3.56 (m; 2H; H5, H6a); 3.46 (dd; 1H; H5'); 3.38 (dd; 1H; H6b; J<sub>6a,6b</sub>=13.01 Hz; J<sub>5,6b</sub>=5.28 Hz); 2.03 (s; 3H; -COCH<sub>3</sub>); 2.01 (s; 3H; -COCH<sub>3</sub>); 2.005 (s; 3H; -COCH<sub>3</sub>); 2.002 (s; 3H; -COCH<sub>3</sub>).

**<sup>13</sup>C-NMR** (CDCl<sub>3</sub>, 100 MHz) δ [ppm]:

171.55, 170.96, 170.93, 170.63 (4C, -COCH<sub>3</sub>); 137.98 (1 quaternary aromatic carbon atom); 134.70 (allyl -CH=CH<sub>2</sub>); 130.64, 129.68, 127.52, (5 tertiary aromatic carbon atoms); 119.09 (allyl =CH<sub>2</sub>); 103.00 (-CHPh); 102.85 (C1'); 100.60 (C1), 79.36 (C4'); 78.69 (C4); 75.28 (C5); 74.47 (C3); 74.06 (C2); 73.24 (C2'); 72.97 (C3); 71.21 (allyl -O-CH<sub>2</sub>-); 69.82 (C6'); 67.82 (C5'); 51.47 (C6); 22.35 (1C; -COCH<sub>3</sub>), 22.12 (1C; -COCH<sub>3</sub>), 22.08 (1C; -COCH<sub>3</sub>), 22.06 (1C; -COCH<sub>3</sub>).

2',3',-Di-O-acetyl-β-D-glucopyranosyl-(1→4)-allyl 2,3-di-O-acetyl-6-azido-6-deoxy-β-D-glucopyranoside **17**



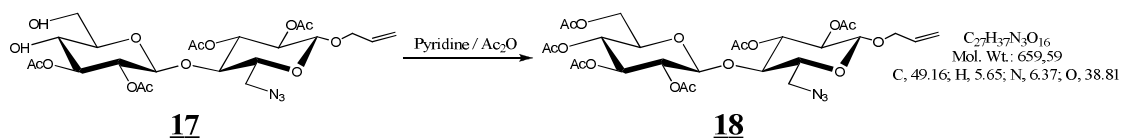
Preparation:

In 250 ml round bottomed flask compound **16** (8.45 g 0.013 mole) was suspended in 100 ml of 60% acetic acid solution and then refluxed at 100°C for 2 hours. After TLC (toluene/ethyl acetate 1:1) had indicated the complete consumption of the educt, the reaction mixture was left to cool to room temperature; subsequently the solution was neutralized with sodium bicarbonate and extracted with chloroform. The chloroform phase was dried over anhydrous  $\text{Mg}_2\text{SO}_4$ , filtered and evaporated under vacuum to give 5.90 g (0.010) mole of **17**, which was directly applied for the next step without further purification.

Characterization:

TLC (EE:T / 1:1):  $R_F$  educt **16** 0.65  
Product **17** 0.15

2',3',4',6'-Tetra-O-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-allyl 2,3-di-O-acetyl-6-azido-6-deoxy- $\beta$ -D-glucopyranoside **18**



Preparation:

In 250 ml round bottomed flask, compound **17** (5.90 g, 0.010 mole) was suspended in 100 ml of 60% acetic acid solution and then refluxed at 100°C for 2 h. After TLC (toluene/ethyl acetate 1:1) had indicated the complete consumption of the educt, the reaction mixture was left to cool to room temperature; subsequently, the solution was neutralized with sodium bicarbonate and extracted with chloroform. The chloroform phase was dried over anhydrous  $\text{Mg}_2\text{SO}_4$ , filtered and evaporated under vacuum to give 6.30 g (0.009 mole) white crystals of **18**, which were recrystallized from isopropanol.

Characterisation:

TLC (EE:T / 1:1):  $R_F$  educt **17** 0.29  
product **18** 0.72

**18**: Optical rotation:  $[\alpha]_D^{20} = -30.40^\circ$  ( $\text{CHCl}_3$ ; 0.05 g/100 ml)

**18**: m.p.: 168-170 °C



**IR:**  $\nu$  [ $\text{cm}^{-1}$ ] ~ 2100 azide accumulated double bond valency oscillation, 2876 (w), 1300 (s)  $=\text{CH}_2$  valency and deformation oscillation, 1740(s)  $-\text{C}=\text{O}$  valency oscillation 1372 (O) $\text{COCH}_3$  valency oscillation.

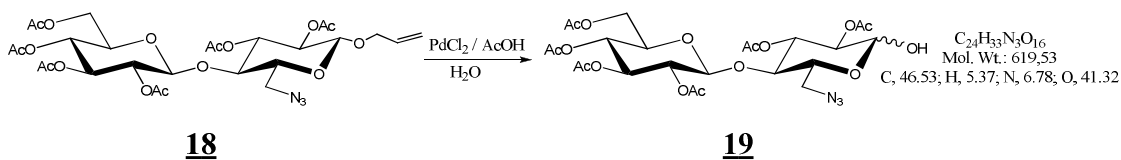
**$^1\text{H-NMR}$**  ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  [ppm]:

5.85 (m; 1H; allyl  $-\text{CH}_2-\text{CH}=\text{CH}_2$ ); 5.27-5.15 (dd; 2H; allyl= $\text{CH}_2$ ); 5.15 (dd; 1H; H3';  $J_{3',4'}=9.50$  Hz); 5.12 (dd; 1H; H3;  $J_{3,4}=9.74$  Hz); 5.03 (dd; 1H; H4;  $J_{4,5}=9.40$  Hz); 4.94 (dd; 1H; H2';  $J_{2',3'}=9.45$  Hz); 4.88 (dd; 1H; H2;  $J_{2,3}=9.45$  Hz); 4.53 (d; 1H; H1';  $J_{1',2'}=7.93$  Hz); 4.50 (d; 1H; H1;  $J_{1,2}=6.99$  Hz); 4.35-4.29 (dd; 2H; allyl= $\text{CH}_2$ ); 4.28 (dd; 1H; H6a;  $J_{5,6a}=4.90$  Hz); 4.03 (dd; 1H; H6b;  $J_{5,6b}=12.43$  Hz;  $J_{6a,6b}=12.43$  Hz); 3.75 (dd; 1H; H4';  $J_{4',5'}=6.83$  Hz); 3.65 (m; 1H; H5); 3.54 (dd; 1H; H5'); 3.52 (dd; 1H; H6a';  $J_{5',6a'}=6.41$  Hz); 3.37 (dd; 1H; H6b';  $J_{5',6b'}=13.18$  Hz;  $J_{6a',6b'}=13.18$  Hz); 2.06 (s; 3H;  $-\text{COCH}_3$ ); 2.01 (s; 3H;  $-\text{COCH}_3$ ); 2.00 (s; 3H;  $-\text{COCH}_3$ ); 1.99 (s; 3H;  $\text{COCH}_3$ ); 1.98 (s; 3H;  $-\text{COCH}_3$ ); 1.95 (s; 3H;  $-\text{COCH}_3$ ).

**$^{13}\text{C-NMR}$**  ( $\text{CDCl}_3$ , 100 MHz)  $\delta$  [ppm]:

171.89, 171.64, 171.20, 170.93, 170.69, 170.23 (6C,  $-\text{COCH}_3$ ); 134.72 (allyl  $-\text{CH}=\text{CH}_2$ ); 119.06 (allyl  $=\text{CH}_2$ ); 102.01 (C1); 100.74 (C1'), 75.89 (C4'); 74.28 (C5'); 73.78 (C3); 73.51 (C3'); 73.43 (C5); 73.10 (C2); 72.90 (C2'); 71.22 (allyl  $-\text{O}-\text{CH}_2-$ ); 69.26 (C4); 62.96 (C6); 51.67 (C6'); 22.07-21.93 (s; 6C;  $-\text{COCH}_3$ ).

2',3',4',6'-Tetra-O-acetyl- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-2,3-di-O-acetyl-6-azido-6-deoxy- $\beta$ -D-glucopyranose **19**



Preparation:<sup>69</sup>

In a round bottom flask, 1.10 g (1.66 mmole) of compound **18**, was dissolved in 60 ml of 88% acetic acid. To this solution, 1.20 g of sodium acetate 1.20 g of PdCl<sub>2</sub> were added. The reaction mixture was stirred for 48 hours, after that TLC showed the complete consumption of **18**. The reaction mixture was neutralized with saturated solution of NaHCO<sub>3</sub>, 50 ml chloroform was added and the mixture filtered over a pad of Celite<sup>®</sup>. The chloroform phase was separated in a separatory funnel, dried over anhydrous MgSO<sub>4</sub>, filtered and evaporated under vacuum to afford 992 mg (1.60 mmole) of **19** with yield of 96%. Column chromatography (EE: T/ 3:2) afforded 794 mg (1.28 mmole) pure crystals of **19** (80% total yield).

Characterization:

TLC (EE: T / 3:2):  $R_F$  educt **18** 0.65  
product **19** 0.24

**19**: Optical rotation:  $[\alpha]_D^{20} = + 40.15^\circ$  (CHCl<sub>3</sub>; 0.050 g/100 ml)

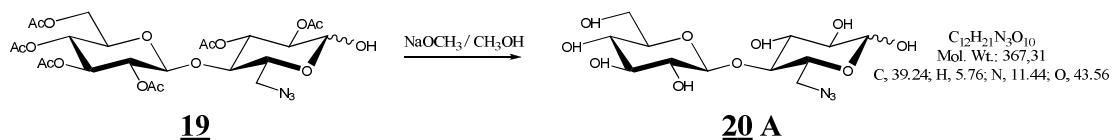
**19**: m.p.: 151 °-152 °C

**IR:**  $\nu$  [ $\text{cm}^{-1}$ ]  $\sim 3600$  –OH valency oscillation,  $\sim 2100$  azide accumulated double bond valency oscillation, 2876 (w), 1300 (s) =CH<sub>2</sub> valency and deformation oscillation, 1740(s) -C=O valency oscillation, 1372 (O)COCH<sub>3</sub> valency oscillation.

**<sup>1</sup>H-NMR** (CDCl<sub>3</sub>, 400 MHz)  $\delta$  [ppm]:

4.80-5.20 (m; 4H; H1, H2; H3, H3'); 4.53 (dd; 1H; H2'; J<sub>2',3'</sub> =8 Hz); 4.50 (dd; 1H; H4'; J<sub>4',5'</sub> =7.99 Hz); 4.32 (dd; 1H; H6a'; J<sub>5',6a'</sub> =4.34 Hz; J<sub>6a',6b'</sub> =12.33 Hz); 4.16 (d; 1H; H1'; J<sub>1',2'</sub> =7.76 Hz); 4.05 (dd; 1H; H6b'; J<sub>5',6b'</sub> =8.22 Hz); 3.80 (dd; 1H; H4; J<sub>3,4</sub> =9.14 Hz); 3.65 (m; 1H; H5; J<sub>4,5</sub> =9.60 Hz); 3.56 (dd; 1H; H5'); 3.52 (dd; 1H; H6a); 3.35 (dd; 1H; H6b; J<sub>5,6b</sub> =5.03 Hz; J<sub>6a,6b</sub> =13.94 Hz); 2.19-1.90 (6 s; 18H; -COCH<sub>3</sub>).

$\beta$ -D-Glucopyranosyl-(1 $\rightarrow$ 4)-6-azido-6-deoxy- $\beta$ -D-glucopyranose **20 A**



Preparation:

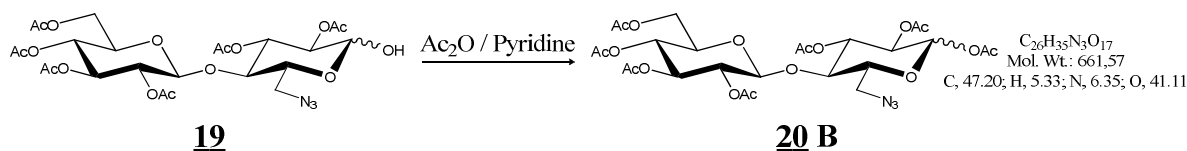
Compound **19** (300 mg 0.49 mmole) was suspended in 10 ml of dry methanol, and a catalytic amount (0.2 ml) of freshly prepared sodium methoxide (230 mg Na /100ml methanol) was added to the reaction mixture. After TLC had indicated the complete conversion of **19** to **20 A**, the reaction mixture was neutralized by Dowex®-H<sup>+</sup>, filtered and evaporated under vacuum to give 177 mg (0.48 mmole) of **20 A**.

Characterization:

TLC (EE: T / 3:2): R<sub>F</sub>      educt **19** 0.25  
   product **20 A** 0.05

**20 A**: m.p.: 155°-158°C

2',3',4',6'-Tetra-O-acetyl-β-D-glucopyranosyl-(1→4)-1,2,3-tri-O-acetyl-6-azido-6-deoxy-β-D-glucopyranose **20 B**



Preparation:

Compound **19** (300 mg, 0.49 mmole) was dissolved in 5.0 ml of dry pyridine. The solution was cooled in an ice-water bath. To the magnetically stirred solution, acetic anhydride ( 5.0 ml) was dropped from a dropping funnel sealed with a drying tube and the mixture stirred further over night. After TLC had indicated the end of the reaction the reaction mixture was poured onto ice , stirred for 30 min. filtered under vacuum and washed several times with distilled water. The product was dried in a desiccator to give 324 mg of product **20 B** (100% yield).

Characterisation:

TLC (EE:T / 3:2):  $R_F$  educt **19** 0.25  
product **20 B** 0.69

**20 B**: Optical rotation:  $[\alpha]_D^{20} = + 49.18^\circ$  ( $\text{CHCl}_3$ ; 0.02 g/100 ml)

**20B**: m.p.: 184-188 °C

**IR**:  $\nu$  [ $\text{cm}^{-1}$ ] 2105 azide accumulated double bond valency oscillation, 1370 (O)COCH<sub>3</sub> valency oscillation, 1740(s) -C=O valency oscillation.

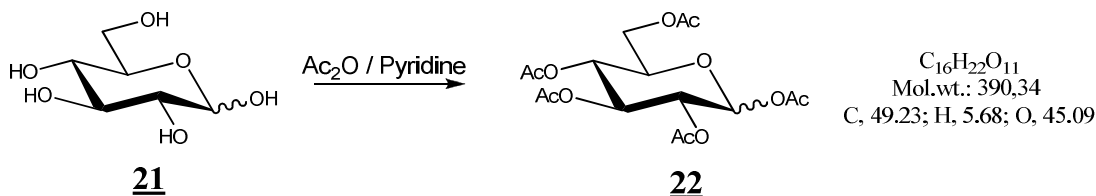
**<sup>1</sup>H-NMR** (CDCl<sub>3</sub>, 400 MHz) δ [ppm]:

5.64 (d; 1H; H1; J<sub>1,2</sub>=8.22 Hz); 5.19 (dd; 1H; H3; J<sub>3,4</sub>=13.79 Hz); 5.15 (dd; 1H; H3'; J<sub>3',4'</sub>=9.14 Hz); 5.05 (dd; 1H; H2'); 5.01 (dd; 1H; H4'; J<sub>4',5'</sub>=9.37 Hz); 4.87 (dd; 1H; H2; J<sub>2,3</sub>=9.14 Hz); 4.54 (d; 1H; H1'; J<sub>1',2'</sub>=7.99 Hz); 4.35 (dd; 1H; H6a'; J<sub>5',6a'</sub>=2.05 Hz); 4.03 (dd; 1H; H6b'; J<sub>5,6b'</sub>=10.38 Hz; J<sub>6a',6b'</sub>=12.11 Hz); 3.87 (dd; 1H; H4; J<sub>4,5</sub>=9.59 Hz); 3.70-3.60 (m; 3H; H5, H5', H6a); 3.36 (dd; 1H; H6b; J<sub>5,6b</sub>=10.05 Hz; J<sub>6a,6b</sub>=13.47 Hz; J<sub>5,6a</sub>=3.66 Hz); 2.10-1.94 (7 s; 21H; -COCH<sub>3</sub>).

**<sup>13</sup>C-NMR** (CDCl<sub>3</sub>, 100 MHz) δ [ppm]:

170.47, 170.22, 169.63, 169.40, 169.26, 168.95, 168.78 (7C, -COCH<sub>3</sub>); 100.39 (C1'); 91.70 (C1), 75.48 (C4); 74.68 (C5'); 72.86 (C5'); 72.33 (C3'); 72.02 (C3); 71.62 (C2'); 70.34 (C4'); 61.54 (C2'); 61.45 (C6'); 49.65 (C6); 20.75, 20.63, 20.58, 20.55, 20.52, 20.51, 20.47 (7 s; 7C; -COCH<sub>3</sub>).

## 1,2,3,4,6-Penta-O-acetyl- $\beta$ -D-glucopyranose **22**



### Preparation :

In a 500 ml round bottom flask, (50g, 0.277 mole) of glucose **21** was suspended in (150 ml) dry pyridine and 150 ml (1.575 mole) acetic anhydride was dropped into the suspension from a dropping funnel sealed with a drying tube (Drierite®). The reaction mixture was cooled in an ice-water bath, stirred with a magnetic stirrer and allowed to come to room temperature. After stirring over night, the content of the flask was poured onto ice and stirred for a further 30 min. The formed product was filtered under vacuum and washed several times with distilled water. The product was stored in a desiccator over Drierite® and gave a total yield 108.0 g (0.277mole) of **22** (100%).

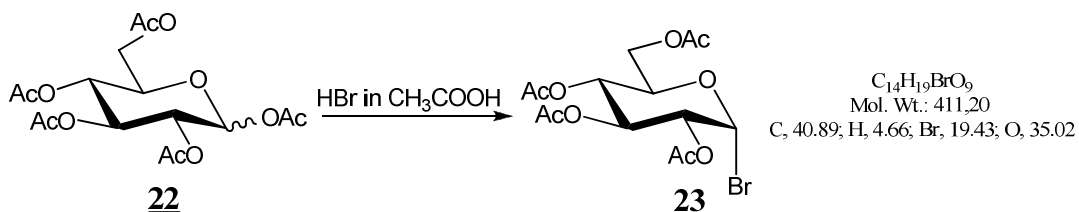
### Characterisation:

TLC (EE:T 3:2):  $R_F$             educt **21** 0.01  
   product **22** 0.70

**22**: Optical rotation:  $[\alpha]_D^{20} = + 30.5^\circ$  ( $CHCl_3$ ; 0.41 g/100 ml)

**22**: m.p.: 87-89°C

## 2,3,4,6-Tetra-O-acetyl-1-bromo-1-deoxy- $\alpha$ -D-glucopyranoside **23**



### Preparation:

100 g of glucose pentaacetate **22** (0.256 mole) was dissolved in 200 ml dry chloroform and magnetically stirred in an ice-water bath. 120 ml of hydrogen bromide in acetic acid (30 wt %) diluted with 20 ml dry chloroform was dropped in over 30 min from a dropping funnel sealed with a drying tube (Drierite®). The reaction mixture was stirred for another 4 h. After TLC indicated the complete consumption of the starting material, the reaction mixture was poured in a separatory funnel onto ice-water and extracted with chloroform. The chloroform layer was washed 4 times with ice-cold water, once with  $NaHCO_3$  and finally with  $NaHSO_4$ . The chloroform layer was then dried over anhydrous  $Mg_2SO_4$ , filtered and evaporated under vacuum to give 101.0 g (0.246 mole) white crystals of product **23**. The product was recrystallized from diisopropylether to give 92.0 g (0.224 mole) of the pure product with a total yield of 87%. After recrystallization the product was stable over several months.

### Characterisation:

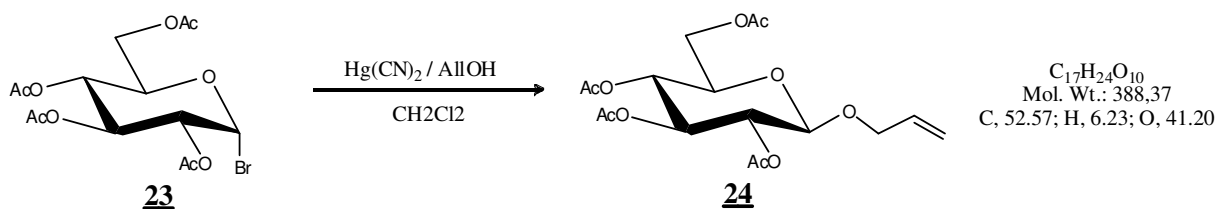
TLC (EE:T 3:2):  $R_F$  educt **22** 0.59  
product **23** 0.70

**23**: Optical rotation:  $[\alpha]_D^{20} = +194^\circ$  ( $CHCl_3$ ; 4.00 g/100 ml)

**23**: m.p.: 89-91 °C



## Allyl-2,3,4,6-tetra-O-acetyl- $\beta$ -D-glucopyranoside **24**



### Preparation :

In a 250 ml round bottom flask, tetra-O-acetyl glucosyl bromide (25.0 g 0.061 mole) **23** was dissolved in 60 ml dry  $CH_2Cl_2$ , 9.0 g of preheated molecular sieves was added. To the magnetically stirred solution 5 g of mercury (II) cyanide and 7.0 ml of dry allyl alcohol were added and the flask was sealed with a drying tube (Drierite®). After 36 hours stirring at room temperature, thin layer chromatography (EE: T / 3:2) indicated the complete consumption of **23**. The reaction mixture was then diluted with 50 ml of  $CH_2Cl_2$  stirred for a further 30 min., the reaction mixture was then filtered over a pad of Celite®, and the filtrate was evaporated under vacuum to give 26.0 g of **24**. After recrystallization from isopropanol the total yield of compound **24** was 19.0 g (0.049 mole, 80 %).

