^{1}H NMR Studies of Molecular Interactions of Carbohydrates in Aqueous Solutions

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Abstract

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The formation and structure of inclusion complexes between α-cyclodextrin (α-CD) and adamantane, 1-adamantanol, 1-(hydroxymethyl)-adamantane, 2-adamantanol and 1,3-adamantanediol in aqueous solutions were studied by ^{1}H NMR spectroscopy using both exchangeable and non-exchangeable protons. Complexes were formed with all adamantane derivatives with the exception of 1,3-adamantanediol. Similarities between the α-CD/adamantane and the α-CD/2-adamantanol complexes were evidenced by the appearance of a narrow and upfield shifted O(3)H signal for α-CD in the complex. α-CD formed 1:1 complexes with 1-adamantanol and 1-(hydroxymethyl)-adamantane. In both cases the O(2)H signal of α-CD was broadened with higher concentration of guest molecule and low temperatures.

^{1}H NMR studies of the hydrogen bonding network in mono-*altro*-β-cyclodextrin and its complex with adamantane-1-carboxylic acid showed that the hydroxy proton chemical shifts, temperature coefficients and vicinal coupling constants could be used to monitor the formation of intermolecular hydrogen bonds and hydration changes. The conformational change undertaken by altrose upon addition of adamantane-1-carboxylic acid allowed a more regular hydrogen bond network between the secondary hydroxyl groups in the CD, as evidenced by the downfield shift for O(3)H of the glucose-units.

In the second part of the thesis, the interactions between di- and trimannosides, substructures of oligomannose-9, and mutants of the HIV inactivating protein cyanovirin-N (CV-N) were studied using saturation transfer difference NMR spectroscopy. In one mutant CV-N^{MutDB}, the carbohydrate-binding site on domain B was suppressed while keeping the domain A intact. In the other mutant, CV-N^{MutDA}, the specificity of domain A for trimannose was altered while domain B was kept intact. Both mutants recognised all di- and trimannosides containing the terminal Manα(1-2)Man epitope. The binding of the mutants were similar, with a slightly stronger affinity for the trisaccharide, Manα(1-2)Manα(1-2)Man*α*OMe in CV-N^{MutDB}. Isothermal titration calorimetry data for CV-N^{MutDB} showed a binding affinity of 3.4 ±0.5 μM for the trisaccharide, which is close to the value derived for the nonamannoside (4.3 ±0.5 μM). No binding parameters were extracted for CV-N^{MutDA} due to the presence of two binding sites. This study confirm previous findings showing that not only the terminal disaccharide but also the linkage to the reducing end residue is important for binding to CV-N and thereby also for antiviral activity.

Keywords: Hydroxy protons, cyclodextrin, conformation, molecular interactions, anti-viral, cyanovirin-N, Man_9, mannosides.

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Till mina föräldrar, Karin och Hasse.

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Appendices

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals, I-IV.
Reprint of Paper II has been made with permission from Elsevier.

I. Bendeby, B., Kenne, L., and Sandström, C. (2004) ^{1}H-NMR studies of the inclusion complexes between α-cyclodextrin and adamantane derivatives using both exchangeable hydroxy protons and non-exchangeable aliphatic protons. *Journal of Inclusion Phenomena and Macrocyclic Chemistry 50*, 173-181.

II. Hakkarainen, B., Fujita, K., Immel, S., Kenne, L., and Sandström, C. (2005) ^{1}H NMR studies on the hydrogen-bonding network in mono-altro-β-cyclodextrin and its complex with adamantane-1-carboxylic acid. *Carbohydrate Research 340*, 1539-1545.

III. Hakkarainen, B., Lahmann, M., Oscarson, S., Matei, E., Kenne, L., Gronenborn, A.M., and Sandström, C. (2006) Atomic mapping of the sugar binding epitopes in one-site and two-site mutants of Cyanovirin-N by Saturation-Transfer Difference NMR Spectroscopy. *Manuscript*

IV. Hakkarainen, B., Kenne, L., Lahmann, M., Oscarson, S., Sandström, C. (2006) NMR study of hydroxy protons of di- and trimannosides substructures of Man-9. *Manuscript*

Abbreviations and symbols

AIDS	Acquired ImmunoDeficiency Syndrome
ATP	Adenosine TriPhosphate
CD	cyclodextrin
CIS	Complex Induced ^{1}H NMR chemical Shifts
CV-N	Cyanovirin-N
δ	chemical shift
Δδ	chemical shift difference
|dδ/d*T*|	temperature coefficient (absolute value)
1D / 2D	one-dimensional / two-dimensional
nD	multi-dimensional
DMSO	dimethylsulfoxide
DQF-COSY	Double-Quantum Filtered COrrelation SpectroscopY
FID	Free Induction Decay
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple-Bond Correlation spectroscopy
HSQC	Heteronuclear Single-Quantum Coherence
HSQC-DEPT	HSQC-Distortionless Enhancement by Polarisation Transfer
HSQC-TOCSY	HSQC-TOtal Correlation SpectroscopY
GlcNAc	*N*-Acetyl-Glucosamine
ITC	Isothermal Titration Calorimetry
$^{3}J_{AX}$	vicinal, three-bond coupling between A and X
kDa	kilo Dalton, 1 Da = 1 u, atomic mass unit
μ	micro,10^{-6}
m	milli, 10^{-3}
M	concentration in mol/dm^{3}
Man	Mannose
Man_9	Oligomannose-9
n	nano, 10^{-9}
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect SpectroscopY
ω	(omega) symbol for dihedral angle of exocyclic hydroxy
ppm	parts per million
ppb	parts per billion

Φ	(phi) symbol for dihedral angle of a glycosidic linkage
Ψ	(psi) symbol for dihedral angle of a glycosidic linkage
ROESY	Rotating-frame Overhauser Effect SpectroscopY
STD	Saturation Transfer Difference
TOCSY	TOtal Correlation SpectroscopY
WATERGATE	WATER suppression by GrAdient-Tailored Exitation
waterLOGSY	Water-Ligand Observed via Gradient SpectroscopY
Å	Ångström, 1 Å = 10^{-10} m

1 Introduction

The periodic table contains 111 elements. Organic chemistry concerns substances containing carbon and hydrogen, but also often oxygen nitrogen and sometimes phosphorus, sulphur and halogens. Yet there is ample variation in the combination of these few atoms. The smallest atom, or element, of the periodic table is hydrogen, H. Carbon, in its sp^3-hybridised form, has the possibility to bind four different atoms or groups of atoms. If doing so this carbon is stereogenic, and can exist in either the *R*-, or *S*-form, from the Latin words *rectus* (right) and *sinister* (left). This is a way to define the geometrical shape of a molecule, that will dictate which part of the molecule will interact with a receptor and if it will interact at all. To obtain knowledge of these interactions is very important. This can be exemplified by the infamous drug thalidomide of which one enantiomer was effective against nausea and the other was teratogenic.

The situation for a chemist of today is very different from the one of a chemist fifty years ago. Then the tools had to be made up and put together by the scientist herself for the specific research aimed to do. Today the tools are ample and a chemist in a large research group has many different techniques to choose from. Another aspect of chemical research today is that it needs to be justified from a biological point of view. The results you obtain have to be valid in/or preferably already be in a biological context. The different research subjects, chemistry, physics, biology and ecology are no longer separate disciplines but interlinked into "life science".

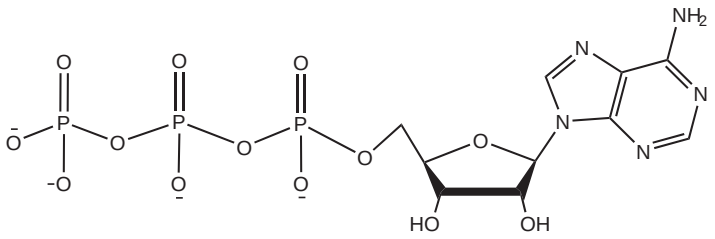
In pharmaceutical industry and other areas where the mechanism of drug action in our body is of interest, it has become increasingly important to understand the interactions of enzymes and cell membranes with substrates and pharmaceuticals. This understanding will facilitate and make the screening of drug candidates more efficient. With the development of for example new NMR spectroscopic methods studies of interactions between molecules in solution have been greatly facilitated.

2 Scope of the thesis

This work has been devoted to the study of molecular interactions in water by nuclear magnetic resonance (NMR) spectroscopy. In the presented studies carbohydrates are involved either as receptors (cyclodextrins) or ligands (di- and trimannoides). In the first part the use of hydroxy protons to study the structures of inclusion complexes between cyclodextrins and small guest molecules was investigated. Together with other methods the information obtained from NMR of hydroxy protons should be very useful to understand the driving forces and binding modes of cyclodextrin complexes. In the second part of this work, the interaction between di- and trimannosides, substructures of Man_9, and mutants of the anti-HIV protein cyanovirin-N (CV-N), was investigated by STD NMR spectroscopy. The NMR data for the hydroxy protons in these di- and trimannoside were collected to determine if additional information on the solution conformation could be obtained.

3 Biomolecules

There are four major classes of biomolecules, proteins, carbohydrates, nucleic acids and lipids. The twenty natural amino acids make up peptides and proteins. Peptides or oligopeptides are small molecules with less than fifty amino acids while proteins or polypeptides contain more than fifty amino acids. Carbohydrates (Hydrates of carbon) are abundant in nature, for example at the surface of cells, as in ATP (adenosine triphosphate, Scheme1) a molecule used for energy transport in biological systems and as structural elements in starch, cellulose or chitin(*1-3*). Nucleic acids, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), are linear polymers consisting of four nucleosides linked by 3´-to-5´-phosphodiester linkages.



Scheme 1. The structure of ATP, adenosine triphosphate. The carbohydrate residue (middle part of the structure) is a ribofuranose unit.

DNA forms a double helix with hydrogen bonds between the nucleobases, adenine with thymine and guanine with cytosine. In RNA thymine is replaced by uracil. Lipids are water-insoluble biomolecules with a variety of biological roles as for example fuel molecules, energy stores, signaling substances and membrane components. There are three major types of lipids, phospholipids, glycolipids and cholesterol. Nucleic acids and lipids will not be discussed further in the thesis. Hydrogen bonding is central for many biological interactions. As mentioned

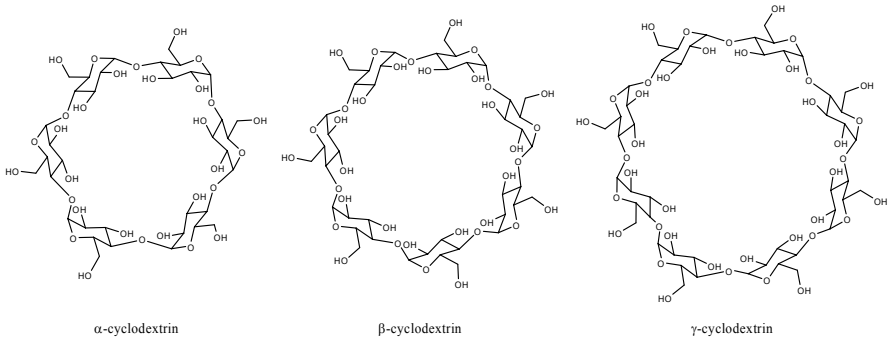
above, hydrogen bonds are formed between the nucleobases of the DNA double helix. Proteins can fold into an α-helix or β-sheet which are held together by inter-residual hydrogen bonds. In carbohydrates, the β(1,4)-linked glucose chains of cellulose interact through hydrogen bonds and form strong fibers(*4*). Hydrogen bonding is also often involved in the macromolecular interactions between a ligand and a receptor.

3.1 Carbohydrates

In 1952 when the chemical nature of the blood group system was elucidated, this also spurred the research on lectins (see 3.2 for definition). In the 1960s, following the establishment of affinity chromatography, a renaissance of carbohydrate chemistry occurred. The development of analytical techniques for structure elucidation has been strong, promoted by the discovery of the importance of carbohydrates in molecular recognition processes(*3, 5*). Carbohydrates are divided into sub-groups according to size and function as well as biological location (lipopolysaccharides and glycoproteins for example). There are monosaccharides, oligosaccharides, up to six sugar moities, polysaccharides have more than six sugars. Carbohydrates have multiple hydroxy groups and can form linear chains, cyclic oligosaccharides or branched structures. To fully describe an oligosaccharide the number of carbons, information on the chirality (L- or D-) and anomeric configuration (α- or β-glycosides) for each monomer and the linkage type between them has to be given.

3.1.1 Cyclodextrins

Cyclodextrins are as the name suggests cyclic oligosaccharides. The most common are α-, β- and γ-cyclodextrins with six, seven and eight α(1,4)-linked glucose units respectively (Scheme 2). The first publication about these structures was written in 1891 by A. Villiers who found a product in his fermented starch which he called "cellulosine"(*6*). In (1903) Franz Schardinger renamed the compound dextrin, thereby the name "Schardinger dextrin". The cyclic nature of the structures were not elucidated until 1936 by Freudenberg and his group(*7*).



Scheme 2. Structures of α-, β- and γ-cyclodextrins.

