Hydroxy Protons in Structural Analysis of Carbohydrates by NMR Spectroscopy and Computational Methods

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Abstract

This thesis describes the use of hydroxy protons in structural analysis of carbohydrates in aqueous solution by NMR spectroscopy.

For aqueous solutions of carbohydrates, using H_2O as the solvent of choice instead of D_2O makes it possible to observe exchangeable protons provided that the proton exchange with bulk water is slow enough. Thus, additional data from exchangeable protons can be acquired in terms of chemical shifts, vicinal coupling constants, temperature coefficients, exchange rates, and NOEs.

The application of the method on the Lewis b, X, and Y oligosaccharides supplied further information about the rigidities of the molecules. Regarding the chemical shift differences, extent of interaction with bulk water molecules and being located around amphiphilic regions were anticipated to play role on magnetic shieldings of hydroxy protons (Articles I-II).

The hydrogen bonds between O(2)H and O(3)H groups on adjacent glucose units in CDs were proved to exist in water solution (Article III), as they had been reported in solid state and DMSO solutions. A weak and transient interaction was also observed between O(2')H and O(3)H in maltose. Using hydroxy protons, the study of intermolecular interactions on the cyclodextrin complexes proved to be useful in providing structural information as chemical shift, temperature coefficient and line-shape of the hydroxy proton signals.

The intermolecular interactions between carbohydrates and nucleotides as revealed by IR studies could not be detected by NMR spectroscopy (Article IV). However, a drastic improvement in the intensity and line-shape of the hydroxy signals from saccharides were encountered upon addition of small amount of purine nucleos(t)ides and nucleobases.

If the reason for the observed upfield and downfield shifts (positive and negative $\Delta\delta$ values) is contemplated, hydration turns out to be the keyword (Article V). When the hydration of a hydroxy proton is hampered by either interactions with acetal oxygens or structural formations (steric effects or perturbed water interactions in amphiphilic regions), the chemical shift of that proton reads an upfield-shifted value in comparison with the hydroxy proton in the corresponding monomeric unit. Likewise, provided that the hydration state is kept the same, a hydroxy proton becomes deshielded when it forms hydrogen bond interaction with another hydroxy group.

Keywords: Hydroxy protons, NMR; *ab initio,* NMR; chemical shift; hydrogen bond; hydration; interaction; conformation; Lewis b, Lewis X, Lewis Y oligosaccharides; cyclodextrins; nucleoside

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This thesis is based on the following original publications, which will be referred to by their roman numerals (**I-V**). Reprints were made with the permissions from the publishers (Elsevier Science and American Chemical Society).

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S. Bekiroglu, C. Sandström, T. Norberg, and L. Kenne,
Carbohydrate Research, 328 (2000) 409-418

II. Structural ^{1}H NMR Study of Lewis X and Y Oligosaccharides in Aqueous Solution Using Hydroxy Protons
Somer Bekiroglu, Lennart Kenne, and Corine Sandström,
article in manuscript

III. ^{1}H NMR Studies of Maltose, Maltoheptaose, α-, β-, and γ-Cyclodextrins, and Complexes in Aqueous Solutions with Hydroxy Protons as Structural Probes
Somer Bekiroglu, Lennart Kenne, and Corine Sandström,
Journal of Organic Chemistry, 68 (2003) 1671-1678

IV. The Effect of Nucleoside Addition on the ^{1}H NMR Spectra of Saccharides
Somer Bekiroglu, Ianric Ivarsson, Lennart Kenne, and Corine Sandström,
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V. The Effect of Hydration on the NMR Chemical Shifts of Hydroxy Protons in Carbohydrates
Somer Bekiroglu, Anders Sandström, Lennart Kenne, Corine Sandström,
article submitted

Abbreviations

1D, 2D, 3D	One-, two-, and three-dimensional
^1H	Proton
^2H, D	Deuterium, nucleus possessing one proton + one neutron
Ac	Acetyl
CD	Cyclodextrin
COSY	Correlation spectroscopy
D_2O	Deuterium oxide, "heavy water"
Da	Dalton
DEPT	Distortionless enhancement by polarization transfer
DFT	Density functional theory
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DQF	Double quantum filtered
FID	Free induction decay
Fuc	Fucose, 6-deoxygalactose
Gal	Galactose
GIAO	Gauge independent (invariable) atomic orbitals
Glc	Glucose
HF	Hartree-Fock
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
Hz	Hertz, frequency unit (s^{-1})
IR	Infra red
Le^b	Lewis b tetrasaccharide
Le^x	Lewis X trisaccharide
Le^y	Lewis Y tetrasaccharide
MD	Molecular dynamics
Me	Methyl
MM	Molecular mechanics
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser and exchange spectroscopy
p	pyranoside, six-membered ring configuration
ppb	parts per billion (10^{-9})
ppm	parts per million (10^{-6})
RNA	Ribonucleic acid
ROESY	Rotating-frame Overhauser enhancement spectroscopy
s	second
THF	Tetrahydrofurane
TOCSY	Total correlation spectroscopy
δ	NMR chemical shift
Å	Ångström $(1 \times 10^{-10}$ meters)

Introduction

Carbohydrates

Carbohydrates, among the four major categories of biomolecules, comprise the class of most abundant organic compounds on the earth. Beside their extensive presence in the biosphere, as polyfunctional and versatile compounds either alone or covalently bonded to proteins or lipids, carbohydrates also carry a variety of vital properties which play many important roles in all forms of life. These can range from being construction materials in cell walls to functioning as information carriers in cellular recognition events, or even from serving as energy stores and metabolic intermediates like starch in plants and glycogen in animals to appearing as integral components of DNA and RNA chains which store and express genetic information. The diversity in their functions is accordingly reflected by their often-complicated structures requiring a better understanding even today.

Carbohydrate chemistry has fallen behind in comparison with the other major compound chemistries until the 1970's, even though carbohydrates have extensively been in the center of our lives. The reasons for this situation are related to the intrinsic difficulties of carbohydrates regarding their chemistry. Carbohydrate molecules have complicated structures that are composed of monomeric units often differing only at one stereogenic center. The monomeric residues also have a broad range of composition (more than 100 different sorts) and type of linkages, increasing the possibility of making complicated structures even with limited number of monosaccharides. Their highly hydrophilic nature adds various challenging difficulties to their isolations, synthesis, and derivatization procedures. However, with the recent advances in chemistry and chemistry related instrumentation, most of the problems that were unattended seem to be controllable and at present carbohydrate chemistry has reached the level that they are even used as starting materials for various syntheses. Similar achievements have taken place in biological studies as well. New findings about carbohydrate dependent markers on cell surfaces assisted in the characterization of various antibiotics and anti-tumor agents. Understanding of complex biological processes is also being helped by such studies unraveling new biosynthetic reactions and enzymatic mechanisms. As a result, carbohydrates are now taking an essential part in the efforts of discovery of new drugs and vaccines against bacterial and viral infections.

Structural Analysis

The importance of 3D structure and activity (function) relationship holds for carbohydrates, as it stands true for all compounds. Therefore, the expanding need for characterization of the widespread biological functions of carbohydrates invokes a major task to understand their 3D structures. Especially studying the conformations shows its vital significance within the carrier role of carbohydrates in cell-cell interactions [1,2]. However, complete structure determination of carbohydrates, including solution conformations and dynamics, is often difficult even for monosaccharides. Since many carbohydrates appear as families of

molecules that differ only in their stereochemistry, very similar and complicated spectral data are observed and important information get concealed. The problem becomes more severe for large oligosaccharides, as the substitution points and the types of linkages of constituent residues increase. The glycosidic bonds, on the other hand, add even more complications to the problems in conformational analysis because of their very flexible character in comparison with glycosyl residues with relatively rigid intra-residue conformations [3].

Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is the principal component in the instrumentation that have been used in this study. This non-destructive technique gives information about molecular structures in terms of spectra of various dimensions. With a coarse simplification, it can be depicted as measuring the resonance response of nuclei to applied electromagnetic radiation (usually of radio wave frequency), provided that the nuclei possess non-zero spin quantum number and are placed in a magnetic field. The sample is typically a compound dissolved in a suitable solvent.

There are other experimental methods, such as fluorescence labeling [4] and optical rotation [5,6] that give useful information for conformational analysis of carbohydrates. However, NMR spectroscopy provides the most complete picture of solution structures of carbohydrates [3,7]. While it is possible to obtain information on the solution conformation and dynamics of a compound, it is also the method of choice for identification of an unknown compound. NMR spectroscopy, despite its relative insensitivity, has the major advantage of being a non-destructive and easy to perform method for quick retrieval of important structural and conformational data. Alternatively, X-ray crystallography is also a powerful tool for both structural and conformational studies in solid state, providing reliable data for only one single conformation. However, the difficulty to obtain single crystals for carbohydrates is eminent. It should also be pointed out that X-ray solid-state structures may prominently deviate from the structures in solution, which mimics the most similar environment for biological systems.

Concerning the experimental conditions employed in NMR spectroscopy, the solvents used are usually deuterated since deuterium nuclei (^{2}H or D) have different resonance frequency than protons (^{1}H), being the most widely exploited nucleus because of its abundance and signal sensitivity. In this manner, it is possible to avoid the solvent signal that would otherwise be overwhelmingly large and distorting in the spectrum. For carbohydrates, D_2O is often the solvent of choice since carbohydrates are highly hydrophilic compounds. DMSO-d_6 and pyridine-d_5 have also been employed. Using different types of solvents actually introduce some complications regarding chemical shift comparisons, because interactions between compounds analyzed and solvent molecules (as well as reference compounds) differ. In fact, not only the chemical shifts but the whole range of possible molecular properties can be altered by solvent interactions [8]. It has been shown that certain hydrogen bonds that were found to be present in DMSO-d_6 solutions do not persist in aqueous solutions [9-12].

In general, the NMR experiments used for conformational studies of carbohydrates are similar to the ones used for other compounds. The type of NMR experiments

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that could be used for this purpose can span a domain from 1D ^{1}H and/or ^{13}C spectra to 2D homo- and hetero-nuclear experiments such as DQF-COSY, TOCSY, NOESY, HSQC, and HMBC [13]. However, there are, of course, subtle differences in the techniques used for different compound groups. For instance, isotopic enrichment is a commonly used and readily available method for proteins, whereas virtually only natural abundance samples are used in the case of carbohydrates.

New types of experiments and methods are continuously being developed. It has been proven useful that the classical experiments can be combined on the cost of time to give multiple evolution time, higher dimensional experiments, *i.e.* 3D, which make the observation of severely overlapping signals [14]. Nevertheless, out of these many different NMR experiments, the information that can be obtained is basically chemical shifts (δ), coupling constants (J), and NOEs. Geminal ($^2J_{XX}$) and vicinal ($^3J_{XX}$) scalar coupling constants are normally used for determination of conformations. For instance, while ring conformations can be analyzed using vicinal proton-proton couplings ($^3J_{HH}$), vicinal homo ($^3J_{CC}$) and heteronuclear ($^3J_{CH}$) scalar couplings through glycosidic bonds provide information about the dihedral angles Φ[C(1)H-C(1)-O(x)-C(x)] and Ψ[C(1)-O(x)-C(x)-C(x)H] or Φ[O5-C1-O(x)-C(x)] and Ψ[C(1)-O(x)-C(x)-C(x-1)] for (1→x) linkage according to the IUPAC rules (Figure 1).
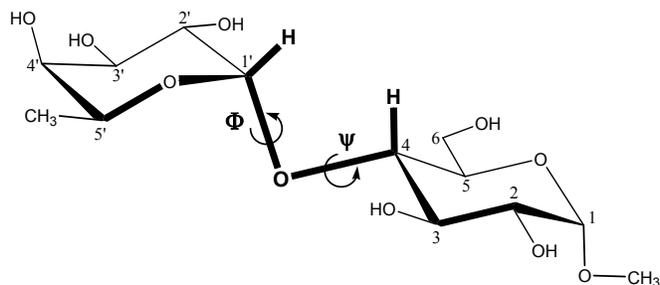
Figure 1. The definitions of Φ and Ψ glycosidic torsion angles and the numbering convention are shown on a disaccharide, α-L-Fuc*p*-(1→4)-α-D-Glc*p*-OMe.

Chemical shifts have also been used in conformational studies [15]. For instance, ^{1}H and ^{13}C chemical shifts, as well as glycosylation shifts, together with computational methods have been used for conformational analysis in carbohydrates [16,17]. However, the primary source for information about overall conformations has been NOE data that provide inter- and intra-residual proton-proton distances on a rather small scale of 5 Å range. In general, these geometry constraints are subsequently used in molecular mechanics and dynamics calculations to obtain a 3D structure, which would be in agreement with experimental data [18-20].

Computational Methods

In fact, what can be acquired from NMR spectroscopy does not correspond to conformational information that can be directly illustrated. The data are rather in the form of structural constraints that can be further treated in order to assess the accessible conformational space of the molecule in question. This is mostly done by molecular mechanics (MM) and/or dynamics (MD) calculations using different methods [20,21].

Carbohydrates once again fall behind proteins and nucleic acids when the extent of implementations of molecular mechanics to biological molecules is taken into consideration. The reason for the difficulties related to the computational treatment of carbohydrates lies in their complex structures. It is already difficult to parametrize a reliable force field for densely packed, highly polar structures, bearing also stereoelectronic effects (anomeric, exo-anomeric, and gauche effects). The relative solution state flexibilities of carbohydrates make this parametrization even more complicated. Among the most commonly used MM and MD force fields and programs in structural studies of carbohydrates, AMBER [22], TRIPOS/Sybyl force field [23], CHARMm [24], GROMOS [25], and MM3 [26] can be mentioned. In addition, there exist different versions of these programs and different modified force fields, which are possible to use. The appropriate choice of force field should be considered in accordance with their different features and with the specific problem(s) addressed [27].

Force field parameters and equations are continuously being improved by making use of new experimental data and new results from more accurate *ab initio*[*] quantum mechanics calculations [28-32]. Quantum mechanical models find applications not only in force field developments but also in conformational studies of moderately sized molecules, as the performance of calculations and computers is improved [33]. Model systems to depict a real problem in terms of simple components are also encountered due to the intrinsic high computer power demands of such calculations [34-36].

In the molecular modeling field, quantum mechanics is one of the most widely used methods with a number of approximation subroutines (*ab initio*, DFT[†], semi-empirical[‡]), which provide trade offs between speed, accuracy, and capacity to answer specific questions. The most commonly used program package for quantum mechanical calculations is Gaussian [37], which was introduced in 1970's and developed continuously over the decades.

The main advantage of quantum mechanical calculations is the possibility to investigate explicitly the electronic properties of molecules. Force field methods do not take into account the electronic motions and hence, the energy of a system

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[*] "From the beginning" in Latin.

[†] Theory based on electron densities and using functionals to calculate electron correlations.

[‡] Quantum mechanical models ignoring core electrons in the system and replacing the lack of electron repulsion information with empirical data.

is defined entirely by nuclear positions. Therefore, if the sought property is related to electronic configurations, quantum mechanics or semi-empirical quantum mechanical schemes are simply the methods of choice. State-of-the-art quantum mechanical methods allow rather accurate *ab initio* calculations of any observable molecular properties ranging from electron affinities of molecules in their excited states [38] to vibrational circular dichroism spectra of chiral molecules [39].

Hydroxy Protons in Conformational Analysis

Importance and Use of Hydroxy Protons

Despite their structural diversity, all carbohydrates carry a common functional group, the hydroxy (–OH) group (Figure 2).
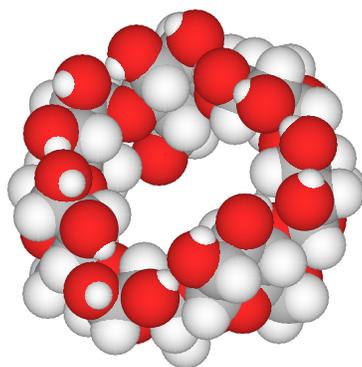

Figure 2. Space-filling model of β-cyclodextrin having many hydroxy groups like all other carbohydrates. Dark grey atoms represent oxygens.