### Characterisation:

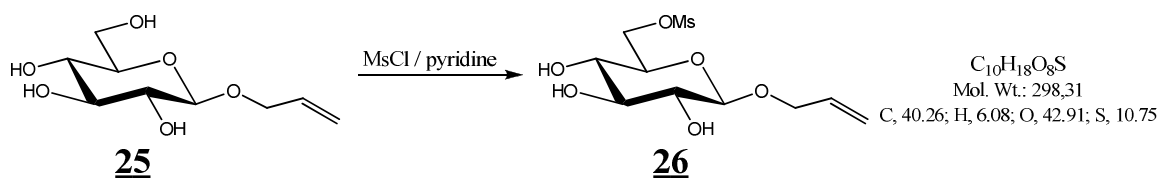
TLC (EE:T/ 3:2):  $R_F$  educt **23** 0.75  
product **24** 0.70

**24**: Optical rotation:  $[\alpha]_D^{20} = -1.7^\circ$  ( $CHCl_3$ ; 0.03 g/100 ml)

**24**: m.p.: 87-88 °C



## Allyl-6-O-methylsulfonyl- $\beta$ -D-glucopyranoside **26**



### Preparation:

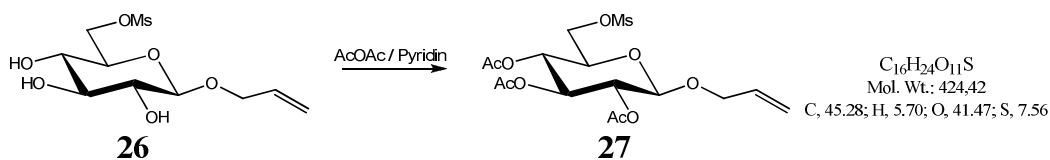
In a 100 ml round bottom flask 4.40 g (0.021 mole) of compound **25** was dissolved in 20 ml dry pyridine. To the magnetically stirred solution 2.48 ml (0.687 mmole) of methanesulfonyl chloride diluted with 10 ml of dry  $\text{CH}_2\text{Cl}_2$  was dropped through a dropping funnel over 4h. The reaction temperature was kept constant at  $-30^\circ\text{C}$  using dry ice. After TLC had indicated the complete consumption of the starting material, the reaction mixture was poured onto ice, stirred for a further 30 min. then extracted with chloroform. The chloroform phase was washed once with  $\text{NaHSO}_4$  then with  $\text{NaHCO}_3$ , dried over anhydrous  $\text{Mg}_2\text{SO}_4$ , filtered and finally evaporated under vacuum to give 4.76 g (0.016 mole) of a syrupy product of **26** with a yield of 76%. Compound **26** was directly applied for the next step without any further purification.

### Characterisation:

TLC (EE:Iso / 10:1):

$R_F$	educt <b>25</b>	0.10
	product <b>26</b>	0.41

## Allyl-2,3,4-tri-O-acetyl-6-O-methylsulfonyl- $\beta$ -D-glucopyranoside **27**



### Preparation:

To the sirupy product **26** (4.76 g 0.016 mole), 20 ml of dry pyridine and 20 ml of acetic anhydride were added. The reaction mixture was stirred in an ice-water bath over night. After TLC had indicated the complete consumption of the educt, the reaction mixture was poured onto ice, stirred, filtered under vacuum and washed several times with distilled water. The product was then recrystallized from 50% ethanol to give 6.70 g (0.016 mole) of pure crystals of **27** with a yield of 100%.

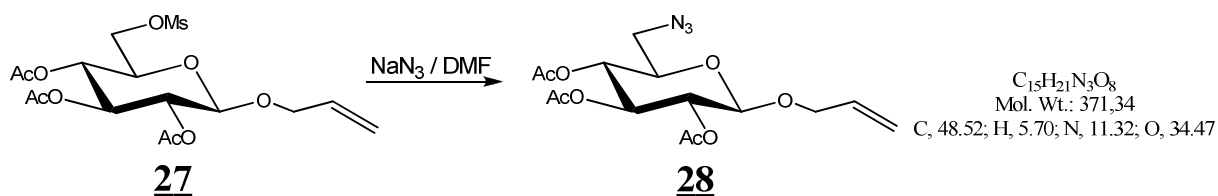
### Characterisation:

TLC (EE: T / 3:2):  $R_F$  educt **26** 0.31  
product **27** 0.73

**27**: Optical rotation  $[\alpha]_D^{20} = -2.8^\circ$  ( $\text{CHCl}_3$ ; 0.021g /100 ml)

**27**: m.p.: 115°- 117°C

## Allyl-2,3,4-tri-O-acetyl-6-azido-6-deoxy-β-D-glucopyranoside **28**



### Preparation:

In a round bottom flask, 6.70 g (0.016 mole) of **27** was dissolved in 50 ml dried DMF. To this solution 5.80 g  $\text{NaN}_3$  was added and refluxed in an oil bath at 120°C for 2h. After TLC had indicated the end of the reaction, the reaction mixture was cooled and evaporated under vacuum. The residue was partitioned between chloroform and water, the chloroform phase separated in a separatory funnel and washed several times with water. The organic layer was then dried over anhydrous  $\text{Mg}_2\text{SO}_4$ , filtered and evaporated under vacuum to afford 5.0 g (0.014 mole) of **28** with a yield of 84%.

### Characterization:

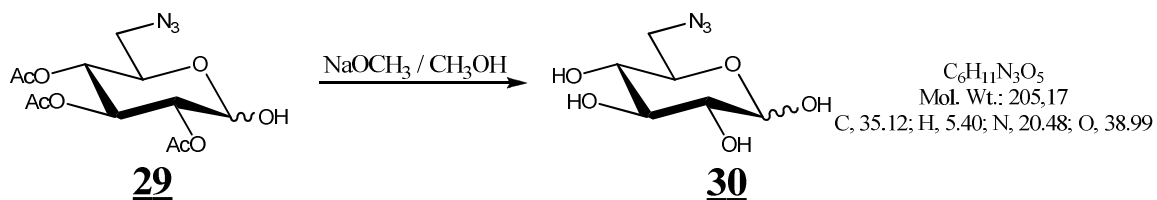
TLC (EE: T / 3:2):  $R_F$  educt **27** 0.70  
product **28** 0.82

**28**: Optical rotation  $[\alpha]_D^{20} = -13.2^\circ$  ( $\text{CHCl}_3$ ; 0.028 g/100 ml)

**28**: m.p.: 119°- 121 °C



## 6-Azido-6-deoxy-D-glucose **30**



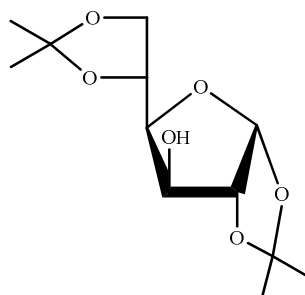
### Preparation:

In a 100 ml round bottom flask, 1.5 g (4.5 mmole) of compound **29** was suspended in 25 ml dry methanol, and a catalytic amount (0.2 ml) of freshly prepared sodium methoxide (230 mg Na / 100 ml methanol) was added to the reaction mixture. After TLC indicated the complete conversion of **29** to **30**, the reaction mixture was neutralized with Dowex<sup>®</sup>-H<sup>+</sup>, filtered and evaporated under vacuum to afford 885 mg (4.31 mmole) of **30** (95%).

### Characterization:

TLC (EE: T / 3:2): R <sub>F</sub>	educt <b>29</b> 0.25
	product <b>30</b> 0.00
TLC ( propanol:water/ 17:3)	educt <b>29</b> 0.80
	Product <b>30</b> 0.63

1,2:5,6-Di-O-isopropylidene- $\alpha$ -D-glucofuranose **31**



$C_{12}H_{20}O_6$   
Mol. Wt.: 260,28  
C, 55.37; H, 7.74; O, 36.88

1,2:5,6-Di-O-isopropylidene- $\alpha$ -D-glucofuranose **31** was purchased from GLYCON Bioch. GmbH, Biotechnology, Luckenwalde, Germany

$^1\text{H-NMR}$  ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  [ppm]:

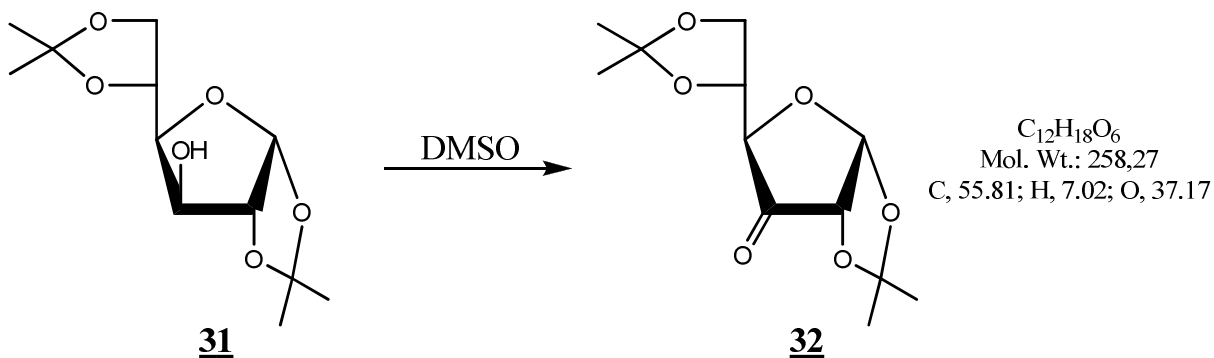
5.94 (d; 1H; H1;  $J_{1,2}=3.79$  Hz); 4.53 (d; 1H; H2;  $J_{2,3}=6.06$  Hz); 4.37-4.30 (m; 2H; H5, H3;  $J_{3,4}=3.03$  Hz,  $J_{4,5}=7.83$  Hz,  $J_{3,-\text{OH}}=3.79$  Hz); 4.20-4.15 (dd; 1H; H6;  $J_{5,6}=6.06$  Hz); 4.09-4.05 (dd; 1H; H4); 4.00-3.95 (dd; 1H; H6';  $J_{5,6'}=7.57$  Hz,  $J_{6,6'}=8.84$  Hz); 2.54 (d; 1H; -OH); 1.49, 1.44, 1.36, 1.31 (4 s; 4x3 H; 4x -CH<sub>3</sub>).

$^{13}\text{C-NMR}$  ( $\text{CDCl}_3$ , 100 MHz)  $\delta$  [ppm]:

111.81, 109.65 (2 quaternary carbon atoms  $(\text{CH}_3)_2\text{-C=}$ ); 105.29 (C1); 85.07 (C2); 81.12 (C4); 75.25 (C3); 73.51 (C5); 67.66 (C6); 26.84, 26.75, 26.17, 25.12 (4 primary carbon atoms -C-CH<sub>3</sub>).



1,2:5,6-Di-O-isopropylidene- $\alpha$ -D-ribo-hexofuranose-3-ulose **32**



Preparation<sup>70</sup> :

To a mixture of 100 ml of acetic anhydride and 400 ml of dry DMSO heated to 50 °C in a 1-liter round bottom flask was added 50 g (0.192 mole) of **31**. The reaction mixture was magnetically stirred and refluxed in an oil bath at 80 °C. After TLC had indicated the complete consumption of **31** (Both educt and product have the same  $R_f$  value; in order to monitor the end of the reaction, a sample of the reaction mixture was reduced to the allofuranose derivative **33**. complete conversion to **33** indicated the complete consumption of **31** ). The solution was concentrated under vacuum and afforded 49.0 g (0.190 mole) syrupy product **32** (99 %). For the characterization of the product, 1.0 g was recrystallized from diisopropylether to afford fine crystals with melting point of 60°-62°C. The rest of the product was applied directly for the next step without further treatment.

Characterization:

TLC (EE:T / 1:1)  $R_f$

educt 0.69

Product 0.69

**32** Optical rotation:  $[\alpha]_D^{20} = + 20.1^\circ$  (CHCl<sub>3</sub>; 0.22 g/100 ml)



**33** m.p      69-71 °C

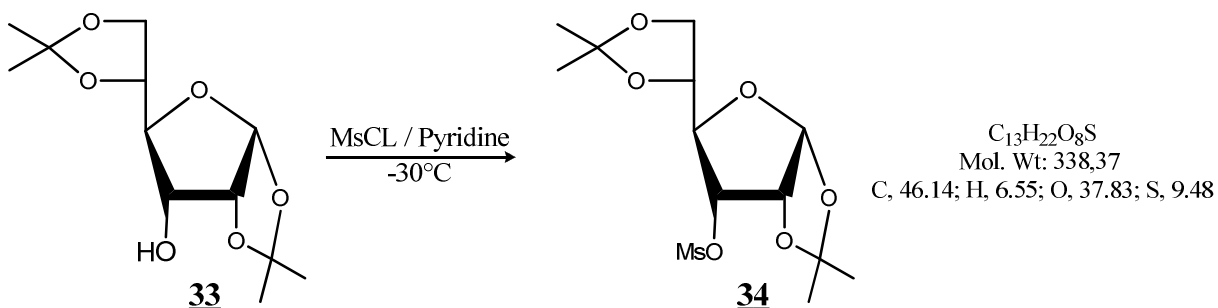
**<sup>1</sup>H-NMR** (CDCl<sub>3</sub>, 400 MHz) δ [ppm]:

5.80 (d; 1H; H1; J<sub>1,2</sub>=3.79 Hz); 4.61 (dd; 1H; H2; J<sub>2,3</sub>=5.30 Hz); 4.33-4.28 (m; 1H; H5; J<sub>5,6</sub>=7.57 Hz, 4.10-3.98 (m; 3H; H3, H6, H6'; J<sub>3,4</sub>=4.55 Hz); 3.83-3.79 (dd; 1H; H4; J<sub>4,5</sub>=8.34 Hz); 4.00-3.95 (dd; 1H; H6'; J<sub>5,6'</sub>=7.57 Hz); 2.53 (d; 1H; -OH; J<sub>3,-OH</sub>=8.59 Hz); 1.57, 1.62, 1.38, 1.36 (4 s; 4x3 H; 4x -CH<sub>3</sub>).

**<sup>13</sup>C-NMR** (CDCl<sub>3</sub>, 100 MHz) δ [ppm]:

112.85, 109.86 (2 quaternary carbon atoms (CH<sub>3</sub>)<sub>2</sub>-C=); 103.94 (C1); 79.76 (C4); 78.96 (C2); 75.61 (C5); 72.52 (C3); 65.89 (C6); 26.56, 26.50, 26.31, 25.27 (4 primary carbon atoms-C-CH<sub>3</sub>).

1,2:5,6-Di-O-isopropyliden-3-O-methylsulfonyl- $\alpha$ -D-allofuranose **34**



Preparation:

In a 250 round bottom flask 5.0 g (0.019 mole) of compound **33** was dissolved in 20 ml of dry pyridine and cooled in ice-water bath. To the magnetically stirred solution, 1,6 ml (0.021 mole) of methyl sulfonyl chloride diluted to 10 ml with dry  $CH_2Cl_2$  was dropped through a dropping funnel over 30 min. After TLC had indicated the consumption of **33**, the reaction mixture was poured onto ice and stirred for a further 30 min. In a separatory funnel **34** was partitioned between  $CHCl_3$  and water. The chloroform phase was washed with saturated solution of  $NaHSO_4$ , then  $NaHCO_3$ , dried over anhydrous  $MgSO_4$ , filtered and finally evaporated under vacuum to afford 5.80 g (0.017 mole) of **34** (90%). The product was recrystallized from 50% ethanol.

Characterization:

TLC (EE:T / 3:2)  $R_f$  educt **33** 0.32

Product **34** 0.69

**34** Optical rotation  $[\alpha]_D^{20} = + 86.0^\circ$  ( $CHCl_3$ ; 0.1 g/100 ml)

**34** m.p.: 128-130°C

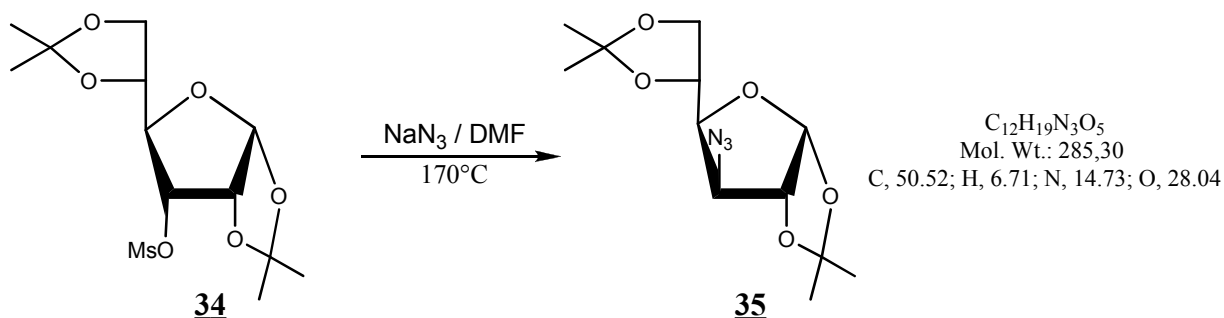
**<sup>1</sup>H-NMR** (CDCl<sub>3</sub>, 400 MHz) δ [ppm]:

5.82 (d; 1H; H<sub>1</sub>; J<sub>1,2</sub>=3.79 Hz); 4.82-4.77 (m; 2H; H<sub>2</sub>, H<sub>3</sub>; J<sub>2,3</sub>=4.80 Hz); 4.34-4.30 (m; 1H; H<sub>5</sub>; J<sub>4,5</sub>=7.83 Hz, J<sub>5,6</sub>=7.84 Hz); 4.15-4.07 (m; 2H; H<sub>4</sub>, H<sub>6</sub>; J<sub>3,4</sub>=4.29 Hz); 3.95-3.91 (dd; 1H; H<sub>6'</sub>; J<sub>5,6'</sub>=8.85 Hz, J<sub>6,6'</sub>=5.81 Hz); 3.14 (s; 3H; -SO<sub>2</sub>CH<sub>3</sub>); 1.58, 1.48, 1.37, 1.36 (4s; 4x3 H; 4x -CH<sub>3</sub>).

**<sup>13</sup>C-NMR** (CDCl<sub>3</sub>, 100 MHz) δ [ppm]:

117.05, 113.76 (2 quaternary carbon atoms (CH<sub>3</sub>)<sub>2</sub>-C=); 103.95 (C<sub>1</sub>); 77.99 (C<sub>3</sub>); 77.15 (C<sub>2</sub>); 77.01 (C<sub>4</sub>); 74.79 (C<sub>5</sub>); 65.61 (C<sub>6</sub>); 38.86 (1 primary carbon atom of the mesyl group -SO<sub>2</sub>-CH<sub>3</sub>); 26.71, 26.66, 26.24, 24.96 (4 primary carbon atoms -C-CH<sub>3</sub>).

## 1,2:5,6-Di-O-isopropyliden-3-azido-3-deoxy- $\alpha$ -D-glucufuranose **35**



### Preparation:

In a 100 ml round bottom flask 3.34 g (9.8 mmole) of compound **34** and 10.7 g  $\text{NaN}_3$  were added to 100 ml dry DMF, the flask was sealed with a drying tube, stirred and heated in an oil bath at 170°C (condenser). When TLC (EE: T / 3:2) had indicated the complete consumption of the educt, the heater was turned off and the solution was cooled to RT. The reaction mixture was evaporated under vacuum to dryness, the residue was partitioned between 100 ml water and 100 ml chloroform, the chloroform phase washed 5 times with water, then dried over  $\text{MgSO}_4$ , filtered and finally evaporated to give 2.46 g (8.6 mmole) of **35** which was purified by column chromatography (EE:T 3:2).

### Characterization:

TLC (EE:T / 3:2)  $R_f$  educt **34** 0.69

Product **35** 0.82

**35** Optical rotation  $[\alpha]_D^{20} = -39.85.0^\circ$  ( $\text{CHCl}_3$ ; 4.8 g/100 ml)

**35** m.p.: 132°- 134°C

**<sup>1</sup>H-NMR** (CDCl<sub>3</sub>, 400 MHz) δ [ppm]:

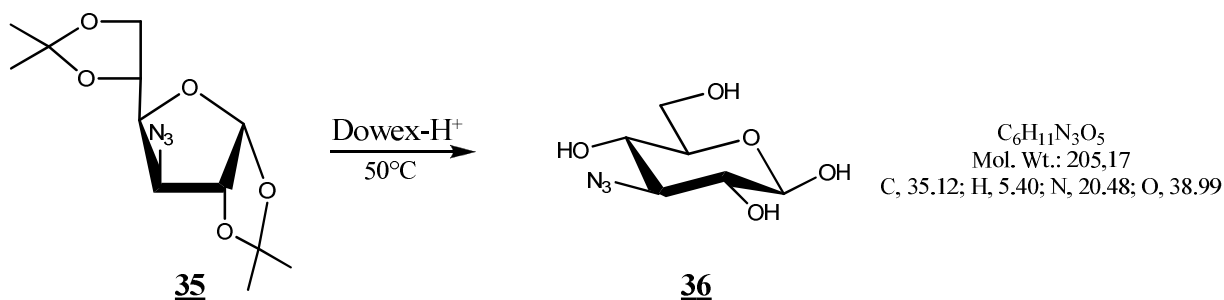
5.85 (d; 1H; H<sub>1</sub>; J<sub>1,2</sub>=3.54 Hz); 4.61 (d; 1H; H<sub>2</sub>, J<sub>2,3</sub>=5.05 Hz); 4.25-4.21 (m; 1H; H<sub>5</sub>; J<sub>4,5</sub>=6.06 Hz); 4.15-4.07 (m; 3H; H<sub>3</sub>, H<sub>4</sub>, H<sub>6</sub>; J<sub>3,4</sub>=3.54 Hz, J<sub>5,6</sub>=8.85 Hz); 3.99-3.96 (dd;

1H; H<sub>6'</sub>; J<sub>5,6'</sub>=4.04 Hz, J<sub>6,6'</sub>=4.80 Hz); 1.50, 1.43, 1.36, 1.32 (4 s; 4x3 H; 4x -CH<sub>3</sub>).

**<sup>13</sup>C-NMR** (CDCl<sub>3</sub>, 100 MHz) δ [ppm]:

112.32, 109.58 (2 quaternary carbon atoms (CH<sub>3</sub>)<sub>2</sub>-C=); 105.05 (C<sub>1</sub>); 83.43 (C<sub>2</sub>); 80.15 (C<sub>4</sub>); 73.05 (C<sub>5</sub>); 67.65 (C<sub>6</sub>); 66.38 (C<sub>3</sub>); 26.86, 26.65, 26.19, 25.15 (4 primary carbon atoms -C-CH<sub>3</sub>).

### 3-Azido-3-deoxy- $\beta$ -D-glucose **36**



#### Preparation:

In a 250 ml round bottom flask, 2.0 g (7.0 mmole) of compound **35** was suspended in 50 ml  $H_2O$ . Dowex<sup>®</sup>-H<sup>+</sup> (3.0 g) was added to this mixture and refluxed at 45 °C. After TLC (EE:T / 3:2) had indicated the consumption of the starting material, the reaction mixture was cooled, filtered and evaporated under vacuum at 40 °C to afford 1.45 g (7.0 mmole) syrupy product of **36**.