The shape of the cyclodextrin molecules can be described as a toroid or a truncated cone. The wider rim is formed by O(2)H and O(3)H hydroxy groups while the narrow rim comprises of the O(6)H. While the outer surfaces of the CDs are hydrophilic, the inner cavities comprising of C(3)H and C(5)H aliphatic ring protons are highly hydrophobic (Figure 1), making them capable of forming inclusion complex with a large variety of smaller hydrophobic molecules.

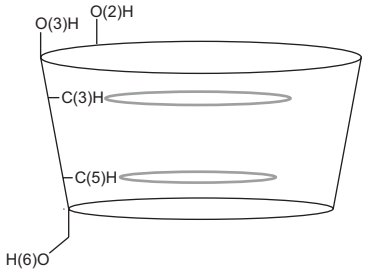


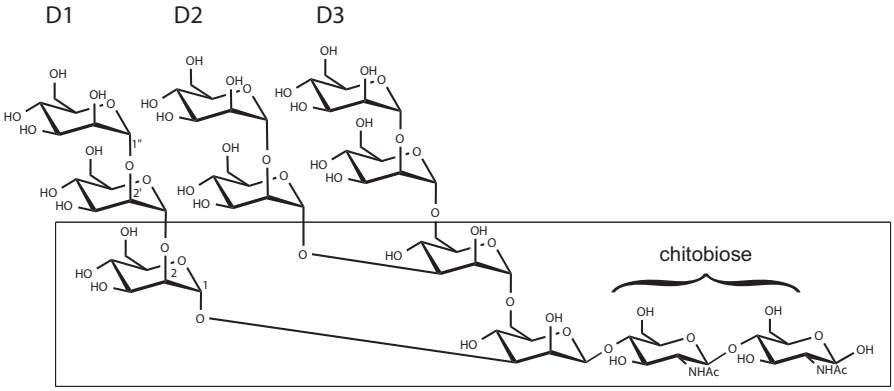
Figure 1. Schematic representation of a CD molecule, showing that the C(3)H and C(5)H protons line the cavity.

CDs are able to alter the physiochemical properties of the guest molecule. This can lead to enhanced solubility of the guest molecules and increase their bioavailability**.** CDs are also used in the pharmaceutical industry as a mean to control the release of active ingredients in drugs(*8*). CDs can also stabilise labile molecules and protect them from degradation by for example light or hydrolysis. In analytical chemistry CDs are used as chiral selector for separation of enantiomeric molecules. Furthermore, CDs or CD derivatives can catalyse certain chemical reactions and are studied as enzyme models(*9*). There is an interesting review on the development and research of CDs and on the present use of CDs by Szejtli(*7*).

Many different techniques are used for the study of cyclodextrin complexes including X-ray crystallography(*10-13*), NMR spectroscopy(*14*), calorimetry(*15*), circular dichroism spectroscopy(*16, 17*), chromatography(*18*). There are no covalent bonds formed or broken during complex formation, and molecules of the complex are in equilibrium with non-complexed molecules in solution. The driving forces for the complex formation have been attributed to the release of water molecules from the cavity, van der Waals interactions, hydrophobic interactions or hydrogen bond formation(*19*). Intermolecular hydrogen bonds are generally thought to be hardly formed in aqueous systems, because of strong hydration of hydrogen bonding sites of both host and guest molecules. Studies of biological systems, however, clearly suggest that hydrogen bonds can be formed in water when the hydrogen bonding sites are located in a microscopically hydrophobic environment and are situated very close to each other. Although examples of inclusion complexes of CDs where hydrogen bonds could participate in complexation have been reported(*20*), no direct evidence for formation of hydrogen bonds in water has been obtained(*21*). In this thesis, the use of hydroxy protons of CDs to monitor intermolecular interactions is described.

3.1.2 Oligomannose-9

Oligomannose-9 (Man_9) is a high mannose type *N*-linked glycan consisting of nine mannose units, divided into three arms, D1-D3 or antennae (Scheme 3). The core pentasaccharide ($Man_3GlcNAc_2$) is common to all *N*-glycans. Glycoproteins are involved in a large number of biological processes and are major components of the surface of mammalian cells. These carbohydrates are involved in cell-cell adhesion, recognition between cells in our immune system and in viral replication (*5*). Man_9 is the major carbohydrate component on gp120 of HIV. Close to 65% of the total amount of carbohydrate moieties on the glycoprotein gp120 of HIV (Scheme 3) are Man_9(*22*). These structures are recognised by our immune system and mediate the fusion between the host cell and the virus(*23*).



Scheme 3. Structure of Man_9, the square marks the core pentasaccharide consisting of three mannose units and chitobiose.

The Man_9 structure is considered relatively flexible, but the core with the chitobiose unit is considered to have restricted conformational freedom(*24, 25*). Since these mannose structures are often directly recognized by the protein binding sites, an understanding of the interaction might begin with knowledge of the possible conformations of these molecules, and of their building blocks.

3.2 Proteins

Lectins

Lectins are proteins that specifically bind carbohydrates and they often contain two or more binding sites.

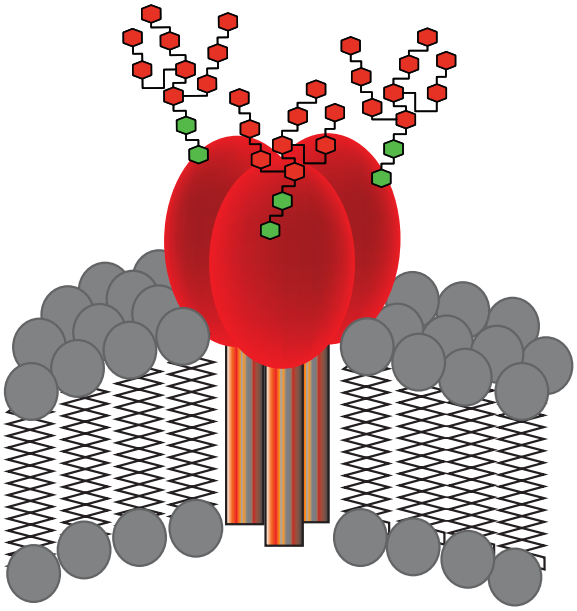
"Lectins bind mono-and oligosaccharides reversibly and with high specificity, but are devoid of catalytic activity, and in contrast to antibodies are not products of an immune response." (*26*)

Lectins are not products of immune response although they are involved in clearance of glycoproteins from circulatory system, adhesion to inflammatory sites and cell-to-cell interactions in the immune system(*5*). Their physiological function is unclear since they display no catalytic activity, with at least one exception in ricin(*26*). Biological sources are for example plants, bacteria, viruses and animals. Lectins are divided into groups depending on for which monosaccharide they exhibit the highest specificity, mannose, galactose or *N*-acetylgalactosamine, N-acetylglucosamine, fucose and N-neuraminic acid.

Cyanovirin-N

Cyanovirin-N (CV-N) is an 11 kDa lectin that was originally isolated from aqueous extract of the cyanobacterium *Nostoc ellipsosporum*(*27*). It has a broad antiviral activity and irreversibly inactivates diverse laboratory strains and isolates of human immunodeficiency virus (HIV) as type 1 (HIV-1), type 2 (HIV-2), simian immunodeficiency virus as well as feline immunodeficiency virus(*27, 28*). It is highly resistant to denaturation and is non toxic at antiviral activity concentration, which makes it a promising candidate for anti-HIV prophylactic(*27-30*). CV-N is currently in clinical trials as a topical microbicidal agent(*31*). It has been found to have antiviral activity against other enveloped viruses such as, measles virus (*29*), Ebola virus(*30*), human herpes virus 6, several strains of influenza viruses(*32*) and Hepatitis C virus(*33*). CV-N specifically binds to high-mannose oligosaccharides of the glycoprotein gp120(*34-36*) present on the surface envelope of HIV(Scheme 4). In this way, CV-N prevents fusion with host cells and also aborts cell-to-cell fusion and thereby the transmission of HIV(*28, 34, 37, 38*).
In solution, CV-N exists either as a compact monomer or a domain swapped dimer(*39-41*). It was found to be a domain-swapped dimer in the crystal form(*40*) and by NMR at neutral pH(*40, 41*). The monomeric form of CV-N is elliptical, ~55Å long and ~25 Å wide. Its 101 amino acids can be divided into two regions with large amino acid sequence similarities(*27, 39*). Domain A of CV-N comprises of residues [1-38/90-101] and domain B of residues [39-89](*42*). By NMR titration experiments and ITC it was found that CV-N has two carbohydrate binding sites with different affinity(*43, 44*). In domain A, a semicircular cleft comprising residues 1-7, 22-26 and 92-95 forms the binding site and in domain B a deeper pocket comprising residues 41-44, 50-56 and 74-78 forms the other binding site. The minimum structure required for recognition in both sites is Manα(1→2)Manα(*37, 43-45*). Studies with larger carbohydrates indicated that the

interactions between CV-N and the sugars were polar/electrostatic, van der Waals and hydrogen bonding interactions(*44-46*).
Since the wild type CV-N (wtCV-N) forms dimers, producing obligate dimers was suggested to adjust the anti-viral activity(*47*). It was also found that the domain swapped dimer of wtCV-N had similar antiviral activity as the monomer(*48*). Further mutations were made to dissect the binding interaction between Man_9 and CV-N and enhance *in vitro* virusidal activity through structure-guided alterations(*49-51*). A mutant, obtained by exchanging proline 51 of the hinge region for a glycine ([P51G]CV-N), stabilised the monomeric form and had no significant differences in anti-HIV and anti-Ebola activities(*31, 48, 51, 52*).



Scheme 4. Illustration of the envelope glycoprotein gp120, with Man_9 structures attached. Man-units are represented by red hexagons and GlcNAc-units by green hexagons.

HIV is the cause of acquired immunodeficiency syndrome, AIDS to which there is yet no cure. In order to prevent transmission of HIV, CV-N could be used as a topical biocide. CV-N will be tested on humans in 2007. In developing countries, the prevention of heterosexual transmission of HIV is complicated by culture, tradition as well as economy. To address these issues and find a working preventive, a transgenic plant (*i. e.* tobacco plant) have been produced that excretes 1.30 g CV-N / kg of leaves and CV-N could be grown and distributed by native farmers(*53*). A second important gene modification is *Lactobacilli jensenii*, a vaginal bacterium that have been shown to produce sufficient amounts of CV-N *in vitro* to sustain a good level of protection(*54*).
Due to the high biological relevance of CV-N, as an aid in preventing the spread of HIV, the interaction with its receptor gp120 and with Man_9, have been the object of intensive research(*31, 35, 37, 39-41, 43-45, 55*).

4 Nuclear Magnetic Resonance

4.1 Historical background

Nuclear Magnetic Resonance (NMR) spectroscopy has been used since the 1950s. Nuclear Inductance was discovered by physicists and later found useful by the chemist community. One report which would lead to development of NMR spectroscopy was published on December 24th 1945 by Purcell, Torrey and Pound under the title "Resonance Absorption by Nuclear Magnetic Moments in a Solid"(*56*). The solid sample was 850 cm^3 of paraffin. Only about a month later, January 29th 1946, Bloch, Hansen and Packard reported detection of Nuclear Induction in water(*57*). The short report is ended by these two sentences:

"We have thought of various investigations in which this effect can be used fruitfully. A detailed account will be published in the near future."(57)

In 1952 Felix Bloch and Edward Mills Purcell received the Nobel Prize in physics(*58*).
Even though the first observation of nuclear magnetic resonance in solids and liquids occurred in 1945/1946, it was not until the 1950s that the chemists realised its potential and started to develop its use for their applications. In his Nobel lecture Purcell mentioned that the chemical environment can be analysed from the chemical shift of a nucleus. Since then, the instruments and methods of NMR spectroscopy have had a rapid development. These rapid developments were in large part due to the discovery by Richard R. Ernst and Weston A. Anderson, that the sensitivity of NMR could be dramatically increased, by applying a short radiofrequency pulse and subsequently measuring the NMR signal as a function of time. The signal obtained is then Fourier transformed, the NMR spectra containing signal related to the different frequencies. Richard R. Ernst also showed that by using 2D FT NMR, studies of larger and more complicated structures could be conducted. The 2D methods were further developed and applied in structural/chemical analysis of biological macromolecules, by Kurt Wütrich and many others. Since 1952, two more NMR related Nobel prizes have been awarded, 1991 to Richard R. Ernst(*59*) and 2002 to Kurt Wütrich(*60*). Today NMR is one of the key instruments in biochemical studies.

4.2 NMR in structural analysis of carbohydrates

The two major methods used for studying the structure and molecular interactions of biomolecules are X-ray crystallography and NMR spectroscopy. The two methods are complementary to each other. One important advantage of NMR is that there is no longer necessity for the crystallisation of proteins to obtain information of intermolecular interactions(*3*).

4.2.1 Structure assignment

Before studying the carbohydrate interactions, the assignment of the NMR signals of the molecules must be achieved: The assignment of ^{1}H and ^{13}C resonances to their corresponding protons and carbon atoms is usually achieved using a combination of different through-bond and through-space 2D NMR experiments. Standard ^{1}H-^{1}H DQF-COSY, ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC are used to assign the different protons and carbons within a residue. The ^{1}H-^{13}C HSQC-DEPT, used in this work, allows the differentiation between CH and CH_3 (positive signals) and CH_2 (negative signals). The HSQC-TOCSY experiment will give additional spreading in the carbon dimension, in this way simplifying the assignment of the individual spin systems when spectral overlaps are present in the proton dimension. The sugar residues are connected using long range through-bond correlation such as ^{1}H-^{13}C HMBC and through-space correlations such as ^{1}H-^{1}H NOESY and ROESY.

