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Suitability of binary mixtures of water with aprotic solvents to turn hydroxyl protons of carbohydrate ligands into conformational sensors in NOE and transferred NOE experiments

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Abstract

The structural analysis of protein-carbohydrate interactions is essential for the long-range aim to sort out entropic/enthalpic factors in the binding process. Of conspicuous clinical interest, this work can also offer the perspective to devise new classes of therapeuticals which interfere with disease-related glycan recognition. We have shown that it is possible to use exchangeable hydroxyl protons of carbohydrate ligands as conformational sensors for defining their bound-state topology by measurements in dimethyl sulfoxide$_{d6}$ (Siebert et al. (2000) *ChemBioChem*, **1**, 181–195). However, the proteins are required to maintain binding capacity in the aprotic solvent. To define conditions to limit its harmful effect on sensitive protein structures while still being able to pick up solvent-exchangeable hydroxyl signals we systematically tested binary solvent mixtures of dimethyl sulfoxide and acetone with water. These solvent mixtures did not preclude to monitor hydroxyl protons of carbohydrate ligands even at temperatures well above 0 °C. Notably, hydrogen bonding of the two tested disaccharides (Galβ1-4Glcα/β and Galα1-3Galα/β or Galα1-3Galβ1-OCH$_3$), which are common lectin ligands, resembled the situation under physiological conditions. Also, a refined topological description for hydroxyl positioning could be achieved for Galα1-3Gal. At least equally important, this approach worked for elucidation of the mistletoe-lectin-bound topology of lactose in its *syn*-conformation with indication for formation of a characteristic interresidual hydrogen bond. These measurements were performed in a binary dimethyl sulfoxide$_{d6}$:water mixture (6:4 ratio, v/v) at −12 °C and encourage to pursue this line of investigation by monitoring in the course of stepwise temperature increases. Our experiments reveal that binary mixtures have favorable properties for the conformational analysis of the free- and bound-state topologies of bioactive ligands.

Introduction

Emerging terms such as *glycomics* or *sugar code* attest the increasing interest in the structural and functional analysis of glycan chains of cellular glycoconjugates (Laine, 1997; Gabius, 2000; Hirabayashi and Kasai, 2000; Gabius et al., 2002). By serving as ligands for tissue receptors (lectins) distinct glycan epitopes are involved in eliciting or mediating important cell activities such as regulation of proliferation/ apoptosis, cell-cell (matrix) interactions or host defense reactions via innate immunity effectors and antigen

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†Prof. Minch died during work on this study; the manuscript written with his final scientific contributions is our mark of respect and wholeheartedly dedicated to him.

uptake/processing (Gabius, 1997, 2001a, b; Kaltner and Stierstorfer, 1998; André et al., 1999; Kopitz et al., 2001; Dahms and Hancock, 2002; Danguy et al., 2002; Kilpatrick, 2002a, b; Lu et al., 2002; Nagy et al., 2002; Rabinovich et al., 2002; Weigel and Yik, 2002). Not surprisingly, pathogens also exploit such determinants as specific docking sites to home in on target cells (Lingwood, 1998; Mulvey et al., 2001). The inherent perspective for medical applications by rational design of lectin ligands calls for precise definition of the carbohydrates' flexibility in solution. Thus, the determination of bioactive conformations has become a challenging task. Fittingly, NMR spectroscopy has increasingly been applied to address these issues, especially to describe bound-state conformations of carbohydrate ligands (Carver, 1993; Gabius, 1998; Poveda and Jiménez-Barbero, 1998; von der Lieth et al., 1998; Jiménez-Barbero et al., 1999; Duus et al., 2000; Imberty and Pérez, 2000; Rüdiger et al., 2000).

Towards this end, interresidual transferred nuclear Overhauser effects (trNOEs) provide important information on the relative positions of the pyranose rings connected by a glycosidic bond. However, the exchange of solvent-sensitive hydroxyl protons often limits this technique to monitoring C-H protons, thereby reducing the precision of calculations of the Φ, Ψ-angles describing the glycosidic linkage. Three lines of evidence have made it appealing to us to consider a solvent change. Building on the pioneering study by Casu et al. (1966) to collect sharp peaks of hydroxyl proton resonances from glucosides in dimethyl sulfoxide (DMSO), the determination of conformational aspects of the glycan chains of glycolipids in this solvent (Dabrowski et al., 1981, 1995, 1998; Yan et al., 1987; Dabrowski and Poppe, 1989) and finally the intriguing experience with receptor-proteins (Dalvit, 1998; Dalvit et al., 1999) and enzymes in organic solvents (Gupta, 1992; Liepinsh and Otting, 1997; Klibanov, 2000, 2001; Mattos and Ringe, 2001), it was tempting to answer the following question: Will trNOE measurements be possible after a solvent change to a polar aprotic medium?

Concerning the behavior of glycans in an aprotic solvent, it was encouraging to note that in the case of histo-blood group A and H determinants the oligosaccharide conformation did not appear to be strongly dependent on this parameter (Yan et al., 1987; Rao and Bush, 1988). This property and the solubility of glycolipids in organic solvents contributed to the popularity of this approach (Dabrowski and Poppe, 1989; Poppe et al., 1990a, b; Siebert et al., 1992). Our recent demonstration that lectins and carbohydrate-binding immunoglobulins with a compact folding pattern retain their binding activity and ligand affinity in DMSO enabled to prove that hydroxyl protons of the carbohydrate ligand can serve as proper sensors for the bound-state topology (Siebert et al., 2000). In the course of this study, we also recognized in the case of the small plant lectin hevein that a complete solvent exchange could be detrimental to a lectin's activity (Siebert et al., 2000). It was thus reasonable to assume that water/aprotic solvent mixtures might offer a window of opportunity: To define a so far unknown solvent/water ratio to exploit the benefit of using an aprotic solvent for turning hydroxyl protons into sensors and still maintain the protein-protecting water properties.

Table 1. Chemical shifts of ring protons of lactose [15 mM] in binary $DMSO_{d6}$: water mixtures at −12 °C

	25:75 ratio $DMSO_{d6}$:water (v/v) chem. shifts (ppm)	63:37 ratio $DMSO_{d6}$:water (v/v) chem. shifts (ppm)	Positions of the chem. shifts in the monosaccharide[a] (ppm)
βGalH1	4.47	4.34	4.48
βGalH2	3.60	3.46	3.41
βGalH3	3.70	3.51	3.56
βGalH4	3.97	3.78	3.84
βGalH5	–	3.68	3.61
βGalH6a	–	3.80	3.70
βGalH6b	–	3.61	3.62
βGlcH1	4.68	4.53	4.51
βGlcH2	3.30	3.16	3.13
βGlcH3	3.69	3.51	3.37
βGlcH4	–	3.79	3.30
βGlcH5	–	3.46	3.35
βGlcH6a	–	3.87	3.75
βGlcH6b	–	–	3.60
αGlcH1	5.43	5.10	5.09
αGlcH2	3.61	3.42	3.41
αGlcH3	3.82	3.72	3.61
αGlcH4	–	3.53	3.29
αGlcH5	3.94	3.84	3.72
αGlcH6a	3.91	3.79	3.63

[a]Bock and Thøgersen, 1982; measured at 400 MHz in D_2O at 23 °C with acetone as internal standard (2.16 ppm).
–: Not assigned due to signal overlap or extensive line-broadening.

Table 2. Chemical shifts of hydroxyl protons of lactose [15 mM] at −12 °C and data sets for chemical shifts and coupling constants of the corresponding monosaccharide signals[a]

	63:37 ratio $DMSO_{d6}$:water (v/v) chem. shifts (ppm)	Positions of the chem. shifts of the monosaccharide[a] (ppm)	J measured for the monosaccharide[a] (Hz)
βGalOH2	6.16	6.38	4.5
βGalOH3	5.69	5.99	6.0
βGalOH4	5.61	5.79	5.2
βGalOH6	5.96	6.16	5.0
βGlcOH1	7.63	–	–
βGlcOH2	6.12	6.46	4.5
βGlcOH3	5.63	6.37	5.0
αGlcOH1	7.00	–	–
αGlcOH2	5.77	6.15	6.5
αGlcOH3	5.45	6.28	5.0
αGlcOH6	5.53	5.92	5.0

[a]Adams and Lerner, 1994; measured in 2:1 ratio (v/v) $acetone_{d6}$: water at 500 MHz and −23 °C.
–: Not determined due to signal overlap or extensive line-broadening.

Table 3. Chemical shifts of the ring protons of lactose [15 mM] in $acetone_{d6}$:water mixture at −12 °C

	15:85 ratio $acetone_{d6}$:water (v/v) chem. shifts (ppm)	2:8 ratio $acetone_{d6}$:water (v/v) chem. shifts (ppm)	Positions of the chem. shifts in the monosaccharide[a] (ppm)
βGalH1	4.48	4.48	4.48
βGalH2	3.59	3.59	3.41
βGalH3	3.70	3.70	3.56
βGalH4	3.95	3.97	3.84
βGalH5	–	3.77	3.61
βGalH6a	–	3.91	3.70
βGalH6b	–	3.86	3.62
βGlcH1	4.70	4.70	4.51
βGlcH2	3.31	3.32	3.13
βGlcH3	3.64	3.63	3.37
βGlcH4	3.69	3.69	3.30
βGlcH5	–	3.69	3.35
βGlcH6a	3.99	3.98	3.75
βGlcH6b	3.86	3.86	3.60
αGlcH1	–	5.27	5.09
αGlcH2	3.60	3.59	3.41
αGlcH3	3.85	3.86	3.61
αGlcH4	3.70	3.70	3.29
αGlcH5	–	3.97	3.72
αGlcH6a	3.91	3.91	3.72
αGlcH6b	–	3.77	3.63

[a]Bock and Thøgersen, 1982; measured at 400 MHz in D_2O at 23 °C with acetone as internal standard (2.16 ppm).
–: Not assigned due to signal overlap or extensive line-broadening.