#### Characterization:

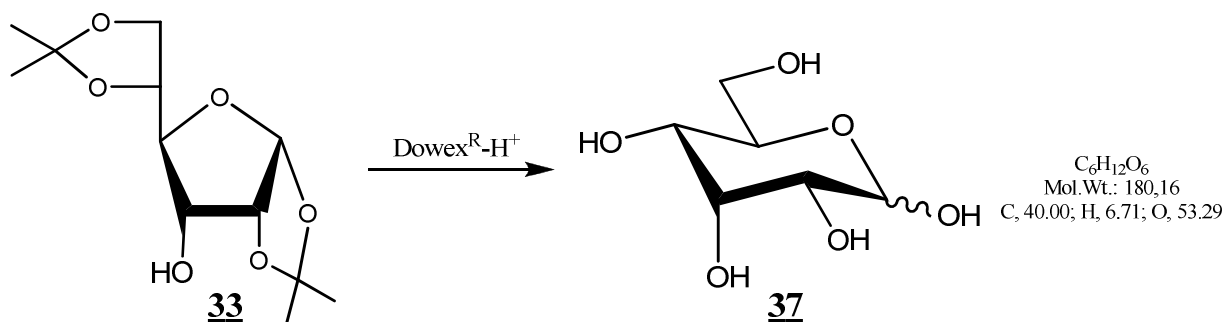
TLC(EE:T / 3:2)

educt **35** 0.82

Product **36** 0.01



## $\beta$ -D-Allose **37**



### Preparation:

In a 250 ml round bottom flask 10.20 g (0.039 mole) of compound **33** was suspended in 100 ml H<sub>2</sub>O. Dowex<sup>®</sup>-H<sup>+</sup> (17.0 g) was added to this mixture and refluxed at 45 °C. After TLC (EE:T / 3:2) has indicated the consumption of the starting materials, the reaction mixture cooled, filtered and the evaporated under vacuum at 40 °C to afford 6.86 g (0.038 mole) of a white crystalline product of **37**. The  $\beta$ -D-allose was recrystallized from 20 ml of 30% ethanol to give needle crystals with m.p. 141 °-142 °C.

### Characterization:

TLC(EE:T / 3:2)	educt <b>33</b> 0.73
	Product <b>37</b> 0.0
TLC(Pr: H <sub>2</sub> O / 17:3:)	educt <b>33</b> 0.85
	Product <b>37</b> 0.68

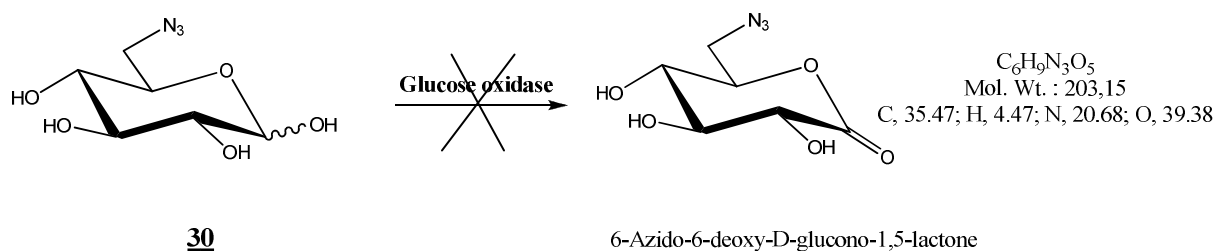
**37** optical rotation.  $[\alpha]_D^{20} = + 14.5^\circ$  (H<sub>2</sub>O; 2.0 g/100 ml)

**37** m.p.: 141 °-142 °C

## 7.5 Enzymatic syntheses description

### 7.5.1 Glucose oxidase catalysis

#### 6-Azido-6-deoxy-D-glucono-1,5-lactone



#### Incubation:

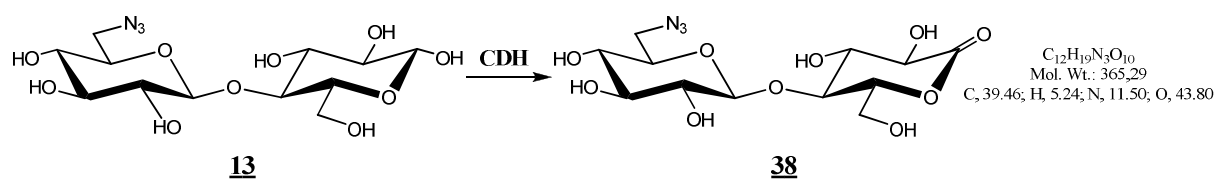
An aqueous solution of compound **30** was prepared (0.5 g in 10 ml H<sub>2</sub>O) in a test tube. To this solution 30 mg of glucose oxidase(105 U) and 3.60 mg of catalase enzyme ( 6.7 U) were added. The pH of the solution was adjusted at 7.0 and the reaction mixture was incubated at RT. Using TLC in propanol/water 17:3 as a mobile phase we could not identify any changes in the substrate.

#### Conclusion:

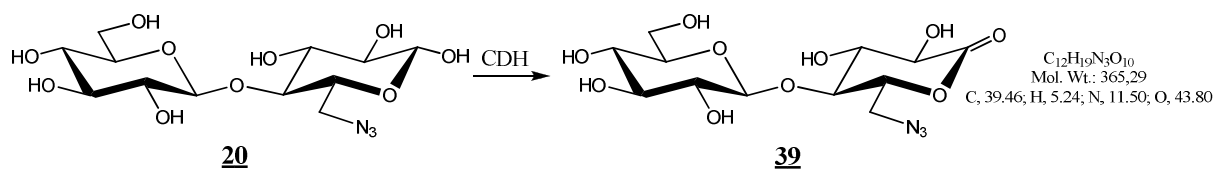
The substitution of hydroxyl group on C6 of glucose with azido group render the molecule incapable of functioning as substrate of glucose oxidase. A conventional chemical oxidation process may be used instead of enzymatic one, for example the oxidation with bromine water or with DMSO.

## 7.5.2 Cellobiose dehydrogenase catalysis

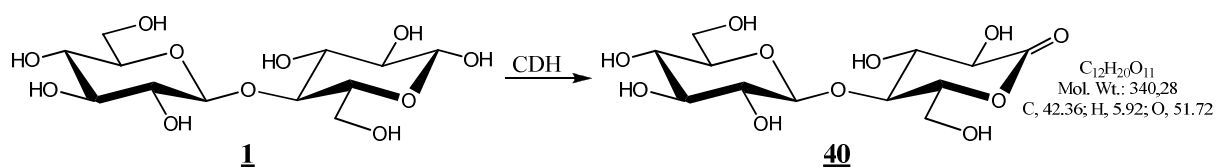
6'-Azido-6'-deoxy- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-gluconic acid delta lactone **38**



$\beta$ -D-Glucopyranosyl-(1 $\rightarrow$ 4)-6-azido-6-deoxy-D-gluconic acid delta lactone **39**



$\beta$ -D-Glucopyranosyl-(1 $\rightarrow$ 4)-D-gluconic acid delta lactone **40**



### Incubation:

In 3 test tubes, the substrates **13**, **20**, and **1** were incubated with the enzyme cellobiose dehydrogenase in the presence of 0.1M potassium hexacyanoferrate shown in Table 3. The incubation was carried out at 30 °C and pH 4.0 using acetate buffer.

Entry	Substrate	mg	Enzyme CDH in $\mu$ l	Catalyst $K_3[Fe(CN)_6]$
1	6'-azido-6'-deoxy-cellobiose	120	660	1.0 ml
2	6-azido-6-deoxy-cellobiose	20	110	1.0 ml
3	Cellobiose	5	30	1.0 ml

Table 3

Samples were withdrawn in one h intervals, and analyzed by TLC using propanol/water 17:3 as a mobile phase.

### Results:

As shown in Figure 33 the spots 1 in red, 2 in blue and 3 in green represent the formed lactones from cellobiose, 6-azido-6-deoxy-D-cellobiose and 6'-azido-6'-deoxy-D-cellobiose respectively. The formation of the product **39** was already completed

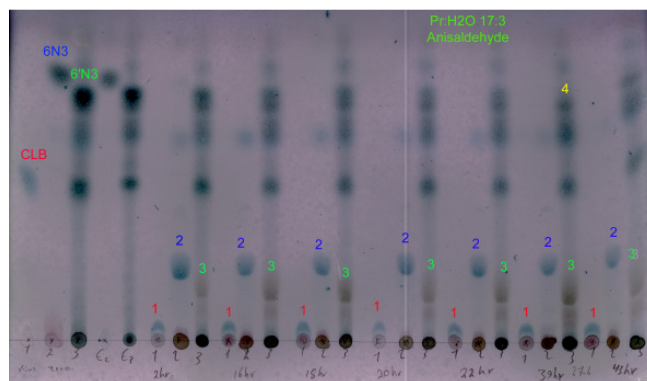


Figure 33

after 2 hours, whereas the formation of compounds **38** and **40** needed 24 hours.

### Conclusion:

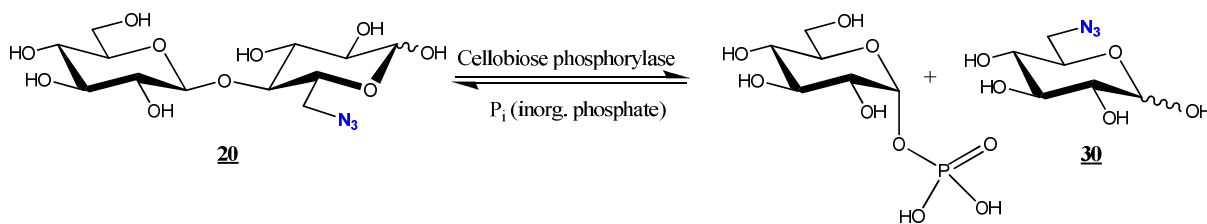
The finding that cellobiose dehydrogenase can oxidize cellobiose analogs with azido group in the six position of both reduced and non-reduced glucose moiety enables the introduction of new precursors for the formation of new biomimetic materials.

## 7.5.3 Cellobiose phosphorylase catalysis

### 7.5.3.1 Phosphorylation of Disaccharides

#### 7.5.3.1.1 Phosphorylation of 6-azido-6-deoxy-D-cellobiose

and formation of  
6-Azido-6-deoxy-D-glucose **30**



#### Incubation:

Compound **20** was incubated with the enzyme cellobiose phosphorylase at 37 °C for 12 hours according to Table 4. The products formed were separated using DEAE- cellulose chromatography.

Substance	Concentration
6-Azido-6-deoxy-D-cellobiose	1.2 ml 0.1 M
Phosphate buffer pH 7.0	0.8 ml
Cellobiose phosphorylase	2.0 ml

Table 4

#### Results:

As shown in Figure 34 the phosphorylation of compound **20** leads to a complete conversion to 6-azido-6-deoxy-D-glucose and glucose 1-phosphate (lanes 5-7). After chromatographic separation with DEAE-cellulose this compound was identical with the chemically prepared compound **30**. In lane 4 it is obvious that more

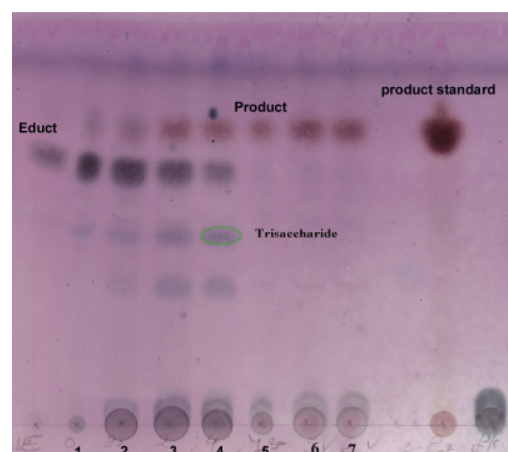


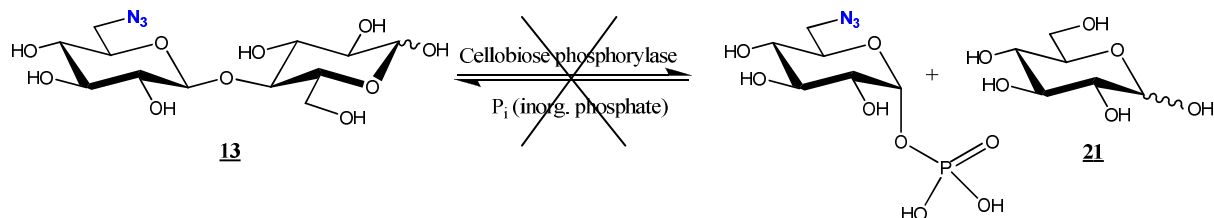
Figure 34

polar products, possibly higher oligosaccharides, are also formed and probably degraded again later.

Conclusion:

The substitution of hydroxyl group by azido group in the 6-position of cellobiose was tolerated by cellobiose phosphorylase. Interestingly, cellobiose phosphorylase seems to be able to use 6-azido-6-deoxy-D-cellobiose as acceptor to form higher oligosaccharides.

### 7.5.3.1.2 Phosphorylation of 6'-azido-6'-deoxy-cellobiose



#### Incubation:

Compound **13** was incubated with the enzyme cellobiose phosphorylase at 37 °C for 12 hours according to Table 5. In the first 2 hours samples were withdrawn for TLC in 15 min. intervals, after that in 2 h intervals.

Substance	Concentration
6'-Azido-6'-deoxy-D-cellobiose	1.2 ml 0.1 M
Phosphate buffer pH 7.0	0.8 ml
Cellobiose phosphorylase	2.0 ml

Table 5

#### Results:

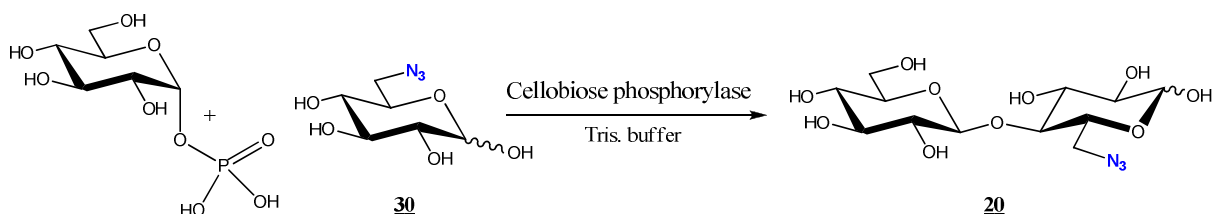
TLC did not indicate any changes in the educt, or the formation of any product.

#### Conclusion:

The 6'-azido-6'-deoxy-cellobiose was expected to be more readily cleaved under catalysis with cellobiose phosphorylase to yield 6-azido-6-deoxy-glucose-1-phosphate and glucose. In our hands such cleavage did not take place indicating that the substitution of the hydroxyl group by azido group at C-6 of the non-reducing glucose unit of cellobiose is not tolerated by the enzyme cellobiose phosphorylase.

## 7.5.3.2 Catalysis of disaccharide formation

### 7.5.3.2.1 Synthesis of 6-azido-6-deoxy-cellobiose



#### Incubation:

6-Azido-6-deoxy-D-glucose **30** was incubated as acceptor with glucose-1-phosphate as donor under catalysis by cellobiose phosphorylase according to Table 6. In this reverse reaction Tris buffer was used instead of phosphate at pH 7.0 and 37°C. Samples were withdrawn in 2 h intervals and were examined on TLC in 1-propanol: water 17:3 as mobile phase.

Substance	Concentration
Glucose-6-N3	0.8 ml 0.1 M
Glucose-1-P	1.2 ml 0.076 M
Cellobiose phosphorylase	2.0 ml
Tris buffer	4.0 ml

Table 6

#### Results:

As shown in Figure **35** the acceptor 6-azido-6-deoxy-D-glucose was able to react with glucose-1-P to give the heterodisaccharide **20**. Analysis of this product showed it to be identical with the sample prepared chemically.

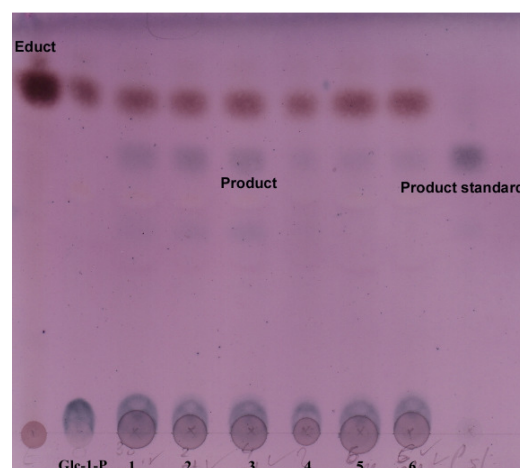


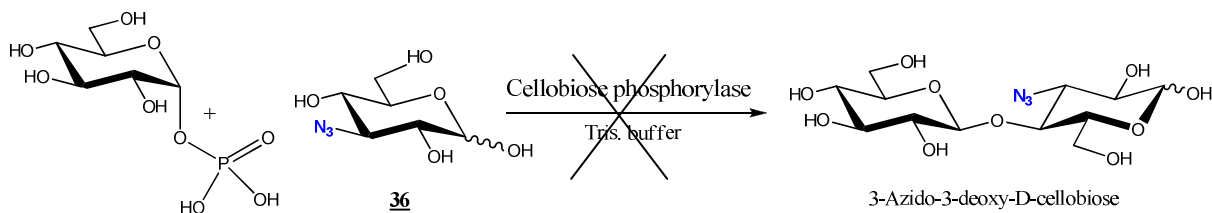
Figure 35



Conclusion:

The ability of 6-azido-6-deoxy-D-glucose to act as a substrate in the reverse action of cellobiose phosphorylase is of great importance because it offers a new way to synthesize heterodisaccharides with  $\beta(1\rightarrow4)$  glycosidic bond. The organic chemical synthesis of such molecules is well known to be difficult.

### 7.5.3.2.2 Synthesis of 3-azido-3-deoxy-D-cellobiose



#### Incubation:

3-Azido-3-deoxy-D-glucose **36** was incubated as glucose acceptor under catalysis by cellobiose phosphorylase with glucose-1-P as a donor. The pH of the solution was adjusted at 7.0 using Tris buffer as shown in Table 7. The incubation continued for 24 h. Samples were withdrawn in 2 h intervals and were examined on TLC in 1-propanol: water 17:3 as mobile phase.

Substance	Concentration
Glucose-3-N <sub>3</sub>	0.8 ml 0.1 M
Glucose-1-P	1.2 ml 0.076 M
Cellobiose phosphorylase	2.0 ml
Tris buffer	4.0 ml

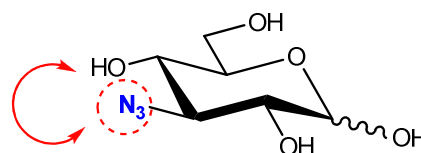
Table 7

#### Results:

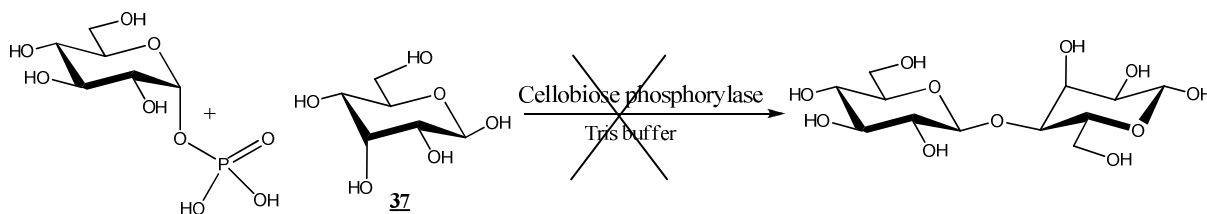
The TLC did not indicate any changes in the educt or the formation of a product.

#### Conclusion:

3-Azido-3-deoxy-D-glucose can not act as a substrate for the reversal action of cellobiose phosphorylase. The location of the azido group at C-3 of glucose may inhibit the formation of  $\beta(1\rightarrow4)$  glycosidic bond as a result of the steric hindrance between C-3 and C-4.



### 7.5.3.2.3 Synthesis of $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-allopyranose



#### Incubation:

$\beta$ -D-Allose **37** was incubated as a glucose acceptor with glucose-1-P as a donor under catalysis by cellobiose phosphorylase. Using Tris buffer the pH of the solution was adjusted at 7.0 as shown in Table 8. The incubation continued for 24 hours and samples were withdrawn in 2 hours intervals during the first 6 hours, then every 6 hours.

Substance	Concentration
$\beta$ -D-Allose	0.8 ml 0.1 M
Glucose-1-P	1.2 ml 0.076 M
Cellobiose phosphorylase	2.0 ml
Tris buffer	4.0 ml

Table 8

Samples were examined on TLC in 1-propanol: water 17:3 as mobile phase.

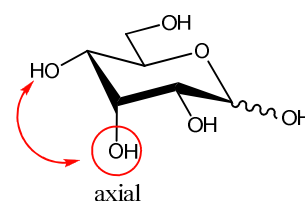
#### Results:

The TLC did not indicate any change in the educt or the formation of a product.

#### Conclusion:

The change of only one stereocentre on  $\beta$ -D-allose namely position 3 hinder the formation of heterodisacchride. Consequently, the  $\beta$ -D-allose cannot act as a substrate for the reversal action of cellobiose phosphorylase.

That means either the equatorial configuration of the hydroxyl on C-3 is essential for the substrate-enzyme binding, or that the axial orientation of the hydroxyl group inhibits the binding.