Even though many hydroxy groups exist in carbohydrates, it can be stated that they have not been at the core of experimental and theoretical exercises. In NMR spectroscopy, deuterated solvents have been used to eliminate the excessive proton signal originating from the solvent. Besides, using deuterated protic solvents, which make ^1H exchange with ^2H possible, has been considered beneficial, since NMR spectra get simpler to interpret due to fewer encountered proton signals.

NMR spectroscopy of carbohydrates already suffers from severe spectral overlap, because most of the monomeric residues differ only in their stereochemistry and their magnetic properties are only little influenced by their position in a polymeric chain. As a result, all the ring proton signals with the exceptions of anomeric protons are to be found in the region of 3.2 - 4.5 ppm.

Another problem in conformational analysis of carbohydrates is that NOEs are scarce. The NOEs that can be observed are mainly intra-residue NOEs that do not supply information about the overall structure of the molecule. The number of

NOEs observed between adjacent residues is normally limited to two and observation of NOEs between two residues, which are not directly connected, is very uncommon. The shortage of inter-residue NOEs worsens the determination of conformations on glycosidic linkages, which determine the majority of molecular flexibility since the structures of glycosyl residues are already relatively well characterized. Moreover, NOEs having an order of millisecond time-scale are measures of time-weighed average distances between protons, generating information about only ensembles of conformations in solution. Taking these difficulties into account, investigation of large carbohydrate structures and their rather flexible conformations becomes an even more challenging task.

The difficulty of working with carbohydrates by NMR spectroscopy can be more clearly understood from the general observation that the size of different biomolecules that today are structurally manageable with NMR spectroscopy varies tremendously. While proteins of 30 kDa scale (*ca.* 200 amino acid residues) and nucleic acids of 12 kDa (*ca.* 40 nucleotides) are possible to characterize by NMR spectroscopy, the maximum manageable size for carbohydrates is only 3000 Da (*ca.* 15 monomeric sugar residues).

To help the problems in structure determination of carbohydrates, there have been studies in which homo and heteronuclear two and three bond spin-spin couplings were measured. Using the two possible heteronuclear proton-carbon scalar couplings ($^3J_{CH}$) through glycosidic linkages (Figure 1), it is not possible to precisely describe the glycosidic torsion angles (Φ and Ψ) [40-42]. Using homonuclear vicinal carbon-carbon scalar couplings through glycosidic bonds provides more data to characterize the glycosidic torsion angles [43,44]. The method seems useful although it is still under development. However, measurement of the couplings requires laborious synthesis of the ^{13}C labeled compounds and at present the method is difficult to use in a general strategy for conformational analysis.

Relaxation time studies give information on the mobility and flexibility behavior of molecules in solution environment [45,46]. The outcomes from this type of measurements are intrinsically complex to evaluate because of the complications related to the resemblance of internal molecular motion time scale and overall tumbling rate, involving anisotropic properties.

Through-space dipole-dipole couplings constitute another potent source of information about molecular structure. However, this information is lost in isotropic environments, unlike relaxation time measurements suffering from anisotropic behaviors in solution. Therefore, special anisotropic settings (dilute liquid crystalline medium) should be used [47,48].

Considering the difficulties and problems mentioned above, it is of high interest to find a way to increase the amount of experimental data that can be obtained from carbohydrates. A possible solution, which is being discussed in this thesis, is hydroxy protons that can be exploited to gain substantial amounts of data.

It has been first shown in 1976 that exchangeable protons of carbohydrates in high concentration samples were possible to observe at sub-zero temperatures [49]. The advantages of making use of exchangeable protons in general and hydroxy protons

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in particular, are numerous [9,50-56]. While the confidence that can be put into conformational analyses can be extended due to increased number of inter-residual NOEs, this method also opens the way for possible chemical shift correlations, and information about conformations around exocyclic C5-C6 bonds [55-58]. Hydrogen bond interactions and hydration effects can also be assessed by the additional data acquired from exchangeable protons in terms of coupling constants ($^{3}J_{HO-CH}$), temperature coefficients (dδ/dT), and exchange rates (k_{ex}) [9-11,50-52,55,56,59-63]. Knowing that hydrogen bonds are important for binding specificities between carbohydrates and proteins, it would not be an unjustifiable assessment to see exchangeable protons as potentially important sources of information in biological events.

Sample Preparation

The two essential conditions, which have to be satisfied for the observation of exchangeable protons in aqueous solution by NMR spectroscopy, are to use H_2O instead of D_2O and to lower the exchange rate of hydroxy protons. Lowering the exchange rate is done by excluding the ionic species that can act as catalysts for the exchange process and employing temperatures as low as -10 °C by addition of acetone [49,59]. The pH of the sample solutions must be adjusted to a value between 5.5 and 7.5 [64]. The optimal pH might change with respect to the nature of the compound. The pH of the sample can be controlled by using buffered water solutions. Trials made with acetate buffer of suitable pH gave nice spectra of hydroxy protons.
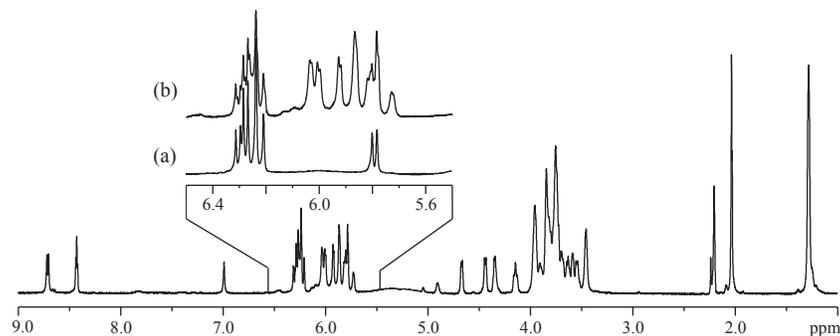
Figure 3. The 1D ^{1}H spectra of α-L-Fuc$p$-(1→2)-β-D-Gal$p$-(1→3)[α-L-Fuc$p$-(1→4)]-β-D-Glc$p$NAc-1-O(CH$_2$)$_2$NHCOCHCH$_2$ before (a) and after (b) resin treatment.

Exclusion of ionic contaminants can be grouped into two phases; removal of (*i*) contaminants (mainly borate anions from glass) coming from the NMR test tube and (*ii*) contaminants that may come with the compound itself. Therefore, NMR sample tubes are soaked for more than 1 h in a 50 mM solution of phosphate buffer, pH 7, to minimize adsorption of impurities from glassware [10]. A ^{1}H NMR spectrum of the sample is then recorded to check if the exchangeable

protons are possible to observe. If the hydroxy protons cannot be observed, the sample is subjected to a purification step using Amberlite mixed ion-exchange resin. Figure 3 shows the 1D ^{1}H spectra recorded for a Lewis b tetrasaccharide derivative in 85% H_2O and 15% fully deuterated acetone (($CD_3)_2CO$ or acetone-d_6) before and after the resin treatment (see article I for details).

The sample compounds are dissolved in a mixture of 85% H_2O and 15% acetone-d_6 by volume. Acetone-d_6 is added to the solution to allow lowering of the sample temperature to around -10 to -15 °C (depending on the nature of the sample) without freezing, and also to provide a deuterium lock signal as well as a reference signal for the chemical shift at $\delta = 2.204$ ppm (acetone-d_5). Since the samples are sensitive to external contamination, the quality of acetone-d_6 is essential. It is advisable to use small batches of high quality acetone-d_6 (usually kept in one-time use ampoules) in order to avoid contamination.

Effect of Acetone-d$_6$ on the Conformation

Because of its relatively weak hydrogen bond donor and acceptor properties [65], acetone-d_6 is an appropriate solvent to employ. However, the question about the influence of acetone on the NMR data obtained for hydroxy protons should be addressed. So far, it has not been possible to study hydroxy protons of carbohydrates in pure water, except at very high concentrations [66] or in supercooled conditions [59]. Therefore, the effect of acetone-d_6 addition was evaluated by an experiment where we observed the hydroxy proton signals of β-cyclodextrin (25 mM) in 95% H_2O/5% D_2O. It has been found that the chemical shifts are comparable in the two solvent systems, 95% H_2O/5% D_2O and 85% H_2O/ 15% ($CD_3)_2CO$ (Article III). It should also be mentioned that a recent study [67] on hydrogen bonding in dicarboxylic acids has shown that even in 90% ($CD_3)_2CO$/10% H_2O mixture, the water content is sufficient to allow full solvation of the intramolecularly hydrogen bonded species.

Experimental Methods and Extraction of Information

In this work, along with 1D proton NMR experiments, DQF-COSY, TOCSY, NOESY, HSQC-DEPT, HSQC-TOCSY, and HMBC experiments were used to obtain homo- (^{1}H-^{1}H) and heteronuclear (^{1}H-^{13}C) correlations to make the ^{1}H and ^{13}C assignments.

Solvent (H$_2$O) Signal Suppression by WATERGATE Pulse Sequence

When water (H_2O) is used to observe exchangeable protons, applying traditional NMR techniques with no solvent signal suppression would result in overwhelming solvent contribution in the recorded FID. As the main component in FID, solvent signal sets the receiver gain to a minimum value to prevent overflow. As a result, the weaker signals coming from the target compound in the solution would be processed insensitively. In other words, the dynamic receiver range would be limited, causing baseline distortions and T_1 noise in 2D experiments.

There are several methods available to suppress the undesired water signal. The common ones are presaturation of the solvent resonance [68], jump-return [69,70],

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and WATERGATE methods [71]. The choice of the method for water suppression depends on the water content of the sample and conditions wanted for the individual experimental setup. There are drawbacks associated with presaturation and jump-return methods, firstly both requiring high quality shimming. When the presaturation pulse is applied, the nuclei interacting with the solvent molecules (exchange) are also being saturated. The jump-return method suffers from the 180° phase shift at the water resonance, making the signals disappear in the tail of the residual water peak [72].
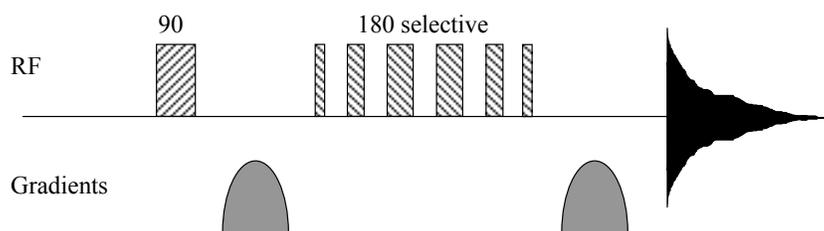

Figure 4. The WATERGATE pulse sequence.

The WATERGATE (WATER suppression by GrAdient-Tailored Excitation) pulse sequence (Figure 4), which can be easily implemented in both 1 and 2D experiments, is superior in efficiency to the other methods [71]. Some modifications of the original WATERGATE pulse sequence have also been introduced [73-76], The WATERGATE pulse sequence [73], which is used in the present studies, makes it possible to eliminate the water signal in the water/substance signal ratio range up to 10^4-10^5 levels.

Temperature Coefficients

Temperature coefficients, dδ/dT, are calculated from the variations of chemical shifts with temperature. NMR spectra are recorded with 5 ºC temperature intervals over the entire range of temperatures at which the exchangeable protons can be observed. The chemical shifts of the proton of interest are plotted versus temperature and the coefficient is calculated as the slope of the line fitted to the data points in ppb/K (or ppb/°C). Temperature changes exert anisotropic magnetic changes upon the hydroxy proton chemical shifts. If a hydroxy proton is involved in a hydrogen bonding interaction, it is expected to have a low temperature coefficient since the anisotropy on the observed proton would be less effective with respect to other hydroxy protons that do not have the same hydrogen bonding property [9]. For saccharides in DMSO, temperature coefficients lower than 3 ppb/°C are usually taken as reference for protons involved in strong hydrogen bonds [51]. Temperature coefficients as low as 4 ppb/°C have been measured for trisaccharides in water solution [55]. As a result, temperature coefficients can

supply useful information about hydrogen bonding interactions, which are utterly important for biological systems and processes [77].

Exchange Rates

In the present context, exchange rate defines the number of proton transfer between a hydroxy group and bulk water in unit time (s^{-1}). Exchange rates, similar to temperature coefficients, can also be used to acquire information about hydrogen bonding interactions. They can indicate steric formations that hamper or facilitate proton transfer.

Exchange rates of hydroxy protons are calculated from NOE cross peak volumes in spectra acquired with several different mixing times, as the ratio of the initial build-up rates of the exchange peaks over the volume of the diagonal peaks at zero mixing time [78-80]. Diagonal peak volumes of zero mixing time experiment are extrapolated from the data generally ranging from 3 to 21 ms. At longer mixing times (> 25 ms) the volumes of exchange cross peaks would not continue growing and might even decrease. Using such short mixing times does not allow significant NOE build-ups due to magnetization transfer. Therefore, the measured volumes are assumed to originate from only chemical exchange.

There are problems associated with this method. The most difficult one is the loss of exchange information due to overlapping signals of the protons. This makes the calculation of cross peak volumes inaccurate, and sometimes impossible. The NOHOSS procedure for discrimination of exchange effects of overlapping exchangeable protons as relayed NOE experiments can be used to circumvent the problem [81]. Still, this method brings forward difficult processing *i.e.* elaborate correction and normalization of the signals. Another important issue to be taken into consideration is that exchange rates of exchangeable nuclei should only be compared within one sample since the variations in contaminant such as trace amount of metal ions and molecular oxygen could occur and in turn would change the NOE intensities.

Coupling Constants

As mentioned earlier, vicinal coupling constants ($^3J_{OH,CH}$) can be used in order to get conformational information. They are indicative to the relative positions (dihedral angle) of nuclei through three-bond coupling. Hydroxy proton vicinal coupling constants are also sensitive to particular conformational preferences and thus to hydrogen bond formation. The correlations between coupling constants and the dihedral angles are expressed by Karplus equations. According to the Karplus equation derived for hydroxy protons [82], vicinal coupling constants of the order of 5.5 ± 0.5 Hz, indicate a free rotation around the C–O bond. Any drastic deviation from this value would suggest a restricted rotation, as it is expected for protons involved in a hydrogen bonding interaction.

The Use of Chemical Shift Differences of Hydroxy Protons

In earlier investigations made on a series of disaccharides [57,58,83], and trisaccharides [55,56], it was shown that chemical shift differences of hydroxy

proton signals could be used for obtaining structural information. The chemical shift differences ($\Delta\delta$ values) were calculated by subtracting the hydroxy proton chemical shift in the corresponding monosaccharides from the chemical shift of the hydroxy proton in the oligosaccharide ($\Delta\delta = \delta_O - \delta_M$, where subscripts O and M stand for oligosaccharide and monosaccharide respectively).

Both negative and positive chemical shift differences ($\Delta\delta$) were measured, the positive differences being smaller in magnitude than the negative differences. When the chemical shift differences ($\Delta\delta$) of hydroxy proton signals were large[*] ($|\Delta\delta| > 0.2$ ppm) and negative *i.e.* an upfield shift of the oligosaccharide signal compared to the chemical shift of the constituent monomeric sugar unit, locations of hydroxy protons were correspondingly recognized to be in the proximity of acetal oxygens or acetamido group. All the hydroxy protons having large negative $\Delta\delta$ values were also found to be in slow chemical exchange with bulk water.

For the hydroxy protons closely interacting with other hydroxy group oxygen atoms, downfield shifts (positive $\Delta\delta$) were observed [55,58]. The presence of hydrogen bonding was not directly correlated to large $\Delta\delta$ values. That is, a hydroxy proton signal might have a large negative or positive chemical shift difference without being involved in a hydrogen bond.