The focus of this study is to exploit the benefits of adding an aprotic solvent to water without any risk for the structural integrity of the receptor. In binary mixtures some properties can not be explained from a simple dilution process. For example, the lowering of the freezing point of a 1:3 (v/v) $DMSO_{d6}$:water mixture to −62 °C, about 80 °C below the freezing point of pure DMSO, is due to not yet fully understood cluster formation of two types of solvent molecules (Singer, 1962; Vaisman and Berkowitz, 1992; Borin and Skaf, 1999; Vishnyakov et al., 1999, 2000; Kirchner and Reiher, 2002). Besides possibly favorable effects on protein stability the physicochemical property of our solvent mixture renders low-temperature experiments possible, an alternative to work with supercooled solutions (Poppe and van Halbeek, 1991, 1994; Adams and Lerner, 1992, 1994; Sheng and van Halbeek, 1995; Bekiroglu et al., 2000).

To approach the aim defined above, we first examined the properties of two binding partners for sugar receptors (animal, bacterial and plant lectins/immunoglobulins) free in solution, i.e., the disaccharides Galα1-3Gal and lactose (Galβ1-4Glc). Their ^{1}H NMR spectra were recorded in varying ratios of the two solvent components. Temperature coefficients of chemical shifts were determined to assess the extent of involvement of hydroxyl protons in hydrogen bonding. Actually, certain carbohydrates are prone to integrate a solvent molecule into their hydrogen-bonding network (Siebert et al., 2000, 2003; Vishnyakov et al., 2000). Temperature variation was also performed to explore whether or not increases in the exchange rate of hydroxyl protons compromise the line-width of the respective resonance signals. This factor might preclude measurements in binary mixtures at temperatures above 0 °C. Our comparative analysis included $DMSO_{d6}$, extending our previous study (Siebert et al., 2000), and also acetone as aprotic part of the binary mixtures. It resulted in measurable solvent-dependent differences concerning rotational equilibria of distinct hydroxyl groups. Monitoring of NOE contacts in-

Table 4. Chemical shifts of the hydroxyl protons of lactose [15 mM] in acetone$_{d6}$:water mixture at −12 °C

	15:85 ratio acetone$_{d6}$:water (v/v) chem. shifts (ppm)	2:8 ratio acetone$_{d6}$:water (v/v) chem. shifts (ppm)	66:34 ratio acetone$_{d6}$:water (v/v) chem. shifts (ppm)
βGalOH2	6.63	6.61	6.38
βGalOH3	6.27	6.20	5.99
βGalOH4	6.13	6.01	5.79
βGalOH6	6.05	6.01	6.16
βGlcOH1	8.04	8.04	–
βGlcOH2	6.68	6.64	6.46
βGlcOH3	6.23	6.11	6.37
αGlcOH1	7.37	7.30	–
αGlcOH2	6.29	6.26	6.15
αGlcOH3	6.02	6.00	6.28
αGlcOH6	5.95	5.93	5.92

–: Not assigned due to signal overlap or extensive line-broadening.

cluding hydroxyl proton resonances was performed to evaluate whether the description of the free-state conformations is improved by this new aspect in the case of the two model ligands. Remarkably, the experimental series enabled us to track down sharp hydroxyl resonances for a distinct water:DMSO$_{d6}$ ratio even in the temperature range above 0 °C. Because this result has implications for studying protein-ligand complexes we tested lactose as ligand. As model receptor, we selected the plant lectin VAA (*Viscum album* L. agglutinin) from mistletoe whose carbohydrate-dependent enthalpically driven binding (Bharadwaj et al., 1999) to cell surfaces triggers intracellular signaling and mitogenic responses in immune and tumor cells (Hajto et al., 1990; Timoshenko et al., 1999, 2001; Gabius et al., 2001; Gabius and Gabius, 2002). The ensuing measurements of trNOE signals from lactose bound to the α/β-galactoside-specific lectin VAA underscored the actual potential which solvent mixtures offer for adding new sensors in trNOE experiments to delineate the bound-state conformation of a ligand.

Materials and methods

Materials

The disaccharides Galβ1-4Glcα/β, Galα1-3Galα/β and Galα1-3Galβ1-OCH$_3$ were obtained from Sigma (Munich, Germany). Acetone$_{d6}$ and DMSO$_{d6}$ were purchased from Merck, Sharp and Dohme (Montreal, Canada). The α/β-galactoside-specific lectin from aqueous extracts of dried mistletoe leaves was purified to electrophoretic homogeneity by affinity chromatography on lactosylated Sepharose 4B, prepared after divinyl sulfone activation, and stored at −20 °C after lyophilization (Gabius, 1990; Kunze et al., 2000). One- and two-dimensional gel electrophoretic analysis, haemagglutination with trypsin-treated and glutaraldehyde-fixed rabbit erythrocytes and solid-phase assays with lactosylated neoglycoprotein or asialofetuin as ligand served as quality control to routinely ascertain purity and activity (Kohnke-Godt and Gabius, 1989; André et al., 1997, 2001).

NMR spectroscopy

750, 500 and 360 MHz ^{1}H-NMR spectra were recorded with Varian Unity 500, Bruker AM and AMX 500 spectrometers at various temperatures and at pH-values between 6 and 10.5. The lyophilized disaccharides (Galβ1-4Galα/β, Galα1-3Galα/β and Galα1-3Galβ1-OCH$_3$) were dissolved in binary mixtures of water (H_2O) with DMSO$_{d6}$ and acetone$_{d6}$, respectively. Similarly, lyophilized mistletoe lectin was dissolved in DMSO$_{d6}$:H_2O mixtures together with lactose. Assignment of the chemical shifts of protons of the two disaccharides was carried out by standard NMR experiments (COSY, RCT, TOCSY, ROESY and NOESY). The 2D ROESY measurements were performed in order to obtain NOE values in the rotating frame to determine the conformations of the disaccharides in the tested solvents in the free state. Lectin-bound conformations were delineated by 1D and 2D trNOE (transferred Nuclear Overhauser Effect) experiments. To avoid calibration errors in the ROESY experiments frequency-offset variation and a distinct spin-lock pulse sequence were applied, as described previously (Gilleron et al., 1998). Various mixing times ranging from 20 to 200 ms or even at 300 and 400 ms were used for ROESY, NOESY and 1D trNOE and 2D trNOESY (transferred NOESY) experiments. Intra- and interresidual proton-proton distance ranges were derived from cross-peak intensities and used for

distance mapping, as outlined elsewhere (Siebert et al., 1992, 1996).

The effective temperatures for the measurements were chosen between −12 °C and 20 °C. Concentrations of the two disaccharides in the range from 15 to 65 mM were tested for the free-state measurements in the binary solutions. For the trNOESY experiments in $DMSO_{d6}$:water (6:4 ratio, v/v) a lectin concentration of 0.3 mM and a lactose concentration of 3 mM was set following initial variations of the molar ratio.

Molecular models

Disaccharide models were constructed using the SWEET-program (http://www.dkfz-heidelberg.de/spec/sweet2/doc/). Intra- and interresidual distances between protons in selected pairs were calculated with the WebLabViewer available free of charge at: http://www.accelrys.com/viewer/

Results and discussion

Binary mixtures of water with either $DMSO_{d6}$ or $acetone_{d6}$ readily dissolved Galβ1-4Glcα/β, Galα1-3Galα/β and Galα1-3Galβ1-OCH_3. ^{1}H NMR spectra were recorded for the two disaccharides at a constant temperature (−12 °C) and a constant sugar concentration (15 mM). The solvent ratio in binary mixtures containing water (H_2O) and $DMSO_{d6}$ or $acetone_{d6}$ was varied to figure out optimal conditions. As compiled in Tables 1–4 for lactose, the assignment of all ring protons and, notably, all hydroxyl protons could be completed in both solvent systems. For comparison we also list the chemical shifts of the βGal, βGlc and αGlc monosaccharide ring protons in D_2O (Bock and Thøgersen, 1982) and of the hydroxyl protons in an $acetone_{d6}$:water mixture (2:1 ratio, v/v) measured at −23 °C (Adams and Lerner, 1994) in Tables 1–3. The alteration of the chemical environment in solvent mixtures is reflected in changes of the shift positions. The data in these tables also teach the lesson that the extent of signal assignment is improved by deliberately testing different ratios of the two solvents. To illustrate the shape of the signals, especially for the hydroxyl resonances, Figure 1 provides an example from the experiments with lactose. The only exception to the otherwise sharp signal profile is the βGlcOH1-proton in the $DMSO_{d6}$:water mixture (6:4 ratio, v/v). The rather small size of this signal is an indication for an exchange process. Thus, the presence of water molecules in the mixture did not significantly impair the possibility to measure hydroxyl signals of the disaccharides.