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## List of abbreviations

<b>aa</b>	amino acid
<b>Ac-</b>	acetyl-
<b>AcOAc</b>	acetic anhydride
<b>ADP</b>	adenosine-diphosphate
<b>AgF</b>	silver fluoride
<b>AgClO<sub>4</sub></b>	Silver perchlorate
<b>AgOTf</b>	Silver triflate
<b>AIBN</b>	Azobisisobutyronitrile
<b>AlIOH</b>	allyl alcohol
<b>AMP</b>	Adenosine monophosphate
<b>BaO</b>	barium oxide
<b>Ba(OH)<sub>2</sub></b>	barium hydroxide
<b>BF<sub>3</sub>·Et<sub>2</sub>O</b>	boron trifluoride etherate
<b>β-G</b>	β-glucosidase
<b>Bn-</b>	benzyl-
<b>Bz-</b>	benzoyl-
<b>BnBr</b>	benzyl bromide
<b>BuNHCl</b>	tetrabutylammonium chloride
<b>Bu<sub>4</sub>NHSO<sub>4</sub></b>	tetrabutylammonium hydrogensulfate
<b>BuOH</b>	n-Butanol
<b>CAP</b>	cellulose acetate phthalate
<b>CBH</b>	Cellobiosylhydrolase
<b>CBM</b>	Carbohydrate binding modules
<b>CBP</b>	Cellobiose phosphorylase



<b>CCl<sub>3</sub>CN</b>	trichloroacetonitrile
<b>CDCI<sub>3</sub></b>	deuterium chloroform
<b>CDH</b>	Cellobiose dehydrogenase
<b>CDP</b>	cytidine-diphosphate
<b>CF<sub>3</sub>COOH</b>	trifluoroacetic acid
<b>CMC Na</b>	carboxymethylcellulose-sodium
<b>COS</b>	Chitosaminooligosaccharide
<b>δ</b>	chemical shift
<b>Da</b>	Dalton
<b>DAST</b>	Diethylaminosulfur trifluoride
<b>DEAE</b>	Diethylaminoethyl
<b>DMF</b>	dimethylformamide
<b>DMSO</b>	dimethylsulfoxide
<b>DS</b>	Degree of substitution
<b>DT</b>	Diphtheria toxoid
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>EG</b>	endoglucanase
<b>EtOH</b>	Ethanol
<b>FAD</b>	Flavin adenine dinucleotide
<b>FMN</b>	Flavin mononucleotide
<b>5FU</b>	5-flurouracil
<b>Gal</b>	galactose
<b>GCL</b>	glycosyl carrier lipid
<b>GDP</b>	guanosine-5-diphosphate
<b>G-1-P</b>	Glucose-1-phosphate
<b>Glc</b>	glucose

<b>GlcNAc</b>	<i>N</i> -acetylglucosamine
<b>GOX</b>	Glucose oxidase
<b>HBr</b>	Hydrogen bromide
<b>HEC</b>	hydroxyethylcellulose
<b>HgBr<sub>2</sub></b>	mercury (II)bromide
<b>Hg(CN)<sub>2</sub></b>	mercury (II)cyanide
<b>HIV</b>	Human Immundeficiency Virus
<b>HOAc</b>	(glacial) acetic acid
<b>HPC</b>	hydroxypropylcellulose
<b>HPMC</b>	hydroxypropylmethylcellulose
<b>HPMCAS</b>	hydroxypropylmethylcellulose-acetate succinate
<b>HPMCP</b>	hydroxypropylmethylcellulose phthalate
<b>H<sub>2</sub>SO<sub>4</sub></b>	sulfuric acid
<b>Hz</b>	Hertz
<b>IgE</b>	Immunoglobulin E
<b>IR</b>	infra-red spectroscopy
<b>J</b>	coupling constant
<b>K<sub>2</sub>CO<sub>3</sub></b>	potassium carbonate
<b>LiAlH<sub>4</sub></b>	Lithium aluminum hydride
<b>MC</b>	methylcellulose
<b>Me-</b>	methyl-
<b>MeCN</b>	acetonitrile
<b>MeOH</b>	methanol
<b>mesyl-</b>	methanesulfonyl-
<b>m.p.</b>	melting point
<b>MS</b>	mass spectroscopy

<b>MsCl</b>	methanesulfonyl chloride
<b>NaBH<sub>4</sub></b>	Sodium borohydride
<b>NaN<sub>3</sub></b>	Sodium azide
<b>NaOAc</b>	sodium acetate
<b>NaOH</b>	sodium hydroxide
<b>NaOMe</b>	sodium methoxide
<b>NH<sub>4</sub>Cl</b>	ammoniumchloride
<b>(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub></b>	ammoniumsulfate
<b>nm</b>	nanometer
<b>NMR</b>	nuclear magnetic resonance spectroscopy
<b><i>p</i></b>	pyranosidic form
<b>PdCl<sub>2</sub></b>	palladium (II) chloride
<b>PhCHO</b>	benzaldehyde
<b>PhCH(OMe)<sub>2</sub></b>	benzaldehyde dimethylacetal
<b>PLC</b>	preparative layer chromatography
<b>PP</b>	pyrophosphate
<b>Pr</b>	n-Propanol
<b>PTS</b>	Proline-threonine-serine
<b>R<sub>F</sub> (value)</b>	relate to front (value)
<b>rpm</b>	Revolutions per minute
<b>ROBn</b>	Benzyl ethers
<b>ROBz</b>	Benzoyl esters
<b>SEM</b>	scanning electron micrograph
<b>S<sub>N</sub>1</b>	Unimolecular nucleophilic substitution
<b>S<sub>N</sub>2</b>	Bimolecular nucleophilic substitution
<b>SnCl<sub>2</sub></b>	tin (II) chloride

<b>T.</b>	Trichoderma
<b>TDP</b>	thymidine-diphosphate
<b>TEMPO</b>	tetramethylpiperidine- <i>N</i> -oxide
<b>THF</b>	tetrahydrofuran
<b>TLC</b>	thin layer chromatography
<b>tosyl-</b>	toluenesulfonyl-
<b>trityl-</b>	triphenylmethyl-
<b>TsCl</b>	toluenesulfonyl chloride (tosyl-chloride)
<b>TsOH</b>	toluenesulfonic acid
<b>UDP</b>	uridine-5-diphosphate
<b>ZnCl<sub>2</sub></b>	zinc chloride

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## Publications

- July 09, 15<sup>th</sup> European Carbohydrate Symposium, Vienna, Austria; Poster presentations entitled "*Reaction of Azidosugars with an Alkyne: Synthesis of Sugar Triazoles as Linking Arms*" and "*Carbohydrate Derived Azides for Pharmaceutical Technical Applications*"
- June 09, "*Novel, Stereoselective Synthesis of 2-Azido-2-deoxy-D-glucose*" and "*Azido Derivatives of Cellobiose: Syntheses and Substrate Properties with Cellobiose Phosphorylase from Clostridium Thermocellum NCIMB 10682*" manuscripts completed, to be submitted for publication
- April 09, "*Carbohydrate Derived Azides as Substrates for Cellobiose Phosphorylase from Clostridium Thermocellum*", Sci Pharm.2009; 7:229
- April 08, The 6<sup>th</sup> World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Barcelona, Spain; Poster presentation entitled "*6-Azido-6-Deoxy-Cellobiose: Organic Chemical and Enzyme-Catalyzed Syntheses*"
- April 07, Pharmaceutical Sciences World Congress, Amsterdam, Holland; Poster presentation entitled "*Syntheis of 6-Azido-6-deoxy- $\beta$ -D-glucopyranose and  $\beta$ -D-Glucopyranosyl(1 $\rightarrow$ 4)-6-azido-6-deoxy- $\beta$ -D-gluopyranose from D-Glucose and D-Cellobiose Respectively and the Enzymatic Confirmation of These Syntheses*"
- February 07, 11<sup>th</sup> Austrian Carbohydrate Workshop, Graz, Austria; Lecture entitled "*Organisch-Chemische und enzymkatalysierte Synthese von 6-Azido-6-desoxy-cellobiose*"

# CURRICULUM VITAE

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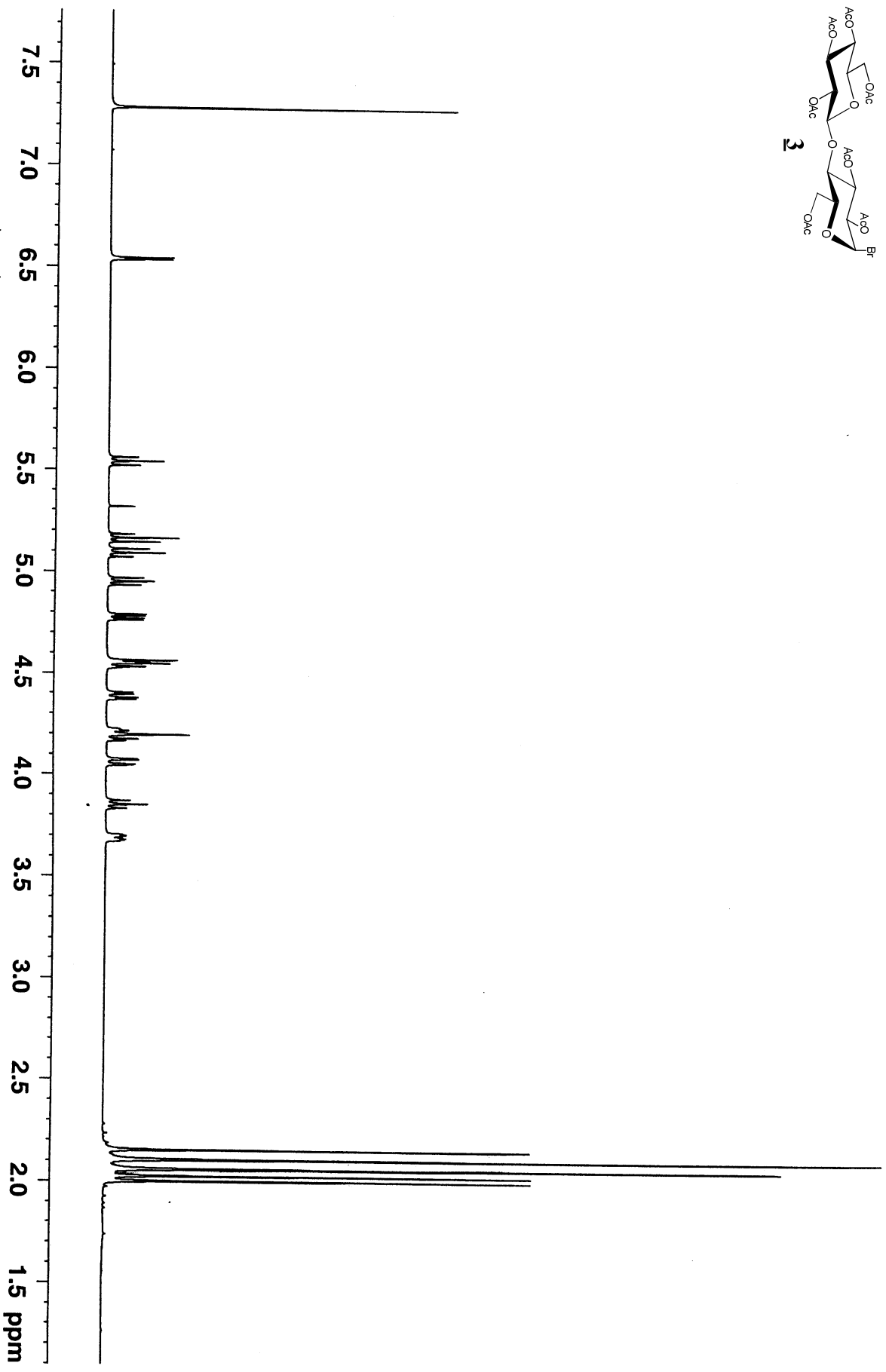
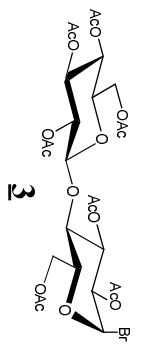
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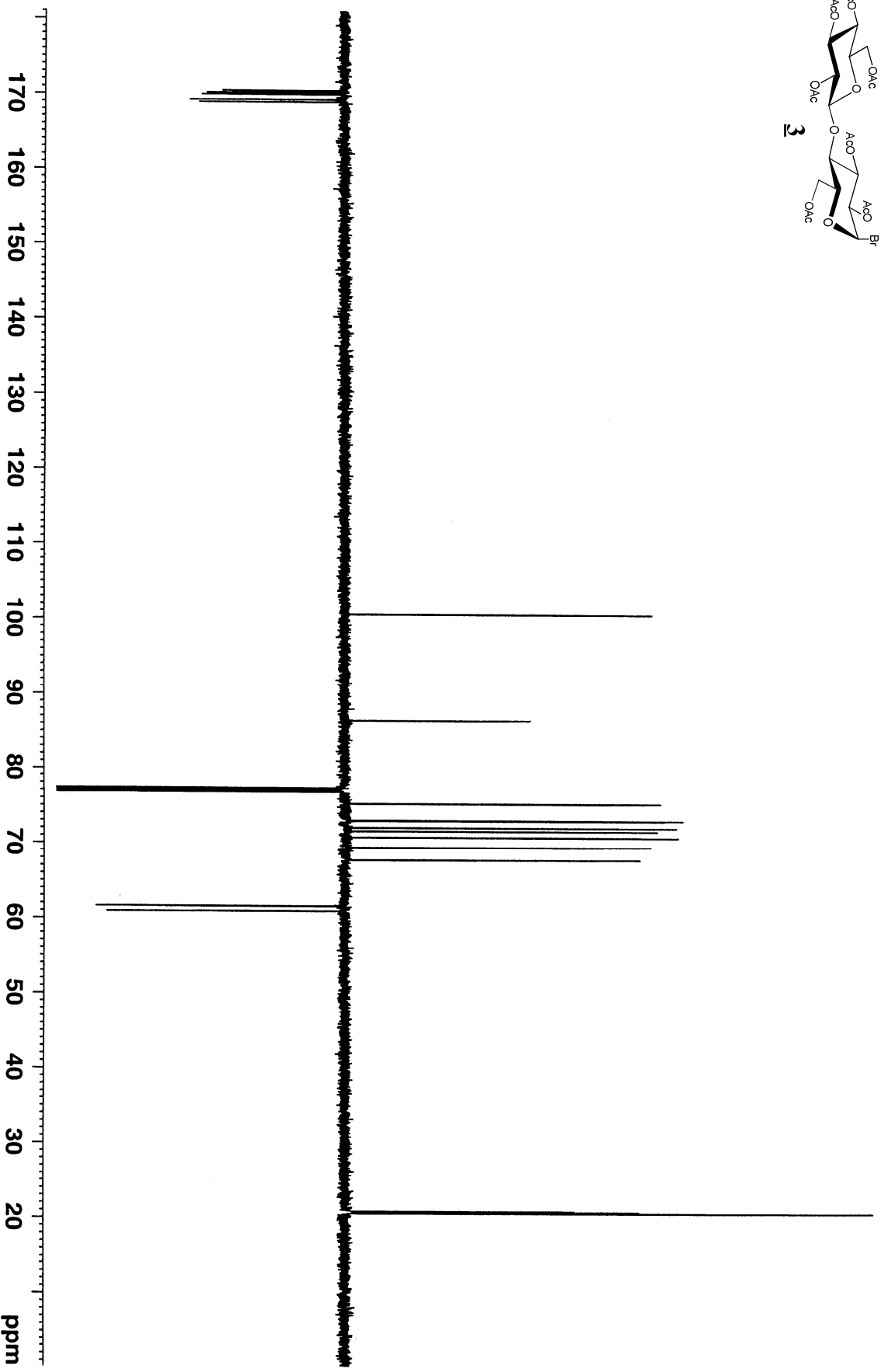
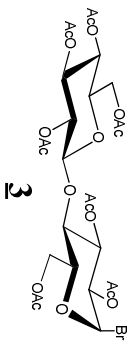
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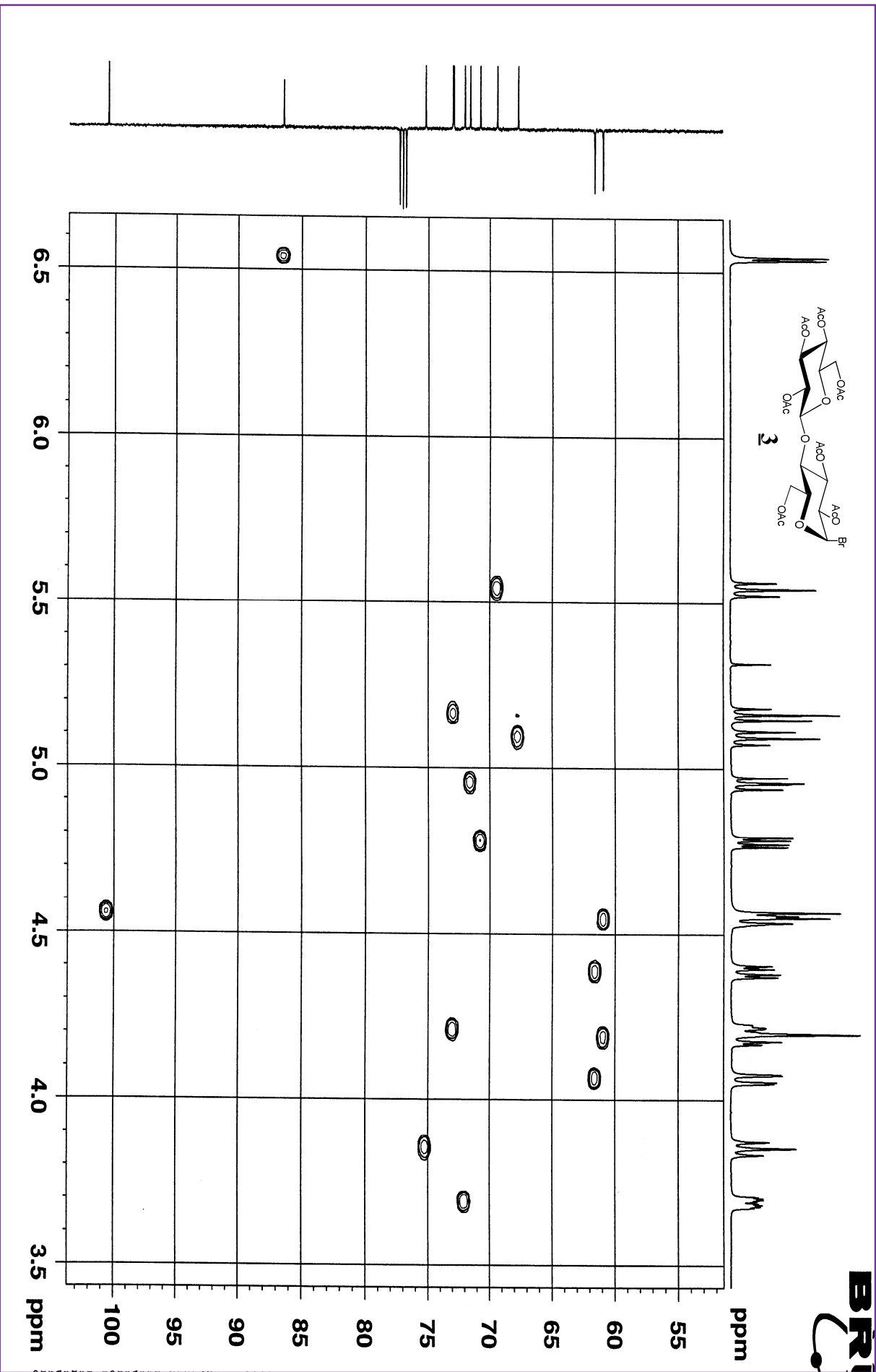
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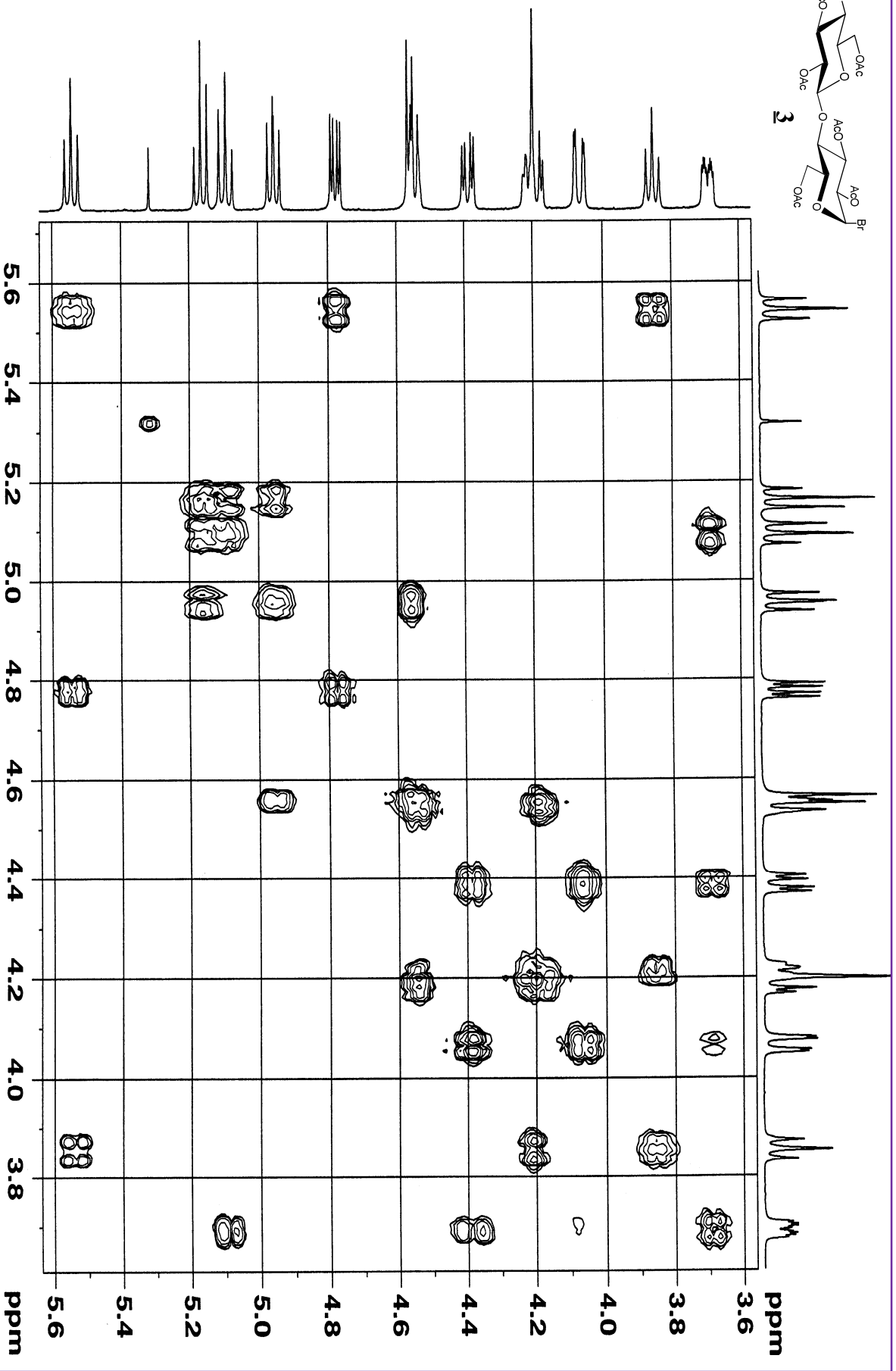
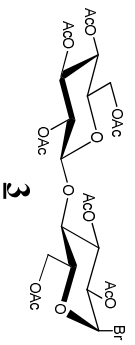