Objectives and Applications

The overall purpose of the present studies is to further explore and exploit the use of hydroxy protons in structural analysis of carbohydrates in aqueous environment. Additionally, it is of substantial interest to understand the origin of the observed chemical shift differences between carbohydrates and their monomeric building blocks. The knowledge about the hydroxy proton behaviors would in turn help us improve and expand their use.

In the earlier studies, the di- and trisaccharides studied by using exchangeable protons were conformationally rather flexible molecules [55-58]. For this reason, it was decided to study the behaviors of hydroxy protons in molecules with less conformational freedom (Articles I and II). Consequently a derivative of the Lewis b (Le[b]), α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-1-O(CH$_2$)$_2$NHCOCHCH$_2$, Lewis X (Le[x]), β-D-Gal*p*-(1→4)[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc, and Lewis Y (Le[y]), α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc, oligosaccharides were selected due to their relatively rigid structures as well as to their biological significance [84-92].

Cyclodextrins (CDs) are very versatile compounds. They have many usage areas due to their unusual physical property of possessing hydrophilic surfaces with hydrophobic inner cavities that lead to inclusion complexes (see the section "Cyclodextrins"). Especially over the last few decades, solution structures of CDs,

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[*] Throughout the text, when a negative $\Delta\delta$ is defined as large, it is not meant to be in accordance with mathematical coherence, but with absolute values.

as well as their complexes, have been studied extensively [93]. The hydrogen bonds between O(2)H and O(3)H groups on adjacent glucose units in CDs had been reported only in solid state and DMSO solutions [93-97]. However, weak hydrogen bonding interactions, which are more likely to occur in aqueous solutions, are difficult to detect by the conventional NMR methods. Complexation induced chemical shifts of non-exchangeable protons are also weak, when the guest molecules constitute only aliphatic properties. It was therefore important to make use of the hydroxy protons in structural studies of α-, β-, and γ-cyclodextrins and their complexes (Article III).

Studies of nucleos(t)ide-carbohydrate interactions can provide structural and binding information that can lead to understand the nucleic acid complexation with carbohydrates *e.g.* DNA interactions with sugar-based antibiotics. In these biologically important processes, hydrogen bonding is considered to have an essential role. In the study of nucleos(t)ide-saccharide interactions (Article IV), using hydroxy proton signals, it was focused on the possibility to get structural and binding properties of these complexes in aqueous solution by NMR spectroscopy. A similar approach using IR spectroscopy had been reported [98].

Finally, it was aimed to find a general rationale for the chemical shift differences and their dependence on structural changes in the theoretical study of the chemical shift behaviors of hydroxy protons (Article V). The nature of the interactions between water and hydroxy groups in the solute molecules had been put forward to be the reason for the observed shift differences. The previous chemical shift observations for hydroxy protons were also contemplated by making use of the chemical shift changes of the methanol hydroxy proton in a series of binary and ternary mixtures with water and ethers.

Structural Analyses of Lewis b, X, and Y Oligosaccharides

Lewis Oligosaccharides

As a subgroup of carbohydrate dependent histo-blood group antigens, the Lewis oligosaccharides (Lewis a, b, X, and Y) are expressed on human erythrocytes. These determinants are found covalently bonded to proteins and/or lipids [99]. Although their discovery dates back to the 1950's, their functions remain unclear even today. Lewis glycoconjugates also attracted special attention as antibody- and lectin-binding [100] and tumor-associated antigens [101].

Conformational Studies of Lewis b; Evidence for Upfield Shifts due to Decreased Hydration

There have been many studies concerning the structure and function of the Lewis b tetrasaccharide (Le^b) [84-92]. The structure of the Le^b derivative, α-L-Fuc$p$-(1→2)-β-D-Gal$p$-(1→3)[α-L-Fuc$p$-(1→4)]-β-D-Glc$p$NAc-1-O(CH$_2$)$_2$NHCOCHCH$_2$, **1**, (Chart 1) can be considered as relatively rigid. Its rigidity mainly depends

on steric factors, hydrophobic interactions and exo-anomeric effect. The orientations of the two fucosyl residues are stabilized by hydrophobic interactions to the N-acetylglucosamine and the galactose residue, respectively.
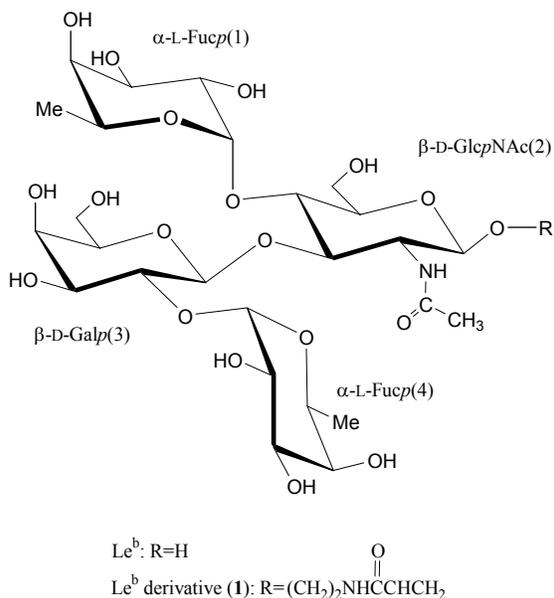

Chart 1. The structure of the Lewis b and the derivative, **1**, showing the numbers designating each residue.

The conditions to observe exchangeable protons by NMR spectroscopy were mentioned in the section for sample preparation. Subsequently, ^{1}H NMR chemical shifts, vicinal coupling constants, temperature coefficients, and exchange rates of the hydroxy protons have been measured. Spectral overlap of hydroxy proton resonances, and overlap with the water signal in the case of O(4)H of Galp(3) precluded the extraction of data for some of the hydroxy protons. The vicinal coupling constants, $^3J_{HO,CH}$, were in the range of 4.8 to 6.8 Hz indicating no preference for a particular conformation. The temperature coefficients values between -6.9 and -12.3 ppb/°C were much larger[*] than the values expected for a hydroxy proton involved in strong hydrogen bonding. Similarly, the exchange rates, which could be measured, indicated that none of the hydroxy protons is protected from exchange with the solvent. These results indicate that no intra-molecular hydrogen bond, which would be taking part in the stabilization of the molecule, exists.

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[*] Negative temperature coefficients throughout the text are referred as large or small with respect to the numerical magnitude of the value, since the negative sign in fact designates the direction of chemical shift change.

Both ROESY and NOESY spectra with various mixing times were recorded to distinguish between the cross-peaks due to dipolar relaxation and chemical exchange. 22 intra- and 5 inter-residue NOEs involving hydroxy protons could be detected. The inter-residue NOEs, O(3)H Gal$p$(3) - C(1)H Fuc$p$(4), O(4)H Gal$p$(3) - C(5)H Fuc$p$(1), O(4)H Gal$p$(3) - C(3)H Fuc$p$(1), O(4)H Gal$p$(3) - C(1)H Glc$p$NAc(2), and O(6)H Gal$p$(3) - C(5)H Glc$p$NAc(2), are specifically important since they are sensitive to conformational changes through glycosidic linkages. Additionally, 3 inter- and 5 intra-residue NOEs could be detected for the amide proton on Glc$p$NAc(2). The extra NOEs obtained from the exchangeable proton resonances confirmed the stacking interactions between Fuc$p$(1) and Gal$p$(3), and between Glc$p$NAc(2) and Fuc$p$(4), and the rigidity of the structure determined by the glycosidic torsions [84-92].

Some hydroxy protons, O(3)H, O(4)H, O(6)H of Gal$p$(3), and O(2)H of Fuc$p$(4), were found to have large negative $\Delta\delta$ values ($|\Delta\delta| \geq 0.2$ ppm) *i.e.* upfield shifted with respect to the chemical shifts in the corresponding methyl glycosides. Since the conformations of individual hydroxy groups become critical when the chemical shift differences are interpreted, a computational analysis using the molecular mechanics program MM3 [102] was performed. In the calculations, previously reported values for the glycosidic torsion angles [91,92] were used. The results of the MM3 calculations showed (Figure 5) that hydroxy protons having large $\Delta\delta$s could not get sufficiently close (< 2.5 Å) to the acetal oxygens with the exception to their own ring oxygen, even when they were deliberately directed toward them. In addition, the positions where O(3)H, and O(4)H of Gal$p$(3) protons could be closest to the acetal oxygens were rather high energy conformations (Figure 5) which made them lean into the sterically crowded region between Fuc$p$(1) and Gal$p$(3). Thus, the large negative $\Delta\delta$ values cannot be explained on the basis of spatial proximity to acetal oxygen atoms. Instead, the shielding was attributed to the orientations of hydroxy protons relative to the hydrophobic/hydrophilic faces of the tetrasaccharide [87]. The hydroxy protons on the outer surface of the tetrasaccharide are most exposed to the bulk water and have small $\Delta\delta$s, indicating similar interaction with the bulk water as in the monosaccharides. The hydroxy groups having large $\Delta\delta$s are found to be within the vicinity of the amphiphilic region composed of primarily the hydrophobic face of Fuc$p$(1) and the hydrophilic face of Gal$p$(3) (Figure 5a). Their hydrogen bonding interactions with bulk water molecules are therefore disturbed [88], causing upfield shifts for the signals when compared to fully hydrated monomer residues.

It is very important to note that O(3)H and O(4)H of Gal$p$(3), having large negative $\Delta\delta$ values, participate in key polar interactions observed in binding studies on Lewis b tetrasaccharide and lectin glycoprotein [87]. This information is significant because it gives direct indication about which hydroxy protons are important for binding processes (see also the section for Lewis Y). It is also put forward that amphiphilic regions create perturbed hydrogen bonding network of water molecules and provide the thermodynamic conditions for molecular recognition in aqueous solutions [88].
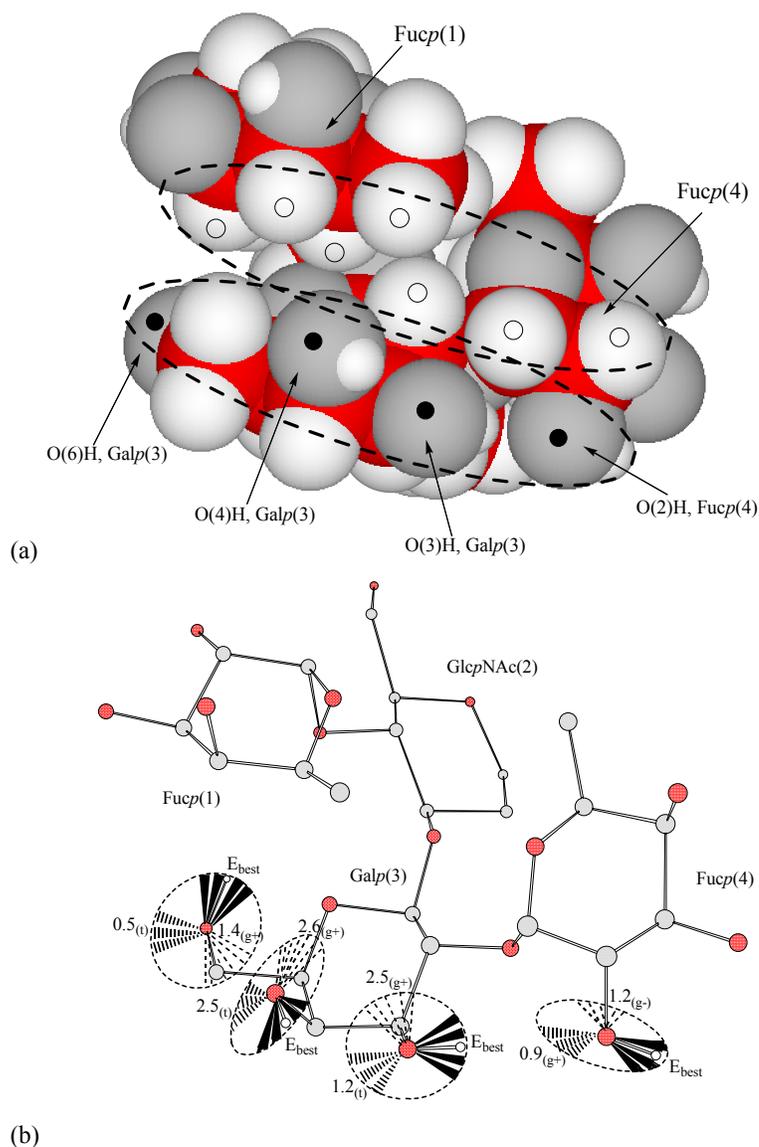
Figure 5. (a) Space filling model of the energetically best structure of the MM3 calculations on **1**. The dashed ellipses show hydrophilic and hydrophobic sides of Fuc*p*(1) and Gal*p*(3), respectively. The hydrogens that are designated with circles and hydroxy groups that are designated with filled circles constitute the two faces creating the amphiphilic region. (b) The elliptic circles designate the rotational orbits of each hydroxy proton with large negative Δδ. The orbits are divided into three major staggered conformational areas as gauche(-), gauche(+), and trans. These conformations are defined with respect to the torsions, C(5)-C(6)-O(6)-O(6)H, C(3)-C(4)-O(4)-O(4)H, and C(2)-C(3)-O(3)-O(3)H on Gal*p*(3) and C(1)-C(2)-O(2)-O(2)H on Fuc*p*(4). The energies of each conformer in kcal mol^{-1} are indicated with stripes of different intensity and represented by only one conformational data point of energy calculations.

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Structural Studies of Lewis X and Y; Further Evidence

Water solutions of Lewis X (Le^x), β-D-Gal$p$-(1→4)[α-L-Fuc$p$-(1→3)]-β-D-Glc$p$NAc, and Lewis Y (Le^y), α-L-Fuc$p$-(1→2)-β-D-Gal$p$-(1→4)[α-L-Fuc$p$-(1→3)]-β-D-Glc$p$NAc, oligosaccharides (Chart 2) have been studied by making use of their hydroxy protons.
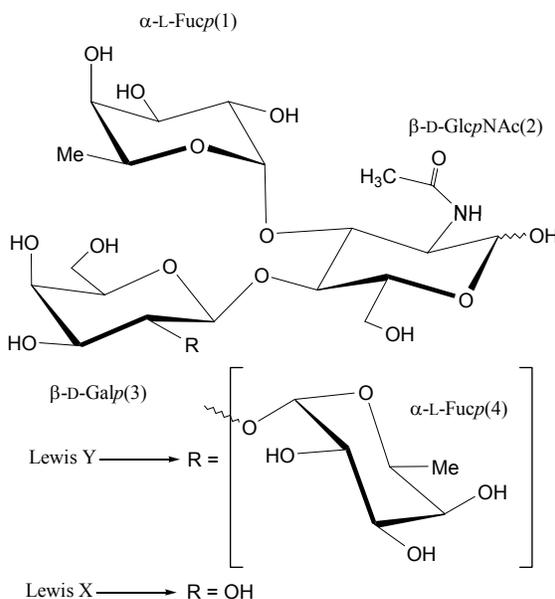
Chart 2. The structures of Lewis X and Y oligosaccharides, showing also the numbering of the residues.

Le^x and Le^y have similar core structures to Le^b tetrasaccharide that was shown to have a series of hydroxy protons with large negative Δδ values. The observed upfield shifts of hydroxy proton signals in Le^b were not attributed to being in the proximity of acetal oxygens. Accordingly, the intention was also to see whether their structural similarity would lead to same type of observation in Le^x and Le^y.