To define their influence and optimal conditions for structural analysis in a mixture we focused the attention on the binary solvent system of water with $DMSO_{d6}$ and evaluated the influence of water content on the resonance signals starting with the 37:63 ratio (water:$DMSO_{d6}$, v/v) and increasing it step by step. In this process, the ensuing alteration of a second factor should not be neglected, Explicitly, line-broadening also depends on the buffer concentration of the aqueous part of the mixture. When using phosphate buffer, sharp signals only occur at a buffer concentration markedly below 10 mM. As illustrated in Figure 2, the spectrum with the given starting concentration of water (buffer) is already in the optimal range for NMR analysis. Considering coupling constants as quality control and source of structural information, the solvent mixture exhibited favorable properties. The coupling constant J_{HH} of βGlcOH1 could only be measured accurately (J_{HH} = 10 Hz) in the mixture of 63:37 ratio ($DMSO_{d6}$:water, v/v). On the other hand, it is remarkable that despite increase in water content up to a ratio of 75:25 ratio (water:$DMSO_{d6}$, v/v) the hydroxyl proton resonances from lactose could still be seen (Figure 2).

An analysis of the impact of the $DMSO_{d6}$ and the lactose concentrations on the chemical shift values of the lactose signals is presented in Table 5. For signal assignment it is essential to know that an increase of the lactose and also of the $DMSO_{d6}$ concentration resulted in an upfield shift of the proton signals of the hydroxyl groups. These concentration-dependent effects are readily seen for example by comparison of columns 1 and 2 or columns 1 and 6 of Table 5, respectively. The chemical shifts compiled in Table 5 depended on the two tested parameters in a predictable way. Regarding the concentration of the disaccharide significant differences in the chemical shift positions were detectable when comparing data obtained with 52 mM and 65.4 mM lactose (columns 1 and 2 of Table 5) in a $DMSO_{d6}$:water mixture of 25:75 ratio (v/v). Such an effect does not occur when performing the measurements in pure solvents. Because solvent mixtures are characterized by interaction of the two types of solvent molecules including the recently described cluster formation between $DMSO_{d6}/H_2O$ molecules (Singer, 1962; Vaisman and Berkowitz, 1992; Borin and Skaf, 1999; Vishnyakov et al., 1999, 2000; Kirchner and Reiher, 2001), this parameter might have a

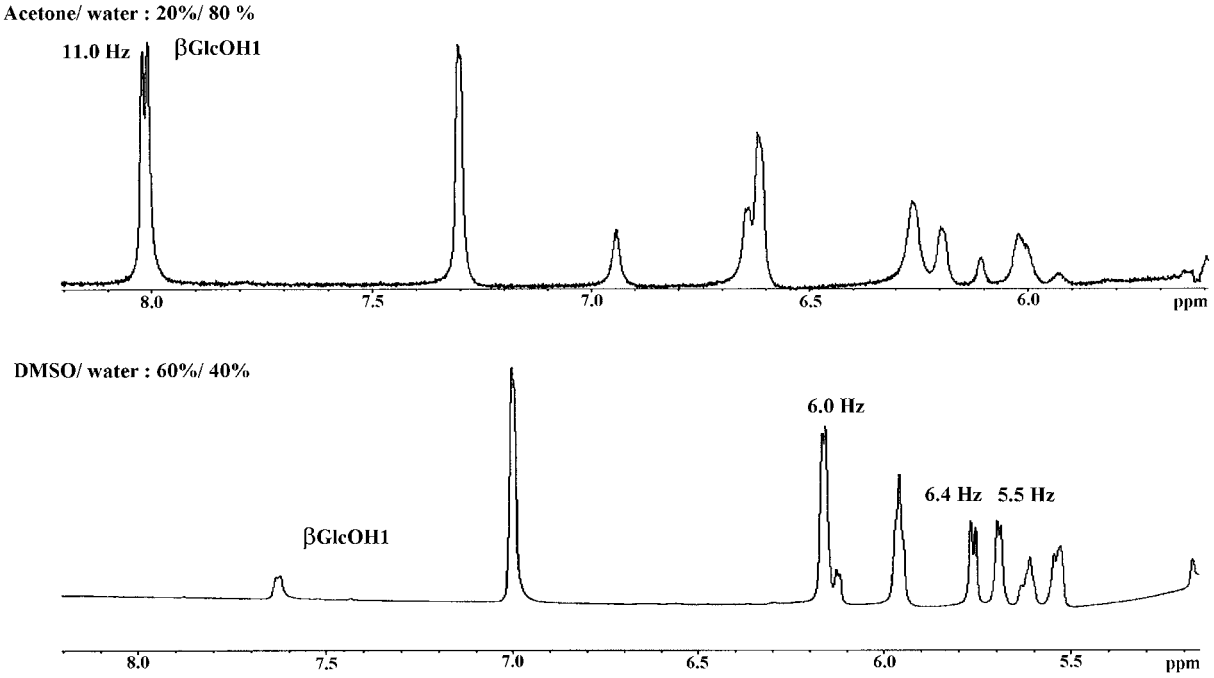

Figure 1. Relevant part (hydroxyl region) of a 1D ^{1}H-NMR spectrum of lactose in binary mixtures of acetone$_{d6}$:H_2O (2:8 ratio, v/v) – top – or DMSO$_{d6}$:H_2O (6:4 ratio, v/v) – bottom-measured at 500 MHz and −12 °C. Signal splitting is indicated for possible peaks.

bearing on the measured NMR-spectroscopic data for this disaccharide.

A factor which should be closely paid attention to in the experiments reported here is the pH value. Although we worked with charge-free carbohydrates, the documented importance of sulfated and sialylated glycans in cell adhesion and phosphorylated glycans for intracellular routing, as reviewed recently (Gabius, 1997; Hooper et al., 1997; Angata and Brinkman-van der Linden, 2002; Dahms and Hancock, 2002), will make it desirable to run experiments also with charged ligands. Thus, we next examined, if the pH will affect the signal pattern of the spectra. Figure 3 indicates that pH variations had a significant influence. At pH values below 6 line-broadening effects are pronounced due to exchange processes. The physiological pH range appeared to establish optimal conditions. Also at pH values above 8 signal broadening was significant.

The next parameter change concerned the temperature. The experiments were performed to eventually carry out trNOE measurements with receptors such as lectins. For this application, the possibility of a temperature increase into the range above water's freezing point would be welcome. Also, it would then no longer be required to work with supercooled solutions of saccharides. Stepwise increases of the temperature from −14.4 and 20 °C led to an upfield shift of the exchangeable proton signals (Table 6, Table 7, Figure 4). However, the signals can still be seen without significant line-broadening effects at temperatures above 0 °C (Figure 4). In this figure we also document the line-splitting of αGlcOH2 and βGlcOH3 at different temperatures. For comparison, the line-splitting of these signals in the two monosaccharides is listed in Table 2. Next, Table 8 shows the temperature dependence of the half-width line-broadenings of selected hydroxyl proton signals of lactose in DMSO$_{d6}$:water or acetone$_{d6}$:water mixtures. Our data indicate that the half-width line-broadening is modulated when raising the temperature. Temperature effects on the exchange rate, viscosity of the mixture and/or mobility of the saccharide can affect this process. Topological parameters of the disaccharide can be calculated to gauge how access to these parameters might enable one to refine the data of the disaccharide's solution structure. For this purpose, the NOE build-up was determined between hydroxyl protons and spatially neighboring protons. The results are listed in Table 9 (for a binary DMSO$_{d6}$:water mixture of 6:4 ratio, v/v) and in Table 10 (for an acetone$_{d6}$:water mixture of 2:8 ratio, v/v). Tables 9 and 10 document that in the case of the DMSO$_{d6}$:water mixture (6:4 ratio, v/v) mixing times

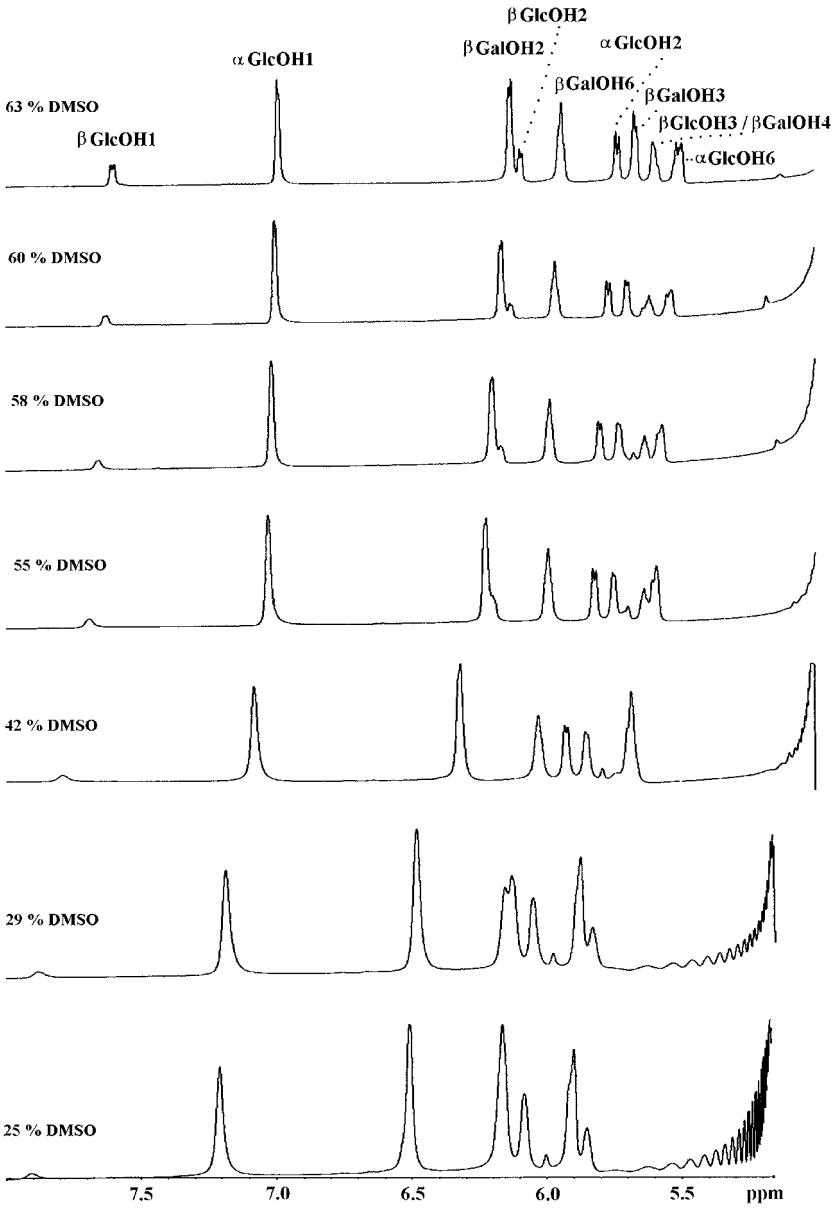

Figure 2. Relevant part of a 1D ^{1}H-NMR spectrum of lactose in binary $DMSO_{d6}$:H_2O mixtures measured at 500 MHz and −12 °C at different volume ratios of $DMSO_{d6}$/water.