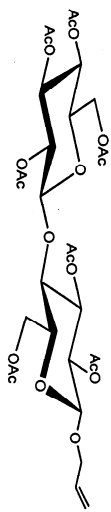
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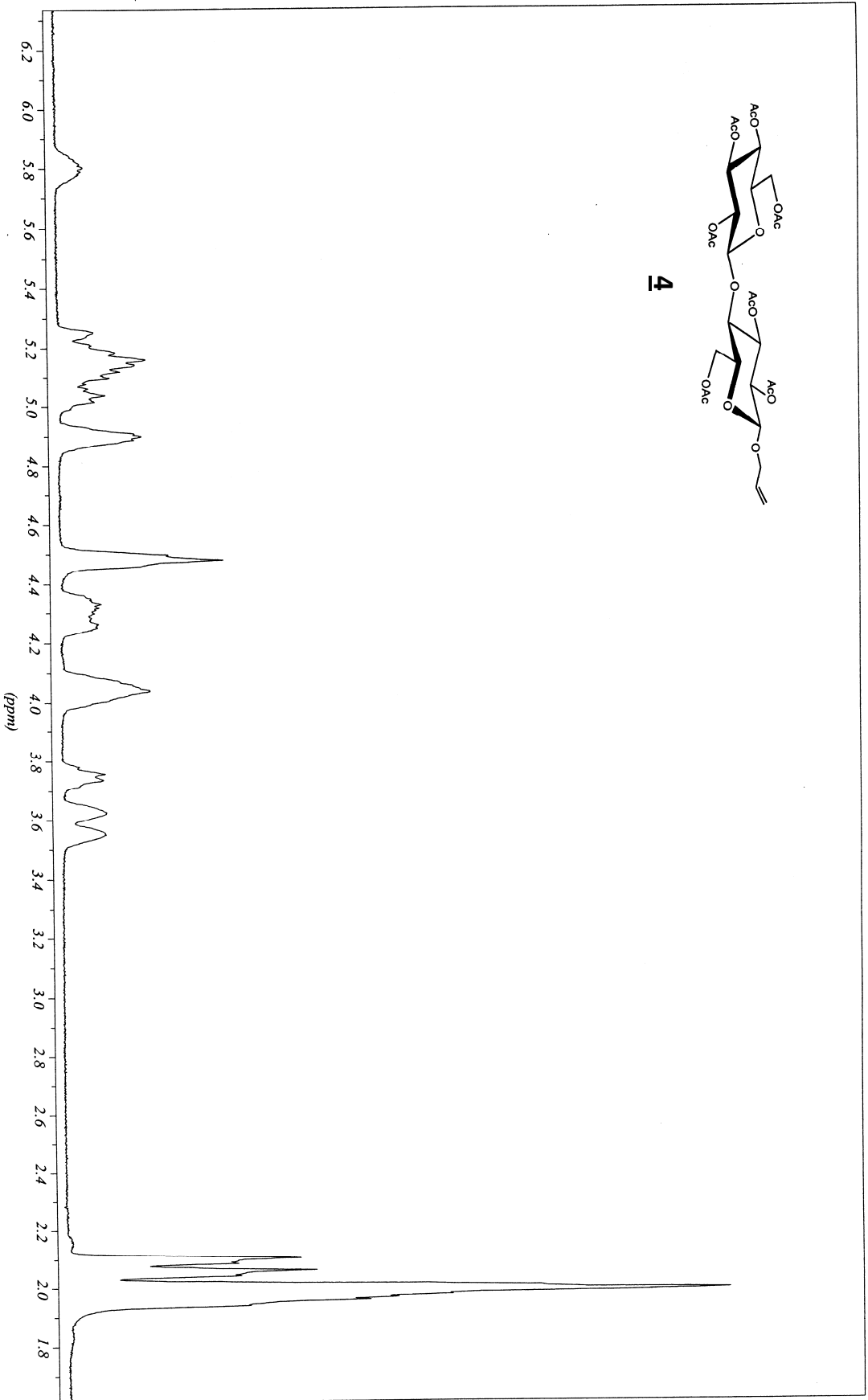


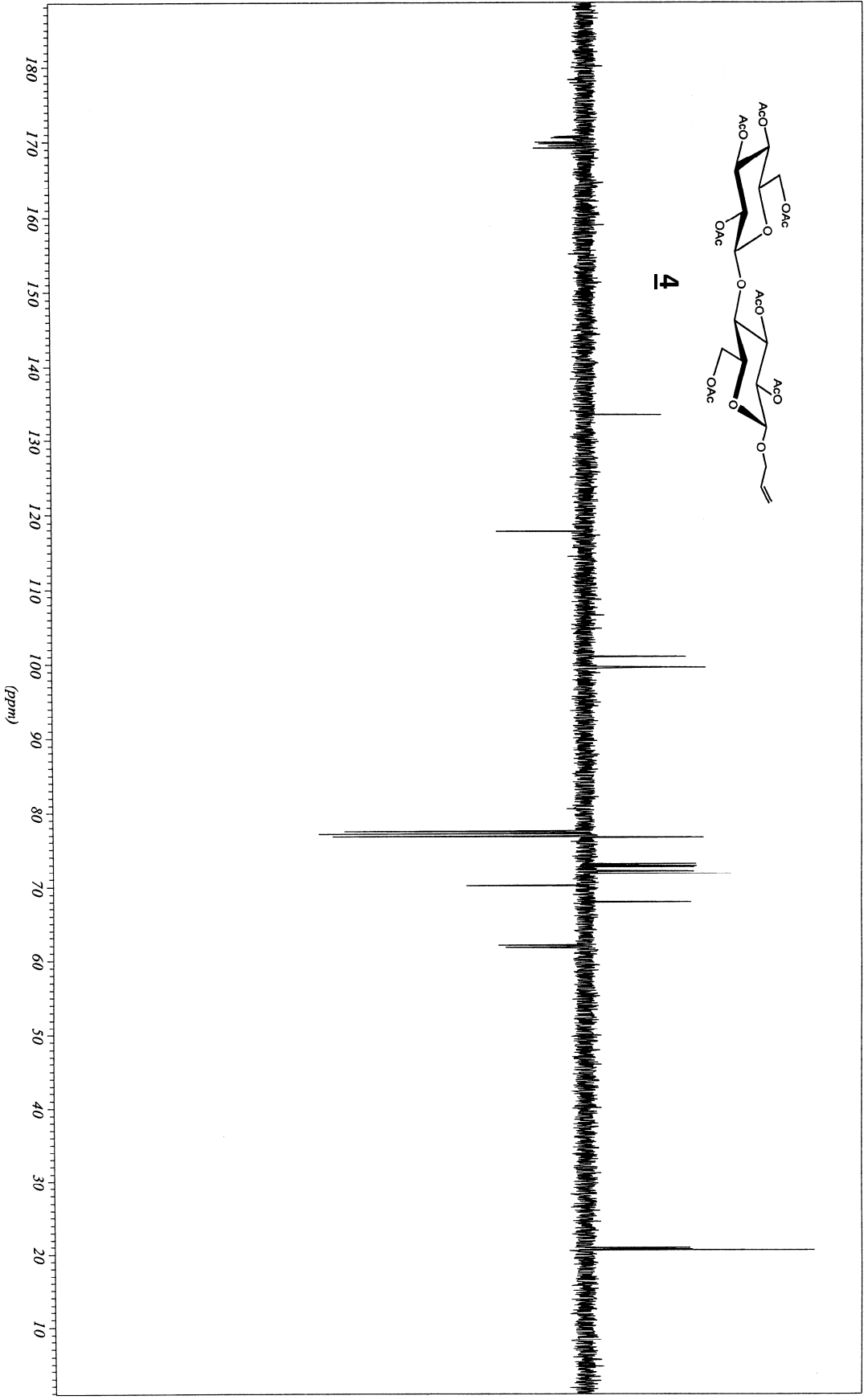


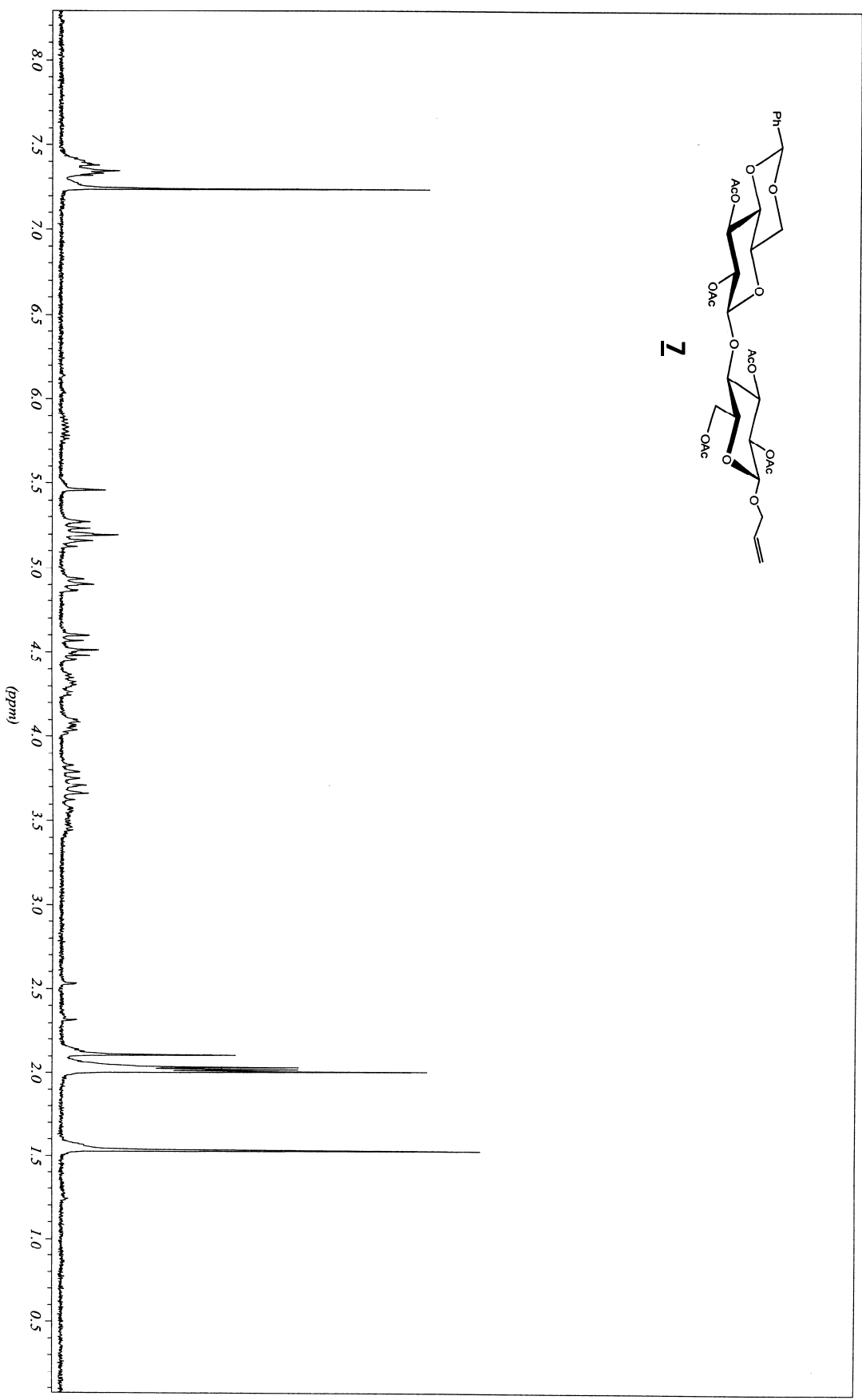
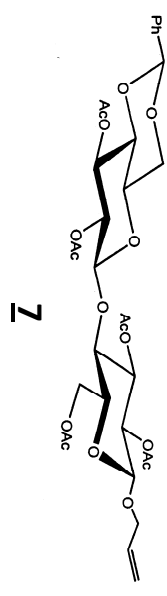




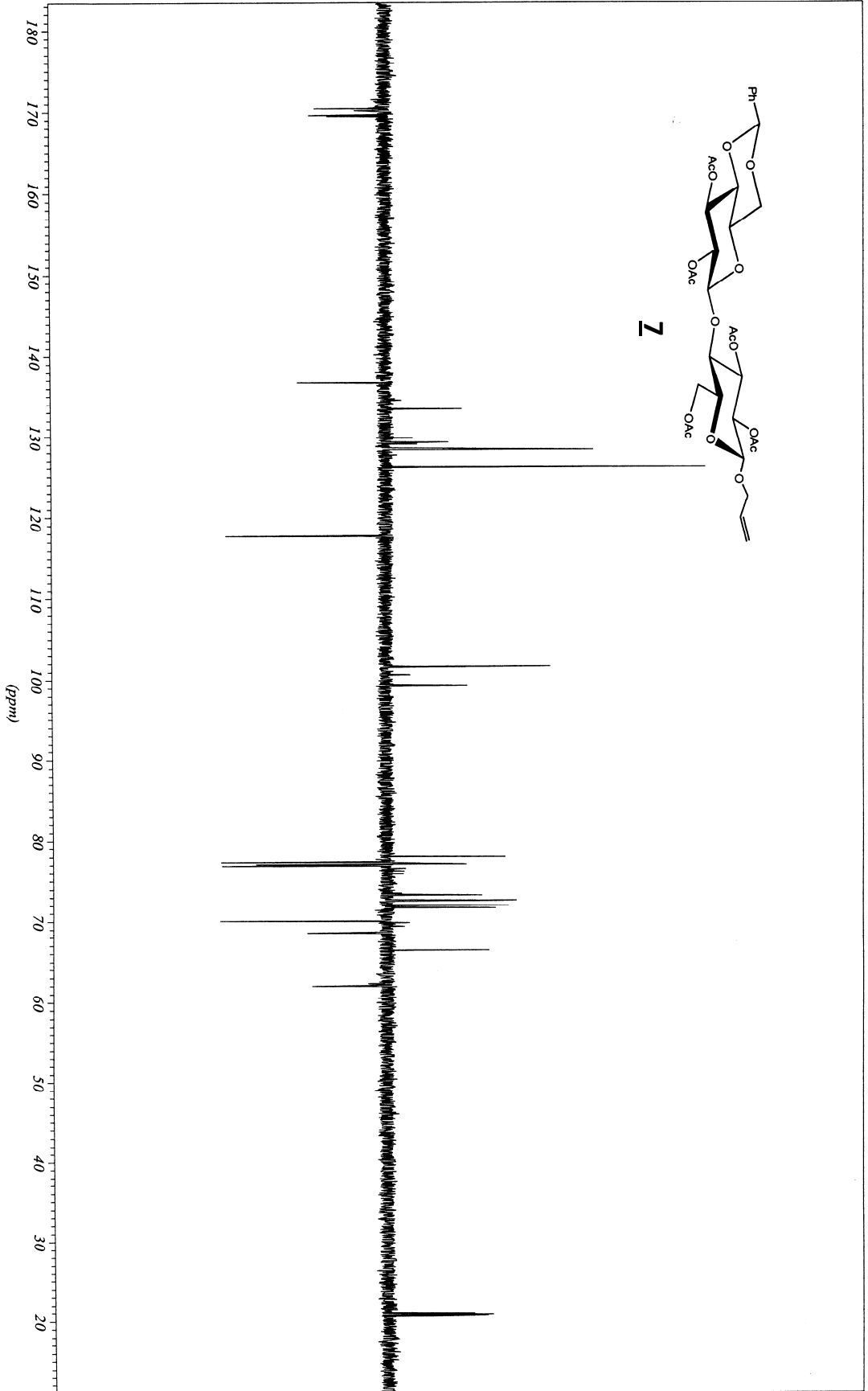
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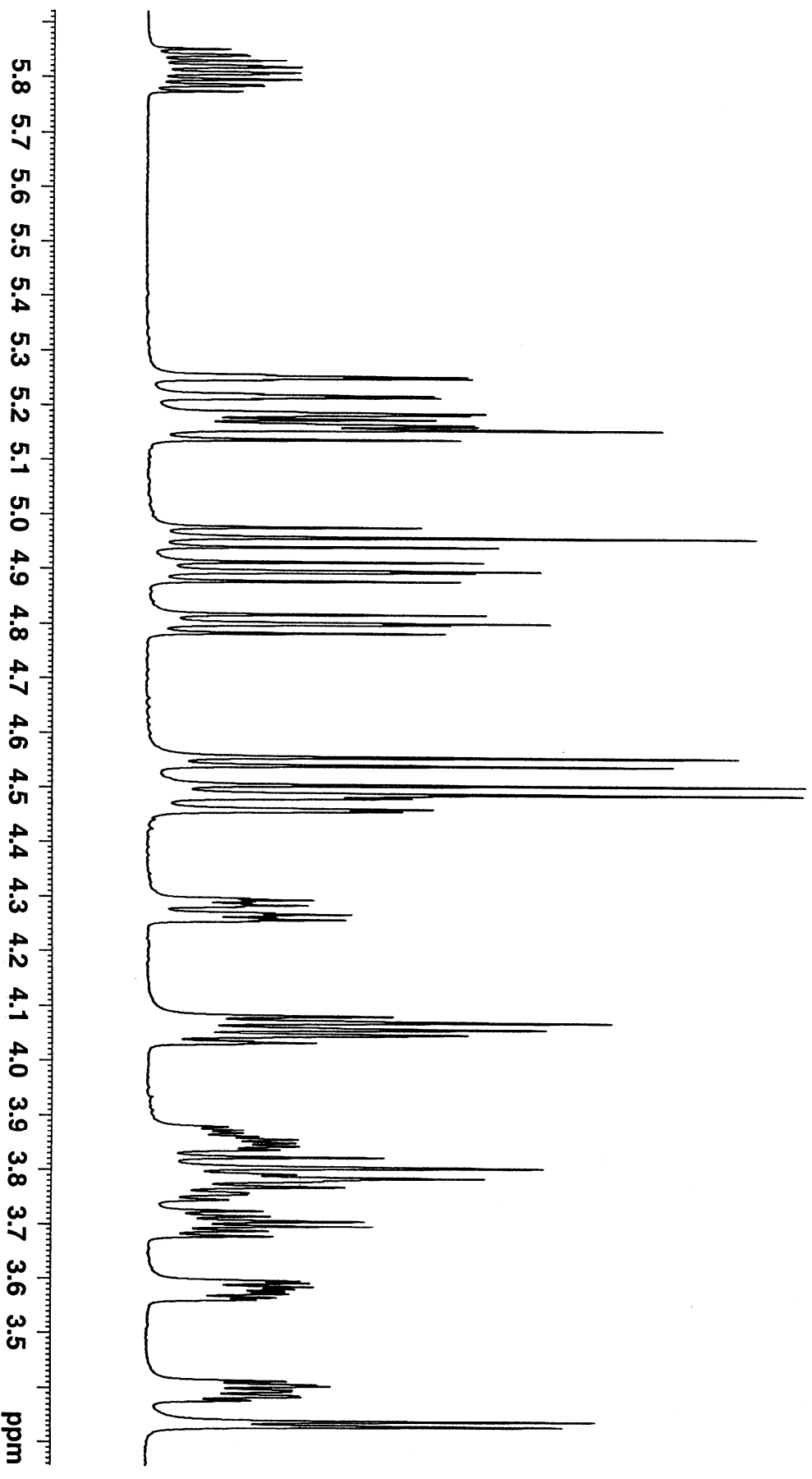
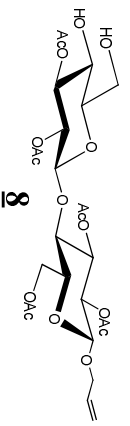


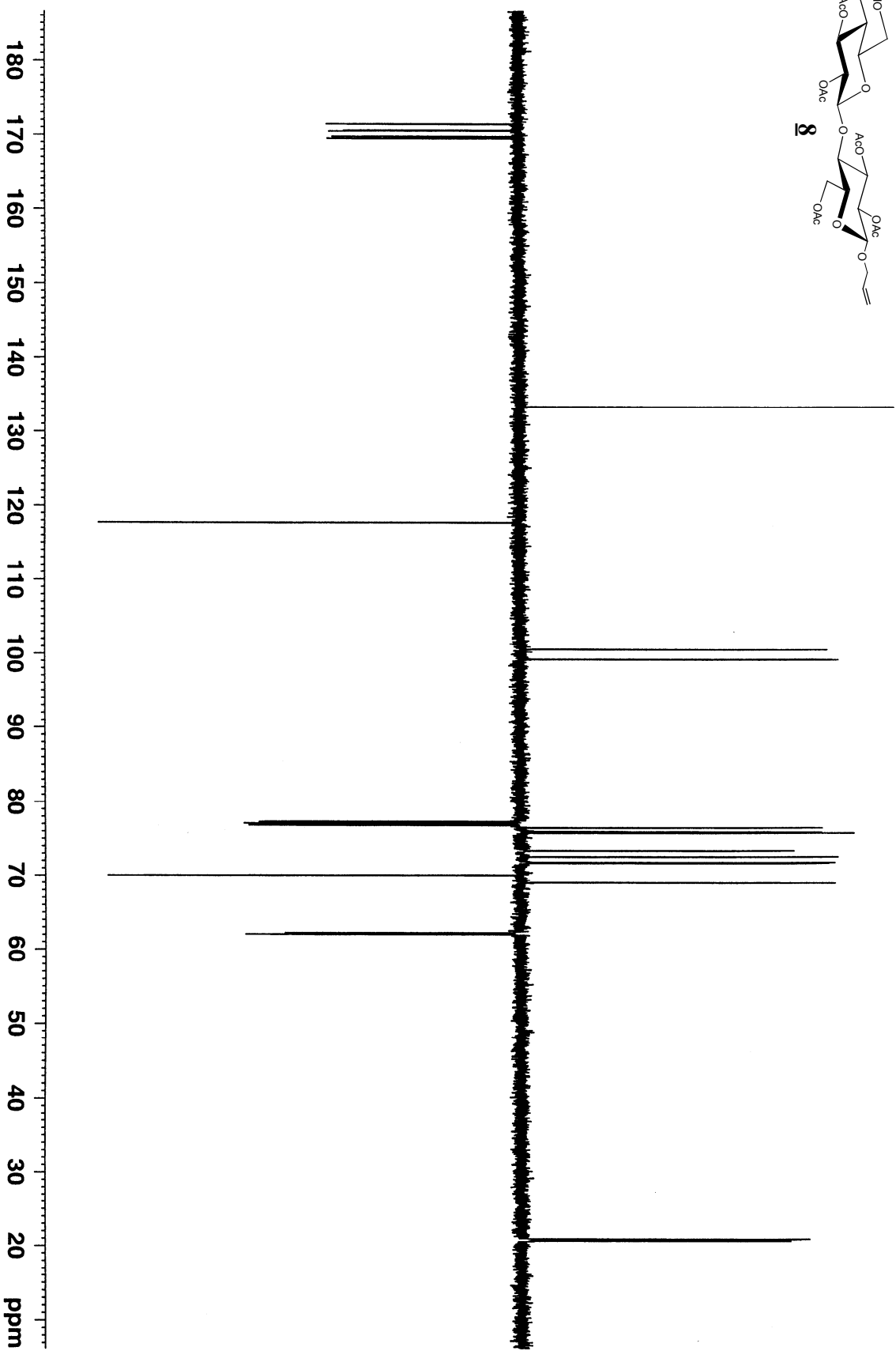
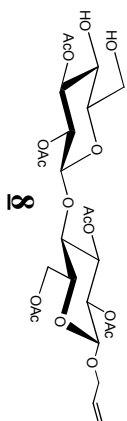