In Table 1, the chemical shifts and Δδ values of the hydroxy protons in Le^x and Le^y are shown. The chemical shifts of O(3)H, O(4)H of Gal$p$(3) and O(2)H of Fuc$p$(4) in Le^y were found to be upfield shifted by 0.184, 0.649, and 0.111 ppm respectively. Concerning the four hydroxy protons (O(3)H, O(4)H, O(6)H of Gal$p$(3), and O(2)H of Fuc$p$(4)) with large negative Δδ values in Le^b, having only three (O(4)H, O(3)H of Gal$p$(3), and O(2)H of Fuc$p$(4)) in Le^y indicates that the stacking interaction between Fuc$p$(1) and Gal$p$(3) might be distorted. The fourth hydroxy proton O(6)H of Gal$p$(3) with large negative Δδ value in Le^b was found to exhibit a slightly positive Δδ in Le^y. However, only O(4)H of Gal$p$(3) in Le^x has a large Δδ of -0.520 ppm.

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Keeping in mind that O(3)H and O(4)H of Galp(3) in Le[b] were also important in its binding to lectin [87], the same type of behavior should not be unexpected from Le[y], because they were also found in the epitope of Le[y] binding to a monoclonal antibody [103].

Table 1. ^{1}H NMR Chemical Shifts (δ) and Chemical Shift Differences ($\Delta\delta$) for the Hydroxy Protons of Le[x] and Le[y] Oligosaccharides

		Lewis Y		Lewis X	
		δ (ppm)	$\Delta\delta$ (ppm)	δ (ppm)	$\Delta\delta$ (ppm)
α-L-Fucp(1)	O(2)H	6.145	0.009	6.018	-0.118
	O(3)H	6.051	0.121	6.107	0.177
	O(4)H	5.905	-0.073	6.020	0.042
D-GlcpNAc(2)	O(6)H(α)	6.016	-0.027	6.068	0.025
	O(6)H(β)	6.140	-0.056	5.999	-0.085
	O(1)H(α)	7.208	-0.107	7.325	0.010
	O(1)H(β)	7.932	-0.043	7.977	0.002
β-D-Galp(3)	O(2)H			6.551	-0.020
	O(3)H	5.938	-0.184	6.118	-0.004
	O(4)H	5.253	-0.649	5.382	-0.520
	O(6)H	6.143	0.021	6.196	0.074
α-L-Fucp(4)	O(2)H	6.025	-0.111		
	O(3)H	6.022	0.092		
	O(4)H	5.901	-0.077		

All measurements were performed on Le[x] and Le[y] oligosaccharides in 85% H_2O/15% $(CD_3)_2CO$ at -10 °C.

It should also be mentioned that O(2)H and O(3)H of Fucp(1) in Le[x] also have large $\Delta\delta$s of -0.118 and 0.177 ppm, respectively. The large negative $\Delta\delta$ value of O(2)H of Fucp(1) was attributed to the interaction with the carbonyl oxygen on GlcNAc(2). Examinations of 3D models built by using glycosidic angles reported in the literature [91] showed that close interactions (*ca.* 2 Å) are possible between O(2)H of Fucp(1) and carbonyl oxygen on GlcNAc(2). The positive large $\Delta\delta$ of O(3)H of Fucp(1) was explained on the basis of the interaction induced by O(2)H of Fucp(1) (see Article V for OH···OH interactions).

The NOEs for the non-exchangeable protons in Le[x] were found to be identical with the published ones, reporting only one inter-residue NOE between C(5)H Fucp(1) and C(2)H Galp(3) [92]. ROESY experiments were also performed to discriminate between the cross-peaks due to dipolar relaxation and those due to chemical exchange. 6 inter- and 24 intra-residue NOEs were detected for the exchangeable protons of Le[x] trisaccharide. Three NOEs, O(4)H(3) - C(4)H(1), O(4)H(3) - Me(1), and O(6)H(3) - C(3)H(1), represent long-range ones between residues which are not directly connected. These NOEs confirm the stacking interaction between Fucp(1) and Galp(3) and also the rigidity of Le[x]. For Le[y], out of 11 inter-residue NOEs, four (O(4)H(3) - C(3)H(1), O(4)H(3) - C(4)H(1) , O(4)H(3) - C(5)H(1),

O(4)H(3) - Me(1)) were long-range NOEs confirming also the stacking interaction between Fuc*p*(1) and Gal*p*(3).

Structural Analysis of Cyclodextrins

Cyclodextrins

As it is ascribed from the name, cyclodextrins (CDs) are cyclic oligosaccharides. The most common ones are α-CD, β-CD, and γ-CD consisting of 6, 7, and 8 α-1,4 linked D-glucopyranose residues, respectively (Chart 3).


2: α-cyclodextrin (6 glucoses)

3: β-cyclodextrin (7 glucoses)

4: γ -cyclodextrin (8 glucoses)
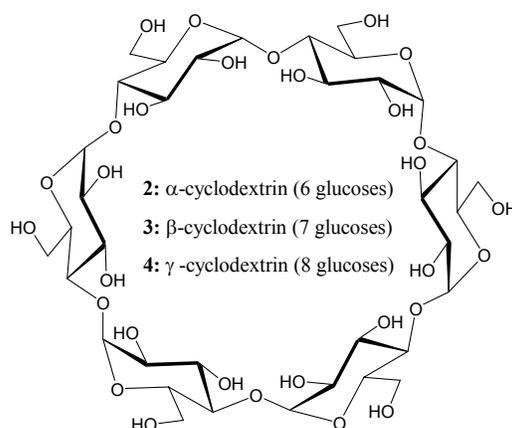
Chart 3. The structures of α-, β-, and γ-cyclodextrins.

These compounds have distinctive torus-like structures with an inner hydrophobic cavity formed by two rings of H3 and H5 protons and a ring of glycosidic oxygens (Figure 6). The bigger rim is composed of the secondary hydroxy groups. The overall rigidity of the structures is mainly determined by hydrogen bonding interactions between O(3)H and O(2)H hydroxy groups on adjacent glucose residues. This hydrogen bond belt is broken in the case of α-CD due to the distorted position of one of the glucose residues, whereas the complete hydrogen bond formation making the molecule rather rigid decreases the solubility of β-CD in aqueous solutions [93]. On the other hand, the primary O(6)Hs placed at the smaller rim of the torus are not participating in intramolecular hydrogen bonds and can therefore rotate to partially block the cavity.

The ^{1}H NMR chemical shifts, coupling constants, temperature coefficients and exchange rates have been measured for the hydroxy protons of aqueous solutions of α-, β-, and γ-CDs, **2 - 4**, (Table 2).
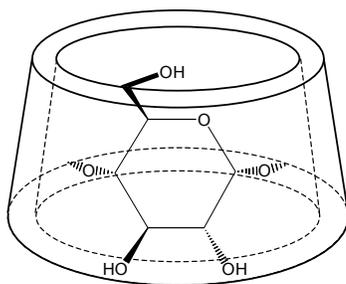
Figure 6. A graphical representation of the torus-like structure of cyclodextrins. Only one glucose unit is shown for clarity. The smaller rim of the torus is composed of primary hydroxy groups whereas the big opening of the torus is arranged by secondary hydroxy groups forming hydrogen bond belt.

The chemical shifts of O(3)H signals in all three compounds were found to be downfield shifted relative to the monomeric methyl glycoside by 0.17 ppm in **2**, and ~ 0.32 ppm in **3** and **4**. The signal of O(2)H in α-CD, **2**, was on the contrary upfield shifted by 0.14 ppm. The other hydroxy protons in **2** - **4** have $|\Delta\delta| < 0.10$ ppm.

Table 2. ^{1}H NMR Chemical Shifts (δ), Chemical Shift Differences ($\Delta\delta$), $^3J_{OH,CH}$ Coupling Constants (J), Temperature Coefficients (dδ/dT) and Exchange Rates (k_{ex}) for the Hydroxy Protons of α-, β- and γ-CD

		δ (ppm)	$\Delta\delta$ (ppm)	$^3J_{HC\text{-}OH}$ (Hz)	dδ/dT (ppb/°C)	k_{ex} (s^{-1})
	O(2)H	6.224	-0.145	6.6	-7.9	7.8
α-CD (**2**)	O(3)H	6.572	0.172	< 3 ^{b}	-8.2	8.0
	O(6)H	6.094	0.088	5.6	-12.3	35.7
	O(2)H	6.401	0.032	6.7	-7.5	2.7
β-CD (**3**)	O(3)H	6.717	0.317	< 3 ^{b}	-8.7	3.0
	O(6)H	6.066	0.060	5.4	-13.3	18.5
	O(2)H	6.435	0.066	7.2	-7.5	6.4
γ-CD (**4**)	O(3)H	6.715	0.315	< 3 ^{b}	-8.3	6.2
	O(6)H	6.020	0.014	5.2	-12.9	47.9

All measurements were performed on 25 mM, 85% H_2O/15% $(CD_3)_2CO$ sample solutions at -10 °C. ^{a} $\Delta\delta$ values are calculated as the difference between hydroxy proton chemical shift in the CD and the corresponding methyl glycoside. A positive $\Delta\delta$ indicates a downfield shift in the CD. ^{b} No splitting is observed despite a very strong Gaussian window function applied on FIDs, and the measured widths at the crowns of the peaks suggest that the couplings are smaller than 3 Hz.

The O(6)Hs have larger temperature coefficients (between -12.3 and -13.7 ppb/°C) than the O(2)H and O(3)Hs (*ca.* -8 ppb/°C). Namely, all values are larger than -7 ppb/°C, indicating no strong hydrogen bonding interactions, which would require temperature coefficients larger than 3 ppb/°C.

The O(3)Hs in **2** - **4** have small (< 3 Hz) $^3J_{OH,CH}$-values. This indicates restricted rotation around the C(3)-O(3) bonds. *J*-values for O(2)H lie between 6.6 and 7.2 Hz that would normally be considered as values suggesting free rotation around the C-O axes. However, these coupling constants are also suitable values for O(2)Hs interacting with O(3)Hs on the adjacent glucopyranosyl units either as hydrogen bond donor or acceptor. In both cases, the dihedral angle, HC(3)-O(3)H, takes up a *gauche* conformation due to the directionality of the hydrogen bond, whereas HC(2)-O(2)H dihedral is more likely to have *gauche* (+ or -) or *trans* conformations (*i.e.* bigger coupling constants) in the case of being acceptor and donor, respectively.

There is a clear difference between primary and secondary hydroxy proton exchange rates. As seen in Table 2, secondary hydroxy protons have significantly slower exchange with water, indicating relatively restricted solvent accessibility. The exchange rates of the hydroxy protons in β-CD, **3**, were much lower than the ones for α-, **2**, and γ-CDs, **4**. However, the relative ratios between secondary and primary hydroxy proton exchange rates are comparable. This observation is not so exciting since it is known that exchange rates are very sensitive to pH, temperature, and catalysis by small traces of impurities. Therefore, individual samples might serve different conditions for the exchange with water, and it should be considered as an indication that the conditions of measurement are not similar.

Finally, in order to discriminate between cross-peaks due to dipolar relaxation and cross-peaks due to chemical exchange, both NOESY and ROESY spectra were recorded. In all the three compounds, a chemical exchange cross-peak is found between the O(2)H and O(3)H signals.

The above data suggest that in aqueous solution, a hydrogen bond exists between O(2)H and O(3)H on adjacent glucose units, as previously reported for α-, β-, and γ-CDs in the solid state and in DMSO solutions [93-97].

Maltose and Maltoheptaose

In maltose, **5**, (Chart 4) two different NMR signals for O(2')H with two *J*-values in DQF-COSY spectrum were observed because of the anomeric configuration (α, β) effect on chemical shifts conveyed by an exchange interaction between O(2')H-O(3)H. In other words, two signals, O(2')H(α) and O(2')H(β), were assigned according to the anomeric configuration (α, β) of the reducing end of maltose, **5**. The information about the exchange interaction was obtained from NOESY and ROESY spectra that were recorded for maltose, **5**, and maltoheptaose, **6,** (Chart 4). A cross-peak due to chemical exchange was present between the O(3)H and the O(2')H signals in β-maltose, indicating that there is a weak and transient (probably water mediated) O(2')H-O(3)H hydrogen bond. The corresponding ROE cross peak was not detected for the α-anomer.

The hydroxy protons in maltose, **5**, and maltoheptaose, **6**, (Chart 4) have small (|Δδ| < 0.10 ppm) Δδ values (Table 3). Their temperature coefficients larger than -8 ppb/°C do not indicate strong hydrogen bonding interaction. It should be mentioned that O(3)H and O(2')H in α-maltose, have somewhat smaller

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temperature coefficients (-10.9 and -10.0 ppb/°C, respectively) than in β-maltose (-12.0 and -11.6 ppb/°C, respectively).


5: maltose
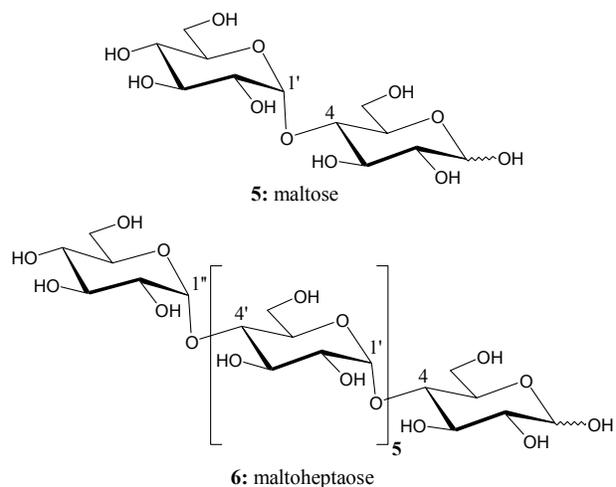
6: maltoheptaose

Chart 4. The structures of maltose and maltoheptaose.

The hydroxy protons of **5** and **6** have $^3J_{OH,CH}$ coupling constants representing conformational averaging (Table 3). The $^3J_{OH,CH}$-values for O(2')H are 8.0 and 6.6 Hz in α- and β-maltose respectively. The coupling constant for O(2)H in methyl α-D-glucopyranoside was 6.0 Hz. Therefore, it is likely that there is conformational change around the C(2')-O(2') bond due to 8.0 Hz coupling.

Spectral overlap impeded the calculations of exchange rates for the majority of the hydroxy protons in **5** and **6**. The exchange rates that could be acquired were similar.

Table 3. ^{1}H NMR Chemical Shifts (δ), Chemical Shift Differences (Δδ), $^3J_{OH,CH}$ Coupling Constants (*J*), Temperature Coefficients (dδ/d*T*) and Exchange Rates (k_{ex}) for the Hydroxy Protons of Maltose and Maltoheptaose

		δ (ppm)	Δδ (ppm)	$^3J_{HC-OH}$ (Hz)	dδ/d*T* (ppb/°C)	k_{ex} (s^{-1})
maltose (**5**)	O(1)H(α)	7.333	0.107	3.2	-10.9	80.7
	O(2)H(α)	6.276	0.094	5.9	-13.6	89.9
	O(3)H(α)	6.380	0.012	5.7	-10.9	*b*
	O(6)H(α)	5.869	-0.071	10.0 *c*	-12.7	*b*
	O(1)H(β)	8.034	0.057	6.2	-11.3	63.4
	O(2)H(β)	6.662	0.097	4.7	-13.9	84.8
	O(3)H(β)	6.485	-0.020	5.1	-12.0	*b*
	O(6)H(β)	5.938	-0.076	9.0 *c*	-12.9	91.8
	O(2')H(α)	6.365	-0.004	8.0	-10.0	*b*

maltose (**5**) continued	O(2')H(β)	6.412	0.043	6.6	-11.6	^{b}
	O(3')H	6.456	0.056	4.4	-13.6	^{b}
	O(4')H	6.490	0.076	5.9	-12.9	^{b}
	O(6')H	6.035	0.029	10.2 ^{c}	-13.7	90.3
maltoheptaose (**6**)	O(1)H(α)	7.299	0.073	3.6	-8.7	142.2
	O(2)H(α)	6.225	0.043	5.5	-11.2	166.6
	O(3)H(α)	6.357	-0.011	6.4 ^{d}	-8.2	^{b}
	O(6)H(α)	5.842	-0.098	9.7 ^{c,d}	-10.8	^{b}
	O(1)H(β)	8.015	0.038	5.9	-9.6	105.1
	O(2)H(β)	6.634	0.069	4.3	-11.6	137.8
	O(3)H(β)	6.471	-0.034	6.4 ^{d}	-9.2	^{b}
	O(6)H(β)	5.909	-0.105	12.4 ^{c,d}	-9.6	^{b}
	O(2')H	6.405	0.036	11.6 ^{d}	-8.1 ^{e}	^{b}
	O(3')H	6.414	0.014	9.2 ^{d}	-8.1 ^{e}	^{b}
	O(6')H	5.935	-0.071	13.4 ^{c,d}	-10.5	^{b}
	O(2")H	6.376	0.007	6.8	-9.3	^{b}
	O(3")H	6.408	0.008	6.5 ^{d}	^{b}	^{b}
	O(4")H	6.475	0.061	7.0 ^{d}	-9.4	^{b}
	O(6")H	6.015	0.009	11.5 ^{c,d}	-11.8	92.0

All measurements were performed on 12.5 mM, 85% H_2O/15% $(CD_3)_2CO$ sample solutions at -10 °C. A single prime designates the non-reducing sugar in **5** and the five glucose units in the middle that have same chemical shifts in **6**. A double prime designates the non-reducing end sugar unit for **6**. ^{b} Severe spectral overlap impeded the extraction of these values. ^{c} These coupling constants are given as the sum of two couplings, $^3J_{O(6)H\text{-}H6a}$ and $^3J_{O(6)H\text{-}H6b}$. ^{d} Values from DQF-COSY spectrum (An overestimation of 2 Hz is obtained. For example for O(2)H(α), 5.5 Hz is read from 1D, 7.5 from 2D DQF-COSY). ^{e} Average values that are measured together for both O(2')H and O(3')H moving together with respect to temperature change.