of up to 200 ms and in the case of $acetone_{d6}$:water mixture (2:8 ratio, v/v) of up to 150 ms turned out to be suitable for the NOE-based measurements without significant distortions caused by spin diffusion.

In combination with the proton-proton coupling constants J_{HH} it was thus possible to delineate the topology of the pendant groups in the free state. Distinct J_{HH} coupling constants of the non-exchangeable protons were measured in both solvent mixtures as reference, i.e., $DMSO_{d6}$:water mixture (6:4 ratio, v/v) and $acetone_{d6}$:water mixture (2:8 ratio, v/v). As expected they were equal: βGlcH1: 7.3 Hz, βGlcH2: 6.8 Hz and βGalH1: 8.1 Hz. Coupling constants of exchangeable protons were then assessed to sort out to what extent this property helps structure analysis. The hydroxyl-group signals were clearly visible in both solvent mixtures. However, in the $acetone_{d6}$:water mixture (2:8 ratio, v/v) they were not sufficiently

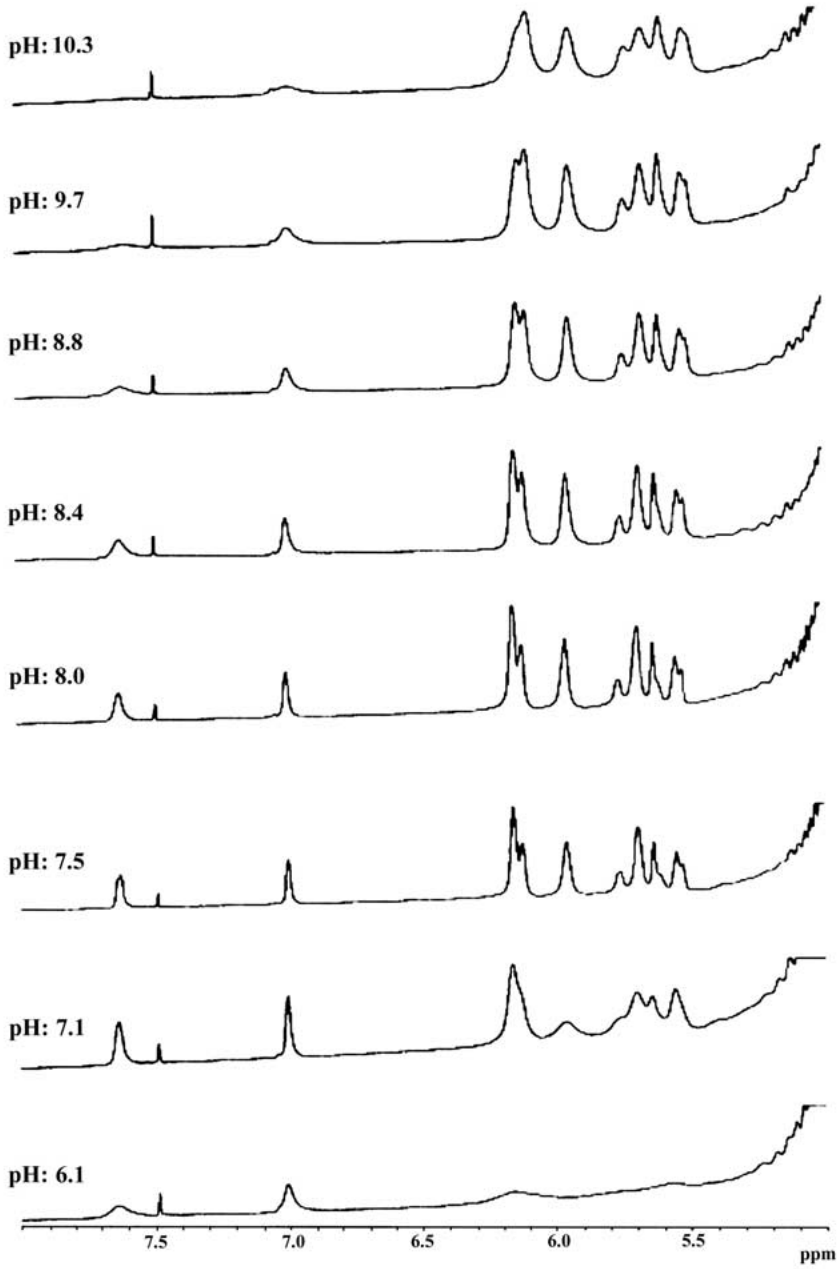

Figure 3. Relevant part of a 1D ^{1}H-NMR spectrum of lactose in binary mixtures of $DMSO_{d6}$:H_2O (6:4 ratio, v/v) measured at 500 MHz and −12 °C at different pH values.

sharp to compare the coupling constants of the pendant groups of lactose reliably under the different conditions. In this context, the coupling constant of βGlcOH1 is an exception. Its value was measured at 11 Hz in the $acetone_{d6}$:water mixture (2:8 ratio, v/v) (Figure 1) and at 10 Hz in the $DMSO_{d6}$:water mixture (63:37 ratio, v/v).

The following step was to analyze the contribution of this new information to the description of interproton distances and hydrogen-bond patterns of the disaccharides. Comparison of the OH-H contacts of lactose in the two solvent mixtures ($DMSO_{d6}$:water (6:4 ratio, v/v) and $acetone_{d6}$:water (2:8 ratio, v/v)), as compiled in Tables 9 and 10, led to the following conclusion:

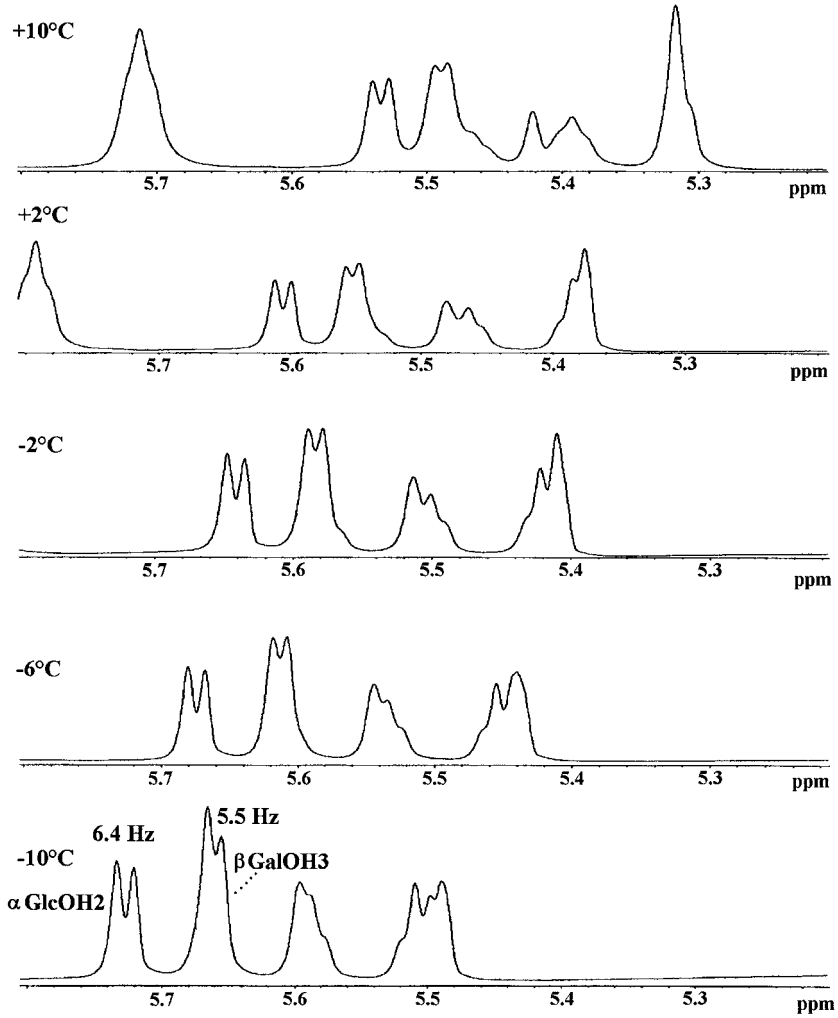

Figure 4. Relevant part of a 1D ^{1}H-NMR spectrum of lactose in a binary mixture of $DMSO_{d6}$:H_2O (6:4, v/v) measured at 500 MHz and different temperatures. Signal splitting is indicated for the αGlcOH2 and βGlcOH3 peaks.