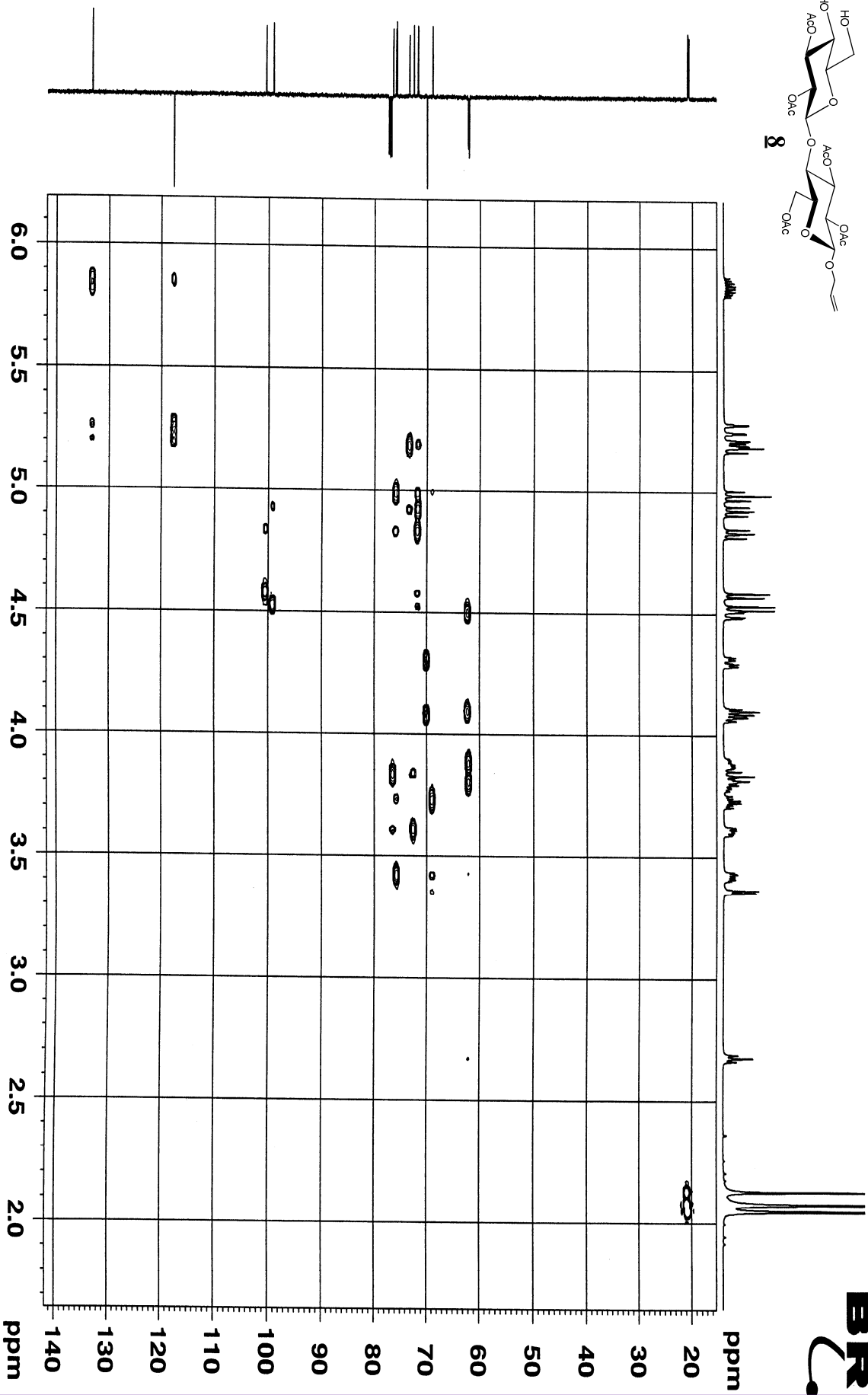
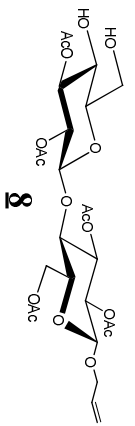


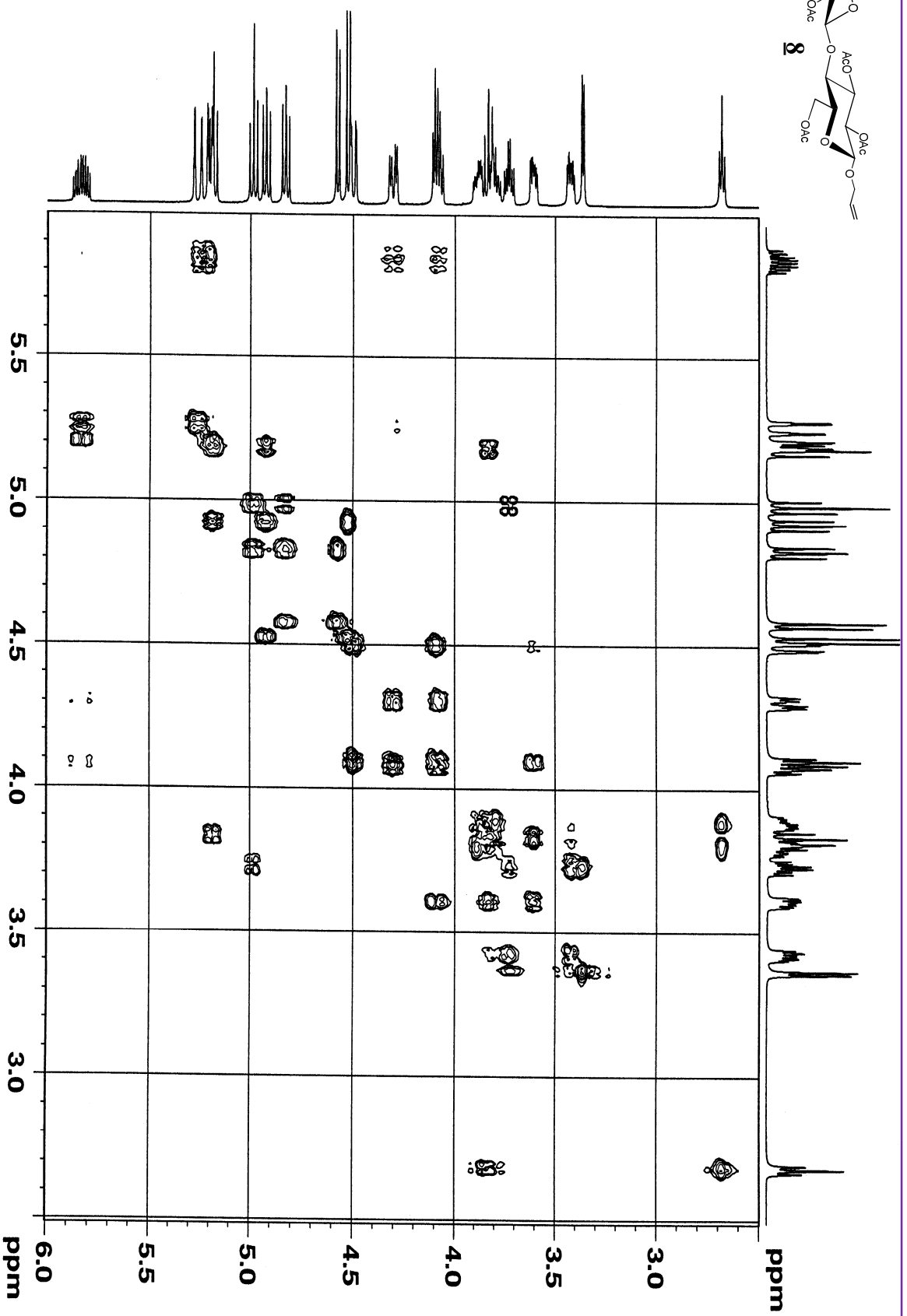
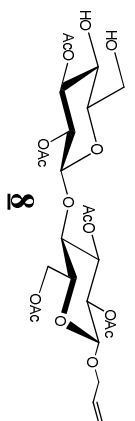


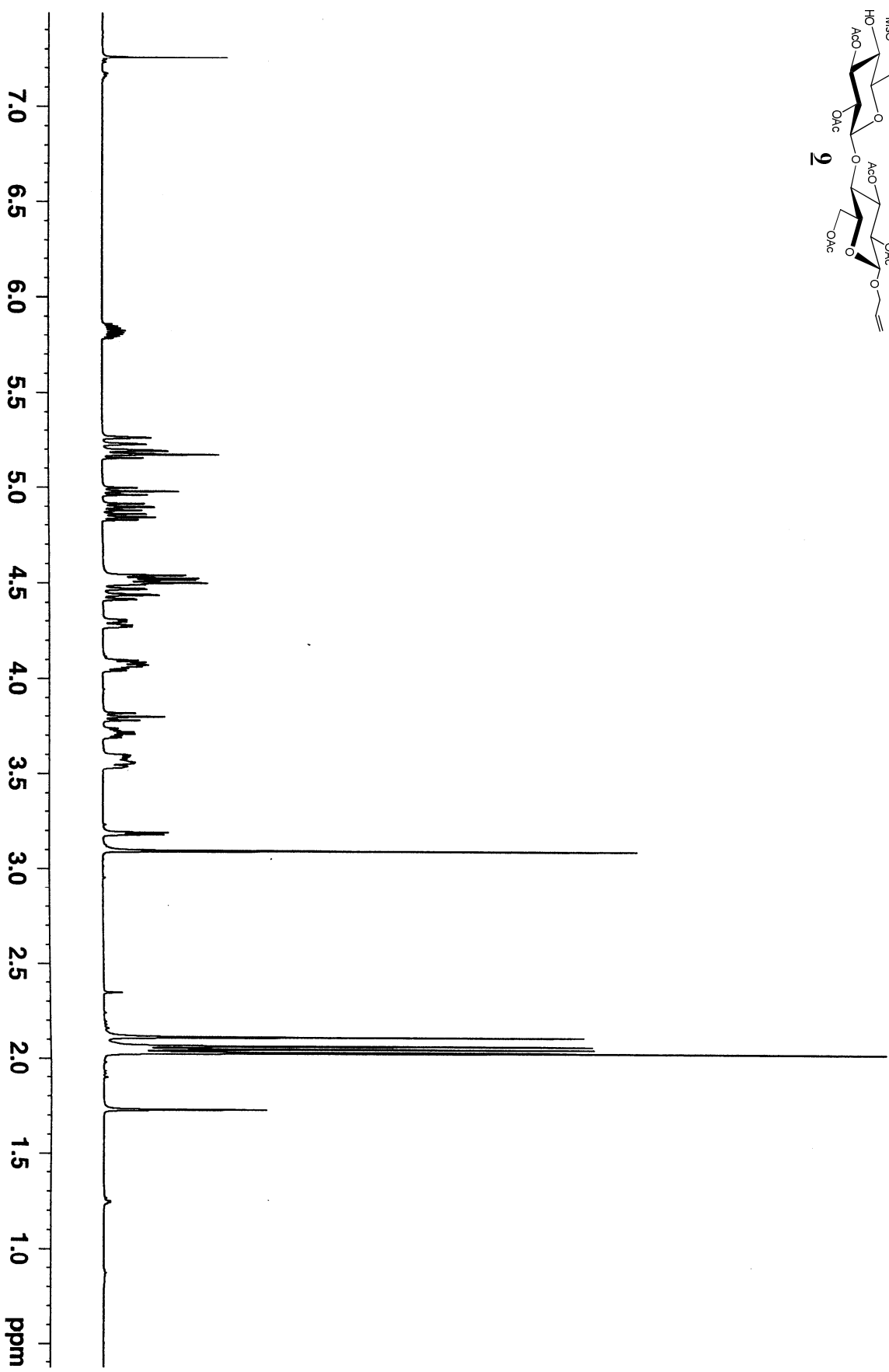
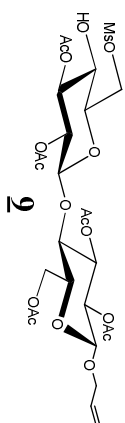


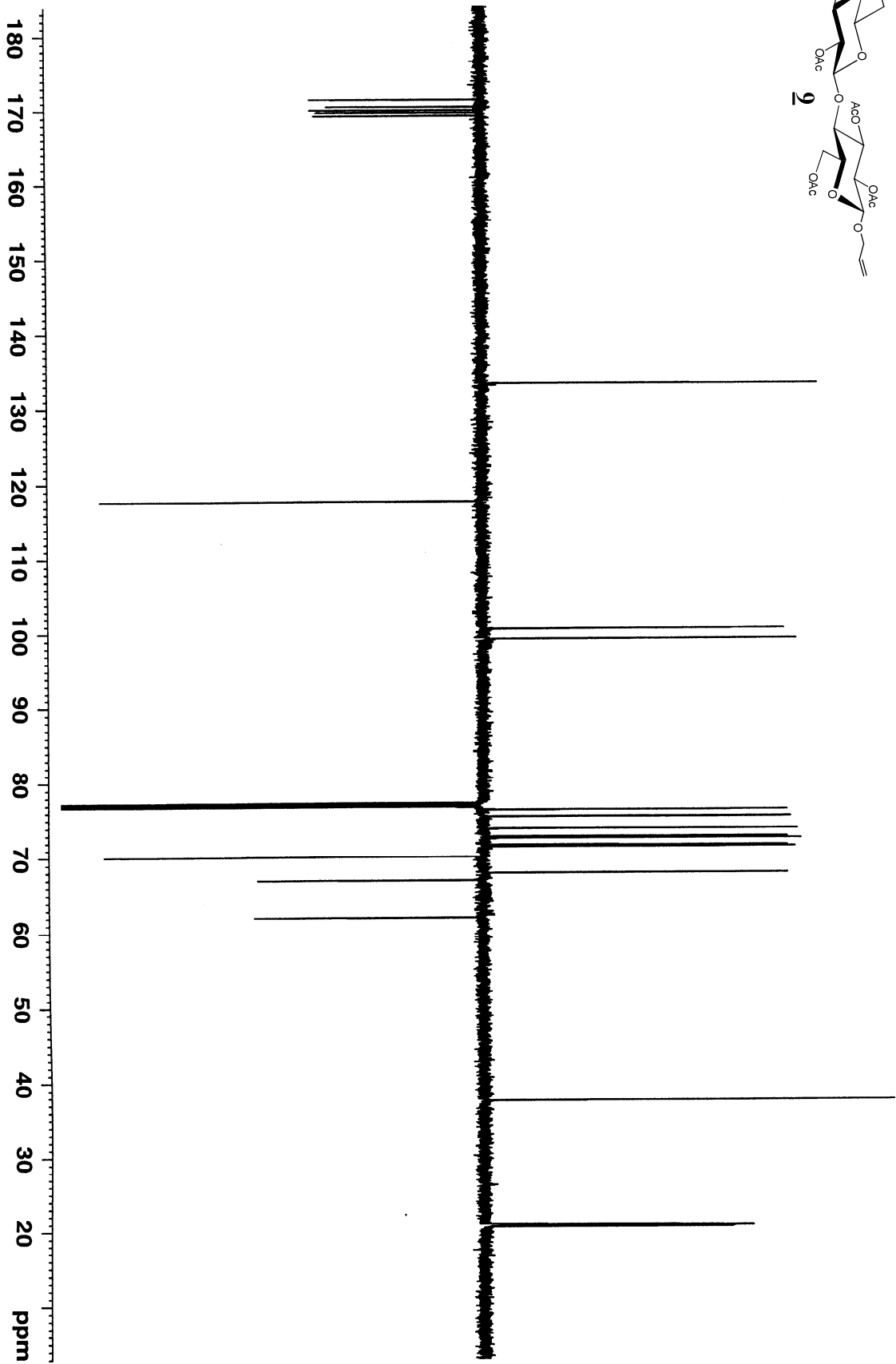
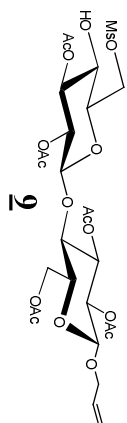


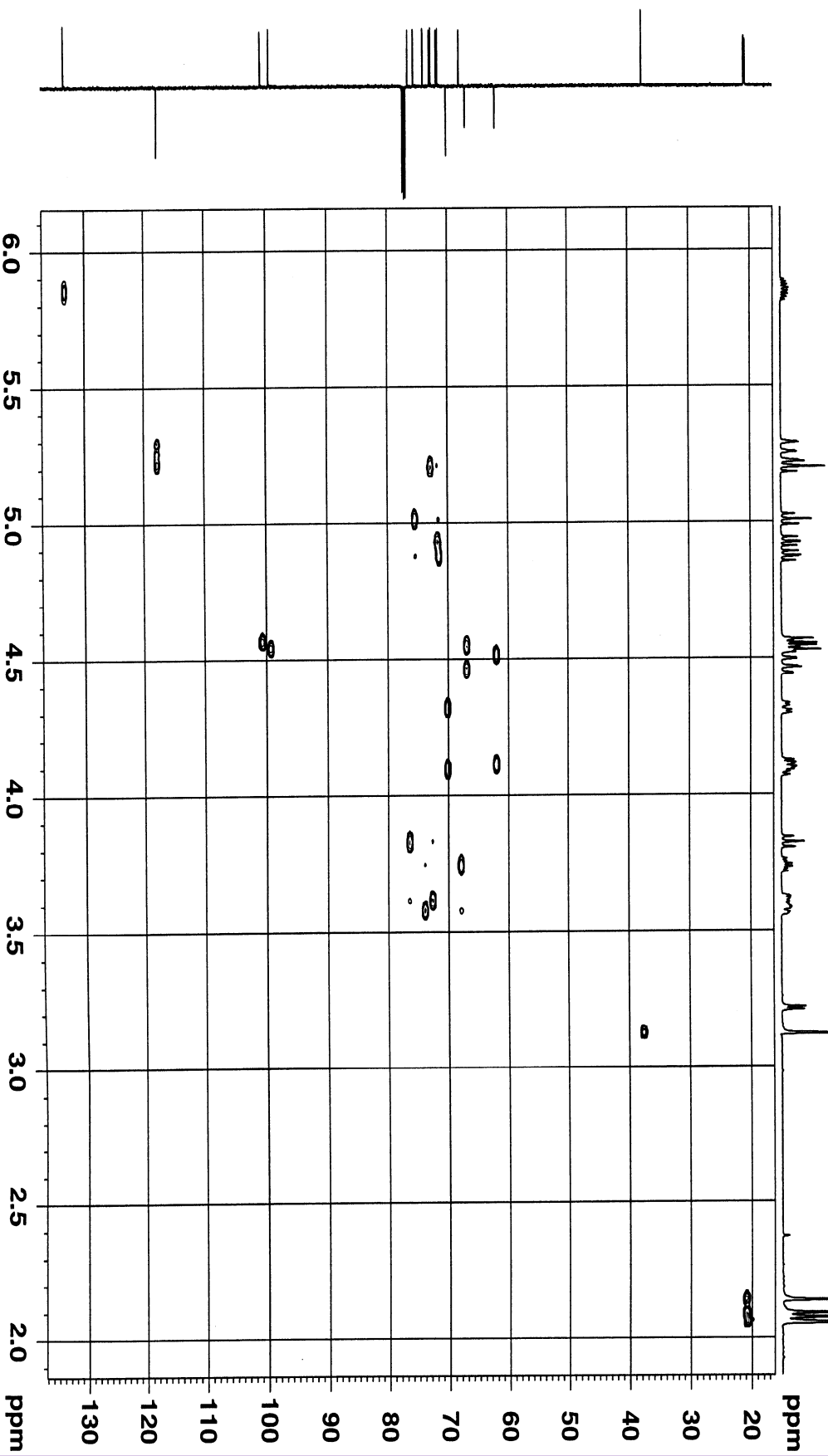
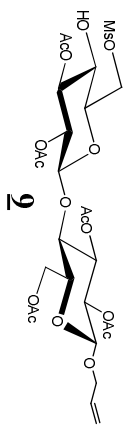






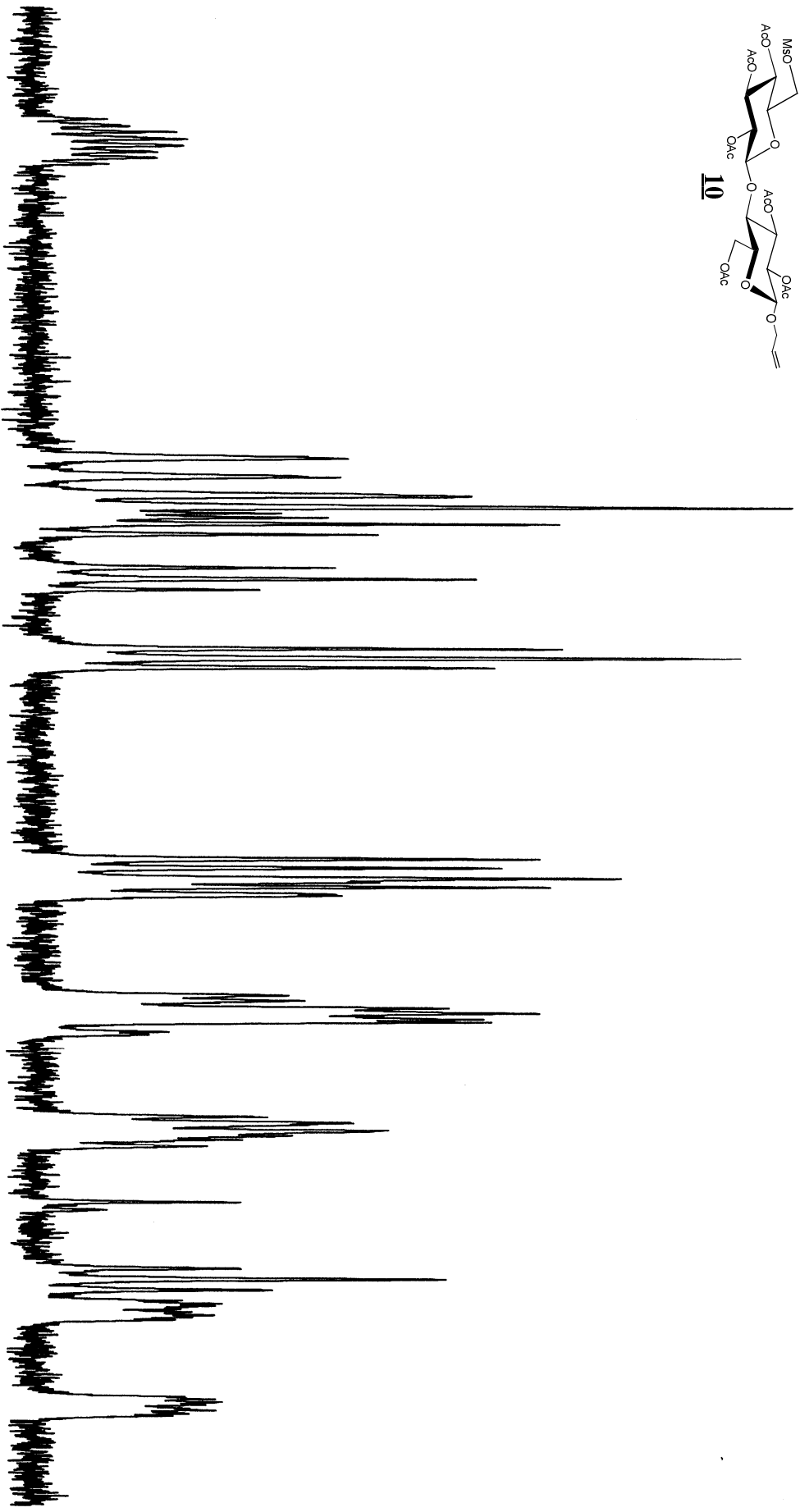
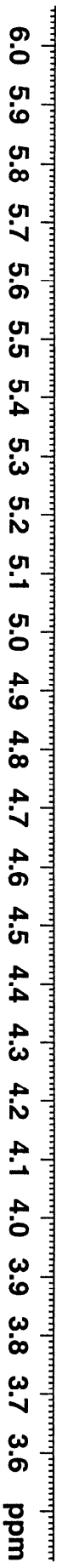
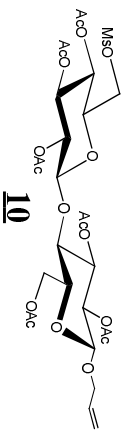