4.2.2 Conformational analysis

Together with the inter-residual homo- and heteronuclear NOEs and ROEs, vicinal heteronuclear J_{CH}, vicinal homonuclear J_{CC} trans-glycosidic coupling constants and residual dipolar couplings can be used to obtain information on the carbohydrate conformation(s)(*61-69*).

The conformation of the glycosidic linkage is described using the Φ (O5'-C1'-O4-C4) and Ψ (C1'-O4-C4-C3) dihedral angles (Figure 2). The exocyclic dihedral angle is described by ω (O5-C5-C6-O6). The Φ^{H} (H1'-C1'-O4-C4) and Ψ^{H} (C1'-O4-C4-H4) dihedral angles describes the same link but the latter describes the relation between the anomeric and the aglycone hydrogen.

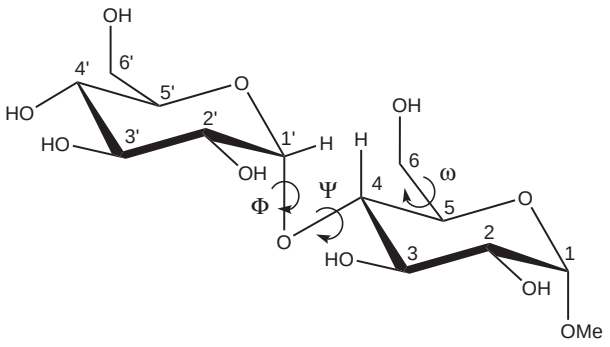


Figure 2. A disaccharide, Glcα(1-4)Glcα*O*Me, with dihedral angles Φ (O5'-C1'-O4-C4) and Ψ (C1'-O4-C4-C3) and the exocyclic dihedral angle ω (O5-C5-C6-O6).

4.3 Hydroxy protons in structural studies of carbohydrates

There are many hydroxy protons in carbohydrates and they are potentially important for conformational studies since they can be for example involved in intra- and intermolecular hydrogen bond interactions. Most of the structural studies of carbohydrates by NMR spectroscopy are done in D_2O solutions. In this solvent, the hydroxy protons are not observed in the ^1H NMR spectra due to exchange with deuterium. However it is of most interest to study the interaction in water, as organic tissue contains water and are surrounded by water. By proper preparation of sample tubes and the right choice of solvent and pH, it is however possible to observe hydroxy protons by NMR(*70, 71*). The study of hydroxy protons in carbohydrates is part of an ongoing project with the aim to show that hydroxy protons can be used as conformational probes for structural studies of carbohydrates free in solution or involved in intermolecular interactions. The use of hydroxy protons in structural studies of carbohydrate is described in a recent review(*72*).

4.3.1 Solvent and sample preparation

4.3.1.1 Solvent

There are a couple of different approaches that can reduce the rate of exchange and thereby make the observation of hydroxy protons possible. To circumvent the problem of rapid exchange of hydroxy protons with the proton from water, a polar aprotic solvent such as DMSO can be used. However, a complete solvent exchange can have an influence on the conformation(s). At room temperature the exchange of the hydroxy protons with water is too fast and a sample temperature below 0 ºC is often required. To avoid freezing the sample, different binary solvent mixtures have been used such as 15% acetone-d_6 / 85% H_2O or 60% DMSO-d_6/ 40% H_2O solution. The acetone-d_6 solution is frequently used and it has been shown that addition of this aprotic solvent to water has small influence on the chemical shifts of hydroxy protons and does not influence the conformation of the carbohydrate(*21, 73, 74*). With an addition of 15% acetone-d_6 temperatures around – 10 ºC can be obtained without freezing the sample(*75*).

4.3.1.2 Sample tubes

A careful sample preparation is usually necessary to be able to observe the hydroxy protons. The NMR sample tubes can be soaked for more than 1 hr with 50-100 mM phosphate buffer at pH 7, in order to minimize the release of impurities from the glassware(*74*).

4.3.1.3 Sample pH

The optimum pH for observation of hydroxy protons is usually around pH 6.5. To adjust the pH, either buffered water solutions or small amounts of HCl and NaOH are added(*70, 71, 73*).

4.3.2 Water suppression

Using H_2O as solvent will result in a large signal originating from the solvent protons while analyte protons are of considerably lower concentration. The concentration of water being ~56 M, hence the concentration of protons is 112 M while the concentration of analyte in the samples are between 1 – 10 mM, The WATERGATE(*76, 77*) pulse sequence (Figure 3) can be implemented into most 1D or nD NMR pulse programs and is widely used to suppress the water signal in NMR spectroscopy of biological samples, or other samples with high content of water.

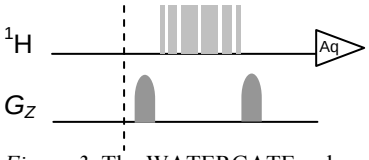


Figure 3. The WATERGATE pulse sequence for solvent suppression with two pulsed field gradients and the binomial-type, hard pulse sequence. The excitation profile of the WATERGATE binomial-type sequence, provide a null at the solvent resonance.

4.4 Hydroxy proton NMR parameters

The different ^{1}H NMR spectroscopic parameters of hydroxy protons that can be used to investigate conformations of carbohydrates are:

- Chemical shifts
- Vicinal $J_{CH,OH}$ coupling constants
- Temperature coefficients
- Rate of exchange with water
- NOEs and ROEs

From these NMR parameters, information on hydration, hydrogen bonding and spatial proximities can be obtained.

4.4.1 Chemical shifts

The chemical shift of hydroxy protons is one of the key parameters used in our studies as they give information on the grade of shielding or deshielding(*70, 71*). The hydroxy proton chemical shift can be used to get information on hydration and hydrogen bond formation in carbohydrates, especially in more conformationally constrained structures. Upfield shifts are indicative of reduced hydration while downfield shifts are observed for hydroxyls in proximity of ring oxygen or other hydroxyls. The observed chemical shift is the combined effect of hydrogen bonding that will give a downfield shift and reduced hydration that gives an upfield shift.

4.4.2 Vicinal $J_{CH,OH}$ coupling constants

Vicinal $J_{CH,OH}$ coupling constants are used to determine the H-C-O-H dihedral angle using the Karplus equation(*78*). Vicinal coupling constant of 5.5 ± 1.5 Hz indicates conformational averaging with free rotation around the C – O bond. Deviations from average values are indications that the hydroxyl is restricted in rotation. Restricted rotation can in turn originate from steric hindrance or hydrogen bonding(*73, 79*).

4.4.3 Temperature coefficients

Temperature coefficient is the temperature dependence of the ^{1}H NMR chemical shift. It gives information on the accessibility of the hydroxy proton by the solvent. Temperature coefficients, $|d\delta/dT|$-value, are high (>10 ppb/ºC) for strongly solvated hydroxy protons while small (<5 ppb/ ºC) if the hydroxy protons are involved in hydrogen bonding or have reduced hydration(*80, 81*). For example, the temperature coefficients for hydroxy protons of a monosaccharide are often around 10 or 12 ppb/ ºC, indicating their accessibility to water(*81*).

4.4.4 Exchange rates

Exchange rate is a measure of how quickly a proton exchanges with the solvent. If a hydroxy proton is protected from the solvent by being directed towards a lipophilic surface of another residue or by acting as a hydrogen bond donor the rate of exchange will be lower compared to that of a proton fully exposed to the solvent. The difficulty to accurately measure the exchange rate, because of the great influence the solvent/sample impurities have on this parameter, is a complication however not impossible to bridge. It is possible to obtain exchange rates from plots of signal intensities in series of NOESY recorded with short mixing times(*82-85*).

4.4.5 NOEs and ROEs

By observation of NOEs between hydroxy protons and other protons, it is possible to detect inter- and intra-molecular interactions(*86*). Small molecules that tumble fast in solution have small positive NOE and intermediate sized molecules have small or no NOEs. Large molecules have negative NOE. In contrasts to NOEs, the ROE are always positive even for intermediate size structures. In NOESY experiments, chemical exchange and dipole-dipole relaxation have the same sign and cannot be distinguished(*75, 87, 88*). In ROESY experiments, on the other hand, the signals due to the two types of interaction have opposite sign. Chemical exchange cross-peaks can also be indicative of spatial proximity or hydrogen bond interaction(*21, 88*).

Molecular Interactions

Cyclodextrin interactions

Chemical shift of non-exchangeable protons and intermolecular NOEs are usually used to study cyclodextrins interactions. From the hydroxy proton chemical shifts of cyclodextrin, temperature coefficients, complex induced chemical shift (CIS),

and intermolecular NOEs/ROEs, the structure of the inclusion complex can be investigated in more details.

Carbohydrate-protein interactions

To be able to develop vaccines, pharmaceuticals and prophylactics, it is necessary to have knowledge about the specific interactions involved during the course of a disease and the specific interactions targeted by the drug. One of the major advantage of NMR spectroscopy is the possibility to study weaker interactions than possible with other methods(*89*).

Two different types of NMR methods are usually used to study interactions between a receptor and a ligand, those detecting the ligand signals and those detecting the receptor signals. One of the most frequently used receptor-based methods rely on the perturbation of the receptor chemical shifts upon binding. A reference spectrum is first recorded without the ligand. In the spectrum recorded with the ligand, binding will give rise to chemical shift changes for the resonances of protein atoms in contact with the ligand. Due to the large size of most receptors, 2D NMR is often required and chemical shift mapping is usually obtained from ^{1}H-^{15}N HSQC and ^{1}H-^{13}C HSQC spectra. Isotope labelling with ^{15}N and/or ^{13}C are often necessary. More precise identification of binding sites can be provided from intermolecular NOEs and from paramagnetic relaxation experiments. In these cases, distances between protein and bound ligand atoms are obtained. More detailed reviews of these techniques can be found in references(*89-91*).

The methods detecting the ligand signals are usually not dependent on the size of the target and isotope labelling is not required. Due to the small size of the ligand molecules, the use of 1D NMR is sufficient. Examples of these methods are line-broadening effects, diffusion editing, WaterLOGSY and saturation transfer difference NMR spectroscopy. Transferred NOE is used to obtain the conformation of the ligand in the bound state. Preferably methods from both categories should be used to obtain information about the ligand, the receptor as well as their interactions(*89-92*).

Saturation transfer difference NMR Spectroscopy

Saturation Transfer Difference (STD) NMR Spectroscopy(*93-95*) is a ligand directed method for studying interactions between for example a protein and its carbohydrate ligands. The technique has no upper size limit for the receptor molecule and it can be applied to any interaction where there is a possibility to selectively irradiate the NMR signals of the receptor(*96, 97*). The STD spectrum is generated by irradiating the protein by a series of pulsed field gradients. Usually for proteins this is done in the aliphatic region where there is a relatively high signal density. The saturation spreads through the protein via spin diffusion and subsequently is transferred to the bound ligand. By exchange the magnetisation is transferred to the ligand free in solution (Figure 4). A difference spectrum is obtained by recording one spectrum with the irradiation frequency set on protein resonances (ON-resonance) and one spectrum with the saturation set at a frequency where there are no protein signals (OFF-resonance), subsequently the latter is subtracted from the first. The ligands that are not bound to the protein will not obtain saturation transfer and the STD NMR spectrum will only contain

signals from ligands in contact with the protein. The resonances of the protons in closest proximity to the receptor molecule will have the strongest enhancement in the spectrum, thereby the method can be used to obtain detailed structural information on the binding epitope of the ligand. However the relaxation time of the individual protons also influences the relative intensity of STD signals(*98*).

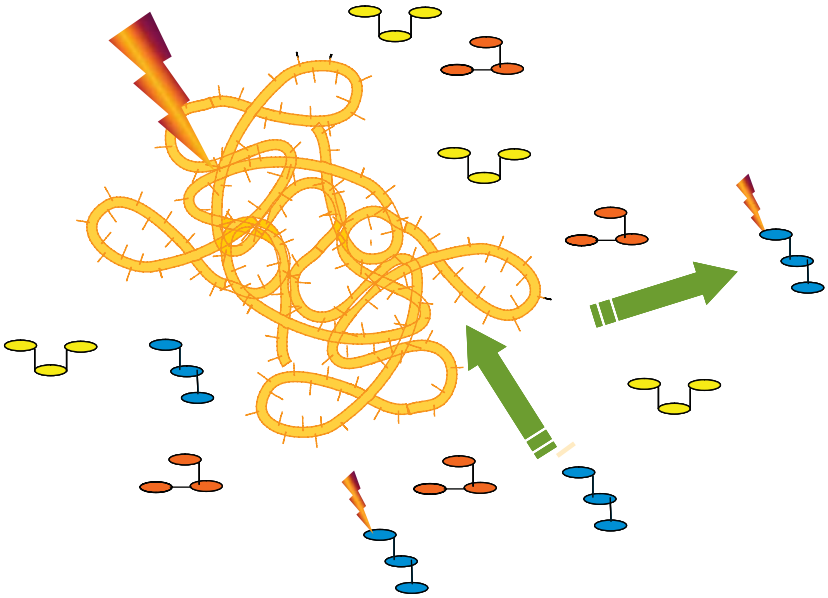


Figure 4. Illustration of the principle of STD NMR. The protein protons are saturated, and the saturation is transferred to binding ligand (blue), the non binding ligands are yellow and orange.