The measured average distances between βGlcOH1-βGlcH1 (2.3 Å) and βGlcOH1-βGlcH2 (2.8–2.9 Å) are nearly equal in both solvents. In addition to the measured coupling constant of βGlcOH1 which has similar size in both solvent mixtures (10 Hz in the $DMSO_{d6}$:water mixture (63:37 ratio, v/v) and 11 Hz in the $acetone_{d6}$:water (2:8 ratio, v/v) mixture) the accordance of the corresponding distances is a second indication that the orientation of the hydroxyl groups was not markedly affected by the solvent change. It is also remarkable that distance parameters were often not altered, e.g., βGlcOH3-βGlcH4 (2.8 Å/ 2.8 Å), or only slightly altered, e.g. βGlcOH2-βGlcH2 (2.7 Å/ 2.9 Å), when changing the solvent (Tables 9 and 10). Whereas these topological parameters were not or not strongly dependent on the solvent properties, other distances proved to be more sensitive to the conditions of the environment, i.e., βGalOH2-βGalH3 (2.4 Å/3.0 Å) and βGalOH3-βGalH3 (2.4 Å/2.8 Å), as determined at a mixing time of 100 ms (Tables 9 and 10). A likely reason for this experimentally detected effect could be found in different conformational preferences of the βGalOH2- and βGalOH3-groups in the two solvent mixtures, which will be discussed in connection with hydrogen-bond formation.

In this context, it is further informative to relate these data to the measured temperature coefficients. The observed differences in the distance separating certain OH—H contacts examined in the two solvent mixtures are in line with the differences between

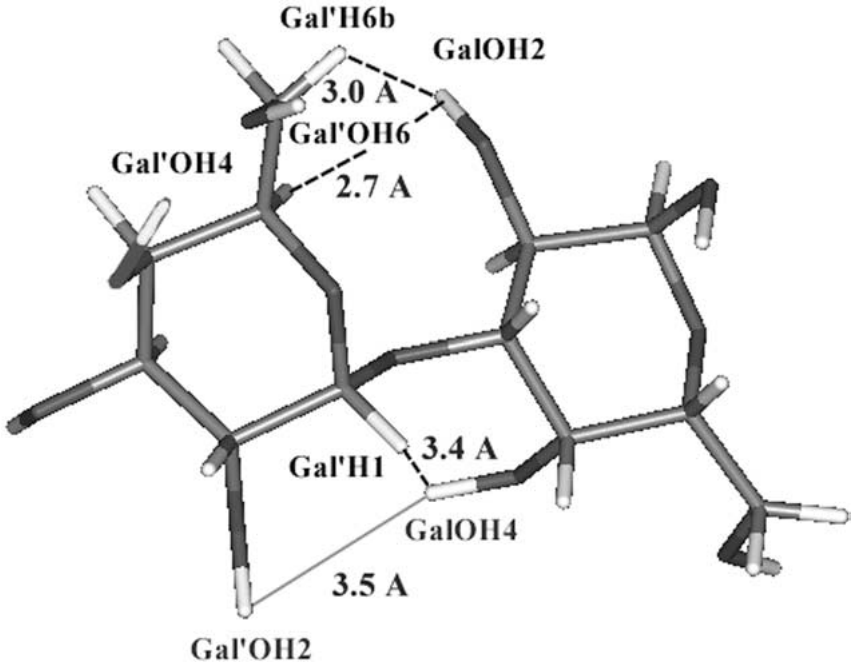

Figure 5. Molecular model of the conformation of Gal′α1-3Galβ derived from the NOE data of the free-state disaccharide dissolved in a binary mixture of $DMSO_{d6}$:H_2O (6:4 ratio, v/v). The measurable interresidual proton distances in which hydroxyl protons are involved are drawn (- - - - -) and their values are given. The distance Gal′OH2 – GalOH4 is also marked (—). Notably, the corresponding NOE was not detectable in the $DMSO_{d6}$:H_2O (6:4 ratio, v/v) solvent mixture.

Table 5. Effects of volume ratio of $DMSO_{d6}$ and concentration of lactose on positions of chemical shifts of hydroxyl protons in binary $DMSO_{d6}$:water mixtures at −12 °C

Lactose (mM)/ DMSO content	52/ 25	65.4/ 25	62.3/ 29.2	59.5/ 33	56.9/ 36.3	52.3/ 36.3	48.4/ 47.1	45.1/ 51.1	42.2/ 54.7	39.6/ 57.7	37.4/ 60.4	32.5/ 62.5	15/ 64.0
βGalOH2	6.63	6.51	6.48	6.45	6.43	6.38	6.33	6.28	6.34	6.20	6.16	6.12	6.07
βGalOH3	6.21	6.09	6.05	6.01	5.98	5.92	5.88	5.82	5.77	5.73	5.69	5.66	5.62
βGalOH6	6.03	6.17	6.16	6.14	6.12	6.10	6.07	6.04	6.01	5.99	5.96	5.93	5.89
βGlcOH1	8.03	7.92	7.89	7.86	7.84	7.80	7.76	7.72	7.69	7.66	7.63	7.60	7.57
βGlcOH2	6.64	6.51	6.49	6.45	6.43	6.38	6.33	6.28	6.21	6.17	6.12	6.08	6.03
βGlcOH3	6.11	6.01	5.98	5.95	5.92	5.87	5.81	5.77	5.72	5.67	5.63	5.60	5.54
βGlcOH6		5.90	5.88	5.85	5.82	5.82	5.79	5.77	5.74	5.64	5.61	5.6	
αGlcOH1	7.32	7.21	7.19	7.16	7.14	7.11	7.08	7.06	7.03	7.02	7.00	6.99	6.97
αGlcOH2	6.28	6.17	6.13	6.09	6.06	6.00	5.94	5.89	5.84	5.80	5.76	5.73	5.68
αGlcOH3	6.11	6.01	5.98	5.95	5.92	5.87	5.81	5.77	5.72	5.67	5.63	5.60	5.54
αGlcOH6	5.96	5.85	5.83	5.81	5.79	5.76	5.71	5.68	5.62	5.57	5.45	5.51	5.46

Table 6. Chemical shifts of hydroxyl protons of lactose in a $DMSO_{d6}$:water mixture (6:4 ratio (v/v)) as a function of the temperature

Temperature (°C)	βGlcOH1	βGlcOH2	βGlcOH3	αGlcOH1	αGlcOH2	αGlcOH3	αGlcOH6	βGalOH2	βGalOH3	βGalOH4	βGalOH6
−12	7.60	6.09	5.69	6.99	5.76	5.51	5.49	6.13	5.93	5.60	5.60
−10	7.59	6.07	5.66	6.98	5.71	5.48	5.47	6.11	5.91	5.58	5.57
−8	7.57	6.05	5.63	6.97	5.69	5.47	5.46	6.10	5.90	5.56	5.56
−6	7.56	6.03	5.62	6.95	5.68	5.46	5.44	6.08	5.87	5.55	5.54
−4	7.54	6.01	5.60	6.94	5.66	5.44	5.42	6.06	5.85	5.53	5.52
−2	7.53	6.00	5.58	6.93	5.64	5.42	5.41	6.04	5.83	5.51	5.50
2	7.50	5.96	5.55	6.90	5.61	5.38	5.38	6.01	5.79	5.48	5.46
6	7.47	5.92	5.52	6.88	5.57	5.35	5.35	5.97	5.75	5.45	5.43
10	7.44	5.88	5.49	6.86	5.53	5.32	5.32	5.94	5.71	5.42	5.39
20	7.36	5.79	5.35	6.79	5.44	5.25	5.23	5.85	5.62	5.35	5.30
k	7.5	6.2	9.1	8.6	8.8	9.9	9.5	7.5	9.1	8.1	8.1

k: Temperature coefficient in $-(\text{ppm/K}) \times 10^{-3}$.

Table 7. Chemical shifts of hydroxyl protons of lactose in a $acetone_{d6}$:water mixture (2:8 ratio (v/v)) as a function of the temperature

Temperature (°C)	βGlcOH1	βGlcOH2	βGlcOH3	αGlcOH1	αGlcOH2	αGlcOH3	αGlcOH6	βGalOH2	βGalOH3	βGalOH4	βGalOH6
−14.4	8.04	6.67	6.13	7.32	6.29	6.04	5.96	6.64	6.21	–	6.04
−11.9	8.01	6.64	6.11	7.30	6.26	6.00	5.93	6.61	6.20	6.01	6.01
−10	8.00	6.62	6.08	7.29	6.23	5.98	5.91	6.59	6.18	6.00	6.00
−8.1	7.98	6.57	6.06	7.27	6.21	5.95	5.88	6.56	6.15	5.97	5.97
−6	7.96	6.55	6.03	7.27	6.18	5.94	5.86	6.55	6.13	5.95	5.95
−4	7.94	6.53	6.01	7.23	6.16	5.91	5.84	6.53	6.11	5.93	5.93
−2	7.92	6.50	5.99	7.23	6.14	5.88	5.82	6.50	6.09	5.90	5.90
0.1	7.89	6.48	5.96	7.19	6.11	–	–	6.48	6.07	5.88	–
2	7.88	6.46	5.94	7.18	6.10	–	–	6.46	6.05	5.86	–
4	7.86	6.44	5.92	7.18	–	–	–	6.44	6.02	5.83	–
6	7.84	6.41	5.90	7.14	–	–	–	6.41	6.00	–	–
8	7.82	6.39	5.87	7.12	–	–	–	6.39	5.98	–	–
10	7.80	6.36	5.85	7.10	–	–	–	6.36	5.96	–	–
k	9.7	9.0	12.4	11.2	11.5	10.3	11.6	11.4	11.1	12.7	10.9

k: Temperature coefficient in $-(\text{ppm/K}) \times 10^{-3}$.
−: Not assigned due to signal overlap or extensive line-broadening.