Structural Comparison of Cyclodextrins with Maltose and Maltoheptaose

Being the building blocks of CDs, the major structural difference between CDs and maltose/maltoheptaose is the flexibility of the linear molecules in solution environment. The $^3J_{OH,CH}$ coupling constant values of < 3 Hz, together with the large Δδ values between 0.17 and 0.32 ppm reflect that the hydrogen bonding interactions between O(2)H and O(3)H on adjacent glucose units in CDs are more pronounced when compared to the ones for maltose, **5**, and maltoheptaose, **6**, showing average $^3J_{OH\text{-}CH}$ and exchange rate values. However, this interaction is, in fact, observed as chemical exchange cross-peak in ROESY spectrum between O(2')H and O(3)H on **5**.

The clear distinction between the temperature coefficients of primary and secondary hydroxy groups in the CDs is not observed for **5** and **6**. Nevertheless, the O(2')H, O(3')H, O(2")H, and O(3")H protons in **6** exhibit comparable temperature coefficient values with the ones for CDs, revealing that maltoheptaose is structurally more similar to CDs than maltose. Except the dδ/dT-value (-11.8 ppb/°C) of O(6")H, the smaller temperature coefficients of O(6)Hs in **5**, relative to

the values of O(6)Hs in **2** - **5**, point out that the secondary hydroxy groups are more subject to interresidual interactions and less exposed to water. Since the dynamic fluctuations of the molecule in solution would be taking place more towards the ends of the molecule, this observation is probably increasing toward the midpoint. This could not be positively confirmed using experimental evidence since only average NMR data could be acquired for the five residues in the middle.

Molecular Interactions

Cyclodextrin Complexes

NMR spectroscopy has numerous advantages over other methods such as UV/visible spectroscopy and calorimetry, since it allows both dynamic (*e.g.* equilibrium constants by NMR chemical shift titrations or relaxation times studies) and structural/conformational analyses (*e.g.* structural data from complexation induced chemical shift (CIS) and NOE/ROE studies) of cyclodextrin complexes.

Table 4. ^{1}H NMR Complexation Induced Chemical Shift Differences (CIS) for the Ring Protons of α-, β-, and γ-Cyclodextrins, **2** - **4**, upon Complexation

		methyl benzoate	adamantane-1-COOH	adamantane-1-OH	L-tryptophane	D-tryptophane
	C(1)H	-0.031	0.003	0.000	-0.004	-0.007
	C(2)H	-0.044	0.004	-0.014	0.000	-0.010
2	C(3)H	-0.182	0.010	0.050	0.000	-0.011
	C(4)H	-0.018	0.003	0.021	-0.012	-0.015
	C(5)H	0.037	0.002	-0.016	0.007	0.004
	C(6)H ^{a}	-0.005	0.003	0.005	0.000	-0.011
	C(1)H	-0.012	0.000	0.004	0.008	0.007
	C(2)H	0.005	0.000	0.005	0.022	0.020
3	C(3)H	-0.039	0.007	0.005	0.032	0.030
	C(4)H	-0.007	0.000	0.007	0.007	0.004
	C(5)H	-0.124	0.012	0.015	-0.032	-0.029
	C(6)H ^{a}	-0.008	0.000	-0.006	-0.018	-0.017
	C(1)H	-0.030	0.000	-0.006	0.000	0.000
	C(2)H	-0.013	0.008	0.000	-0.006	-0.005
4	C(3)H	-0.080	-0.003	-0.025	0.008	0.002
	C(4)H	-0.023	0.000	-0.005	-0.006	-0.005
	C(5)H	-0.104	-0.011	-0.005	0.010	0.005
	C(6)H ^{a}	-0.022	0.000	0.000	0.000	0.000

^{a} For each cyclodextrin molecule, the chemical shifts for the two C(6)Hs protons were measured from the joint signals of both C(6)H' and C(6)H" protons.

In the NMR studies of cyclodextrin complexes in solution, the interactions and their properties are usually characterized by NMR chemical shift titrations of non-exchangeable nuclei. These titration methodologies are helpful in complexation

studies as well as structural studies of the complexes. Chemical shift changes upon complexation (CIS) are especially informative when the shielding or deshielding exerted by the guest molecule is extensive [93].

Table 4 shows that the remotely located ring protons, H1, H2, H4 and H6, have small CIS when complexed with methyl benzoate, **7**. The H3 proton in α-CD and the H5 proton in β- and γ-CD are shielded up to 0.18 ppm. These observations are in good agreement with previous studies and confirm the formation of inclusion complexes [93]. In comparison with upfield shifts measured for H3 protons in β- and γ-CD, H5 show larger CIS, indicating that guest molecules are more immersed into the cavity. The other guest molecules adamantane-1-carboxylic acid, **8**, adamantane-1-ol, **9**, L-, **10**, and D-tryptophane, **11**, do not cause large CIS for conclusive interpretation (Table 4). The structures of the guest molecules, **7** - **11**, can be seen in Chart 5.


7: methyl benzoate   **8:** adamantane-1-carboxylic acid   **9:** adamantane-1-ol

10: L-tryptophane   **11:** D-tryptophane
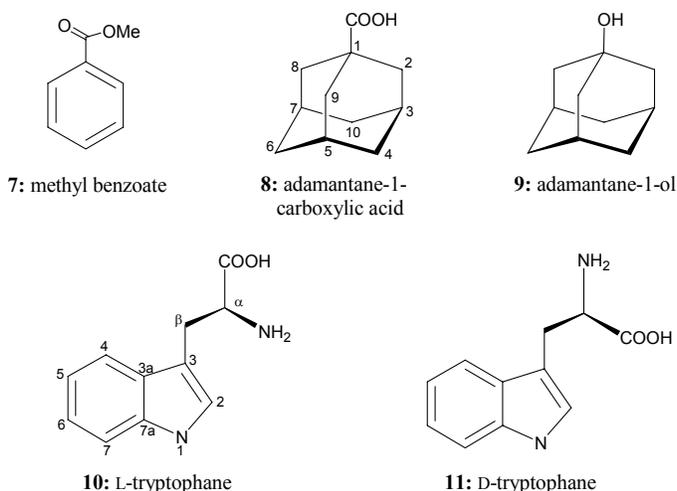
Chart 5. The structures of guest molecules **7** - **11**, showing also the numbering conventions.

The changes in chemical shifts and the temperature coefficients measured for the hydroxy proton signals in α-, β-, and γ-CDs, **2** - **4**, upon addition of one equivalent of **7** - **11** are shown in Table 5. Methyl benzoate, **7**, and adamantane-1-carboxylic acid, **8**, lead to relatively small CIS (between 0.01 and 0.06 ppm) for the hydroxy proton signals in **2** - **4**. These data (CIS, dδ/dTs, and line-shape of the signals) do not indicate intermolecular hydrogen bonding interaction, while a hydrogen bond was found between the carboxylate function of the guest and the O(2)H group of α-CD in a computational study [104] on the complexation of α-CD with adamantane-1-carboxylate. However, it should be noted that the guest molecule in this study [104] was the deprotonated form of **8**. Similarly, as a recent NMR study showed [105], in DMSO p-methyl benzoate (p-CH$_3$-C$_6$H$_4$COO$^-$) interacts with β-CD through hydrogen bonding but the carboxylic acid form (p-CH$_3$-C$_6$H$_4$COOH) does not.

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Table 5. ^{1}H NMR Complexation Induced Chemical Shift Differences (CIS) and Temperature Coefficients ($d\delta/dT$) for the Hydroxy Protons of α-, β-, and γ-Cyclodextrins, **2** - **4**, upon Complexation

		methyl benzoate		adamantane-1-COOH		adamantane-1-ol		L-tryptophane		D-tryptophane	
		CIS	$d\delta/dT$	CIS	$d\delta/dT$	CIS	$d\delta/dT$	CIS	$d\delta/dT$	CIS	$d\delta/dT$
2	O(2)H	-0.063	-6.5	0.010	- 8.5	-0.226[a]	-3.8	0.039	-7.8	0.038	-7.9
	O(3)H	0.019	-8.5	0.039	- 8.7	-0.050	-7.0	0.034	-8.5	0.031	-8.7
	O(6)H	-0.001	-12.0	0.034	- 12.8	-0.002	-12.0	0.049	-12.1	0.048	-12.6
3	O(2)H	-0.023	-6.4	-0.015	- 6.9	0.001	-7.0	0.108	-8.3	0.088	-8.6
	O(3)H	0.006	-8.4	0.069	- 8.6	0.122	-9.5	0.130	-9.6	0.123	-10.1
	O(6)H	0.015	-12.5	0.022	- 12.5	0.024	-12.7	0.035	-12.5	0.032	-12.6
4	O(2)H	0.022	-7.4	0.009	- 7.5	0.005	-7.0	0.015	-7.4	0.013	-7.7
	O(3)H	0.051	-8.5	0.035	- 8.2	0.033	-7.9	0.010	-8.2	0.015	-8.5
	O(6)H	0.006	-12.0	0.016	- 12.6	0.005	-12.2	0.023	-12.3	0.018	-12.6

CIS values (ppm) were calculated as the hydroxy proton chemical shifts in α-, β-, and γ-cyclodextrins, **2** - **4**, subtracted from the chemical shifts of the hydroxy proton signals in the 12.5 mM, 1:1 molar ratio complexes at -10 °C. [a] Values (in ppb/°C) calculated from the fitted line of the temperature dependence of chemical shifts (Chemical shifts of (OH)$_{adamantane-1-ol}$ are 5.955, 5.950 ppm with β- and γ-CD respectively, whereas the calculated δ value is 5.990 ppm with α-CD).

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The complex of adamantane-1-ol, **9**, with α-CD shows unique NMR properties. It is seen in Figure 7 that addition of the guest compound **9** does not alter significantly the chemical shift and the shape of the O(3)H and O(6)H signals in α-CD, **2**. However, the O(2)H signal of **2** was not observed at -10 °C because of extensive line broadening due to complexation (T_2 shortening). Its chemical shift was then calculated to be 5.990 ppm, using a fit on the chemical shift values acquired from the spectra recorded at higher temperatures (-5 to 25 °C). This calculated value is upfield shifted by 0.226 ppm in comparison with the shift for the O(2)H in uncomplexed α-CD.
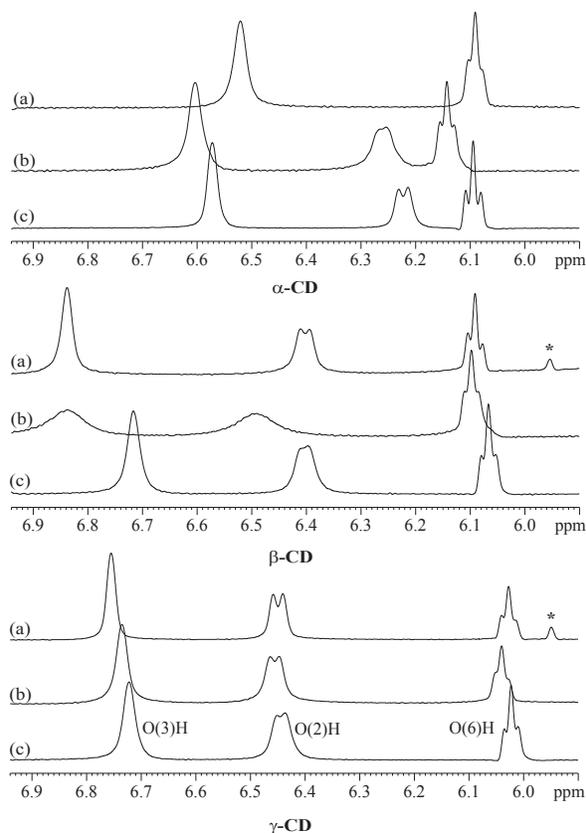

Figure 7. ^{1}H NMR spectral changes observed at -10°C for the hydroxy protons of α-, β-, and γ-CDs upon addition of one equivalent of (a) adamantane-1-ol and (b) D-tryptophane. (c) ^{1}H NMR reference spectra of CDs alone. The O(2)H signal of α-CD with adamantane-1-ol is too broad to be visible. An asterisk designates the adamantane-1-ol OH signals reading 5.955, 5.950 ppm for β- and γ-CD respectively.

At temperatures above 0 °C, the exchange of hydroxy protons with water are generally too rapid to obtain sharp signals, as mentioned earlier. However, this statement did not hold for the complex of α-CD/adamantane-1-ol. When the temperature is decreased from 25 to -10 °C, the O(2)H signal becomes gradually

broader and finally vanishes (Figure 8). Therefore it can be concluded that the sharpening of the signal is surpassed by the broadening effect of the complexation process.

The low temperature coefficient of -3.8 ppb/°C for O(2)H in α-CD/adamantane-1-ol complex implies a reduced water accessibility to the region. This is also supported by the calculated chemical shift for O(2)H, which was found to be upfield shifted. The effect of limited hydration on hydroxy proton chemical shifts was also encountered in previous studies [55,58,83](Articles I and II). Most likely sharing the same broadening effect due to complexation as considered for O(2)H, the hydroxy proton signal of adamantane-1-ol is not detected in the NMR spectrum recorded in the temperature interval from 25 to -10 °C. Furthermore, the signals from non-exchangeable protons in adamantane-1-ol, together with H3 of α-CD, are also strongly broadened upon complexation.