This highly versatile technique is frequently used in screening for drug candidates. Screening mixtures of ligands with dissociation constants K_D ranging from 10^{-3} – 10^{-8} M is possible(*99, 100*). The structure of the large receptors does not have to be determined to study eventual interaction or binding affinities. Binding constants can also be obtained from STD NMR experiments(*94*). The STD sequence can be used in combination with other pulse programs and has been added to 1D and 2D TOCSY, COSY, HMQC and HMBC (*94, 101, 102*). It has been shown to work on membrane bound protein with very complex ligand mixtures(*97*). When the target molecule is small or has elongated structure, making the saturation by spin diffusion less efficient, extra care have to be taken to find appropriate settings for the experiments(*103*).
Further advantages of STD are that small amount of protein can be used. STD NMR was applied for atomic mapping of the interactions between mutants of the antiviral agent CV-N (11 kDa) and di- and trimannoside ligands (MW 356 and 519) that are substructures of Man_9.

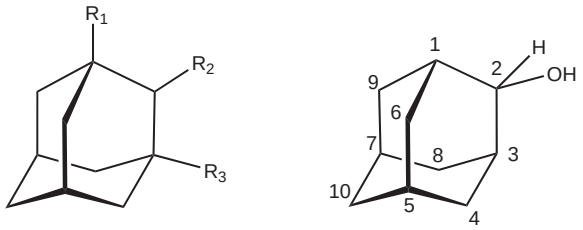
5 Results and Discussion

5.1 Papers I and II – Hydration and hydrogen bonding in complexes between cyclodextrins and adamantane derivatives

The existence of a hydrogen bond network between the secondary hydroxyl groups in α-, β- and γ-CDs is well established by X-ray crystallography, by NMR spectroscopy in DMSO solutions and was also shown to be present in aqueous solutions(*21, 104-106*). The driving forces to form inclusion complexes are electrostatic, van der Waals and hydrophobic interactions, and hydrogen bonding. NMR studies on inclusion complexes formed by CDs are usually done using the complexation induced ^{1}H NMR shifts (CIS) and NOEs of the C(3)H and C(5)H signals. The aim of these studies was to see if additional conformational information could be obtained using the temperature coefficients (|dδ/dT|-values), CIS and NOEs of hydroxy protons of CDs. Adamantane and derivatives were selected due to their low solubility that should favour complex formation and due to the presence of hydroxy groups at different positions that could allow formation of intermolecular hydrogen bonds.

5.1.1 Paper I – ^{1}H NMR studies of the inclusion complexes between α-cyclodextrin and adamantane derivatives using both exchangeable hydroxy protons and non-exchangeable aliphatic protons.

The compounds investigated were α-cyclodextrin (α-CD) (Scheme 2), and adamantane as well as derivatives such as 1-adamantanol, 2-adamantanol, 1-hydroxymethyl-adamantane and 1,3-adamantanediol (Scheme 5).



adamantane (R1-R3 = H),
1-adamantanol (R1=OH, R2,R3=H)
1-hydroxymethyl-adamantane (R1=CH2OH, R2,R3=H)
1,3-adamantanediol (R1,R3=OH, R2=H)

Scheme 5. Structure of adamantane and derivatives. The structure on the right is 2-adamantanol.

The stoichiometry of the α-CD – adamantane complex could not be determined because of the low solubility of adamantane. At a molar equivalent of 0.11 adamantane and at temperatures below 10 ºC, two set of signals from α-CD were observed, one corresponding to the free form and one to α-CD in complex with adamantane (Figure 5). The O(3)H of α-CD in complex with adamantane had a narrow line and was upfield shifted by 0.33 (Table 1). This together with the low

|dδ/dT|-value was attributed to reduced hydration of O(3)H caused by the expulsion of water from the hydrophobic cavity upon partial inclusion of adamantane.

Table 1. ^{1}H NMR chemical shifts (δ, ppm), CIS (ppm), and temperature coefficients (|dδ/dT|, [ppb/ºC]) for the hydroxy protons of α-CD alone and in the presence of adamantane analogues at the stoichiometric ratio 1:1.

	α-CD	O(2)H	O(3)H	O(6)H	O(3c)H
α-CD	δ	6.22	6.57	6.09	
	|dδ/dT|	8.0	8.6	12.5	
adamantane	CIS	0.01	0.01	0.01	-0.33
	|dδ/dT|	8.2	8.9	13.1	4.7
1-adamantanol	CIS	-0.05	-0.03	-0.01	
	|dδ/dT|	12.7[a] / 5.5[b]	7.3	11.7	
1-(hydroxymethyl)-adamantane	CIS	-0.04	-0.03	-0.01	
	|dδ/dT|	9.7[a] / 5.7[b]	7.7	12.6	
2-adamantanol	CIS	0	0	0	-0.21
	|dδ/dT|	10.0	8.1	12.1	1.2
1,3-adamantanol	CIS	0	0	-0.01	
	|dδ/dT|	7.7	8.1	12.2	

[a] Calculated for temperatures below 0 ºC.
[b] Calculated for temperatures above 0 ºC.

A strong NOE was observed between C(3)H of α-CD and H2 of adamantane, while weaker NOEs were observed between C(3)H of α-CD and H1 of adamantane and between C(5)H of α-CD and H2 of adamantane. For O(3)H of α-CD in the complex an NOE was observed to H2 of adamantane. These NOEs confirmed the formation of a partial inclusion complex.

A plot of the chemical shifts of C(3)H and C(5)H of α-CD as a function of the concentration of 1-adamantanol and 1-(hydroxymethyl)-adamantane suggested formation of 1:1 complexes. Only one set of resonances was observed for the host and guest, indicating fast exchange between free and complexed form on the NMR time scale. Strong intermolecular NOEs between C(3)H of α-CD and H2, H3 and H4 of the guest together with weak C(5)H – H3, H4 NOEs confirmed partial inclusion of the guest into the hydrophobic cavity of α-CD.
The NMR data for O(3)H and O(6)H of α-CD were similar for the two complexes to those in α-CD alone. Some characteristic features were observed for the O(2)H signal. Thus, addition of the guest molecule resulted in a broadening of the O(2)H signal. When the temperature was increased, the O(2)H signal was getting sharper while usually hydroxy proton signals become broader upon increasing temperature, due to faster exchange. The temperature coefficient was not linear in the temperature range –10 – 10 ºC and a change in slope were observed at 0 ºC. The temperature coefficient was large at low temperature suggesting more access to water.

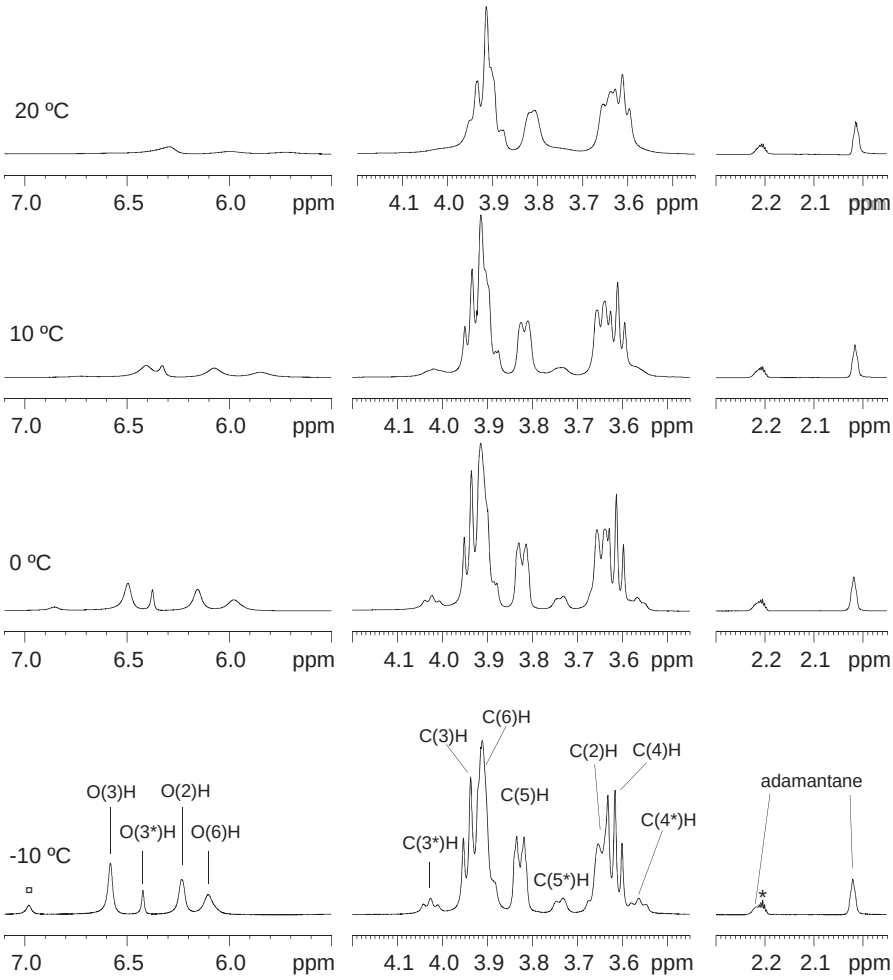


Figure 5. Regions of the ^{1}H NMR spectra of α-CD with 0.11 molar equivalent of adamantane recorded at different temperatures. ¤ Hemiacetal of acetone-d_6, * acetone-d_5.

The stoichiometry of the α-CD/2-adamantanol complex, 1:1 or 2:1 could not be determined unambiguously. At high temperature, one set of NMR-signals for α-CD and 2-adamantanol was observed while at temperatures below 5 ºC two sets of resonances were observed for 2-adamantanol. Exchange cross-peaks between the two sets of signals were present in the ROESY spectra. One set of signals (A, Table 2) was only slightly downfield shifted (<0.1 ppm) if compared to 2-adamantanol alone. The other set of signals was shifted downfield by more than 0.2 ppm (set B). Below 5 ºC, the NMR signals of C(3)H and C(5)H of α-CD were very broad. Set A of 2-adamantanol had NOEs from H4, H6 and H9 to C(3)H of α-CD. No intermolecular NOE involving C(5)H of α-CD or H5, H7, H8 and H10 of 2-adamantanol was found, suggesting only partial inclusion of the guest into the α-CD cavity. There was no intermolecular NOE involving the B set of 2-adamantanol. As observed with adamantane, an additional α-CD O(3)H signal appeared at temperatures below 5 ºC. This O(3)H signal was upfield shifted by

0.21 ppm and had a very small |dδ/d*T*|-value (1.2 ppb/ ºC). This was attributed to reduced hydration due to partial inclusion of 2-adamantanol and expulsion of water from the α-CD cavity. No definite conclusion on the structure of the inclusion complex could be drawn from the data. Complexes with a 2:1 α-CD/guest ratio have been reported, with for example 1-bromoadamantane and adamantane-1-carboxylic acid(*107, 108*). It was suggested that if the guest molecule is asymmetric the high-affinity part of the molecule is included into one α-CD and the low-affinity part is included in a second α-CD(*107*).

Table 2. ^{1}H NMR chemical shifts (δ, [ppm]), of 2-adamantanol alone and of 2-adamantanol in complex with α-cyclodextrin at – 10 °C.

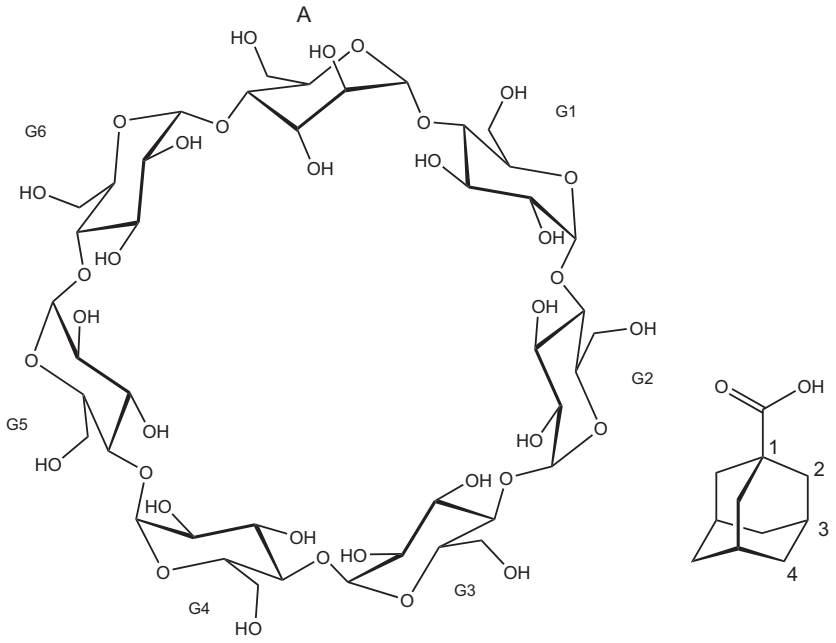
	2-adamantanol	2-adamantanol with α-CD		
H	e/a	set A	set B	CIS
1, 3	1.863	1.876	2.189	0.326
2	3.918	3.985	4.176	0.258
4, 9	1.704 / 1.978	1.717 / 1.871	1.926 / 2.048	0.222 / 0.070
5, 7	1.780	1.812	2.076	0.296
6	1.716	1.735	1.921	0.205
8, 10	1.540 / 1.991	1.562 / 1.997	1.771 / 2.437	0.231 / 0.446

[a] The numbering is shown in Scheme 3
[b] Difference in chemical shift between set B and free 2-adamantanol.