Table 8. Half-width line-broadenings of selected lactose signals [Hz] measured in $DMSO_{d6}$:water (6:4 ratio (v/v)) or $acetone_{d6}$:water mixtures (2:8 ratio (v/v))

Temperature (°C)	βGlcOH3 $DMSO_{d6}$	αGlcOH2 $DMSO_{d6}$	βGalOH3 $DMSO_{d6}$	βGlcOH1 $acetone_{d6}$	βGlcOH3 $acetone_{d6}$	αGlcOH2 $acetone_{d6}$	βGalOH3 $acetone_{d6}$
−10	12.5	10.8	13.5	16.7	12.5	20.1	16.7
−6	10.8	11.8	12.7	16.9	12.4	23.4	17.2
−2	12.7	10.9	12.6	17.1	13.0	25.8	17.4
2	11.8	11.9	12.5	17.0	13.1	30.9	17.7
10	10.9	12.8	12.5	17.5	13.3	–	18.0

−: Not determined since the signal disappeared due to exchange effects.

Table 9. NOE values and calculated interproton distances at various mixing times for lactose [15 mM] in a binary $DMSO_{d6}$:water mixture (6:4 ratio, v/v) at 500 MHz and −12 °C

	100 ms	Dist. (Å) (100 ms)	200 ms	Dist. (Å) (200 ms)	300 ms	400 ms
βGalH1/βGalH3	7.2	2.7	11.8	2.7	14.9	16.0
βGalH1/βGlcH4	7.1	2.7	11.5	2.7	3.4	4.0
βGalOH2/βGalH2	22.3	2.2	33.6	2.3	37.7	29.6
βGalOH2/βGalH3	16.0	2.4	20.1	2.5	23.4	31.7
βGalOH2/βGalOH3	4.3	2.9	4.4	3.2	4.8	3.9
βGalOH3/βGalH2	9.9	2.6	16.7	2.5	20.2	19.4
βGalOH3/βGalH3	15.6	2.4	20.2	2.5	27.4	21.9
βGalOH3/βGalH5	3.6	3.0	7.7	2.9	8.6	7.0
βGalOH6/βGalH4	9.2	2.6	12.2	2.7	11.4	10.8
βGalOH6/βGalH6b	4.2	3.0	4.7	3.1	5.8	5.9
βGlcOH1/βGlcH1	21.3	2.3	29.8	2.3	29.6	28.8
βGlcOH1/βGlcH2	5.7	2.8	8.4	2.9	9.7	10.1
βGlcOH1/βGlcH3	2.9	3.1	–	–	6.3	8.3
βGlcOH2/βGlcH1	2.4	3.2	2.9	3.4	3.6	–
βGlcOH2/βGlcH2	8.0	2.7	11.8	2.7	13.2	12.0
βGlcOH2/βGlcH3	4.0	3.0	6.5	3.0	9.3	5.4
βGlcOH3/βGlcH2	4.0	3.0	6.5	3.0	7.6	8.2
βGlcOH3/βGlcH3	16.7	2.3	21.9	2.4	28.2	29.7
βGlcOH3/βGlcH4	6.0	2.8	8.7	2.8	7.4	6.7
βGlcOH6/βGlcH6	4.5	2.9	6.6	3.0	6.9	7.0
αGlcOH1/αGlcH1	12.7	2.5	19.3	2.5	22.2	22.4
αGlcOH1/αGlcH5	4.2	3.0	5.7	3.0	8.0	6.6
αGlcOH3/αGlcH2	6.2	2.8	9.1	2.8	9.8	9.3
αGlcOH3/αGlcH3	5.2	2.9	5.3	3.1	6.4	6.6
αGlcOH3/αGlcH4	9.0	2.6	16.6	2.5	18.3	15.7
αGlcOH6/αGlcH5	–	–	6.1	3.0	14.2	7.9
αGlcOH6/αGlcH6	13.8	2.4	21.0	2.5	22.4	20.6

–: Not detected due to an insufficient NOE response.

the temperature coefficients k calculated from the ppm/K (chem. shift/ T) data derived for lactose in $DMSO_{d6}$:water (6:4 ratio, v/v) or in $acetone_{d6}$:water (2:8 ratio, v/v) mixtures (Tables 6 and 7). The calculated k-values for lactose in $DMSO_{d6}$:water (6:4 ratio, v/v) were in general lower than the k-values for lactose in $acetone_{d6}$:water (2:8 ratio, v/v). Assuming that the strength of hydrogen bonds will be enhanced in pure $DMSO_{d6}$ relative to that in water, the size of the temperature coefficients k should increase by raising the volume ratio of water in the mixture. It is thus important to note that mixtures apparently have the advantage to suppress hydrogen-bonding characteristics for the disaccharide present in an aprotic solution which can be considered as physiologically irrelevant or even misleading. The primary role of water appears to be to disrupt intramolecular hydrogen binding, as theoretically inferred elsewhere (Kirschner and Woods, 2001). In this respect, a graphic example has been published: An intramolecular hydrogen bond for OH3—O5′ in methyl β-cellobiose could be seen in $DMSO_{d6}$ that was hardly detectable in H_2O-CH_3OD (4:1, w/w) (Leeflang et al., 1992; Kroon et al., 1994). By referring to data from the literature we can get further information concerning a relationship between the presence of water and the k values for lactose as part of an oligosaccharide. In detail, the k-values of the Galβ1-4Glc part of the glycan chain of ganglioside

Table 10. NOE values and calculated interproton distances at various mixing times for lactose [15 mM] in a binary acetone$_{d6}$:water mixture (2:8 ratio, v/v) at 500 MHz and −12 °C

	100 ms	Dist. (Å) (100 ms)	150 ms	Dist. (Å) (150 ms)	200 ms	300 ms
βGalH1/βGlcH4	–	–	0.53	2.6	1.43	2.64
βGalH1/βGalH6a	–	–	0.36	2.8	0.94	1.51
βGalH1/βGalH6b	–	–	0.42	2.8	1.60	2.88
βGalOH2/βGalH2	0.74	2.4	0.86	2.4	0.31	0.15
βGalOH2/βGalH3	0.21	3.0	0.24	3.0	0.54	0.72
βGalOH3/βGalH3	0.28	2.8	–	–	0.66	0.73
βGalOH4/βGalH4	0.60	2.5	0.67	2.5	–	0.84
βGalH4/βGalH3	–	–	0.66	2.5	–	–
βGlcH1/βGlcH2	–	–	0.67	2.5	0.51	1.07
βGlcH1/βGlcH3	0.47	2.6	–	–	4.72	7.31
βGlcH1/βGlcH5	0.23	2.9	0.48	2.6	2.24	3.80
βGlcOH1/βGlcH1	0.85	2.3	1.09	2.3	1.30	0.62
βGlcOH1/βGlcH2	0.26	2.9	–	–	0.26	0.37
βGlcOH2/βGlcH2	0.22	2.9	0.44	2.7	0.47	0.70
βGlcOH3/βGlcH4	0.31	2.8	–	–	0.33	0.52
βGlcH4/βGlcH2	–	–	0.43	2.7	4.91	7.22
βGlcOH6/βGlcH6a	0.21	3.0	–	–	0.12	0.07
βGlcOH6/βGlcH6b	–	–	0.28	2.9	–	–
αGlcOH1/αGlcH5	0.22	2.9	–	–	0.45	0.50

–: Not detected due to an insufficient NOE response.

GM_3 in pure $DMSO_{d6}$ (Siebert et al., 1992) revealed that even the relatively large values between 4.2 and 5.8 are significantly below those presented in Tables 6 and 7. Temperature coefficients of OH-protons which are involved in stable hydrogen bonds (see below) are generally smaller than 2 ($k < 2$). This is only the case for βGalOH2 (0.8) and βGlcOH3 (1.7) of the Galβ1-4Glc part of the GM_3 ganglioside in pure $DMSO_{d6}$ (Siebert et al., 1992).