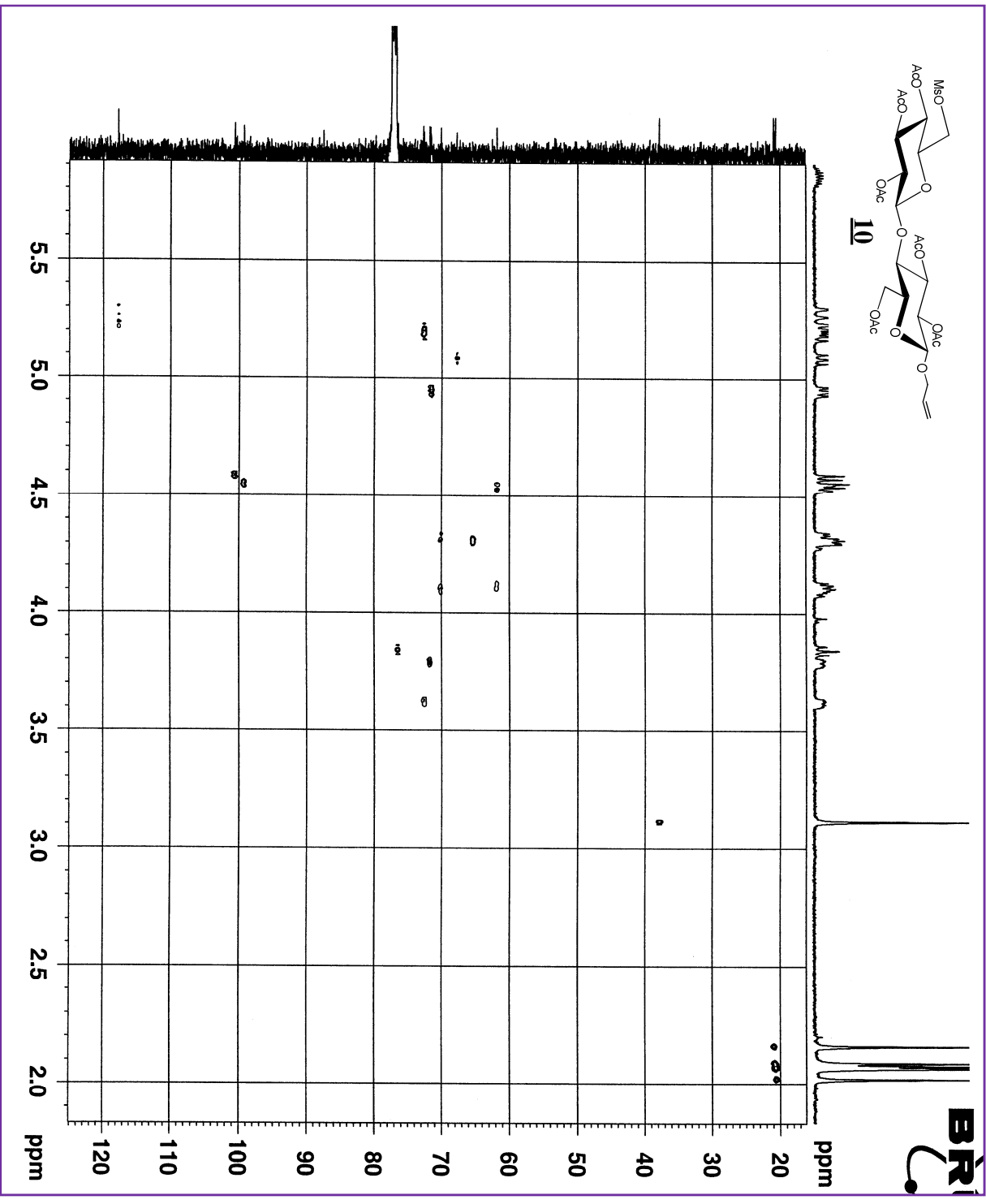
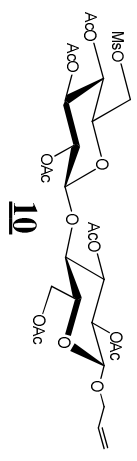




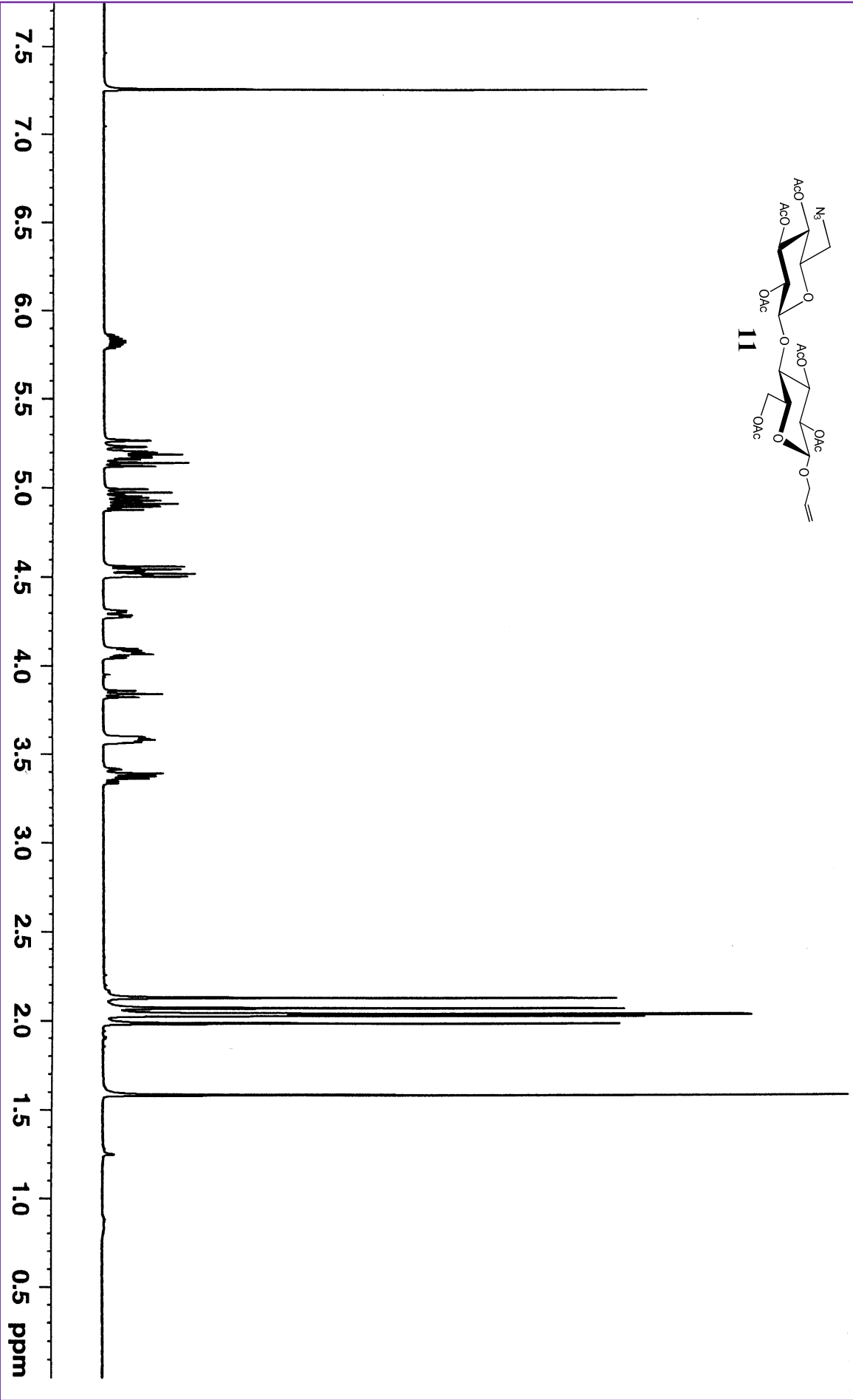
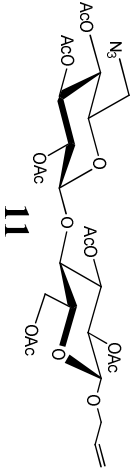


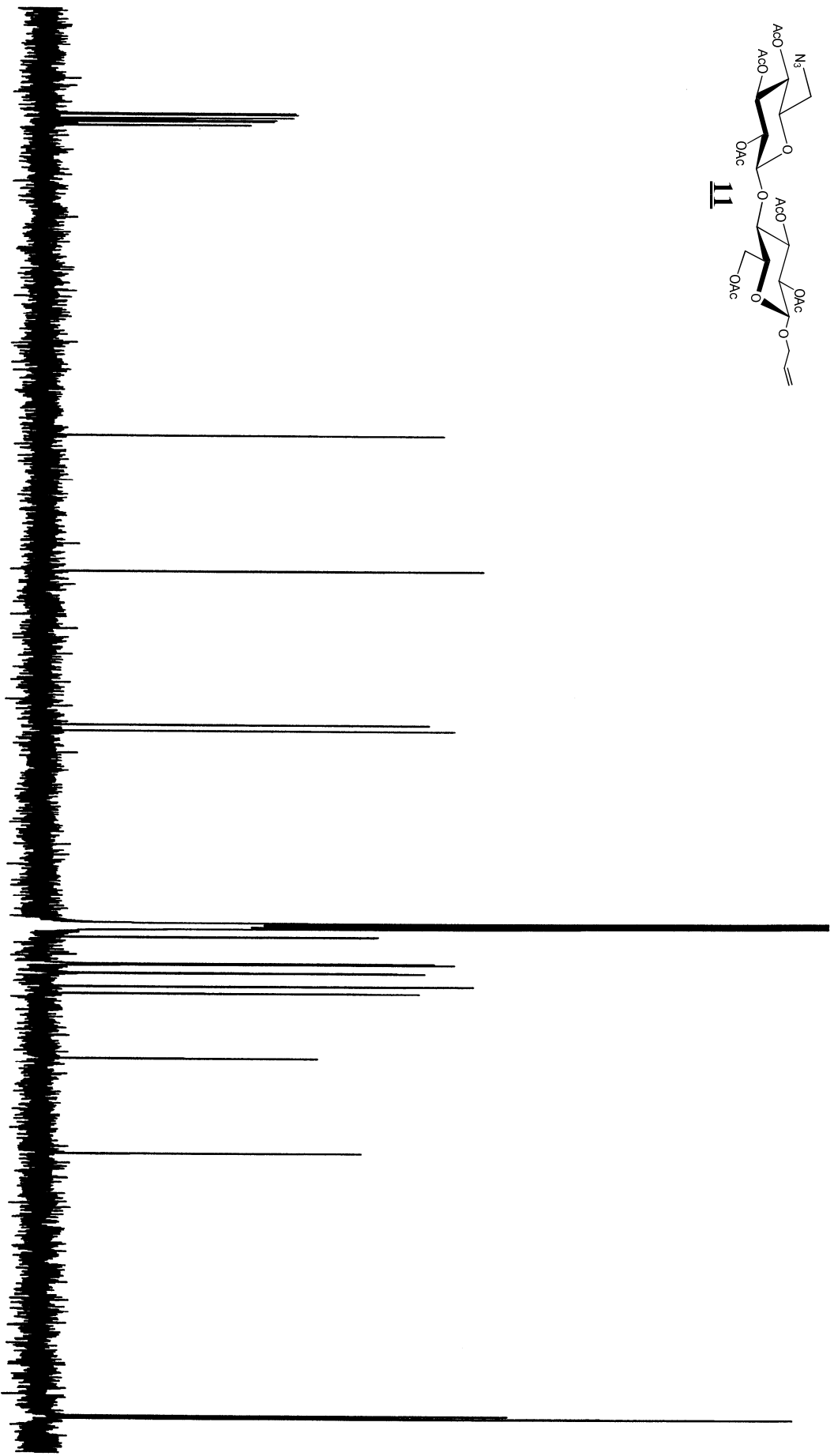
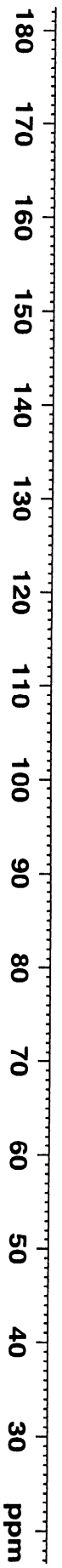
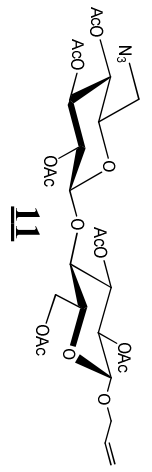


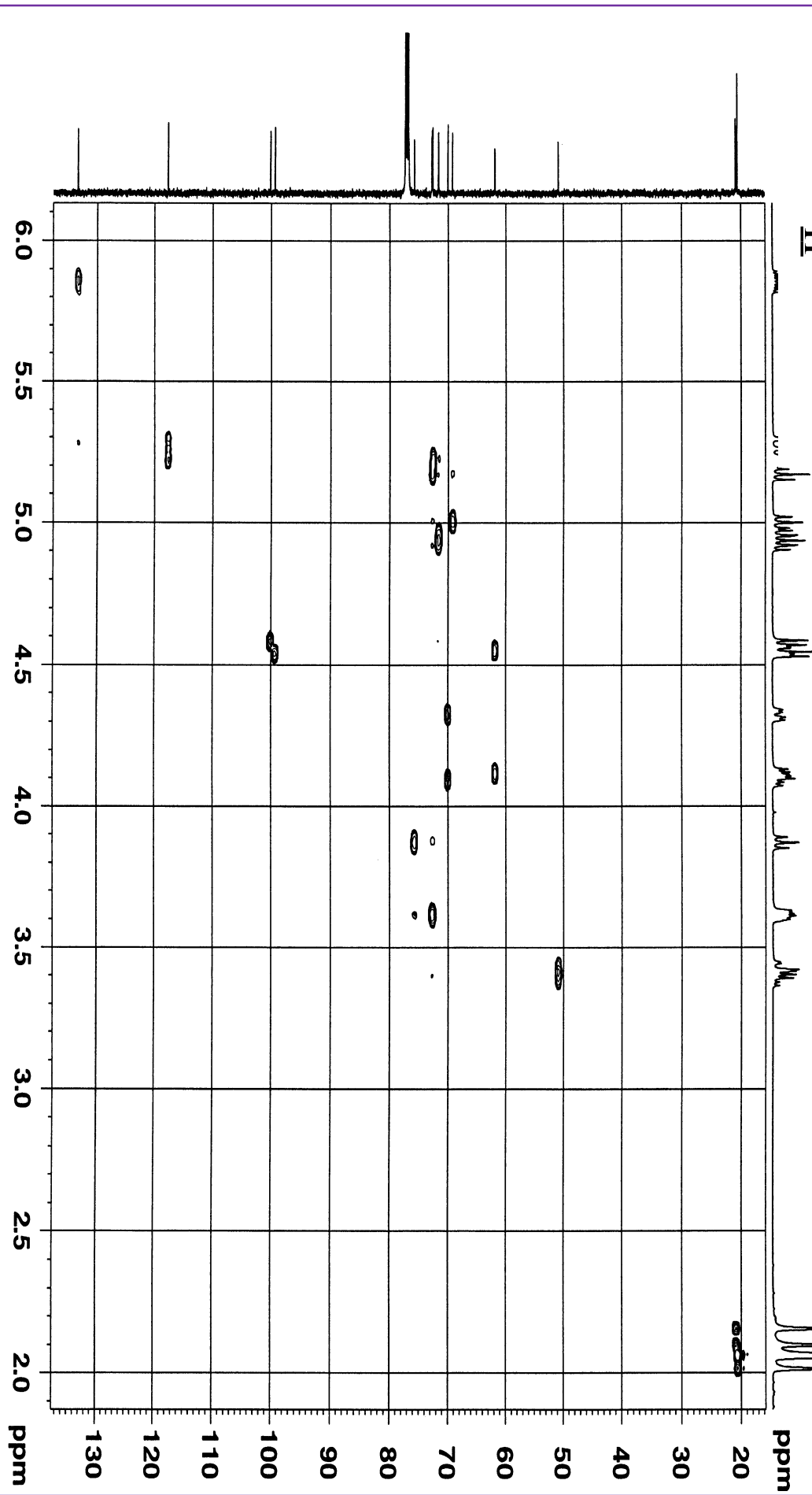
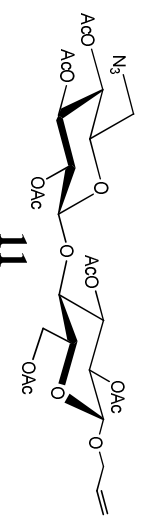


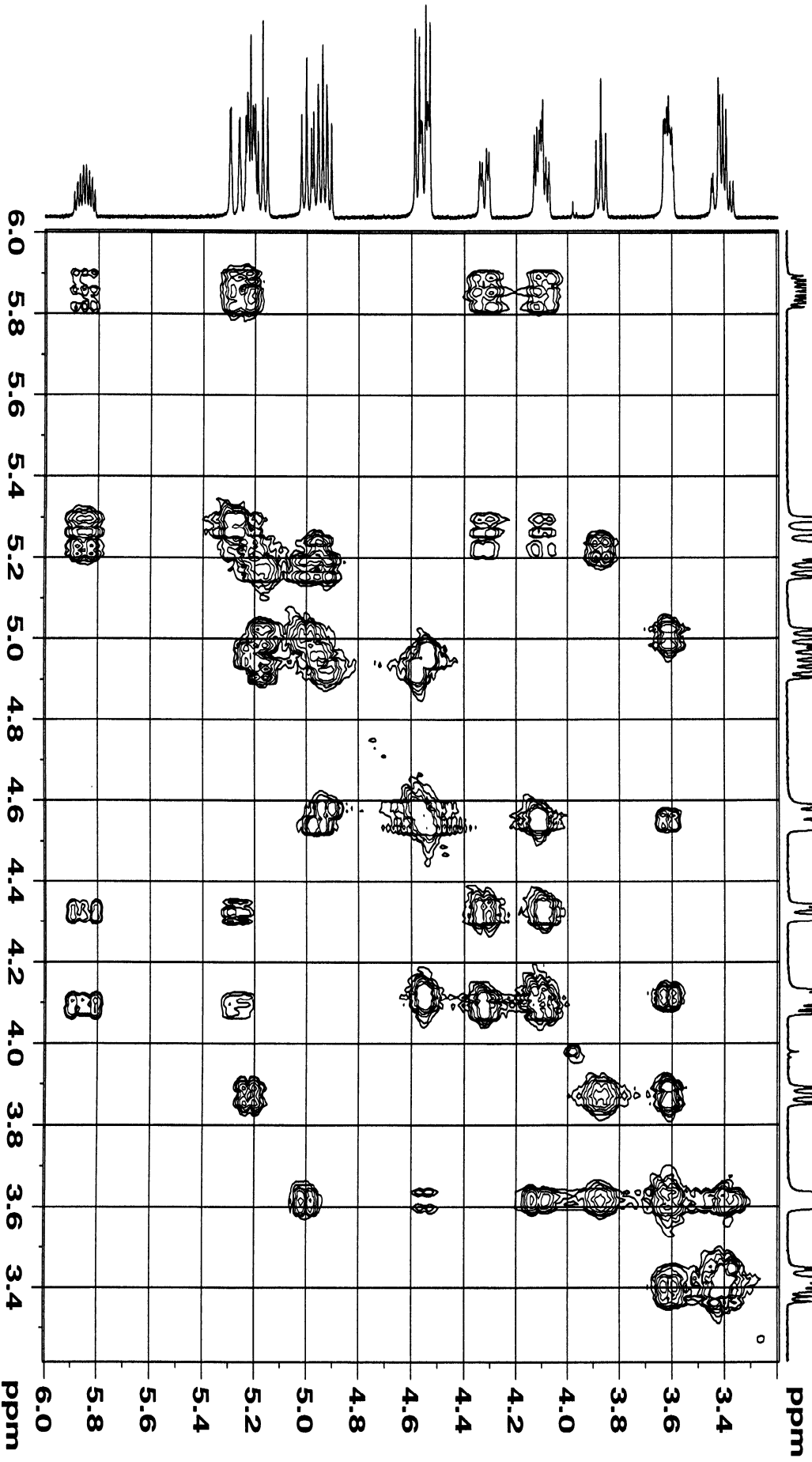
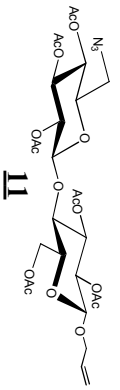