The very small CIS and the absence of intermolecular NOEs indicated that no complex was formed between α-CD and 1,3-adamanatanediol. The chemical shifts, temperature coefficients and linewidth of the hydroxy protons of α-CD in presence of 1,3-adamantanediol were very similar to those in α-CD alone.
Thus, when changes in NMR parameters are observed for α-CD hydroxy protons these changes can be used to detect formation and identify the structure of inclusions complexes.

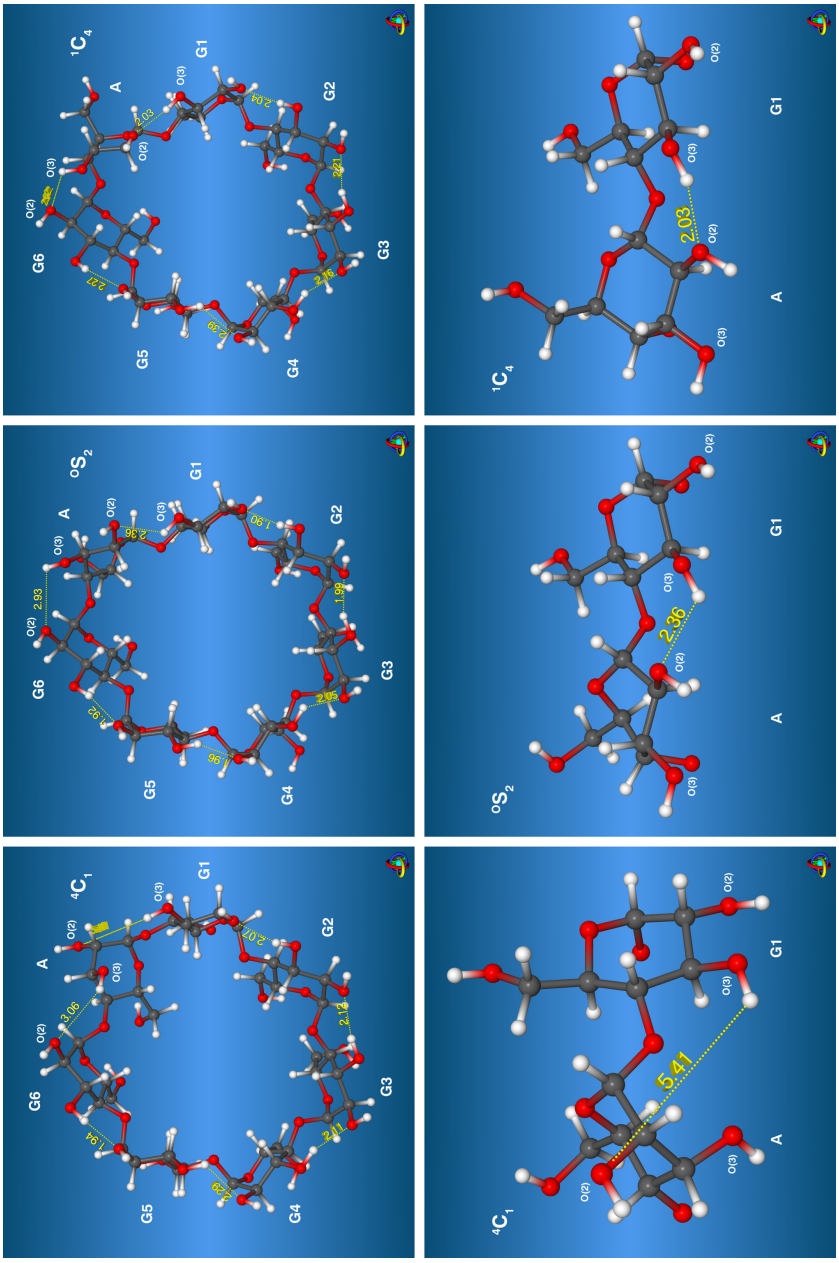
5.1.2 Paper II – ^{1}H NMR Studies on the hydrogen-bonding network in mono-altro-β-cyclodextrin and its complex with adamantane-1-carboxylic acid

CDs are only able to form static "lock and key" type complexes(*109*). On the other hand, cyclic oligosaccharides in which one or several glucose units are replaced by an α-D-altrose residue are highly flexible since altrose can adopts various conformation. Mono-*altro*-β-cyclodextrin (Scheme 6) is one of the first examples of a cyclic oligosaccharide that can mimic an "induced-fit" type mechanism by producing an adaptable host (*110-114*). It was shown that in D_2O solutions, the altrose residue adopts preferentially the $^{1}C_4$ conformation. Inclusion of adamantane-1-carboxylic acid resulted to a shift to 80 % $^{O}S_2$ conformation (Scheme 7)(*115*). We have investigated using NMR of hydroxy protons how the incorporation of altrose and its conformational changes influence the hydrogen bond network in cyclodextrin.



Scheme 6. Left: Structure of mono-*altro*-β-cyclodextrin with the Alt-unit (A) and the Glc-units (G1 – G6). Right: Structure of adamantane-1-carboxylic acid.

The spin systems in TOCSY were assigned to the different O(2)H, O(3)H and O(6)H in the COSY spectrum (Figure 6). The sequence of the G2-G5 residues was obtained from inter-residual NOEs between the anomeric proton of one residue and the C(4)H of the next residue. The G6 was assigned from NOE between its anomeric proton and C(4)H of A. In the HMBC spectrum, H1 of A gave a cross peak to C4 of G1. In the HSQC-TOCSY spectrum, the spin system C(5)H-C(4)H-C(3)H-C(2)H was identified at the chemical shift of this C(4). Thereby the corresponding O(2)H, O(3)H and O(6)H as well as C(1)H and C(6)H resonances were assigned. For mono-*altro*-β-cyclodextrin in the complex, A and G1-G6 residues were assigned by following the chemical shift changes of the O(2)H signals at increasing amounts of adamantane-1-carboxylic acid. TOCSY and DQF-COSY spectra recorded at –10 ºC were then used as before to obtain the chemical shifts.



Scheme 7. Ball and stick models of mono-*altro*-β-cyclodextrin with altrose (A) in different conformations ($^4C_1$, $^OS_2$, and $^1C_4$, from bottom to top). On the right are closer views of A with O-2···3-OH (H····O distances given in Å) to the neighbouring G1, indicated with dotted lines. These graphics were generated with the program MolArch$^+$ using structures proposed in Ref. (*115*).

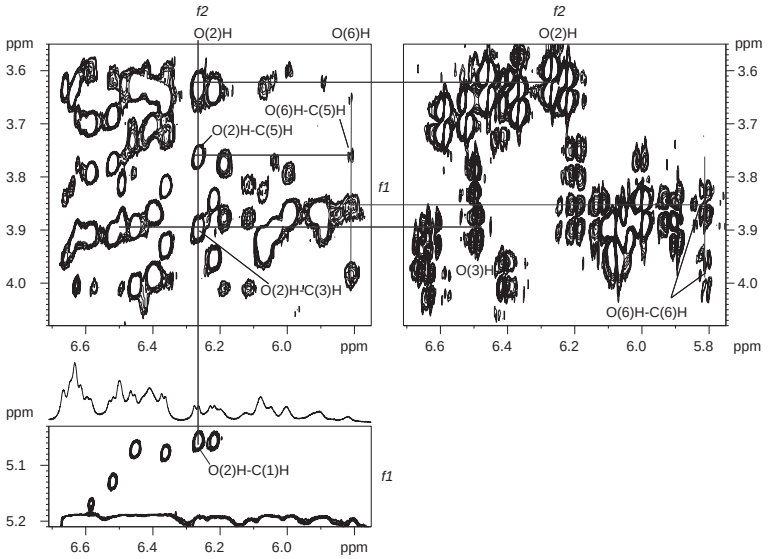


Figure 6. Left: Expansions of the TOCSY spectrum of mono-*altro*-β-cyclodextrin of Glc-units. In *f2* the hydroxy protons are shown and in *f1* the ring protons. Right: Corresponding region in the DQF-COSY spectrum.

In β-cyclodextrin (β-CD), O(2)H and O(6)H have chemical shifts similar to those in methyl-α-D-glucopyranoside (Glc of Table 3), while the O(3)H is deshielded by 0.30 ppm(*21*). From the values of chemical shift, temperature coefficient, coupling constant and rate of exchange, it was proposed that O(3)H is involved as the donor in an intermolecular hydrogen bond with O(2)H of the next glucose residue. In mono-*altro*-β-cyclodextrin, all Glc-residues O(3)H have chemical shifts between 6.61 and 6.66 ppm except for O(3)H of G1 which has a chemical shift of 6.49 ppm. These values can be explained by inspection of the 3D models built with A adopting the three conformations $^4C_1$, $^OS_2$ and $^1C_4$ (Scheme 7), using reported dihedral angles (*110, 115*). In the $^4C_1$ chair, the predominant conformation for free mono-*altro*-β-cyclodextrin, the distance between O(2) of A and O(3)H of G1 is 5.41 Å, which is too far for hydrogen bond interaction (Scheme 7). This was well represented by the chemical shift of O(3)H signal of G1, which has a value close to that in the monosaccharide. The O(3)H of G2 – G6 have distances that allow hydrogen bonding to O(2) of the next residue. These protons were indeed deshielded by more than 0.2 ppm, compared to the monosaccharide (Table 3). The deshielding were however not as large as for O(3)H of β-CD indicating that the hydrogen bonding network is disrupted throughout the whole glucose chain and not only around A. The O(3)H of G2, G3 and G5 have small (<3 Hz) coupling constants, the remaining O(3)H $^3J_{CH,OH}$-values could not be measured. All |dδ/dT|-values are larger than 3 ppb/ºC, indicating that the hydrogen bond interactions are relatively weak.

Table 3. ^{1}H-NMR chemical shifts (δ), Δδ, temperature coefficients (|dδ/d*T*|) and coupling constants ($^{3}J_{OH,CH}$) for hydroxy protons of mono-*altro*-β-cyclodextrin at –10 ºC in 85% H_2O / 15% $(CD_3)_2CO$). Values for methyl α-D-glucopyranoside (Glc) and β-CD are reported for comparison.

	O(2)H				O(3)H				O(6)H		
	δ	Δδ[a]	|dδ/d*T*|	$^{3}J_{OH,CH}$	δ	Δδ[a]	|dδ/d*T*|	$^{3}J_{OH,CH}$	δ	Δδ[a]	|dδ/d*T*|
	ppm	ppm	ppb/ºC	Hz	ppm	ppm	ppb/ºC	Hz	ppm	ppm	ppb/ºC
G1	6.27	-0.11	7.0	6.1	6.49	0.08	9.6		5.81	-0.20	9.6
G2	6.22	-0.16	6.6	7.7	6.66	0.25	8.4	<3	6.08	0.07	11.2
G3	6.36	-0.01	7.1	6.2	6.64	0.23	8.1	<3	6.07	0.06	11.7
G4	6.52	0.14	8.3	7.3	6.62	0.21	7.6		6.04	0.03	11.6
G5	6.59	0.21	9.1	6.6	6.61	0.20	6.5	<3	5.99	-0.01	11.2
G6	6.45	0.08	10.4	7.0	6.63	0.22	7.3		5.89	-0.12	11.6
A	6.49		8.7		6.11		11.7		6.19		10.4
β-CD	6.40	0.02	7.1	6.7[b]	6.71	0.30	8.1	<3[b]	6.06	0.05	12.7
Glc	6.38		12.1	6.0[c]	6.41		11.2	5.1[c]	6.01		12.6

[a]Values obtained by subtracting the chemical shift of methyl α-D-glucopyranoside.
[b] Taken from reference (*21*).
[c] Taken from reference (*116*).

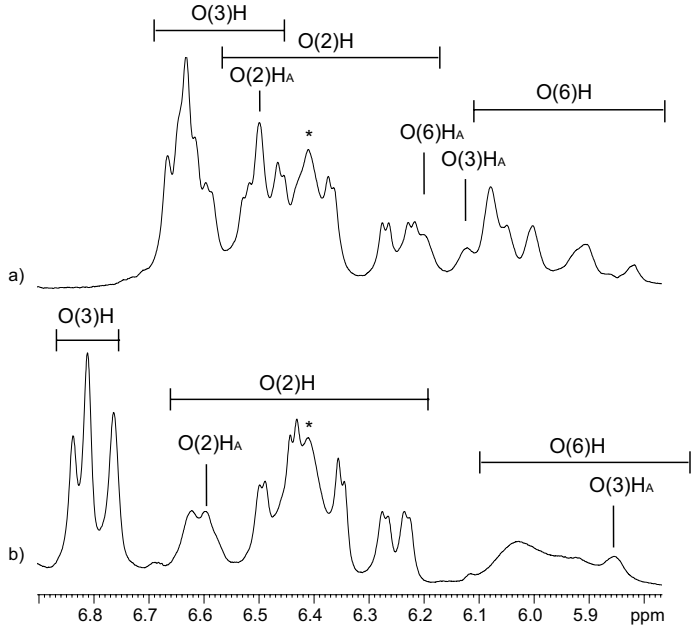


Figure 7. Expansion of the hydroxy protons region of ^{1}H NMR spectrum of a) mono-*altro*-β-cyclodextrin and b) mono-*altro*-β-cyclodextrin in complex with adamantane-1-carboxylic acid, at pH 6.3.