With regard to the topology of the glycosidic linkage, the free-state conformations could readily be attributed to the energy valley of the global minimum on the basis of the detectable GalH1-GlcH4 contact listed in Tables 9 and 10. Remarkably, this topology equals that of the *syn*-state conformation of the Galβ1-4Glc-linkage in oligosaccharides, with dihedral angles of the glycosidic linkage Φ of about 60° and Ψ of about 0° in water (Φ: H1-C1-O-C2, Ψ: C1-O-C2-H2) (Breg et al., 1989; Grönberg et al., 1994; Asensio and Jiménez-Barbero, 1995; Asensio et al., 1995a, b, 1999; Espinosa et al., 1996, 1998; Casset et al., 1997; Alonso-Plaza et al., 2001) and in pure $DMSO_{d6}$ (Dabrowski and Poppe, 1989; Acquotti et al., 1990; Poppe et al., 1990a, b; Ejchart et al., 1992; Siebert et al., 1992). The question arises as to whether there are further detectable constraints with involvement of hydroxyl protons, which are suitable for defining the Φ, Ψ angles of the glycosidic linkage. Based on the mentioned data obtained the hydrogen bond between GlcOH3—GalO5 in the lactose part of the GM_3 ganglioside in pure $DMSO_{d6}$ characterized by a small coupling constant (3.1 Hz) and temperature coefficient (1.7 [−(ppm/K) × 10^{-3}]) (Siebert et al., 1992), might be a possible candidate. In the $DMSO_{d6}$:water mixture (6:4 ratio, v/v), however, this constraint was significantly weaker than in pure $DMSO_{d6}$ as indicated by a coupling constant of 10 Hz and a temperature coefficient of 7.5 [−(ppm/K)×10^{-3}] (Table 6) for the GlcOH3 proton, rendering it insufficient for improving the quality of conformational description. Furthermore, the measurements question the relevance of this contact under physiological conditions. While there are cases with differences in the preferred orientations of hydroxyl protons when comparing lactose in $DMSO_{d6}$:water (6:4 ratio, v/v) and in acetone$_{d6}$:water (2:8 ratio, v/v) (see Tables 9 and 10), it can be con-

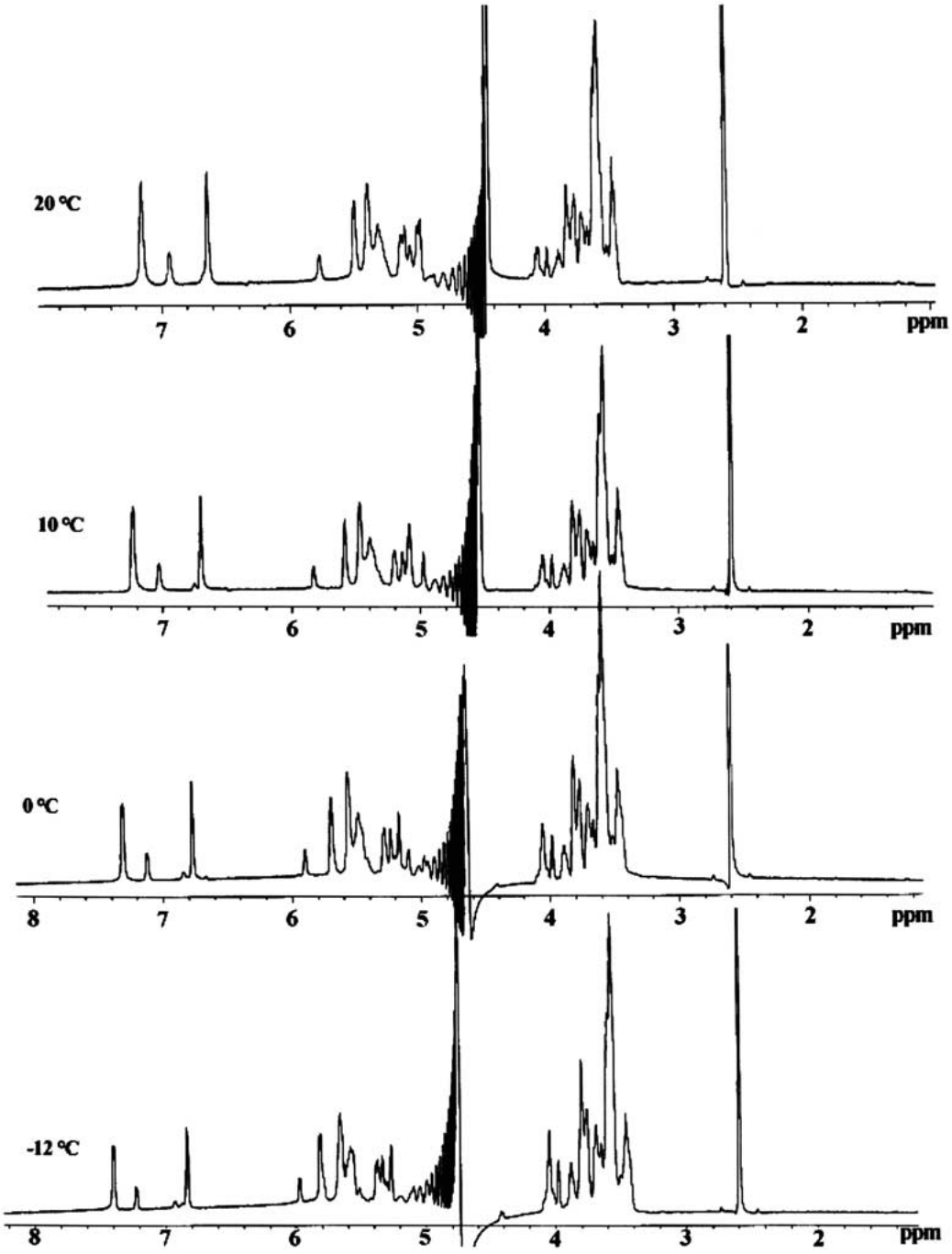

Figure 6. Relevant part of a 1D ^{1}H-NMR spectrum of Gal′α1-3Gal in $DMSO_{d6}$:H_2O (6:4 ratio, v/v) measured at 500 MHz and various temperatures.

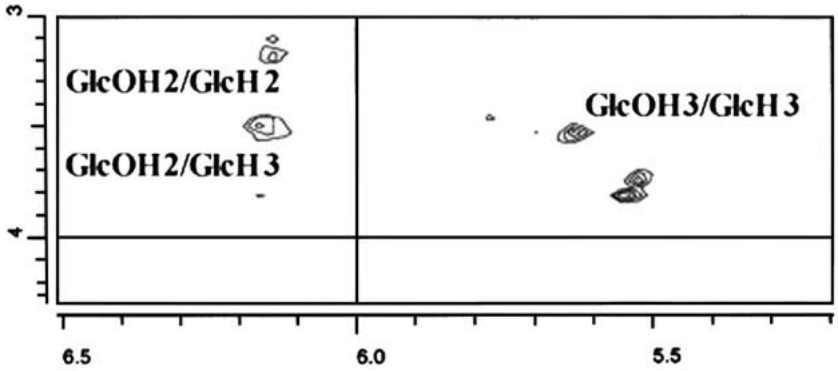

Figure 7. Relevant part of a 2D trNOESY spectrum of lactose in a binary mixture of $DMSO_{d6}$:H_2O (6:4 ratio, v/v) in the presence of VAA measured at 500 MHz and −12 °C with a mixing time of 200 ms, showing intraresidual contacts involving hydroxyl protons.

cluded that no stable intramolecular hydrogen bond is present in the tested mixtures. In summary, this analysis of proton signals enabled to monitor hydroxyl orientation in lactose. The new information failed to significantly add precision to the determination of the Φ/Ψ-angles of the glycosidic bond beyond the already given levels in this case. However, this result cannot be generalized. An improvement was detectable in the case of Gal′α1-3Galα/β and Gal′α1-3Gaβ1-OCH_3 (the Gal′-labeling is used to distinguish between the two Gal-residues). The chemical shifts of the proton resonances in the binary mixture of $DMSO_{d6}$ and water (6:4 ratio, v/v) can be directly correlated with those in pure $DMSO_{d6}$ solution, which we had reported previously (Siebert et al., 2000).

For Gal′α1-3Galα/β and Gal′α1-3Galβ1-OCH_3, too, k-values were calculated on the basis of our experimental data (Figure 6). The values are in the same range as those obtained for lactose in a $DMSO_{d6}$:water mixture (6:4 ratio, v/v), listed in Table 6. The k-value determined for the GalOH1 proton of Gal′α1-3Gal in the temperature interval between −12 and 20 °C is 7.2 [−(ppm/K) × 10^{-3}]. No evidence for the existence of intra- and intermolecular hydrogen bonds was found for Gal′α1-3Galβ1-OCH_3 in this solvent mixture. To complete the conformational analysis, the NOE values were measured (Table 11) and the calculated interproton distances obtained for Gal′α1-3Galα/β or Gal′α1-3Galβ1-OCH_3 (also shown in Table 11) were used to determine the dihedral angles of the preferred orientations of distinct pendant groups.

Previously, we had found for this ligand of sugar receptors how the quality of the topological description was refined by measurements in the aprotic solvent for the ligand free in solution and bound to human immunoglobulin G molecules (Siebert et al., 2000). The interproton distance between Gal′OH2 and GalOH4 of the Gal′α1-3Gal linkage was determined to be 3.1 Å in pure $DMSO_{d6}$ solution (Siebert et al., 2000). In the $DMSO_{d6}$:water mixture (6:4 ratio, v/v) this NOE contact could no longer be detected. The corresponding interproton distance must therefore be larger than 3.4 Å. In this instance, the recruitment of the otherwise elusive hydroxyl protons to this analysis enabled an elaborate conformational description. Figure 5 illustrates the involvement of distinct hydroxyl protons in interresidual contacts which are the new

Table 11. NOE values and calculated interproton distances at the mixing time of 100 ms for Galα1-3Gal [15 mM] in a binary $DMSO_{d6}$:water (6:4 ratio, v/v) mixture at 500 MHz and −12 °C