In the case of adamantane-1-ol/β-CD complex, the O(3)H signal was downfield shifted by 0.122 ppm. The chemical shifts of O(2)H and O(6)H were almost unchanged with respect to their shifts in only β-CD sample. The hydroxy protons of the complex had slightly smaller temperature coefficients than in β-CD alone, while their signal shapes were comparable.
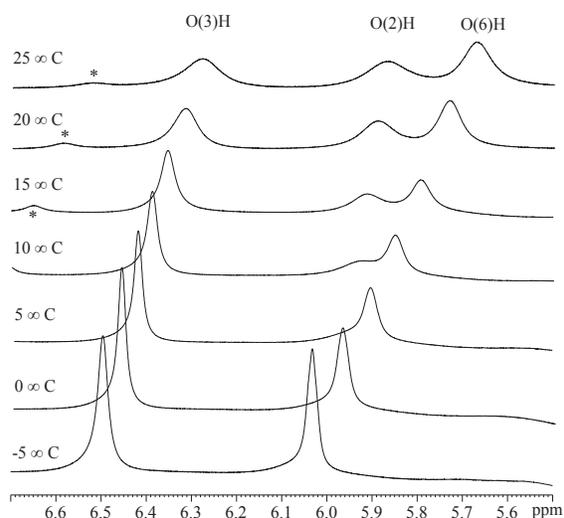

Figure 8. ^{1}H NMR spectra showing the temperature dependence from 25 to -5 °C of the hydroxy protons in the α-CD/adamantane-1-ol (1:1) complex. Hydroxy proton signals of the hydrate form of acetone are designated by an asterisk.

The effect of D-tryptophane addition on the exchangeable proton signals of α-, β- and γ-CDs are also shown in Figure 7. In terms of CIS and broadening, it was not observed significant spectral changes for α- and γ-CD complexes. The minor CIS-values observed for α-CD (~0.04 ppm) are larger than those for γ-CD (~ 0.02 ppm). This could be attributed to the size difference between the two, resulting in

closer interaction for the smaller cavity of α-CD. However, these small CIS-values observed for α-CD hydroxy protons when complexed with L- and D-tryptophane do not indicate any hydrogen bonding interactions as reflected also in a calorimetry and NMR study of α-CD/L-tryptophane complex [106].

The signals of the O(2)H and O(3)H protons in β-CD are broader upon complexation with D-tryptophane or L-tryptophane. Their chemical shifts are also downfield shifted by up to *ca.* 0.13 ppm. The O(6)H protons sitting on the remotely located smaller rim of β-CD seem to be unaffected by the complexation with the amino acids. Since cooperative hydrogen bonding causes considerable stabilization in complexes [107], such hydrogen bonds can be considered for β-CD/tryptophane complexes. Two-centered hydrogen bonds can take place between the O(2)H and O(3)H of β-CD and the NH_3^+ and COO^- groups of tryptophane (Figure 9), explaining the similar chemical shift, broadening, and temperature coefficient behaviors of both O(2)H and O(3)H protons. There are in fact small differences, which cannot be used conclusively, between L- and D-tryptophane complexes with β-CD (Table 5). The somewhat larger CIS-values and smaller temperature coefficients in L-tryptophane relative to D-tryptophane cannot be, at present, recognized as indications to chirality recognition or relative strength of hydrogen bonding. Additionally, intermolecular NOEs were observed between H3 of β-CD and the aromatic, and α-, β-protons of both L- and D-tryptophane.
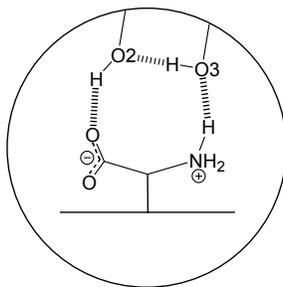

Figure 9. Schematic representation of a two-centered hydrogen bonds between the O(2)H and O(3) of β-CD and the NH_3^+ and COO^- groups of tryptophane.

When simple 3D models of the γ-CD molecule and its complexes with the guest molecules, **7 - 11**, were analyzed, it was seen that the γ-CD molecule is large enough not to allow any short distance contacts with its hydroxy protons. As a consequence of this structural property, it was not observed any chemical shift, temperature coefficient, and line-shape distortions for the γ-CD complexes (Table 5, Figure 7).

Nucleoside Interactions

Interactions between sugar molecules and nucleotides have been observed in high sugar concentrations, by IR spectroscopy studies [98,108]. The interaction takes place through hydrogen bonding between hydroxy protons of the sugar molecules

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and nitrogen atoms in the nucleotides. This posed a question whether it was possible to detect these complex formations by NMR spectroscopy, making use of hydroxy protons. In our study, methyl β-D-glucopyranoside, methyl β-D-galactopyranoside, D-glucose, and sucrose were used to represent carbohydrates. Adenosine and guanosine as nucleosides, adenine and guanine as nucleobases were employed to investigate their effect on the NMR spectra of the carbohydrates.
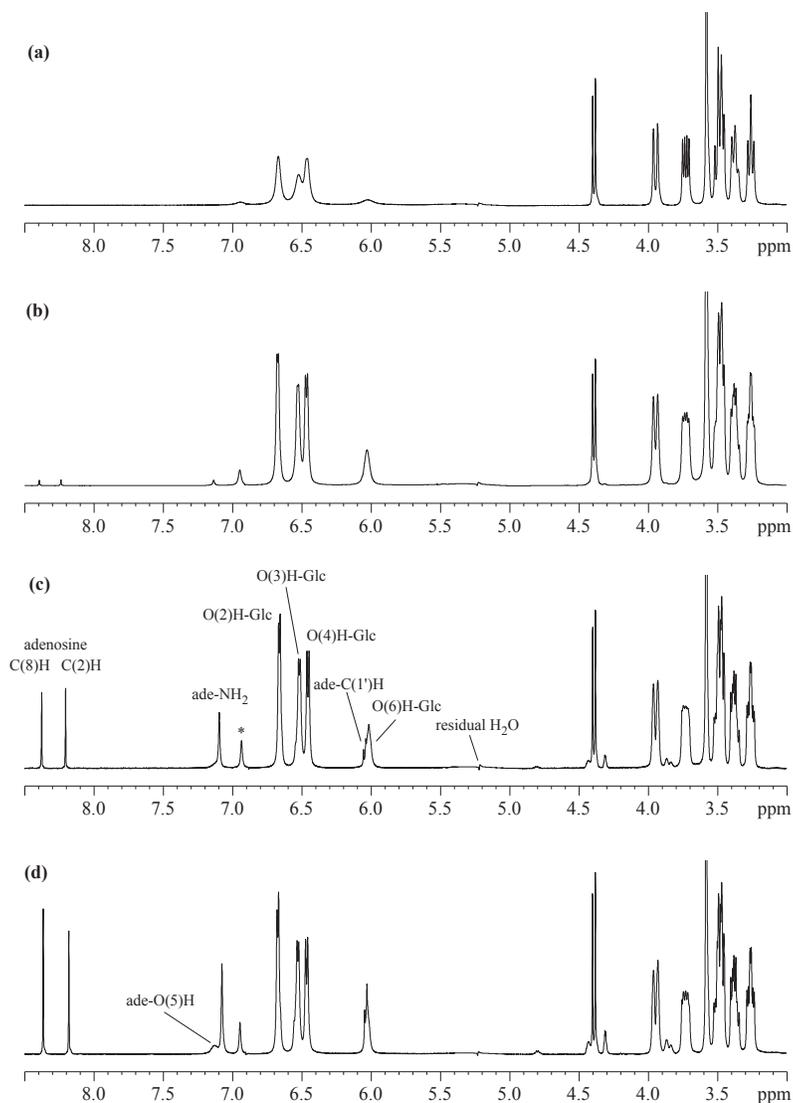

Figure 10. ^{1}H NMR spectra of (a) methyl β-D-glucopyranoside (50 mM in 15% $(CD_3)_2CO$/85% H_2O, -5 °C), (b) - (d) after addition of 1, 5, and 10 mM adenosine respectively. An asterisk indicates the OH signal of the hydrate form of acetone. Addition of adenosine leads to sharpening of this signal as well.

^{1}H NMR spectrum of a 50 mM solution of methyl β-D-glucopyranoside at -5 °C and pH 6.9 shows that the hydroxy proton signals appearing in the region δ 5.5 - 7.5 ppm are broad and not resolved (Figure 10a). When adenosine is added to the sample (1 mM equivalent concentration) the hydroxy proton signals became more intense and sharper, making it possible to observe the splitting due to three-bond couplings ($^{3}J_{CH,OH}$) to the ring protons (Figure 10b). No pH change was observed upon addition. When continued to add more adenosine (5 mM in Figure 10c and 10 mM in Figure 10d), the spectral changes observed were only slight intensity enhancements. The NMR spectral observations that were made for the effect of adenosine addition to β-D-glucopyranoside were true for the corresponding cases where the same experiments were repeated with β-D-galactopyranoside, D-glucose and sucrose.

The ^{1}H NMR chemical shifts, vicinal coupling constants ($^{3}J_{HC,OH}$) and temperature coefficients were measured for the hydroxy protons of methyl β-D-galactopyranoside solutions (50 and 100 mM) before and after addition of adenosine. The reason for the observed intensity enhancements of hydroxy proton signals should have an influence on one of these parameters, which are known to be sensitive to hydrogen bonding interactions. Additionally, the chemical shift changes of the C(2)H and C(8)H protons of adenosine was determined. The ^{15}N chemical shifts of adenosine were also acquired from 2D ^{1}H-^{15}N HSQC and HMQC spectra to detect possible interactions that could take place through hydrogen bonds. It is also important to consider the possibility that the nucleoside (nucleobase) might chelate metal ions catalyzing the exchange of hydroxy protons with bulk water.

The 1D proton spectrum of β-galactopyranoside is shown in Figure 11a. It is seen that its hydroxy proton signals are broad, almost invisible in the spectral region from 5.5 to 7.0 ppm. However, the intensities of these signals increase drastically after addition of 1 % (by molarity) adenosine (Figure 11b). The signals are intensified further up to 2 % adenosine and remain constant upon further adenosine addition (Figure 11c-11e). It was observed that in the presence of 6 % adenosine, the integrals of hydroxy proton signals are equal to those of non-exchangeable protons, indicating that the hydroxy proton signals are fully developed. Further addition up to 10 % adenosine in the sample did not affect the spectrum. Throughout the addition procedure, it was also observed that none of the NMR parameters (chemical shifts, coupling constants ($^{3}J_{HO-CH}$), temperature coefficients) for hydroxy protons change. The measurements of the ^{15}N chemical shifts of adenosine alone and together with methyl β-D-galactopyranoside did not show any significant (< 0.5 ppm) shift changes. With increased adenosine concentration the chemical shifts of C(2)H, C(8)H and NH$_2$ protons were upfield shifted. However, the same behavior was observed for adenosine sample alone. C(2)H and NH$_2$ were shielded by 0.04 ppm and C(8)H by 0.02 ppm when the concentration of adenosine was increased from 4 mM to 16 mM. These shieldings might be attributed to the stacking interactions between nucleobases.

The same intensification effect on the hydroxy proton signals was also encountered for the mixtures where guanosine, adenine, and guanine were added to methyl β-D-galactopyranoside. This suggests that sugar molecules are

interacting with nucleoside molecules through the nucleobase parts. It was also shown in the IR study [98] that nucleotides are interacting with hydroxy protons of the sugars via the nitrogen atoms in their nucleobases.
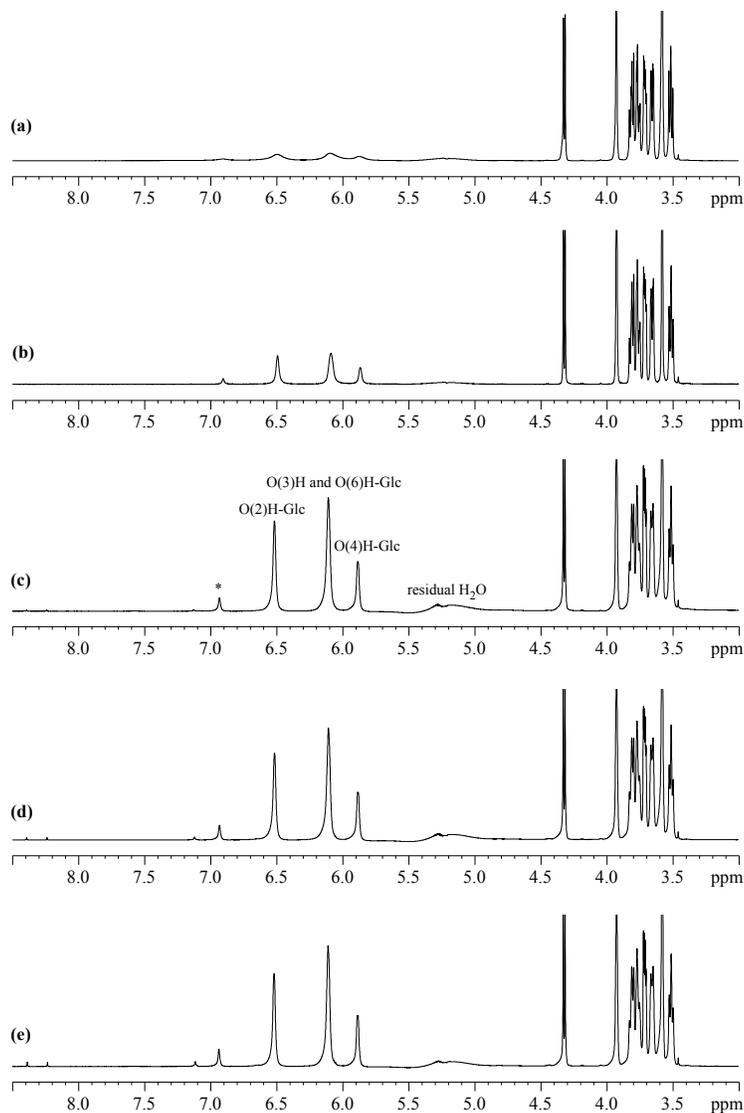

Figure 11. ^{1}H NMR spectra of (a): methyl β-D-galactopyranoside (15% $(CD_3)_2CO$/85% H_2O, -5 °C), (b) - (e) after addition of 1, 2, 3, and 6 % adenosine. An asterisk indicates the OH signal of the hydrate form of acetone.

The pH values of the methyl β-D-galactopyranoside, methyl β-D-glucopyranoside, D-glucose, sucrose and adenosine solutions were in the neutral region (6.8 - 7.2). The pH of sugar solutions did not change more than 0.1 units upon addition of

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adenosine. When the hydroxy proton signals from 1 mM solution of methyl β-D-galactopyranoside in acetate buffer of pH 5.5 were observed, they were found sharper due to slower exchange with water at pH 5.5. Subsequently, adenosine was added to the buffer solution and it was seen that the hydroxy proton signals did not improve. Outside the pH range of 5 - 8, the hydroxy proton signals are not observed due to rapid exchange with water. Experiments performed under acidic and basic conditions revealed that hydroxy proton signals did not improve upon addition of adenosine. Therefore, pH changes are not the reason for the improvement of hydroxy proton signals when adenosine is added.

The effect of metal ions on the hydroxy proton signals were investigated by adding of $MgCl_2$, $CaCl_2$, $CuCl_2$ salts (2 mM) to saccharide samples (25 mM). When the NMR spectra of respective salt saccharide samples were acquired, it was seen that the deliberate addition of metal ions at neutral pH did not affect the quality of the hydroxy proton signals. Broadening of hydroxy proton signals was observed when the salts, $AlCl_3$ or $FeCl_2$, were added presumably due to the capacity of these salts to change the pH. Effect of $MnCl_2$ as a source of paramagnetic ions was also checked on methyl β-D-glucopyranoside samples. The decay in the signal intensities was observed for both exchangeable and non-exchangeable protons. The subsequent addition of adenosine improved neither the exchangeable nor the non-exchangeable proton signals. These observations suggest that the effect of nucleoside addition is not taking place by chelating the metal ions. Finally it can be concluded that the hydrogen bond interactions between sugars and nucleotides [98,108] should be too weak to be detected by NMR spectroscopy accounting for chemical shift, coupling constants and temperature coefficient differences.