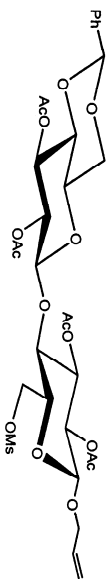




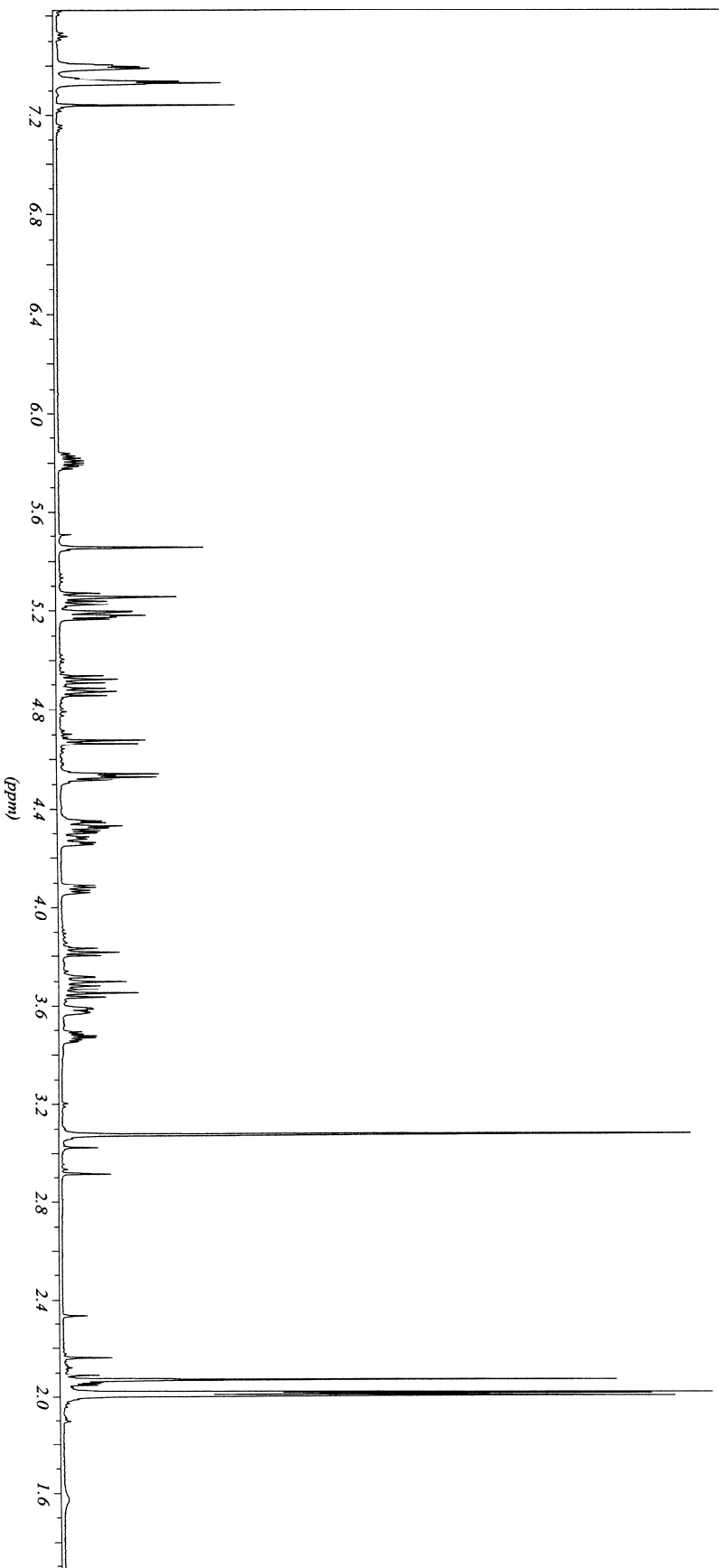


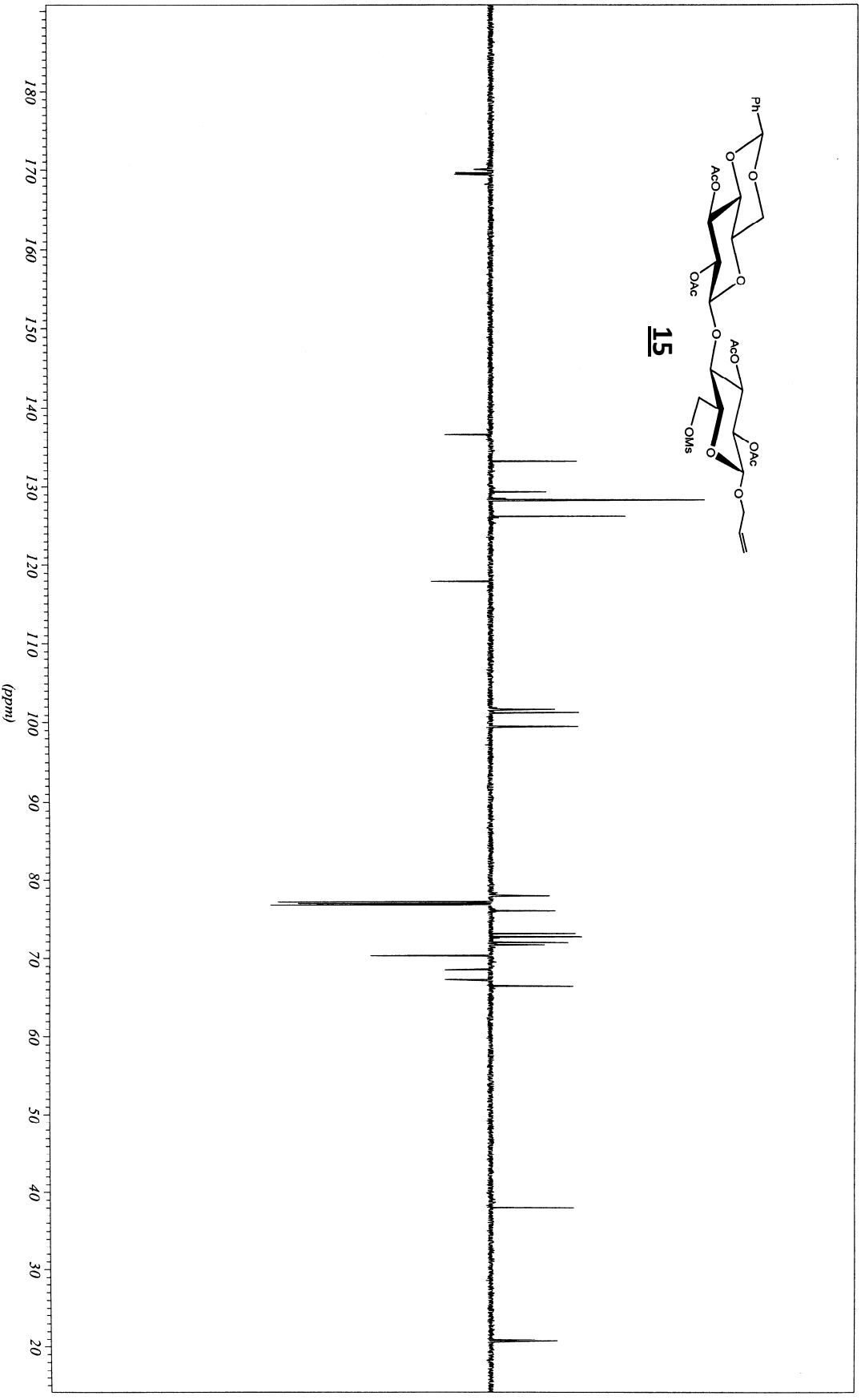


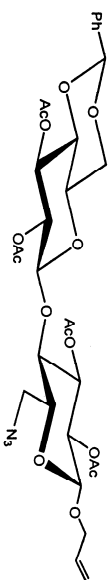




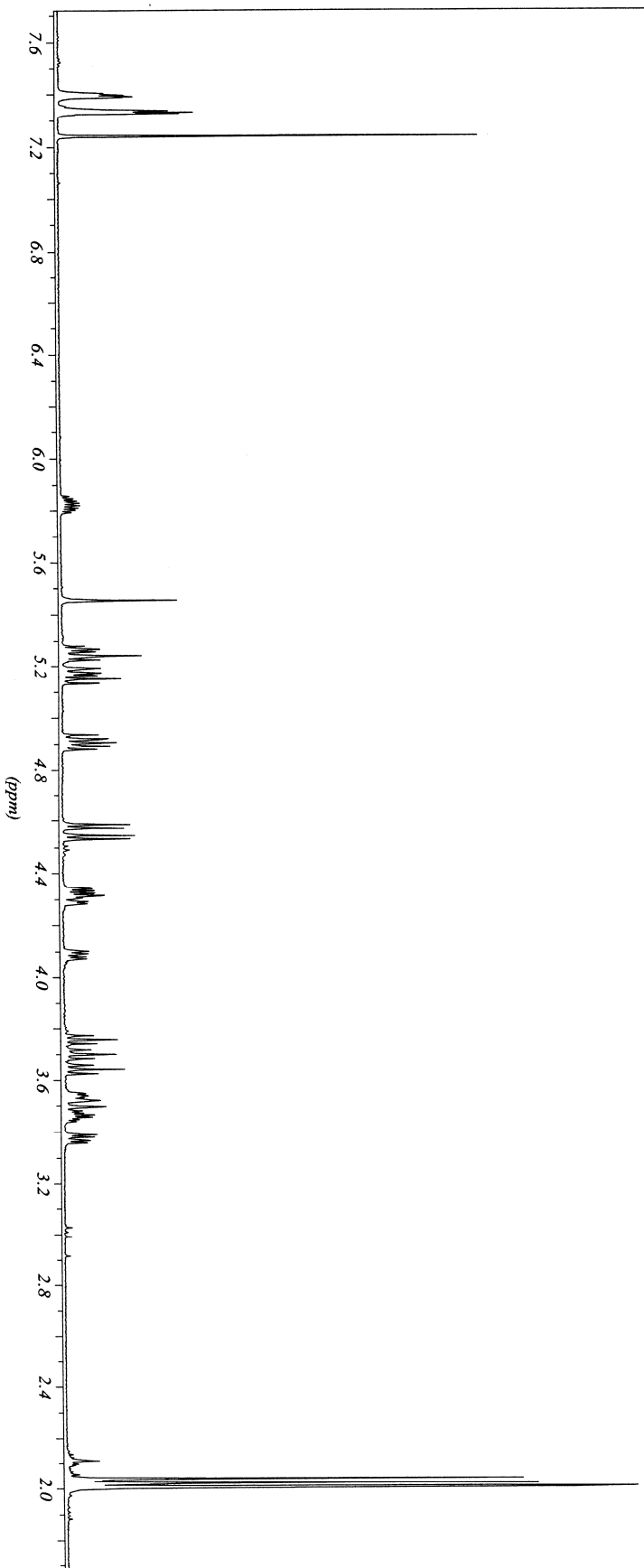
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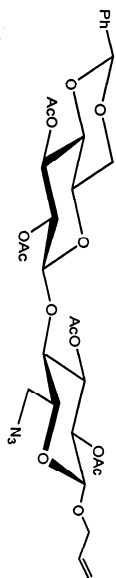




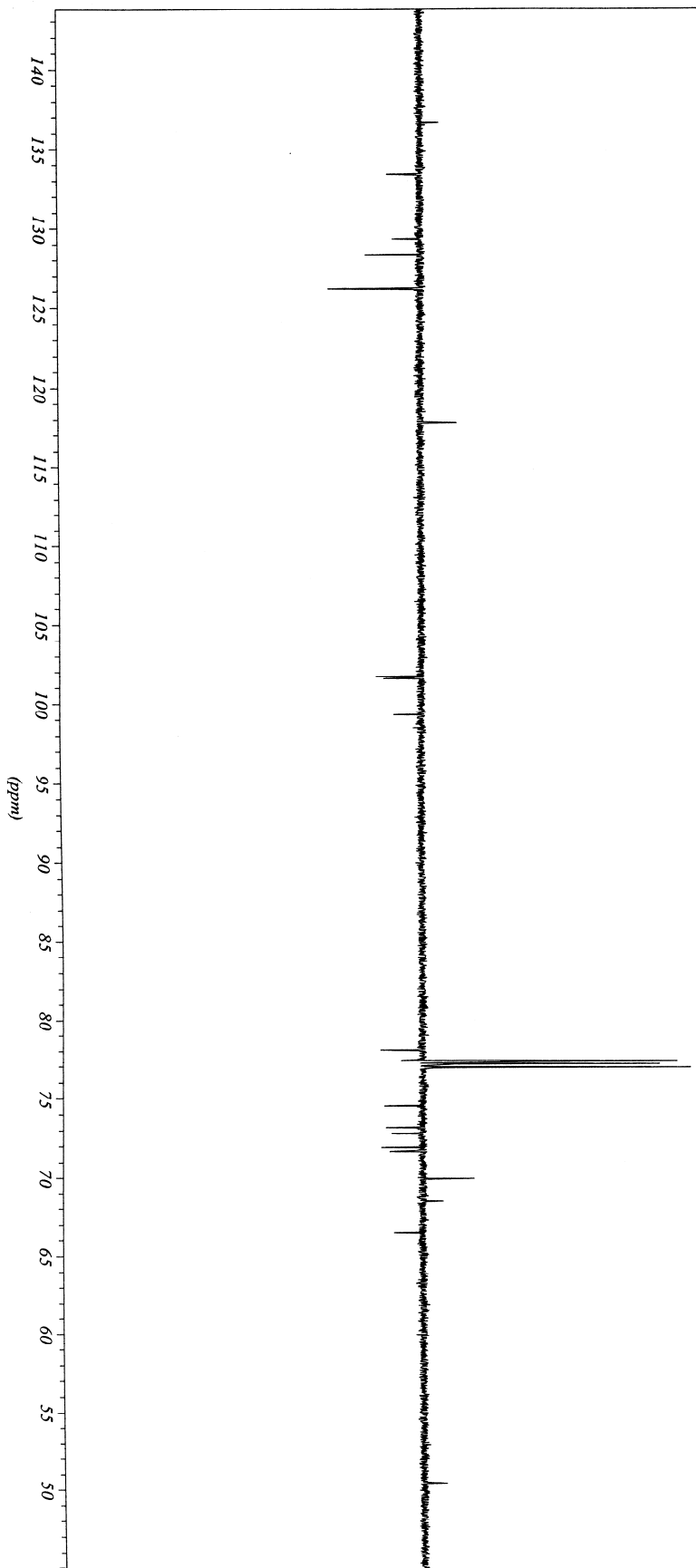


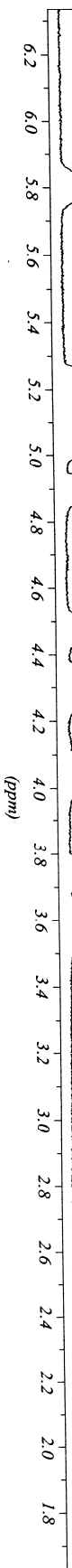
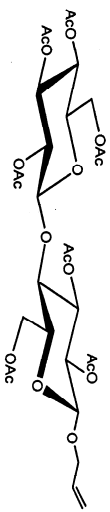
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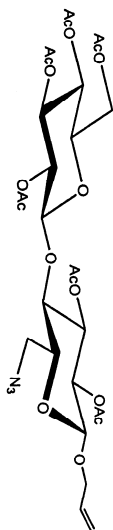




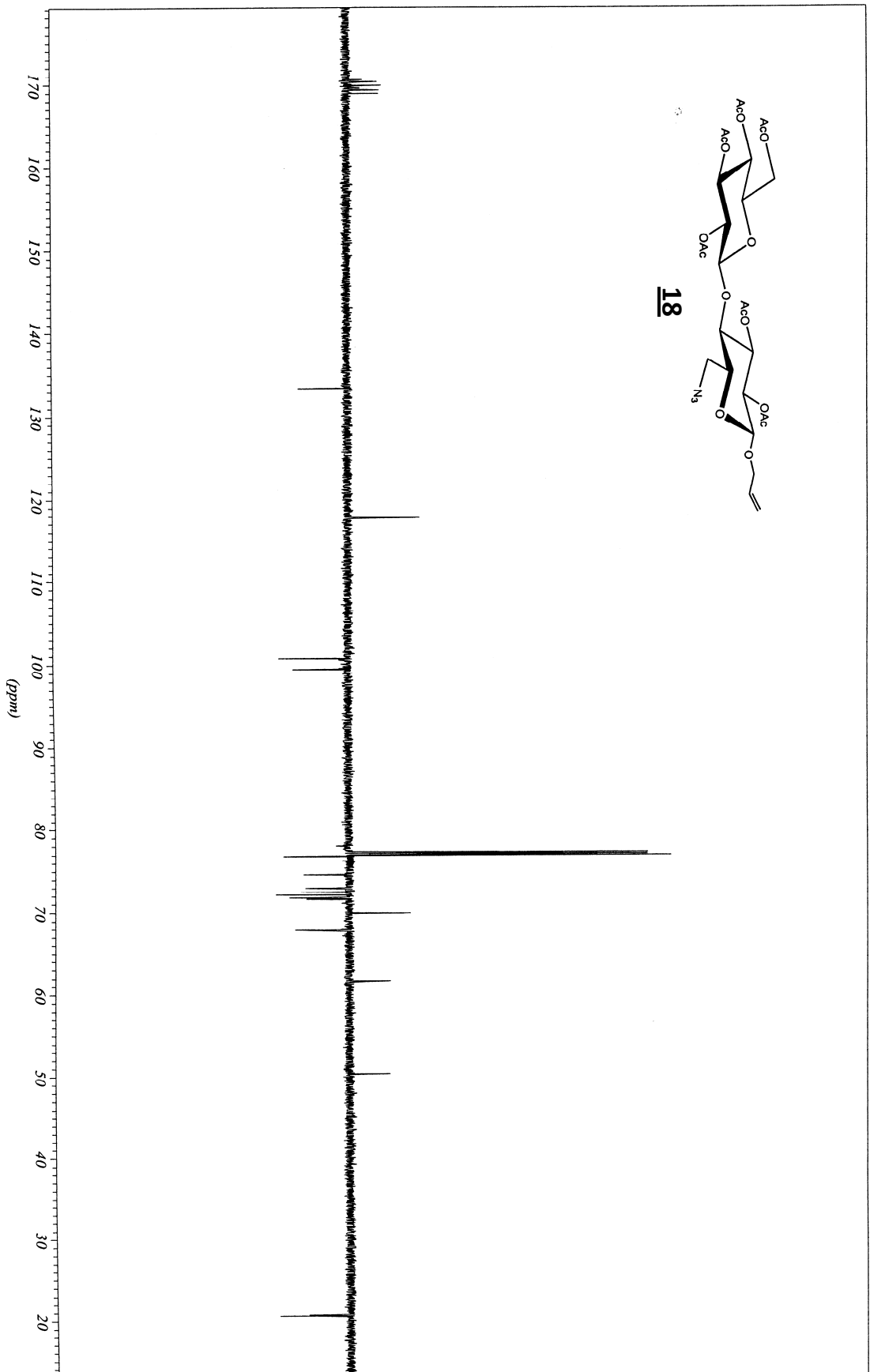
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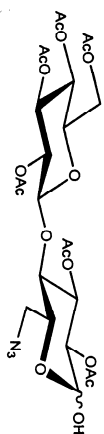




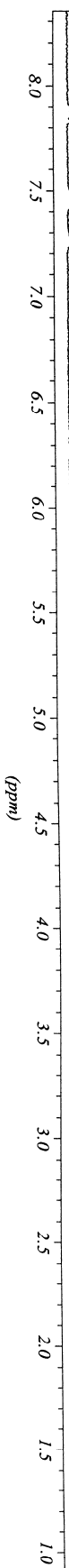


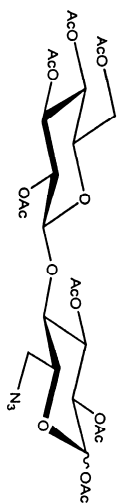
**18**



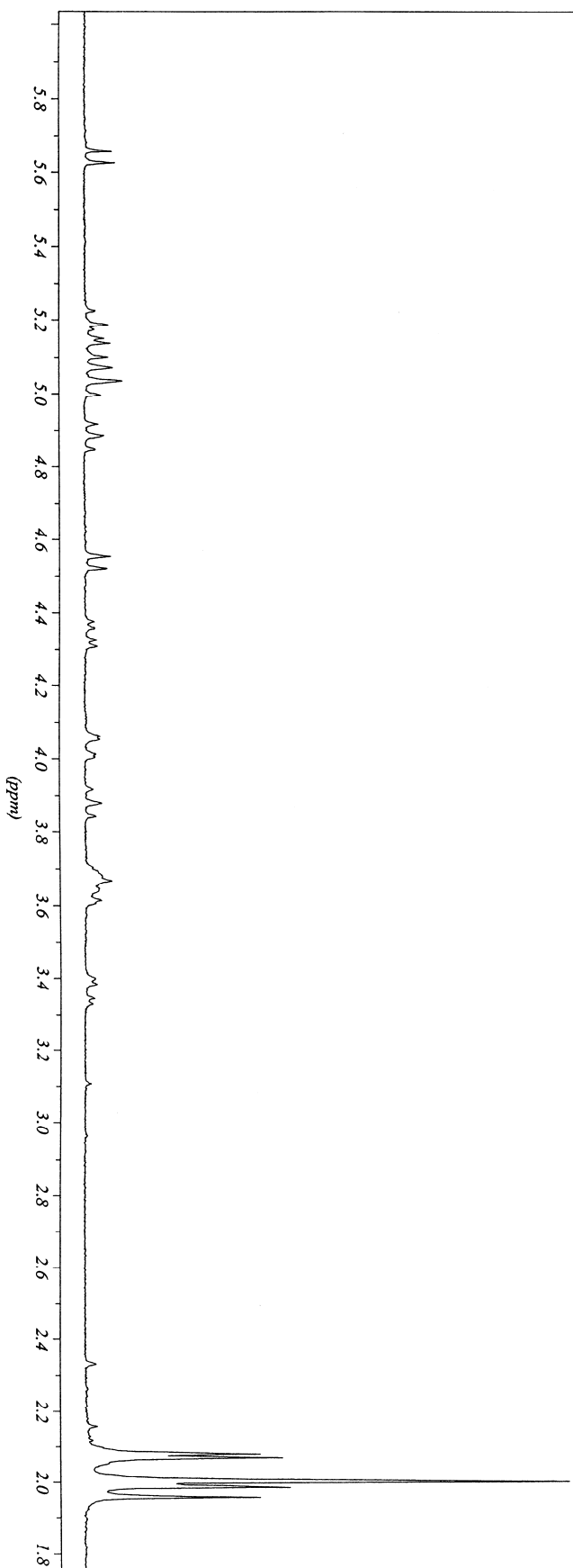


**19**

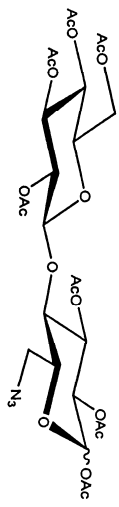




**20B**







**20B**