	100 ms	Dist. (Å)
αGalOH2/αGalH2	7.9	2.4
αGalOH2/αGalH3	7.5	2.4
αGalOH3/αGalH3	9.2	2.3
αGalOH3/αGalH2	2.5	2.9
αGalOH4/αGalH4	3.7	2.7
αGalOH4/αGalH2	1.9	3.0
αGalOH4/αGalOH6	9.8	2.3
βGalOH1/βGalH2	10.3	2.3
βGalOH1/βGalH2	4.9	2.6
βGalOH2/βGalH2	12.5	2.2
βGalOH2/βGalH3	7.0	2.4
βGalOH2/αGalH6b	1.9	3.0
βGalOH2/αGalH5	3.7	2.7
βGalOH4/αGalH1	0.9	3.4

sensors for the conformational analysis of the Gal′α1-3Gal linkage. Evidently, the Gal′H1-GalOH4 and the Gal′H5-GalOH2 as well as the GalH6b-GalOH2 contacts can be reconciled with the *syn*-state topology of the Gal′α1-3Gal linkage. The global minimum conformation of the glycosidic linkages was determined with refined precision in a small area around ($\Phi = -30$, $\Psi = -30$). Molecular mechanics calculations and molecular dynamics simulations as well as NMR measurements in water and in an aprotic solvent had indicated that this topology is the preferred low-energy conformation in aqueous solution or in an aprotic solvent (Lemieux et al., 1980; Hindsgaul et al., 1982; Poppe et al., 1990b, 1991; Li et al., 1999; Siebert et al., 2000; Corzana et al., 2002; Tempel et al., 2002). Our previous study on this tumor- and xenoreactive antibody fraction from human serum in pure $DMSO_{d6}$ and a recent crystallographic study on the plant lectin GSI-B_4, which share Galα1-3Gal specificity (Dong et al., 1995, 1997), had demonstrated that the *syn*-state topology is selected for binding by these two receptor proteins (Siebert et al., 2000; Tempel et al., 2002). Regarding conformational analysis it is further instructive to consider the GalNAcα1-3GalNAc-linkage. In this case a number of interresidual contacts leading to NOEs in which an N-acetyl group is involved play the role of the exchangeable hydroxyl protons (Poppe et al., 1991). As this example attests, the solvent mixture offers the possibility to pick up new constraints for a refined topological analysis of the free ligand.

Since the temperature could be raised considerably above the freezing point without losing the information from the hydroxyl protons of lactose (Tables 6, 7, Figure 4), similar experiments were carried out with Gal′α1-3Galα/β and Gal′α1-3Galβ1-OCH_3. Actually, it turned out to be possible to measure OH-proton signals for the Gal′α1-3Gal linkage in a $DMSO_{d6}$:water mixture not only at low temperatures (−12 °C) but also at temperatures above 0 °C in the same quality (Figure 6). With our method a temperature window into the commonly set range from 20°– 40 °C for biomacromolecules has now been opened. It is interesting to test whether it can be used to run meaningful trNOE experiments with a lectin at various temperatures. Special reasons can require to perform measurements at lower temperatures. Due to unfavorable signal overlap and desirable modulation of the exchange rate between ligand and receptor to achieve the optimal trNOE conditions NMR measurements had to be carried out at 5 °C for the plant lectin concanavalin A (Sayers and Prestegård, 2002).

In order to demonstrate the suitability of our approach using hydroxyl protons as sensors for the conformational analysis of the bound ligand by trNOESY in mixtures of aprotic media and water, we chose lactose and the mistletoe lectin (VAA) as model system. Consequences of binding of lactose to the lectin were analyzed in a $DMSO_{d6}$:water mixture (6:4 ratio, v/v). We were able to detect distinct trNOE signals at −12 °C (Figure 7). In view of our aim to recruit hydroxyl protons as additional sensors for a refined topological analysis of the ligand's bound state the trNOE signals involving GlcOH2 and GlcOH3 are of special interest. This aspect of the bound ligand's structure provided information adding to our previous analysis in D_2O (Gilleron et al., 1998). The experimental access to the topological definition of the positions of the two hydroxyl groups, as discussed in the next paragraph, argued in favor of a mutual influence of the binding process on these exchangeable Glc-protons (for comparison to data obtained by chemical mapping with lactose derivatives such as O-methyl-bearing ligands (Rüdiger et al., 2000)).

Using the intraresidual trNOE signals (Figure 7), the positioning of the two OH-groups from the βGlc-residue could be determined and compared with that of the free state. Most importantly, the GlcOH3 group adopted a position in the bound state, which is in agreement with formation of the GlcOH3- - -GalO5 hydrogen bond, discussed above. Its occurrence in our binary mixtures is apparently restricted to the bound state, as indicated by the comparison of the data in pure $DMSO_{d6}$ with our results in the mixtures. By the way, systematic measurements with β-lactoside derivatives in $DMSO_{d6}$ or water had revealed that this contact is not essential to let the disaccharide adopt its low-energy *syn*-state conformation (Rivera-Sagredo et al., 1991a, b; Fernandez and Jiménez-Barbero, 1993). When further examining the ligand's groups in the complex, the βGlc2OH-group orientation in the bound state was fixed in a distinct way. Obviously, the hydroxyl rotation was restricted after being accommodated into the binding site. The derived information on the bound-state structure especially of the mentioned hydroxyl groups is visualized in Figure 8. It equals the *syn*-conformation, as likewise seen in pure $DMSO_{d6}$ for the Gal′α1-3Gal linkage in the free state and for the Gal′β1-3Gal linkage in the presence of VAA at 30 °C (Siebert et al., 2000). The interresidual Gal′H1-GalH3 and Gal′H1-GalH4 contacts had

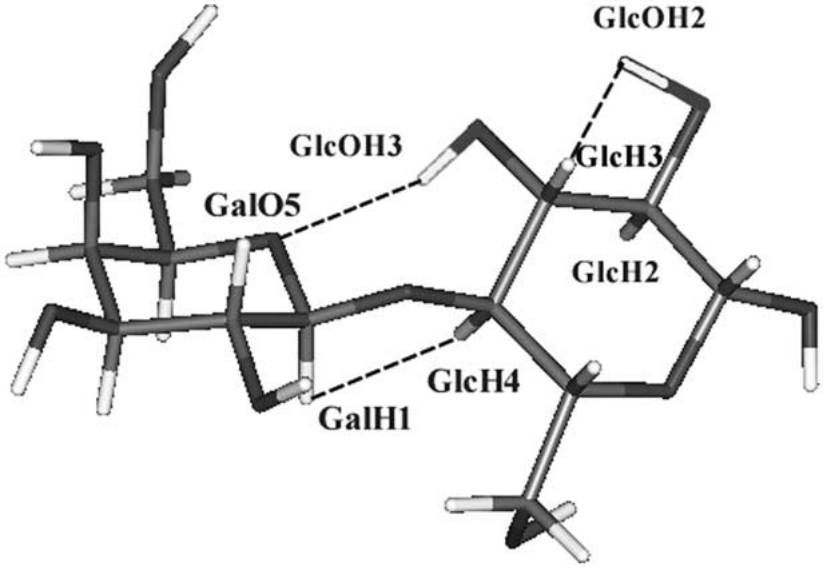

Figure 8. Molecular model of Galβ1-4Glcβ (lactose) derived from the NOE data of the VAA-bound disaccharide dissolved in a binary $DMSO_{d6}$:H_2O mixture (6:4 ratio, v/v). The experimentally determined orientations of the hydroxyl protons GlcOH2 and GlcOH3 are shown.

also been detectable for both disaccharide linkages (Gal′α1-3Gal and Gal′β1-3Gal) in a preliminary monitoring with this lectin in a binary solvent mixture containing water:$DMSO_{d6}$ (6:4 ratio, v/v) (Siebert et al., 2000). In the case of lactose, the measurable interresidual distance between Gal′H1-GlcH4 of about 2.7 Å is indicative for a bound-state conformation reminiscent of the global minimum conformation of the free state (Asensio et al., 1999; Alonso-Plaza et al., 2001). The fact that VAA accommodated β-galactosides in the *syn*-state in water (Alonso-Plaza et al., 2001) proves that neither the ligand nor the protein were subject to distortions of bioactive conformations. This result underlines that NOE and trNOE measurements in mixtures can characterize bioactive conformations at a refined level relative to measurements in water. It encourages to proceed to test our strategy on lectin-carbohydrate complexes at higher temperatures to define the upper temperature limit for successful trNOE measurements. Similarly, the use of mixtures may restrict the harmful effect of the aprotic solvent on the binding capacity of small lectins such as the tested plant protein hevein and carbohydrate-binding peptides obtained by phage-display screening (Siebert et al., 2000, 2002).

Conclusions

It was our aim to answer the question whether mixtures of water with an aprotic solvent could provide the opportunity to recruit water-exchangeable hydroxyl protons of the carbohydrate ligand as confomational sensors while limiting any harmful effect of the aprotic solvent to sensitive structural aspects of the reactants. By variations of the disaccharide concentrations, of the ratio between solvent and water, the pH value as well as the temperature we defined suitable conditions for conformational analysis of ligands free in solution. The measured hydrogen bonding within each of the two tested disaccharides in the solvent mixtures corresponded to the data obtained under physiological conditions. The example of the Gal′α1-3Gal compound underscored the potential for refinement of the description of conformational aspects by this approach. Notably, it is suitable even at temperatures well above 0 °C. To prove its usefulness for analyzing protein-carbohydrate interactions, we documented binding of lactose in the low-energy *syn*-state to a plant lectin, being able to define hydroxyl group positioning with refined precision. We conclude that binary solvent mixtures harbor favorable properties for the NMR-spectroscopic analysis of the bound-state topology of a ligand in receptor-ligand complexes. Access to new structural information can be helpful to guide ligand synthesis to lowering the entropic penalty during bind-

ing to the receptor protein, thereby improving the property of carbohydrate ligands to target drugs, to block cell adhesion or to localize receptor sites glycohistochemically in diagnostic procedures (Gabius, 1988, 2001a; Rüdiger et al., 2000; Yamazaki et al., 2000; Camby et al., 2001).

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