After addition of one equivalent of adamantane-1-carboxylic acid, the chemical shifts of O(2)H in G1-G6 were not changed significantly. The O(3)H of G3-G6 were deshielded by more than 0.15 ppm (Figure 7b). The O(3)H of G1 was deshielded by as much as 0.27 ppm (Table 4). This large downfield shift, almost similar to those observed for the remaining O(3)H, might be due to hydrogen bonding interaction with O(2) of A, which now have change its conformation to 80% $^{O}S_2$. The distance between the O(3)H of G1 and O(2) of A is only 2.36 Å (Scheme 7).

Table 4. ^{1}H-NMR chemical shifts (δ), Δδ and temperature coefficients (|dδ/d*T*|) for hydroxy protons of mono-*altro*-β-cyclodextrin in complex with adamantane-1-carboxylic acid at –10 ºC in 85% H_2O / 15% $(CD_3)_2CO$). Values for methyl α-D-glucopyranoside and β-CD are reported for comparison. The data for O(6)H was collected for solutions at pH 7.4.

	O(2)H				O(3)H			O(6)H		
	δ	Δδ[a]	|dδ/d*T*|	$^3J_{OH,CH}$	δ	Δδ[a]	|dδ/d*T*|	δ	Δδ[a]	|dδ/d*T*|
	ppm	ppm	ppb/ºC	Hz	ppm	Ppm	ppb/ºC	ppm	ppm	ppb/ºC
G1	6.27	-0.11	6.5	6.6	6.76	0.35	9.5	5.88	-0.13	11.5
G2	6.23	-0.15	6.8	5.7	6.69	0.28	7.0	6.08	0.07	12.0
G3	6.35	-0.03	6.5	6.6	6.81	0.40	10.5	6.07	0.06	11.9
G4	6.49	0.12	7.7	6.1	6.84	0.43	8.7	6.05	0.04	12.4
G5	6.62	0.25	10.7	[b]	6.76	0.35	6.7	6.01	0.00	11.2
G6	6.44	0.06	6.9	7.2	6.81	0.40	10.7	5.98	-0.03	12.4
A	6.59		9.7	[b]	5.85		10.2	[b]		[b]
β-CD	6.40	0.02	7.1		6.71	0.30	8.1	6.06	0.05	12.7
Glc	6.38		12.1		6.41		11.2	6.01		12.6

[a]Values obtained by subtracting the chemical shift of methyl α-D-glucopyranoside.
[b]Not measured due to spectral overlap.

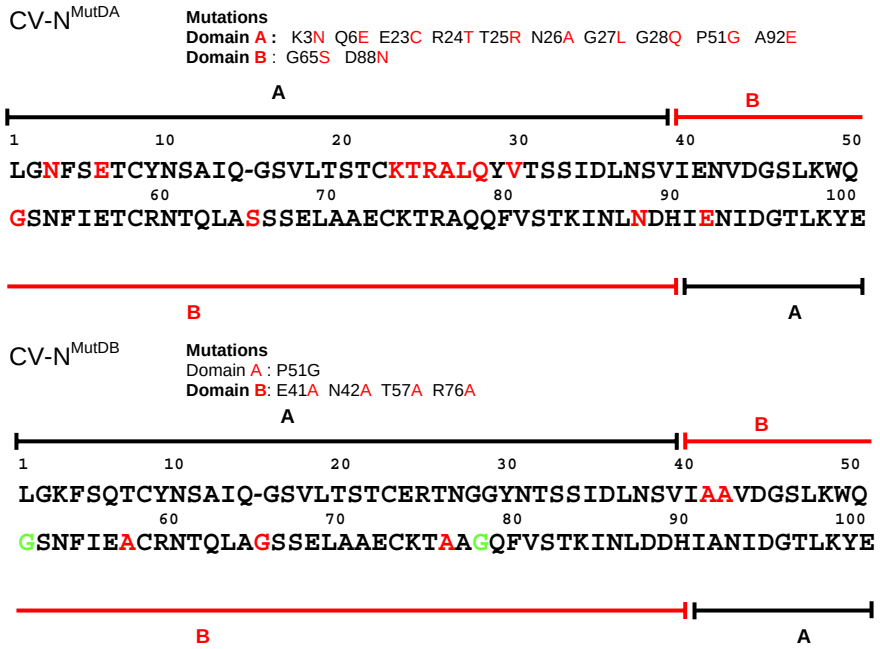
The distance between O(3)H of A and O(2) of G6 is larger when A adopts the $^{O}S_2$ conformation (2.93 Å compared to 2,62 Å). This was reflected by the upfield shift of O(3)H of A that indicate weaker hydrogen bonding. The vicinal coupling constants and temperature coefficients were not significantly changed upon complex formation.

Confirmation on the formation of the inclusion complex was obtained from the intra-molecular ROEs between C(3)H of mono-*altro*-β-cyclodextrin and H3 and H4 of adamantane-1-carboxylic acid. C(5)H did not have any intermolecular ROE suggesting a partial inclustion of the guest molecule into the cavity. The change in altrose conformation from $^4C_1$ to $^{O}S_2$ causes the cavity to change to a more elliptical shape which allows the inclusion of the guest molecule. This shape allows a more regular hydrogen bond network and this can be seen from the chemical shift of O(3)H of the glucose residues. The conformational change of A can be observed from $^3J_{CH,CH}$-values while intermolecular NOEs give information about the inclusion complex. The monitoring of chemical shift of the hydroxy protons give structural information on hydrogen bonding.

5.2 Papers III and IV

Paper III- Atomic mapping of the sugar binding epitopes on one-site and two- site mutants of Cyanovirin-N by saturation transfer difference NMR spectroscopy

Due to aggregation problem, it is not possible to study the binding of Man_9 to CV-N in solution and instead interactions between CV-N and substructures of Man_9 have been investigated(*36, 43, 44*). It has been shown that the smallest oligosaccharide required for binding is the disaccharide Manα(1-2)Man(*35, 37, 43-45, 49*), domain B exhibiting a slightly higher affinity than domain A (K_A 7.2 ± 4 x 10^6 M^{-1} and 6.8 ± 4 x 10^5 M^{-1}). ITC experiments performed on the trimannosides forming the three arms D1-D3 of Man_9 showed that the α(1,2)-linked trimannosides had highest affinity for domain A. The two other trisaccharides were binding to both domains with lower affinity(*37*).
A previous STD NMR study on the atomic mapping of the interaction between CV-N and these di- and trimannosides has shown how the carbohydrates bind to CV-N(*117*). Not only the terminal disaccharide, but also the reducing mannose residue or the linkage to it was demonstrated to influence the affinity to CV-N. Since CV-N has two separate binding sites both being simultaneously occupied by the ligands in solution, the STD NMR spectra might have however represented only an average picture of the interaction of the sugars with the two binding sites.
To be able to differentiate between the two binding sites on the protein, mutants of CV-N were designed (Scheme 8)(*51*) In one mutant, CV-N^{MutDB}, the sugar-binding site in domain B was completely abolished. In the other mutant, CV-N^{MutDA}, the binding site on domain A was modified in such a way that the specificity for trisaccharides should be altered. Since CV-N^{MutDB}, has only one binding site, it should be possible using STD NMR to identify without ambiguity the binding epitope on the sugar involved in the interaction with site A.
The STD experiments were run at two different temperatures (10 and 25 °C) with different irradiation frequencies and irradiation times. At 10 °C the STD signals were more intense due to slower molecular motion. The irradiation frequency was chosen to ensure that the ligand protons were not directly saturated while the protein signals were fully saturated. Experiments with irradiation frequency set to 7, 2, 1.3, 0.6, 0.4, 0, -1 and -5 ppm were recorded. At 0.4 ppm a good saturation of the proteins were obtained while direct saturation of the ligand *O*-methyl was avoided. The optimum irradiation time was determined from build up of STD intensities with increasing saturation times and maximum intensities were obtained with a 4s saturation (Figure 8). A comparison of intensities at different temperatures show that the STD effect was higher at lower temperature(*98*).
A high, 100-fold, ligand excess was used since it has been shown to afford higher intensities in the STD spectra. However STD spectra run on samples containing only ligand, showed residual signals with relative intensities as in a normal 1D spectrum. This has been reported previously and correction was made by subtracting these spectra from the spectra obtained in the presence of protein(*118*).



Scheme 8. Amino acid sequences in the CV-N mutants, CV-N^{MutDA} and CV-N^{MutDB} respectively. Above each of the two proteins are the list of mutations.

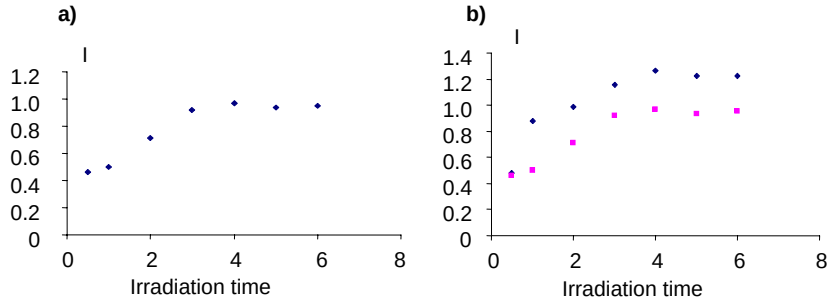


Figure 8. The build-up of STD signals (I) with increasing saturation times for a) H2' of Manα(1-2)Manα*O*Me at 10 ºC in the presence of CV-N^{MutDA} and b) at 10 ºC and 25 ºC in the presence of CV-N^{MutDB}.

Large signals were observed in the STD NMR spectra of the disaccharide Manα(1-2)Manα*O*Me in the presence of both mutants (Figure 9). Since the signal are caused by magnetisation transfer from the protein to the ligand, it is an indication that the Manα(1-2)Manα*O*Me is recognised by both CV-N mutants. The largest STD effect was observed for H2', H3' and H4' on the non reducing end, showing that these protons have the closest contact with the proteins. Figure 9 shows that the STD enhancements are larger for CV-N^{MutDB}.

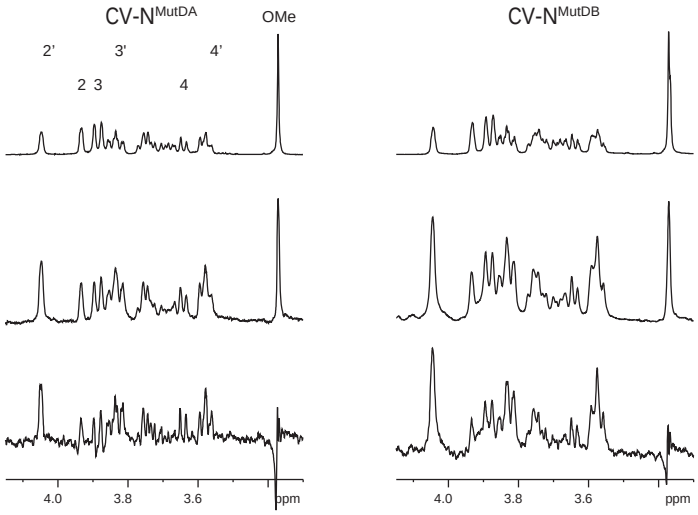


Figure 9. Region of the 1D ^{1}H NMR spectra of Manα(1-2)Manα*O*Me at 10 ºC, (top) reference spectra, (middle) STD spectra and (bottom) STD spectra corrected for high ligand concentration.

The two trisaccharides, Manα(1-2)Manα(1-3)Manα*O*Me and Manα(1-2)Manα(1-6)Manα*O*Me have signals in the STD spectra, indicative of binding to the mutants (Figures 10 and 11). The STD NMR spectra for Manα(1-2)Manα(1-3)Manα*O*Me were very similar with both mutants. Stronger enhancement are observed for H2", H3", H4" of the non reducing end and for H4' of the penultimate mannose residue (Figure 10). For Manα(1-2)Manα(1-6)Manα*O*Me similar enhancements are observed but the intensities of the STD signals are higher in the presence of CV-N^{MutDB} (Figure 11).

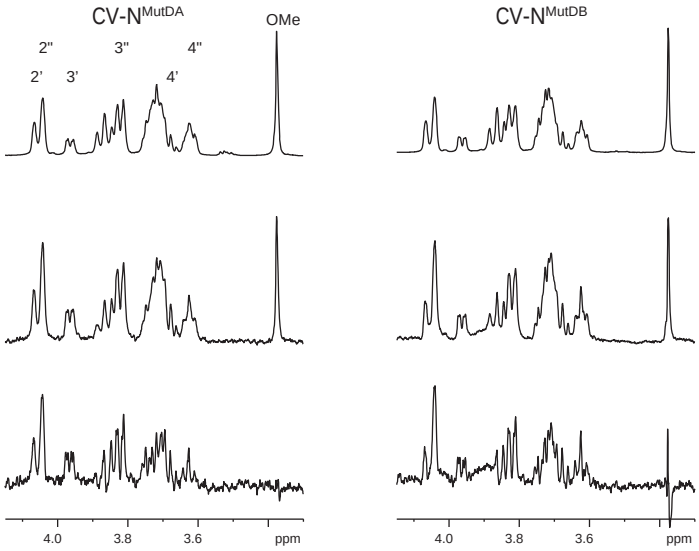


Figure 10. Region of the 1D ^{1}H NMR spectra of Manα(1-2)Manα(1-3)Manα*O*Me at 10 ºC, (top) reference spectra, (middle) STD spectra and (bottom) STD spectra corrected for high ligand concentration.