Hydroxy Protons Chemical Shifts

Attempt to Calculate Hydroxy Proton Chemical Shifts

Gathering the information from the conformational study of the Lewis b derivative (Article I) and from the previous studies [55-58,83], the hypothesis, which is explaining the reasons for the observation of large Δδs of hydroxy protons as induced from the extent of interactions with bulk water, could be placed. This might also be realized as if, depending on their locations, magnetic shieldings of hydroxy protons are regulated by the nature of bulk water interactions. In order to study this hypothesis, as well as the applicability of chemical shifts calculations on hydroxy protons, density functional theory (DFT) and Hartree-Fock (HF) quantum mechanical calculations were performed on the disaccharides, β-L-Fuc*p*-(1→4)-α-D-Gal*p*-OMe, **12**, β-L-Fuc*p*-(1→4)-α-D-Glc*p*-OMe, **13**, and β-L-Fuc*p*-(1→3)-α-D-Glc*p*-OMe, **14** (Figure 12). The procedure involved first a comprehensive MM3 conformational analysis [109] followed by DFT geometry optimization using the B3LYP functional [110] and standard 6-31G(d) basis set with TIGHT option for more restricted convergence criteria. Finally the gauge-independent atomic orbital (GIAO) NMR calculations [111] with both DFT and HF methods using 6-311++G(2d,2p) basis set were performed on the disaccharides. All calculations were done by using the Gaussian 98 program package [37] with default settings for

the methods and basis sets. It was experimentally determined by NMR spectroscopy that the hydroxy protons, O(3)H of Gal*p*, **12**, O(6)H of Glc*p*, **13**, and O(2)H of Glc*p*, **14**, had large negative chemical shift differences (Δδs) of -0.636, -0.478, -0.315 ppm respectively (Figure 12).
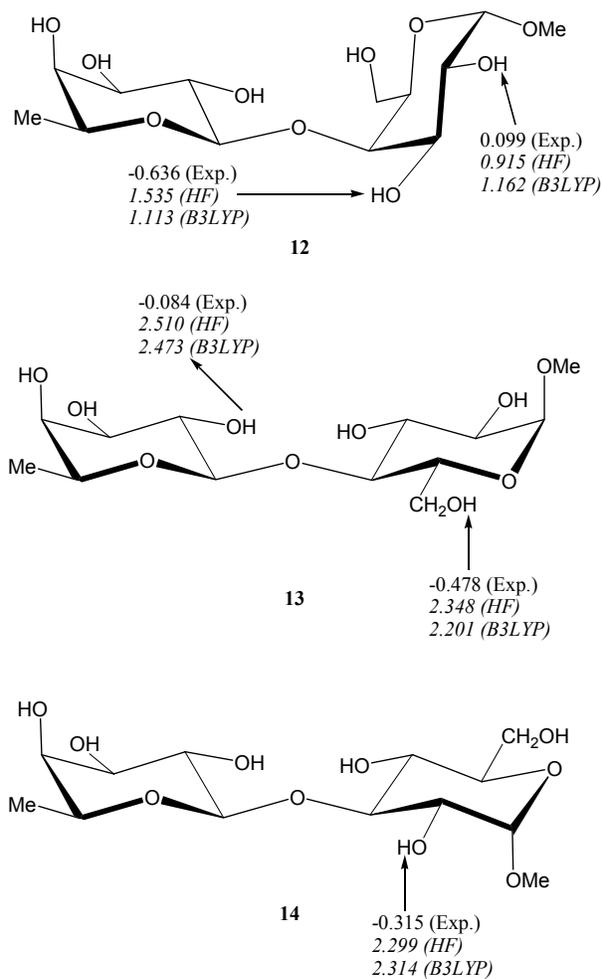

Figure 12. The structures of the disaccharides showing the experimental (plain text) and calculated (italic) Δδ values for the hydroxy protons having either experimentally or computationally large Δδs.

The computational Δδs of hydroxy protons were also calculated by subtracting the calculated chemical shift of a hydroxy proton on corresponding monosaccharide from the calculated chemical shift on the disaccharide. The computational

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procedure for calculating the chemical shifts of hydroxy protons on monosaccharides was the same as the one described for the disaccharides. The results from the calculations have shown that the chemical shifts of the three hydroxy protons, which were found to be upfield shifted experimentally, were downfield shifted.

Apart from O(3)H of **12**, O(6)H of **13**, and O(2)H of **14**, the chemical shifts of O(2)H of **12** and O(2')H of **13** were also calculated to be downfield shifted (Figure 12). The reason for the downfield shift calculated for the O(2)H of **12** was the conformational difference between the monosaccharide (α-D-Gal*p*-OMe) and disaccharide **12**. While the O(2)H was making hydrogen bond with the oxygen atom of OMe group in the monosaccharide, this conformation is reverted in the disaccharide to form a hydrogen bond with O(3) oxygen instead. The O(2')H of **13** was calculated to be downfield shifted because of the hydrogen bonding it forms with O(3)H in the disaccharide. Nevertheless, it is remarkable that the hydroxy protons experimentally found to have large negative $\Delta\delta$ values also got large positive $\Delta\delta$ values computationally. The common property making them to be downfield shifted was that they were involved in hydrogen bonding interactions.

The above discrepancies were attributed to the absence of solvent in the calculations. It was neither explicitly nor implicitly possible to include solvent molecules for the calculations of NMR shieldings.

Methanol Model Systems

Binary Mixtures

A series of mixtures of structurally simple compounds assumed to represent the interactions of hydroxy groups with bulk water, and acetal oxygens was examined. NMR spectra were recorded for methanol/water, methanol/diethylether, methanol/tetrahydrofurane (THF), methanol/dioxane mixtures, since these molecules can be considered as to mimic carbohydrate hydroxy groups interacting with water (methanol/water) or acetal oxygens (methanol/ethers). The proton chemical shifts were measured on these binary mixtures with varying molar ratios using the same conditions, -10 °C and 15% acetone-d_6, as used in previous studies. In Figure 13, it is seen that the hydroxy proton chemical shift of methanol was downfield shifted upon addition of water, whereas it was upfield shifted upon addition of ethers. This observation is in agreement with the hypothesis stating that replacement of strong hydrogen bonds with water by weaker hydrogen bond interactions with ether oxygens results in the upfield shifts experimentally observed for oligosaccharides [55-58,83] (as well as Articles I - III).

In the binary mixtures, the proton chemical shifts, except for the methanol hydroxy proton and water protons, do not change significantly upon changes in molar concentrations. This can be ascribed to the fact that the chemical shift of the methanol hydroxy proton and water protons in methanol/water mixtures change because they are directly involved in hydrogen bonding interactions. That is, the hydrogen bond interactions in the binary mixtures take place mainly between the

methanol hydroxy group and corresponding water or ether oxygens as donor and acceptors respectively.
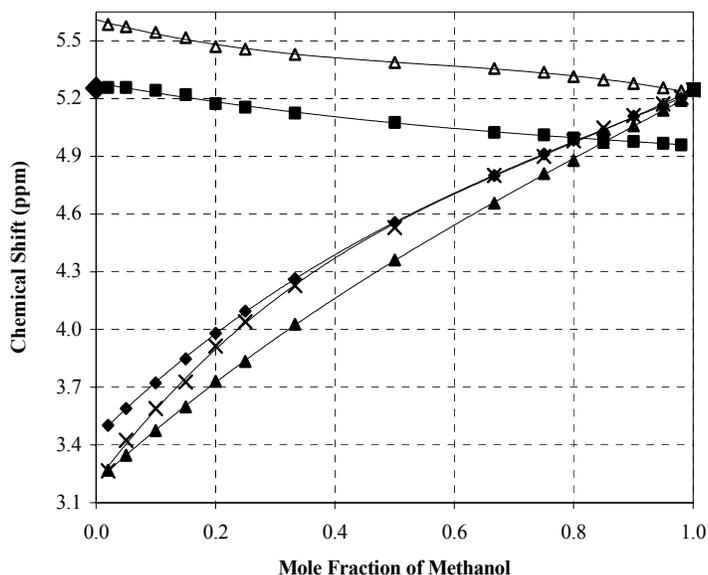

Figure 13. Chemical shift of the hydroxy proton of methanol as a function of the mole fraction of methanol in water (△), diethylether (✕), tetrahydrofurane (◆) and dioxane (▲). The chemical shift of water proton as a function of the mole fraction of methanol is also shown (■). The hydroxy proton chemical shift of methanol only sample and water chemical shift for water only sample are designated by a larger filled square (■) and larger filled tilted square (◆) respectively. The fitted lines are to show the trends of change.

Ternary Mixtures

In the binary systems, the individual effects of water and ether molecules on methanol hydroxy proton chemical shift are studied. However, it is also crucial to show that the presence of water would not interfere with the upfield shifting effect of ethers. This ternary system would also represent the simultaneous interactions of a hydroxy proton (methanol hydroxy proton) with water and acetal oxygens (dioxane). To achieve this, two ternary mixture series of methanol/water/dioxane were prepared. Two constant mole fractions (0.04 and 0.40) of methanol were used to make two series of ternary mixtures. In Figure 14, it is seen that the effect of dioxane concentration on the chemical shift of methanol hydroxy proton is not linear. As observed in the binary mixtures, increased dioxane concentration puts the chemical shift of methanol hydroxy proton to an upfield-shifted position. This observation applies to both mole fractions of methanol (0.04 and 0.40). However, the amount of upfield shift observed is also dependent on methanol concentration, that is, it is different for two series. When smaller amount of methanol (0.04 mole

fraction) is present, the upfield shift observed for its hydroxy proton is more pronounced.
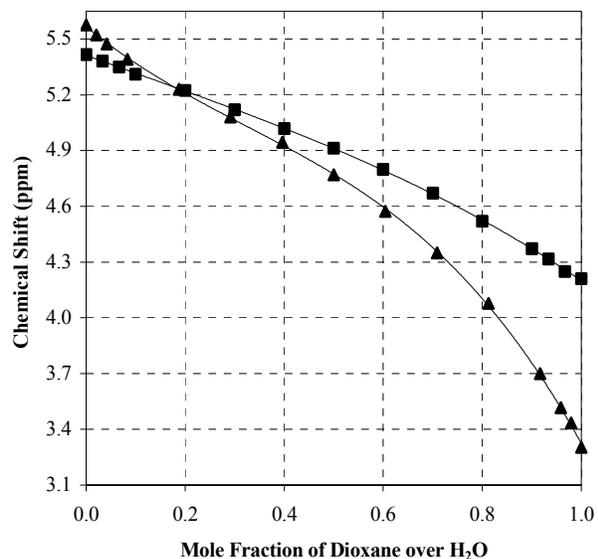

Figure 14. Chemical shift of the hydroxy proton of methanol as a function of the mole fraction of dioxane over water ($n_{dioxane}$ / ($n_{dioxane}$ + n_{water})). The triangles show the data points for the series of mixtures having 0.04 mole fraction of methanol whereas the squares represent the data points for 0.40 mole fraction of methanol. The fitted third degree polynomial curves are to show the trends of change.

Finally, it can be stated that interplay between hydrogen bond interactions and reduced hydration is the decisive factor for the chemical shift of a hydroxy proton. While additional (the ones other than solvation hydrogen bonds observed for monosaccharides) hydrogen bonds change the chemical shift of a hydroxy proton downfield, reduced hydration (lack of hydrogen bonds with bulk water) results in upfield shift. These two factors contribute to the value of chemical shifts in an opposite manner and are clearly reflected in the chemical shift changes from both binary and ternary mixtures. Therefore, by measuring $\Delta\delta$ values, it is possible to obtain information about hydration and/or hydrogen bonding interactions of hydroxy groups in carbohydrates.

Concluding Remarks

The use of hydroxy protons in structural studies yields additional information. The rigid structures and the stacking interactions of Lewis oligosaccharides [84-92] were confirmed by the additional NOEs involving hydroxy protons (Articles I and

II). Concerning the chemical shift differences, extent of interaction with bulk water molecules and being located around amphiphilic regions were anticipated to play role on magnetic shieldings of hydroxy protons.

The hydrogen bonds between O(2)H and O(3)H groups on adjacent glucose units in CDs were proven to also exist in water solution (Article III). A weak and transient interaction was also observed between O(2')H and O(3)H in maltose. Using hydroxy protons, the study of intermolecular interactions on the cyclodextrin complexes proved to be useful in providing structural information in the forms of chemical shift, temperature coefficient and line-shape of the hydroxy proton signals.

The intermolecular interactions between carbohydrates and nucleotides as revealed by IR studies [98,108], could not be detected by NMR spectroscopy (Article IV). However, a drastic improvement in the intensity and line-shape of the hydroxy proton signals from saccharides were encountered upon addition of small amount of purine nucleos(t)ides and nucleobases.

If the reason for the observed upfield and downfield shifts (positive and negative $\Delta\delta$ values) is contemplated, hydration turns out to be the keyword (Article V). When the hydration of a hydroxy proton is hampered by either interactions with acetal oxygens or structural formations (steric effects or perturbed water interactions in amphiphilic regions), the chemical shift of that proton gets an upfield-shifted value in comparison with the hydroxy proton in the corresponding monomeric unit. Likewise, provided that the hydration state is kept the same, a hydroxy proton becomes deshielded when it forms hydrogen bond interaction with another hydroxy group.

The introduction of hydroxy protons into the field of carbohydrate structure analysis has shown its potential and importance. It is likely that by improved understanding of the nature of hydroxy protons, even small differences in $\Delta\delta$s, CIS, temperature coefficients, and exchange rates will become frequently applicable in structural analysis.

Proposals for Further Studies

- The use of the method should be extended to obtain more data from different kinds of molecules and complexes *i.e.* proteins, nucleosides, carbohydrates and their interactions where applicable.

- Computational studies (MD and/or quantum mechanics) should be employed to learn more about the hydration/chemical shift relationship.

- There are still many unknown aspects of alcohol/water mixtures, concerning the chemical shift behaviors. This is especially crucial for the quantification of encountered effect of component on each other's chemical shifts.

References

1. J.M. Harlan, and D.Y. Liu, *Adhesion: Its Role in Inflammatory Disease*, New York, WH Freeman, 1992.

2. H. Lis, and N. Sharon, *Chemical Reviews*, 98, **1998**, 637-674.

3. S.W. Homans, *Progress in Nuclear Magnetic Resonance Spectroscopy*, 22, **1990**, 55-81.

4. K.G. Rice, P.G. Wu, L. Brand, and Y.C. Lee, *Biochemistry*, 32, **1993**, 7264-7270.

5. E.S. Stevens, *Biopolymers*, 34, **1994**, 1395-1401.

6. E.S. Stevens, *Biopolymers*, 34, **1994**, 1403-1407.

7. C.A. Bush, M. Martin-Pastor, and A. Imberty, *Annual Review of Biophysics and Biomolecular Structure*, 28, **1999**, 269-293.

8. A. Molinaro, C. De Castro, R. Lanzetta, E. Manzo, and M. Parrilli, *Journal of the American Chemical Society*, 123, **2001**, 12605-12610.

9. L. Poppe, and H. Vanhalbeek, *Journal of the American Chemical Society*, 113, **1991**, 363-365.

10. B. Adams, and L. Lerner, *Journal of the American Chemical Society*, 114, **1992**, 4827-4829.

11. B.R. Leeflang, J.F.G. Vliegenthart, L.M.J. Kroonbatenburg, B.P. Vaneijck, and J. Kroon, *Carbohydrate Research*, 230, **1992**, 41-61.

12. J. Kroon, L.M.J. Kroonbatenburg, B.R. Leeflang, and J.F.G. Vliegenthart, *Journal of Molecular Structure*, 322, **1994**, 27-31.

13. P.K. Agrawal, *Phytochemistry*, 31, **1992**, 3307-3330.

14. J.O. Duus, C.H. Gotfredsen, and K. Bock, *Chemical Reviews*, 100, **2000**, 4589-4614.

15. K. Bock, A. Brignole, and B.W. Sigurskjold, *Journal of the Chemical Society-Perkin Transactions 2*, **1986**, 1711-1713.

16. H. Baumann, B. Erbing, P.E. Jansson, and L. Kenne, *Journal of Chemical Society, Perkin Transactions I*, **1989**, 209-217.