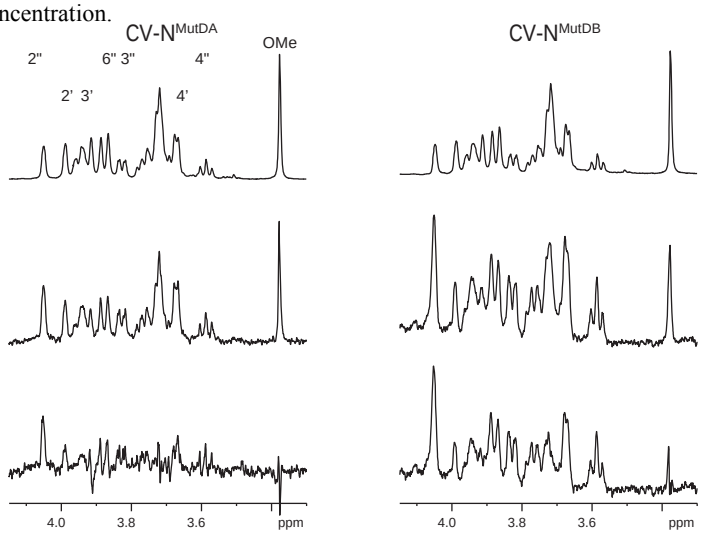


Figure 11. Region of the 1D ^{1}H NMR spectra of Manα(1-2)Manα(1-6)Manα*O*Me at 10 ºC, (top) reference spectra, (middle) STD spectra and (bottom) STD spectra corrected for high ligand concentration.

There was no signal enhancement in the STD spectra of Manα(1-3)[Manα(1-6)]Manα*O*Me or Manα(1-2)Manα(1-2)Manα*O*Me in the presence of CV-N^{MutDA} or CV-N^{MutDB} (Figures 12 and 13 respectively). The absence of signal in the STD spectra is due to either to lack of binding or to strong binding. To determine if the two ligands were binding or not, competition STD NMR experiments were performed(*94, 119*). Upon raising the temperature to 25 ºC small STD enhancements were obtained for Manα(1-2)Manα(1-2)Manα*O*Me in the presence of CV-N^{MutDA}, but not in the presence of CV-N^{MutDB}.

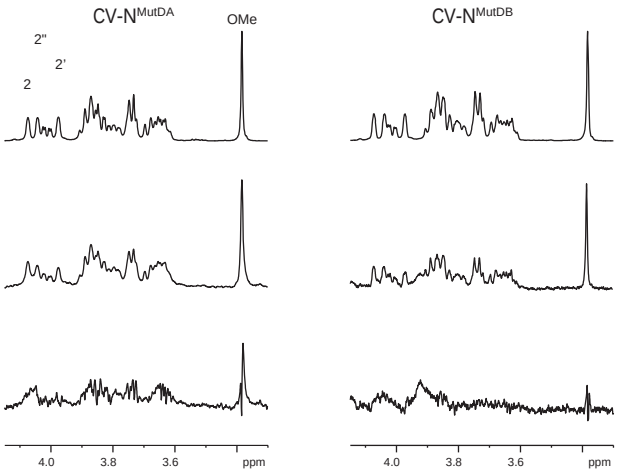


Figure 12. Region of the 1D ^{1}H NMR spectra of Manα(1-3)[Manα(1-6)]Man*O*Me at 10 ºC, (top) reference spectra, (middle) STD spectra and (bottom) STD spectra corrected for high ligand concentration.

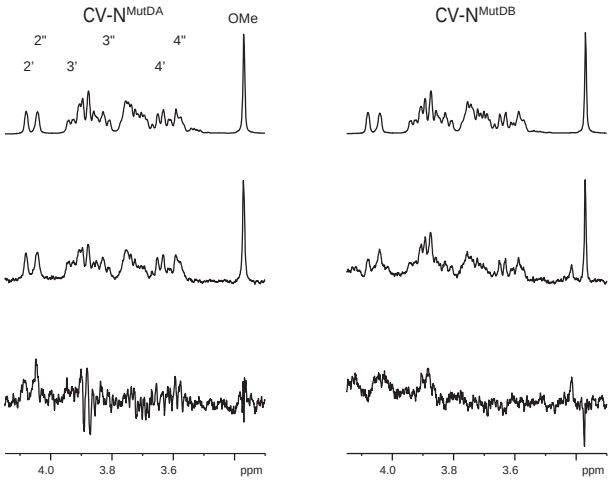


Figure 13. Region of the 1D ^{1}H NMR spectra of Manα(1-2)Manα(1-2)Manα*O*Me at 10 ºC, (top) reference spectra, (middle) STD spectra and (bottom) STD spectra corrected for high ligand concentration.

In the competition studies a medium affinity ligand is used. An STD NMR spectrum of this ligand is first recorded. The other ligand is added to the solution and a new STD NMR experiment is performed. A significant reduction or disappearance of the STD signal of the medium affinity ligand will prove the presence of a high affinity ligand competing for the same binding site.
The STD NMR spectra of Manα(1-2)Manα(1-6)Manα*O*Me in the presence of each protein show no large reduction of signals upon addition of the core trisaccharide Manα(1-3)[Manα(1-6)]Manα*O*Me (Figure 14). This suggests that Manα(1-3)[Manα(1-6)]Manα*O*Me does not interact with either CV-N^{MutDA} nor CV-N^{MutDB}.

These results are in good agreement with previous studies showing that Manα(1-2)Manα epitope is required for binding(*37, 43, 44, 117*).

For Manα(1-2)Manα(1-2)Manα*O*Me, two competition experiments were performed, one with Manα(1-2)Manα(1-6)Manα*O*Me and one with Manα(1-2)Manα*O*Me. In both competitions studies, a clear reduction of the STD signals of the medium affinity ligand was observed upon addition of Manα(1-2)Manα(1-2)Manα*O*Me. Figure 15 shows the competition experiment performed with the disaccharide. These experiments indicate that Manα(1-2)Manα(1-2)Manα*O*Me is a high affinity ligand for both CV-N mutants.

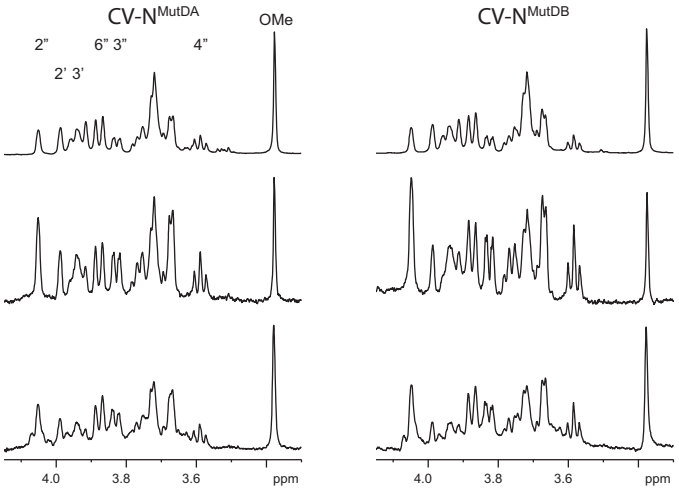


Figure 14. The top spectra are reference ^{1}H NMR spectra of Manα(1-2)Manα(1-6)ManαOMe in the presence of each protein, the middle spectra are the STD spectra of the same sample and the bottom spectra are STD spectra after addition of Manα(1-3)[Manα(1-6)]ManαOMe.

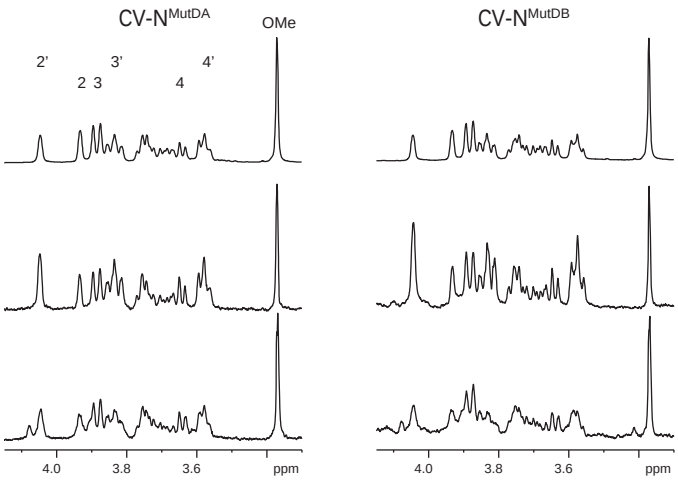


Figure 15. The top spectra are the reference ^{1}H NMR spectra of Manα(1-2)Manα*O*Me in the presence of each protein, the middle spectra are the STD of the same sample and the bottom spectra are STD spectra recorded after addition of Manα(1-2)Manα(1-2)Manα*O*Me.

Isothermal titration calorimetry experiments were performed by our collaborators for the linear α(1,2)-linked trimannoside with CV-N^{MutDB} in order to determine the binding constant. A negative ΔH value, of – 8.22 kcal/mol (Table 5), indicated that the binding is driven by enthalpic contributions, with a strong unfavourable entropic contribution, which is consistent with loss in rotational, translational and conformational freedom for the trisaccharide upon complex formation. The ΔG value of – 7.6 kcal/mol was obtained by analysis of the binding isotherm and fits a one site model satisfactory. This is also consistent with data for the CV-N^{mutDB} interactions with Man_9(*51*). No attempt was made to obtain binding parameters for CV-N^{MutDA} due to the existence of two binding sites.

Table 5. Overall thermodynamic parameters recovered from the binding data of CV-N^{mutDB} with Manα(1-2)Manα(1-2)Manα*O*Me, (Man_3) using one-site model.

	Enthalpy ΔH (kcal/mol)	Entropy TΔS (kcal/mol)	Free energy ΔG (kcal/mol)	Affinity K_d (μM)
CVN^{mutDB}- Man_3	-8.22 ± 0.03	- 6.54 ± 0.03	-7.57± 0.08	3.4±0.05
CVN^{mutDB}- Man_9*	-11.12 ± 0.1	-3.68 ± 0.06	-7.431 ± 0.04	4.3 ± 0.3

* Taken from Ref. (*51*).

The fact that similar results were obtained for both mutants with the disaccharide and trisaccharides, Manα(1-2)Manα(1-3)Manα*O*Me and Manα(1-2)Manα(1-6)Manα*O*Me, suggests that the binding modes are similar for the two binding sites. Additionally we could show that the binding affinity of the α(1,2)-linked trimannoside, Manα(1-2)Manα(1-2)Manα*O*Me, were higher for both mutants which confirms the previous results(*117*) that showed that not only the link between the two terminal residues are important for binding but also the link

between the reducing end and middle residue. This further suggests that the conformation around the glycosidic linkage causes the observed selectivity. In binding, the carbohydrate adopts a stacked conformation for which the α(1,2)-linked trisaccharide is more compact compared to the trisaccharide with α(1,3)- and α(1,6)-linkages to the reducing end residue.
These observations can be explained with the aid of the map of mutations in the two mutants (Scheme 8). The polar residues of Domain B which are most crucial for carbohydrate binding, Glu41, Asn42, Thr57 and Arg76 are replaced with non-polar alanine residues. Hydroxy groups of the carbohydrate ligands are thereby prevented from participating in polar interactions and hydrogen bonding with the binding site in domain B, which have been suggested to play a key role in the binding interactions(*42, 45, 46*). The binding site in Domain A is still able to bind carbohydrates according to data from STD NMR, however, it was shown that the mutations result in a complete loss of antiviral activity(*51*).

The mutations in CV-N^{MutDA} were made to eliminate the preference for the D1 arm of Man_9. According to Bewley, Lys3, Gln6, Thr7, Glu23, Thr25 and Asn93 are involved in either water mediated or direct hydrogen bonding with hydroxyls of the disaccharide Manα(1→2)Man. In the mutant, four of these residues were replaced with amino acid residues that either lack hydrogen bond donor or acceptor capacity or have shorter side-chains. The aim of the mutations was to obtain a binding site similar to that in domain B and thereby eliminate the preference for the Manα(1-2)Manα(1-2)Manα*O*Me trisaccharide while retaining the recognition for the Manα(1-2)Man epitope. The STD NMR experiments showed that CV^{MutDA} binds to the disaccharide Manα(1-2)Manα*O*Me as well as to the trisaccharides Manα(1-2)Manα(1-3)Manα*O*Me, Manα(1-2)Manα(1-6)Manα*O*Me and Manα(1-2)Manα(1-2)Manα*O*Me.

5.3 Paper IV – NMR study of hydroxy protons of di- and trisaccharides, substructures of Man_9

The solution conformations of α(1,2)-, α(1,3)- and α(1,6)-linked di- and tri-mannosides, building blocks of Man_9, have been widely investigated by NMR spectroscopy and molecular modelling due to the importance of the molecule in recognition processes(*25, 120-124*). The importance of conformation in molecular recognition is exemplified in the interaction between Manα(1-2)Manα*O*Me and CV-N. The disaccharide adopts a conformation in which one mannose residue is stacked over the other residue, giving a compact structure that can penetrate into the binding sites on the protein(*45*). The disaccharides Manα(1-3)Manα*O*Me and Manα (1-6)Manα*O*Me did not bind to CV-N while the trisaccharides forming the three branches of Man_9 had different binding affinities(*37*).
The NMR studies on these compounds have been dealing with the non-exchangeable protons, and we have investigated the hydroxy protons to determine if information on hydrogen bonds and hydration could be obtained.

Most of the hydroxy protons in Manα(1-2)Manα*O*Me, Manα(1-3)Manα*O*Me and Manα(1-6)Manα*O*Me have chemical shifts that are very similar to those in the monosaccharide methyl α-D-mannoside (Table 6). Exceptions are found for the O(3)H signal of the reducing end in Manα(1-2)Manα*O*Me and for O(2)H and O(4)H of the reducing end in Manα(1-3)Manα*O*Me. These protons have positive Δδ values larger than + 0.15 ppm. Positive Δδ indicate that the hydroxy protons experience a downfield shift in the disaccharide relative to the same proton in the corresponding monosaccharide. In Manα(1-6)Manα*O*Me, none of the hydroxy protons has large Δδ values.

Table 6. Chemical shift (δ, ppm) at –10 ºC, temperature coefficients (|dδ/dT|, ppb/ºC) and chemical shift differences compared to monosaccharide (Δδ, ppm) for each disaccharide.

			O(2)H	O(3)H	O(4)H	O(6)H
Manα*O*Me		δ	6,29	6,15	6,37	6,06
		dδ/dT	12,9	13,1	12,6	14,7
Manα(1-2)Manα*O*Me	H	dδ/dT		14,2	12,8	14,9
		Δδ		**0,16**	-0,02	-0,06
	H’	dδ/dT	13,3	13,0	13,1	16,1
		Δδ	-0,04	-0,06	-0,01	0,03
Manα(1-3)Manα*O*Me	H	dδ/dT	14,9		13,1	15,7
		Δδ	**0,23**		**0,22**	0,04
	H’	dδ/dT	13,6	13,5	13,5	13,8
		Δδ	0,04	0,03	0,05	-0,04
Manα(1-6)Manα*O*Me [a]	H	dδ/dT	9,5	13,2	10,6	
		Δδ	-0,03	0,02	0,00	
	H’	dδ/dT	11,8	11,5	13,9	14,3
		Δδ	0,02	-0,00	0,03	-0,02

[a] The Δδ - values were measured at – 7 ºC.

The trisaccharide Manα(1-2)Manα(1-2)Manα*O*Me has two O(3)H, one on the reducing and one at the penultimate end, showing a positive Δδ. In Manα(1-2)Manα(1-3)Manα*O*Me, three hydroxy protons, O(2)H and O(4)H of the reducing end and O(3)H at the penultimate sugar have positive Δδ. In Manα(1-2)Manα(1-6)Manα*O*Me, O(3’)H has a positive Δδ while in the branched Manα(1-3)[Manα(1-6)]Manα*O*Me, the O(2)H and O(4)H are experiencing a downfield shift. No hydroxy protons from the Manα(1-6)Manα*O*Me moieties in Manα(1-2)Manα(1-6)Manα*O*Me or Man(1-3)[Manα(1-6)]Manα*O*Me have large Δδ values. Thus, the changes in chemical shift observed when forming a disaccharide from a monosaccharide are conserved in the trisaccharides, and no additional changes are observed when building the trisaccharides from the two constituent disaccharide subunits.

According to previous studies, positive Δδ might be due either to the effect of glycosylation or to the proximity of the hydroxy proton to a hydroxyl group from a neighbouring sugar(*123*). For CH proton, the effect of glycosylation on chemical shifts is well known and results usually in a deshielding of the protons across the glycodic bond as well as of the protons at the two neighbouring sites of the aglycon. The magnitude of the deshielding depends on the type of monosaccharide, anomeric linkage, and conformation around the glycosidic bond. The main causes for this deshielding are the steric repulsion between hydrogen and the fixation of oxygen lone-pairs close in space to the hydrogen in

question(*125*). In the di- and trimannosides, the hydroxy protons exhibiting a large positive Δδ are located at the neighbouring sites of the aglycon (O(3)H in Manα(1-2)Manα*O*Me and O(2)H, O(4)H in Manα(1-3)Manα*O*Me, and therefore these Δδ might be due to the proximity of the protons to the glycosidic linkage oxygen with more directed lone pairs. We have shown previously that large positive Δδ could also be correlated to spatial proximity to another hydroxyl group. In some cases, the spatial proximity between the two hydroxyl groups was confirmed by chemical exchange cross-peaks in the ROESY spectra. These effects were observed for O(2')H and O(3)H in maltose and in cyclodextrins(*21*).

Table 7. Chemical shift (δ, ppm) at –10 ºC, temperature coefficients (|dδ/dT|, ppb/ºC) and chemical shift differences compared to monosaccharide (Δδ, ppm) for each trisaccharide.

			O(2)H	O(3)H	O(4)H	O(6)H
Manα*O*Me		δ	6,29	6,15	6,37	6,06
		dδ/dT	12,9	13,1	12,6	14,7
Manα(1-2)Manα(1-2)Manα*O*Me	H	dδ/dT		11,4	10,2	12,4
		Δδ		0,15	0,02	-0,07
	H'	dδ/dT		10,7	11,0	12,7
		Δδ		0,13	0	-0,00
	H"	dδ/dT	10,4	10,2	10,7	12,9
		Δδ	-0,05	-0,07	-0,01	-0,03
Manα(1-2)Manα(1-3)Manα*O*Me	H	dδ/dT	13,8		13,5	14,4
		Δδ	**0,15**		**0,20**	-0,01
	H'	dδ/dT		12,6	12,3	13,3
		Δδ		**0,16**	0,00	-0,12
	H"	dδ/dT	13,0	13,0	11,2	15,1
		Δδ	-0,04	-0,06	-0,04	-0,13
Manα(1-2)Manα(1-3)Manα*O*Me	H	dδ/dT	9,9	11,9	9,8	
		Δδ	-0,08	-0,04	-0,04	
	H'	dδ/dT		11,7	12,0	12,4
		Δδ		0,14	-0,01	-0,11
	H"	dδ/dT	11,7	10,4	11,2	13,4
		Δδ	-0,04	-0,06	-0,02	-0,04
Manα(1-3)[Manα(1-6)]Manα*O*Me[a]	H	dδ/dT	15,3		12	
		Δδ	**0,17**		**0,20**	
	H'	dδ/dT	13,5	14,7	13,0	14,0
		Δδ	0,03	0,04	0,04	-0,06
	H"	dδ/dT	13,5	14,7	13,0	14,0
		Δδ	0,03	0,04	0,04	-0,06

[a] The Δδ - values were measured at – 9 ºC

The branched trisaccharides Glcβ(1-3)[Glcβ(1-4)]Galα*O*Me, Glcβ(1-3)Fucα(1-4)]Galα*O*Me and Glcβ(1-3)[Fucβ(1-4)]Galα*O*Me, were characterized by a downfield shift of O(2)H Gal and a chemical exchange between O(2)H Gal and O(2)H Glc*(125)*. Even in disaccharides, where higher conformational flexibility exists, downfield shift and chemical exchange were observed between O(2)H Glc and O(2)H Gal in Glcβ(1-3)Galα*O*Me and between O(2)H Fuc and O(3)H Gal in Fucα(1-4)Galα*O*Me*(126)*. For all these compounds, the coupling constants and temperature coefficients values indicated however that no strong hydrogen bonding interaction was involved. It is therefore possible that these interactions are present for geometrical reason in the minimum energy conformations, but they do not stabilize the conformation. One should however note that downfield shifts

are not systematically measured for all hydroxy protons situated on each side of the glycosidic linkage.
With the α(1,6)-linkage, only small Δδ values were observed, suggesting that the hydroxy protons do not have, at least for a significant amount of time, any close contact to non-protonated oxygen or to hydroxyl group. This linkage involves the exocyclic hydroxymethyl group of one of the monosaccharide subunit. This extra bond in the linkage reduces the steric interactions between the two sugars and enhances their conformational flexibility. Thus the data confirm other studies showing that small Δδ can be good indicators of conformational flexibility and/or of absence of close contacts with other functional groups in the molecule.
All hydroxy protons in the di- and trimannosides have high temperature coefficient |dδ/dT|-values (Table 6 and 7) indicating that they are fully hydrated and not involved in strong intermolecular hydrogen bond interaction. NOESY and ROESY experiments run at several mixing times showed no dipolar or chemical exchange between hydroxy protons that could support the existence of interaction involving O(3)H in Manα(1-2)Manα*O*Me and O(2)H or O(4)H in Manα(1-3)Manα*O*Me. For Manα(1-3)Manα*O*Me however, an NOE was found between O(4)H of the reducing end and H5 on the non reducing end. To get some insight on whether the positive Δδ's were due to glycosylation effects and/or to spatial proximities between hydroxyl groups, previously reported conformational studies were examined.

The α(1,2)-linkage has been shown to adopt two interconverting conformations (Φ/ Ψ = +70º/-105º and Φ/Ψ= +65º/-170º, with Φ = O5'-C1'-O1'-C2, Ψ = C1'-O1'-C2-C1) and inspection of these conformations shows that O(3)H has no close contact to hydroxyl groups on the neighbouring sugar(*25*). Thus, the downfield shift measured for O(3)H might only reflect glycosylation shift due to proximity to the lone pair of the glycosidic oxygen. According to Ref. (*123*) the potential energy surface as a function of the dihedral angles Φ_H = H1'-C1'-O1'-C2 and Ψ_H = C1'-O1'-C2-H2 have a global minimum for the α(1,2)-linkage at Φ_H / Ψ_H = -47°/20°, which also is in agreement with the above mentioned review(*25*).
A recent study, performed in the gas phase(*124*), on the structural preferences of the α(1,3)- and α(1,6)-dimannosides has shown that for the α(1,3)-linkage, only one conformer is significantly populated (Φ_H/Ψ_H = -32º/52º). In this conformation, there is a relatively strong O(2)H-O6' hydrogen bond, in which O6' is the acceptor. Table 6 shows that O(4)H also have a positive Δδ. Inspection of the 3D structure, shows however that the distance between O(4) and O(2') is too large for inter residual contact (5.8 Å). In this model the closest oxygen to O(4) is the O1' of the link which make it more probable that the chemical shift difference for O(4)H is due to glycosylation shift and not to an interaction with hydroxyls on the other residue.
In general the trisaccharides with α(1,2)- and α(1,3)-linkages have slightly lower Δδ-values than those measured in the disaccharides. The differences however are too small to allow an interpretation of the data. The largest deshielding in the trisaccharides is for O(4)H of the reducing end residue of Manα(1-2)Manα(1-3)Manα*O*Me and Manα(1-3)[Manα(1-6)]Manα*O*Me which is 0.20 ppm in both cases. Thus, despite the fact that O(4)H of the reducing end is between two glycosidic linkages in the branched trisaccharide, its Δδ-value is the same as in the

linear Manα(1-2)Manα(1-3)Man*α*OMe trisaccharide. This show the higher degree of flexibility in the 1,6-linkage.

6 Concluding remarks

It has been shown that the NMR data obtained from hydroxy protons can be used to obtain information on hydrogen bonding and hydration changes in cyclodextrins upon formation of inclusion complexes. For α-CD in complex with adamantane derivatives, it was clearly demonstrated that the hydroxy proton chemical shifts are very good indicators of hydration changes. With mono-*altro*-β-cyclodextrin in complex with adamantane-1-carboxylic acid, changes in chemical shifts could be correlated to changes in the hydrogen bond network in the cyclodextrin.

The STD NMR spectroscopy study on the interaction between mutants of CV-N and oligomannosides showed that despite the mutations both proteins have the ability to recognise the Manα(1-2)Man epitope. The H2, H3 and H4 protons on the reducing end had the closest contact with the binding site on domain A of the protein. The two mutants have different affinity for the trisaccharide Manα(1-2)Manα(1-2)Man*α*OMe, CV-M^{MutDB} having the stronger affinity.

Future aspects

A direct continuation of this work will be to perform STD NMR studies on 2'-, 3'-, 4'- and 6'-deoxy-analogues of the disaccharide Manα(1-2)Man*O*Me. This would allow the determination of the hydroxyl groups that are required for binding to CV-N.

Due to difficulties in observing hydroxy protons in NMR spectroscopy, studies dealing with carbohydrate interactions are usually performed in D_2O solutions and only the CH non-exchangeable protons are studied. If it was possible to observe the hydroxy protons, one could directly identify the hydroxyl groups that are in contact with the protein and thereby the key functional groups necessary for binding. Thus, it would be of great interest to develop methods in which hydroxy protons are observed in STD NMR spectroscopic experiments.

7 References

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