17. N.K. Kochetkov, G.M. Lipkind, A.S. Shashkov, and N.E. Nifant'ev, *Carbohydrate Research*, 221, **1991**, 145-168.

18. I. Tvaroska, *Current Opinions in Structural Biology*, 2, **1991**, 661-665.

19. R.J. Woods, *Current Opinion in Structural Biology*, 5, **1995**, 591-598.

20. A. Imberty, and S. Perez, *Chemical Reviews*, 100, **2000**, 4567-4588.

21. T.L. James, *Current Opinion in Structural Biology*, 4, **1994**, 275-284.

22. W.D. Cornell, P. Cieplak, C.I. Bayly, I.R. Gould, K.M. Merz, D.M. Ferguson, D.C. Spellmeyer, T. Fox, J.W. Caldwell, and P.A. Kollman, *Journal of the American Chemical Society*, 117, **1995**, 5179-5197.

23. M. Clark, R.D. Cramer, and N. Vanopdenbosch, *Journal of Computational Chemistry*, 10, **1989**, 982-1012.

24. B. Brooks, R. Bruccoleri, B. Olafson, D. States, S. Swaminathan, and M. Karplus, *Journal Of Computational Chemistry*, 4, **1983**, 187-217.

25. Groningen Molecular Simulation (GROMOS) Library Manual , W.F. Van Gunsteren, and H.J.C. Berendsen, BIOMOS, Groningen, The Netherlands, 1987.

26. N.L. Allinger, Y.H. Yuh, and J.H. Lii, *Journal of the American Chemical Society*, 111, **1989**, 8551-8566.

27. S. Perez, A. Imberty, S.B. Engelsen, J. Gruza, K. Mazeau, J. Jimenez-Barbero, A. Poveda, J.F. Espinosa, B.P. van Eyck, G. Johnson, A.D. French, M. Louise, C.E. Kouwijzer, P.D.J. Grootenuis, A. Bernardi, L. Raimondi, H. Senderowitz, V. Durier, G. Vergoten, and K. Rasmussen, *Carbohydrate Research*, 314, **1998**, 141-155.

28. S. Reiling, M. Schlenkrich, and J. Brickmann, *Journal of Computational Chemistry*, 17, **1996**, 450-468.

29. H. Senderowitz, and W.C. Still, *Journal of Organic Chemistry*, 62, **1997**, 1427-1438.

30. W. Damm, A. Frontera, J. TiradoRives, and W.L. Jorgensen, *Journal of Computational Chemistry*, 18, **1997**, 1955-1970.

31. F.A. Momany, and J.L. Willett, *Journal of Computational Chemistry*, 21, **2000**, 1204-1219.

32. R. Eklund, and G. Widmalm, *Carbohydrate Research*, 338, **2003**, 393-398.

33. F.A. Momany, and J.L. Willett, *Carbohydrate Research*, 326, **2000**, 210-226.

34. J.R. Kneisler, and N.L. Allinger, *Journal of Computational Chemistry*, 17, **1996**, 757-766.

35. I. Tvaroska, and J.P. Carver, *Theochem-Journal of Molecular Structure*, 395, **1997**, 1-13.

36. F.J. Luque, J.M. Lopez, M.L. de la Paz, C. Vicent, and M. Orozco, *Journal of Physical Chemistry a*, 102, **1998**, 6690-6696.

37. Gaussian 98 (Revision A.3), M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, V.G. Zakrzewski, J. Montgomery, J. A., R.E. Stratmann, J.C. Burant, S. Dapprich, J.M. Millam, A.D. Daniels, K.N. Kudin, M.C. Strain, O. Farkas, J. Tomasi, V. Barone, M. Cossi, R. Cammi, B. Mennucci, C. Pomelli, C. Adamo, S. Clifford, J. Ochterski, G.A. Petersson, P.Y. Ayala, Q. Cui, K. Morokuma, D.K. Malick, A.D. Rabuck, K. Raghavachari, J.B. Foresman, J. Cioslowski, J.V. Ortiz, A.G. Baboul, B.B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. Gomperts, R.L. Martin, D.J. Fox, T. Keith, M.A. Al-Laham, C.Y. Peng, A. Nanayakkara, C. Gonzalez, M. Challacombe, P.M.W. Gill, B.G. Johnson, W. Chen, M.W. Wong, J.L. Andres, M. Head-Gordon, E.S. Replogle, and J.A. Pople, Gaussian Inc., Pittsburgh, PA, 1998.

38. J.B. Foresman, and H.B. Schlegel, *Application of the CI-Singles method in predicting the energy, properties and reactivity of molecules in their excited states*, The Netherlands, Kluwer Academic, 1993.

39. J.R. Cheeseman, M.J. Frisch, F.J. Devlin, and P.J. Stephens, *Chemical Physics Letters*, 252, **1996**, 211-220.

40. T. Rundlof, A. Kjellberg, C. Damberg, T. Nishida, and G. Widmalm, *Magnetic Resonance in Chemistry*, 36, **1998**, 839-847.

41. L. Poppe, W.S. York, and H. Vanhalbeek, *Journal of Biomolecular Nmr*, 3, **1993**, 81-89.

42. I. Tvaroska, M. Hricovini, and E. Petrakova, *Carbohydrate Research*, 189, **1989**, 359-362.

43. M.J. Milton, R. Harris, M.A. Probert, R.A. Field, and S.W. Homans, *Glycobiology*, 8, **1998**, 147-153.

44. B. Bose, S. Zhao, R. Stenutz, F. Cloran, P.B. Bondo, G. Bondo, B. Hertz, I. Carmichael, and A.S. Serianni, *Journal of the American Chemical Society*, 120, **1998**, 11158-11173.

45. A.G. Palmer, J. Williams, and A. McDermott, *Journal of Physical Chemistry*, 100, **1996**, 13293-13310.

46. P. Dais In *Advances in Carbohydrate Chemistry and Biochemistry, Vol 51*, 1995; Vol. 51.

47. P.J. Bolon, and J.H. Prestegard, *Journal of the American Chemical Society*, 120, **1998**, 9366-9367.

48. T. Rundlof, C. Landersjo, K. Lycknert, A. Maliniak, and G. Widmalm, *Magnetic Resonance in Chemistry*, 36, **1998**, 773-776.

49. J.M. Harvey, M.C.R. Symons, and R.J. Naftalin, *Nature*, 261, **1976**, 435-436.

50. H. Vanhalbeek, and L. Poppe, *Magnetic Resonance in Chemistry*, 30, **1992**, S74-S86.

51. L. Poppe, R. Stuikeprill, B. Meyer, and H. Vanhalbeek, *Journal of Biomolecular Nmr*, 2, **1992**, 109-136.

52. L. Poppe, and H. Vanhalbeek, *Journal of the American Chemical Society*, 114, **1992**, 1092-1094.

53. J.R. Brisson, S. Uhrinova, R.J. Woods, M. vanderZwan, H.C. Jarrell, L.C. Paoletti, D.L. Kasper, and H.J. Jennings, *Biochemistry*, 36, **1997**, 3278-3292.

54. R. Harris, T.J. Rutherford, M.J. Milton, and S.W. Homans, *Journal of Biomolecular Nmr*, 9, **1997**, 47-54.

55. C. Sandstrom, H. Baumann, and L. Kenne, *Journal of the Chemical Society-Perkin Transactions 2*, **1998**, 2385-2393.

56. C. Sandstrom, H. Baumann, and L. Kenne, *Journal of the Chemical Society-Perkin Transactions 2*, **1998**, 809-815.

57. C. Sandstrom, G. Magnusson, U. Nilsson, and L. Kenne, *Carbohydrate Research*, 322, **1999**, 46-56.

58. I. Ivarsson, C. Sandstrom, A. Sandstrom, and L. Kenne, *Journal of the Chemical Society-Perkin Transactions 2*, **2000**, 2147-2152.

59. L. Poppe, and H. Vanhalbeek, *Nature Structural Biology*, 1, **1994**, 215-216.

60. S.Q. Sheng, and H. Vanhalbeek, *Biochemical and Biophysical Research Communications*, 215, **1995**, 504-510.

61. D.R. Bundle, H. Baumann, J.R. Brisson, S.M. Gagne, A. Zdanov, and M. Cygler, *Biochemistry*, 33, **1994**, 5183-5192.

62. B. Adams, and L.E. Lerner, *Magnetic Resonance in Chemistry*, 32, **1994**, 225-230.

63. S.Q. Sheng, R. Cherniak, and H. van Halbeek, *Analytical Biochemistry*, 256, **1998**, 63-66.

64. R. Konrat, M. Tollinger, G. Kontaxis, and B. Krautler, *Monatshefte Fur Chemie*, 130, **1999**, 961-982.

65.   L.C.G. Freitas, J.M.M. Cordeiro, and F.L.L. Garbujo, *Journal of Molecular Liquids*, 79, **1999**, 1-15.

66.   G. Batta, and K.E. Kover, *Carbohydrate Research*, 320, **1999**, 267-272.

67.   J. Lin, and P.A. Frey, *Journal of the American Chemical Society*, 122, **2000**, 11258-11259.

68.   D.J. Hoult, *Journal of Magnetic Resonance*, 21, **1976**, 337-342.

69.   M. Gueron, P. Plateau, and M. Decorps, *Progress in Nuclear Magnetic Resonance Spectroscopy*, 23, **1991**, 135-209.

70.   M. Gueron, and P. Plateau, *Journal of American Chemical Society*, 104, **1982**, 7310-7311.

71.   M. Piotto, V. Saudek, and V. Sklenar, *Journal of Biomolecular Nmr*, 2, **1992**, 661-665.

72.   S. Braun, H.-O. Kalinowski, and S. Berger, *100 and More Basic NMR Experiments*, Weinheim, VCH, 1996.

73.   V. Sklenar, M. Piotto, R. Leppik, and V. Saudek, *Journal of Magnetic Resonance Series a*, 102, **1993**, 241-245.

74.   G. Lippens, C. Dhalluin, and J.M. Wieruszeski, *Journal of Biomolecular Nmr*, 5, **1995**, 327-331.

75.   C. Dalvit, S.Y. Ko, and J.M. Bohlen, *Journal of Magnetic Resonance Series B*, 110, **1996**, 124-131.

76.   M.L. Liu, X.A. Mao, C.H. Ye, H. Huang, J.K. Nicholson, and J.C. Lindon, *Journal of Magnetic Resonance*, 132, **1998**, 125-129.

77.   G.A. Jeffrey, and W. Saenger, *Hydrogen Bonding in Biological Structures*, New York, Springer-Verlag, 1994.

78.   J. Jeener, B.H. Meier, P. Bachmann, and R.R. Ernst, *Journal of Chemical Physics*, 71, **1979**, 4546-4553.

79.   S. Macura, and R.R. Ernst, *Molecular Physics*, 41, **1980**, 95-117.

80.   A. Kumar, G. Wagner, R.R. Ernst, and K. Wüthrich, *Journal of American Chemical Society*, 103, **1981**, 3654-3658.

81.   B.R. Leeflang, and J.F.G. Vliegenthart, *Journal of Magnetic Resonance*, 89, **1990**, 615-619.

82.   R.R. Fraser, M. Kaufman, P. Morand, and G. Govil, *Canadian Journal of Chemistry*, 47, **1969**, 403-409.

83.   L. Kindahl, C. Sandstrom, T. Norberg, and L. Kenne, *Journal of Carbohydrate Chemistry*, 19, **2000**, 1291-1303.

84.   R.U. Lemieux, *Chemical Society Reviews*, 7, **1978**, 423-452.

85.   R.U. Lemieux, K. Bock, L.T.J. Delbaere, S. Koto, and W.S. Rao, *Canadian Journal Of Chemistry*, 58, **1980**, 631-653.

86.   R.U. Lemieux, and K. Bock, *Arch. Biochem. Biophys.*, 221, **1983**, 125-134.

87.   R.U. Lemieux, *Chemical Society Reviews*, 18, **1989**, 347-374.

88.   R.U. Lemieux, *Accounts of Chemical Research*, 29, **1996**, 373-380.

89.   O. Hindsgaul, T. Norberg, J. Le Pendu, and R.U. Lemieux, *Carbohydrate Research*, 109, **1982**, 109-142.

90. U. Spohr, and R.U. Lemieux, *Carbohydrate Research*, 174, **1988**, 211-237.

91. A. Imberty, E. Mikros, J. Koca, R. Mollicone, R. Oriol, and S. Perez, *Glycoconjugate Journal*, 12, **1995**, 331-349.

92. A. Geyer, G. Hummel, T. Eisele, S. Reinhardt, and R.R. Schmidt, *Chemistry-a European Journal*, 2, **1996**, 981-988.

93. H.J. Schneider, F. Hacket, V. Rudiger, and H. Ikeda, *Chemical Reviews*, 98, **1998**, 1755-1785.

94. M. Onda, Y. Yamamoto, Y. Inoue, and R. Chujo, *Bulletin of the Chemical Society of Japan*, 61, **1988**, 4015-4021.

95. J.C. Christofides, and D.B. Davies, *Journal of Chemical Society, Chemical Communications*, **1982**, 560-562.

96. M. St-Jacques, P.R. Sundararajan, K. Taylor, and R.H. Marchessault, *Journal of American Chemical Society*, 98, **1976**, 4386-4391.

97. B. Klar, B. Hingerty, and W. Saenger, *Acta Crystallographica, Section B*, 36, **1980**, 1154-1165.

98. L. ElMahdaoui, J.F. Neault, and H.A. TajmirRiahi, *Journal of Inorganic Biochemistry*, 65, **1997**, 123-131.

99. M.J. King, *Biochimica Et Biophysica Acta-Reviews On Biomembranes*, 1197, **1994**, 15-44.

100. R.S. Singh, A.K. Tiwary, and J.F. Kennedy, *Critical Reviews in Biotechnology*, 19, **1999**, 145-178.

101. J. Le Pendu, S. Marionneau, A. Cailleau-Thomas, J. Rocher, B. Le Moullac-Vaidye, and M. Clement, *Apmis*, 109, **2001**, 9-31.

102. MM3 , 1996, Department of Chemistry, University of Georgia, Athens, Georgia 30602, USA.

103. M. BlaszczykThurin, R. Murali, M.A.J. Westerink, Z. Steplewski, M.S. Co, and T. KieberEmmons, *Protein Engineering*, 9, **1996**, 447-459.

104. V. Rudiger, A. Eliseev, S. Simova, H.J. Schneider, M.J. Blandamer, P.M. Cullis, and A.J. Meyer, *Journal of the Chemical Society-Perkin Transactions 2*, **1996**, 2119-2123.

105. K. Kano, N. Tanaka, and S. Negi, *European Journal of Organic Chemistry*, **2001**, 3689-3694.

106. J. Nishijo, and M. Tsuchitani, *Journal of Pharmaceutical Sciences*, 90, **2001**, 134-140.

107. T. Hayashi, T. Miyahara, N. Koide, Y. Kato, H. Masuda, and H. Ogoshi, *Journal of the American Chemical Society*, 119, **1997**, 7281-7290.

108. H.A. Tajmirriahi, M. Naoui, and S. Diamantoglou, *Journal of Biomolecular Structure & Dynamics*, 12, **1994**, 217-234.

109. I. Ivarsson, A. Sandstrom, and L. Kenne, unpublished results.

110. A.D. Becke, *Journal of Chemical Physics*, 98, **1993**, 5648-5652.

111. R. Ditchfield, *Molecular Physics*, 27, **1974**, 789-807.

Corrigenda for Articles I and III

Page/Paragraph/Line	Reads	Should read
Article I		
409/1/6	Glc*p*NAc	Gal*p*
411/2/13	(≤ 10.21 ppm)	($|\Delta\delta| \leq 0.2$ ppm)
Article III		
1673/1/7	$\Delta\delta < 0.1|$ ppm	$|\Delta\delta| < 0.1$ ppm
1674/1/16	glycopyranosyl	glucopyranosyl

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