The use of chemical shifts of hydroxy protons of oligosaccharides as conformational probes for NMR studies in aqueous solution. Evidence for persistent hydrogen bond interaction in branched trisaccharides

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The ^{1}H NMR chemical shifts, vicinal coupling constants, temperature coefficients, exchange rates with solvent and NOEs have been measured for the hydroxy protons of a series of 15 branched trisaccharides in aqueous solution. These compounds are methyl α-D-galactopyranosides substituted at the 3- and 4-positions with either L-fucosyl or D-glucosyl groups. While most of the hydroxy protons in the trisaccharides have chemical shifts similar to those in the corresponding methyl monosaccharides ($\Delta\delta \leq \pm 0.20$ ppm), some hydroxy protons are found to exhibit large upfield shifts. The NMR data together with HSEA and MM2 calculations show a correlation between these large upfield shifts and the proximity of the hydroxy group to oxygen atoms. The largest upfield shifts are observed for hydroxy protons which are in close proximity to ring oxygens O(5). This dependency of chemical shifts of hydroxy protons on the electronic environment might be used as a conformational probe to improve the determination of conformation of oligosaccharides. The NMR data also show that in three branched trisaccharides, α-D-Glc*p*-(1→3)-[α-D-Glc*p*-(1→4)]-α-D-Gal*p*-OMe, β-L-Fuc*p*-(1→3)-[α-D-Glc*p*-(1→4)]-α-D-Gal*p*-OMe and α-D-Glc*p*-(1→3)-[β-L-Fuc*p*-(1→4)]-α-D-Gal*p*-OMe, there is a persistent hydrogen bond interaction between the O(2′)H of the 3-linked sugar residue and the O(5″) of the 4-linked sugar residue. In the three compounds, a large upfield shift relative to the constituent methyl monosaccharide is observed for the hydroxy proton O(2′)H involved in hydrogen bonding with the O(5″) oxygen. Additional information about the conformation could also be obtained from the inter-residue NOEs involving the exchangeable hydroxy protons. These additional NOEs are in good agreement with a conformation in which the O(2′)H proton and the O(5″) oxygen atoms are involved in a hydrogen bond interaction.

Introduction

It is well recognized that NMR chemical shifts depend on the electron densities around the nuclei which can be influenced by the surrounding environment, and therefore the chemical shifts could contain important structural information. Since in carbohydrates, the effect of the conformation around the glycosidic bond is often limited to the atoms involved in, or close to, the glycosidic linkage, the determination of the tertiary structure by NMR has been dominated by the measurement of coupling constants to derive torsion angles and inter-residue NOEs to derive distance constraints. But since only a limited number of inter-residue NOEs can be observed in ^2H_2O solutions, only poorly defined tertiary structures which represent average conformations can be obtained. In the last few years, it has been shown that by using H_2O solutions instead of ^2H_2O solutions, the number of NOE cross-peaks can be increased by observing the exchangeable hydroxy protons.[1–7] The measurement of coupling constants, exchange rates with water and temperature dependence of the chemical shifts of hydroxy proton signals can also give important additional structural information in terms of hydrogen bond interactions.[1–4,7–9] The existence of persistent hydrogen bonds in aqueous solution has however been questioned, and it has been shown for example that strong hydrogen bonds which were found to exist in DMSO solutions do not persist in aqueous solutions,[10,11] and also that different intramolecular hydrogen bonds can be formed.[12] It is thus often believed that the existence of hydrogen bonding in aqueous solution only reflects in part the presence of a stable conformation, in which the hydroxy groups are located by chance within hydrogen-bonding distance. But it is also possible that hydrogen bonds play a deciding role in selecting between two or more conformations of otherwise similar energies. Too few data involving hydroxy protons for conformational studies are yet available[1–15] to answer these questions and this work is a part of a study done to investigate if and how the NMR data obtained for the hydroxy protons can be used to better characterize the conformation of oligosaccharides in aqueous solution.

In a previous study,[7] we have evaluated different experimental ^{1}H and ^{13}C NMR techniques involving the hydroxy protons which could be used for conformational analysis and determination of hydrogen bond interactions in aqueous solution. The study was done for two branched trisaccharides, α-D-Glc*p*-(1→3)-[β-D-Glc*p*-(1→4)]-α-D-Gal*p*-OMe, **5**, and β-L-Fuc*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-α-D-Gal*p*-OMe, **12** (Scheme 1). We showed that by combining the data obtained from the scalar coupling constants, temperature coefficients, exchange rates and NOEs of the hydroxy protons, it was possible to detect hydrogen bonding interactions between the hydroxy groups of the two non-reducing sugars, and to increase the total number of observed NOEs.

In this study, we have measured the chemical shifts, vicinal $^3J_{H,OH}$ coupling constants, temperature coefficients, rate of exchange with water and NOEs for the hydroxy protons of a series of 15 additional branched trisaccharides which are methyl α-D-galactopyranosides with [α/β]-L-fucosyl or [α/β]-D-glucosyl groups at the 3- and 4-positions (Scheme 1). We chose these compounds because they represent a family of compounds with all possible anomeric combinations, and are also

α-D-Glc*p* (1→4)
α-D-Gal*p*-OMe **1**
α-D-Glc*p* (1→3)

α-D-Glc*p* (1→4)
α-D-Gal*p*-OMe **2**
β-D-Glc*p* (1→3)

α-D-Glc*p* (1→4)
α-D-Gal*p*-OMe **3**
α-L-Fuc*p* (1→3)

α-D-Glc*p* (1→4)
α-D-Gal*p*-OMe **4**
β-L-Fuc*p* (1→3)

β-D-Glc*p* (1→4)
α-D-Gal*p*-OMe **5**
α-D-Glc*p* (1→3)

β-D-Glc*p* (1→4)
α-D-Gal*p*-OMe **6**
β-D-Glc*p* (1→3)

β-D-Glc*p* (1→4)
α-D-Gal*p*-OMe **7**
α-L-Fuc*p* (1→3)

β-D-Glc*p* (1→4)
α-D-Gal*p*-OMe **8**
β-L-Fuc*p* (1→3)

α-L-Fuc*p* (1→4)
α-D-Gal*p*-OMe **9**
α-D-Glc*p* (1→3)

α-L-Fuc*p* (1→4)
α-D-Gal*p*-OMe **10**
β-D-Glc*p* (1→3)

α-L-Fuc*p* (1→4)
α-D-Gal*p*-OMe **11**
α-L-Fuc*p* (1→3)

α-L-Fuc*p* (1→4)
α-D-Gal*p*-OMe **12**
β-L-Fuc*p* (1→3)

β-L-Fuc*p* (1→4)
α-D-Gal*p*-OMe **13**
α-D-Glc*p* (1→3)

β-L-Fuc*p* (1→4)
α-D-Gal*p*-OMe **14**
β-D-Glc*p* (1→3)

β-L-Fuc*p* (1→4)
α-D-Gal*p*-OMe **15**
α-L-Fuc*p* (1→3)

Scheme 1

good models for many naturally occurring carbohydrates. Their conformational analysis by HSEA and GESA approaches has been reported,[16,17] and it was shown that α-D/β-L-glycosides on the one hand and α-L/β-D-glycosides on the other hand have similar conformations around the glycosidic bond. In this work, we show that the chemical shifts of hydroxy protons are very sensitive to the electronic environment, and that persistent hydrogen bond interaction can exist in aqueous solution.

Results and discussion

Assignment of hydroxy proton resonances

A prerequisite for the observation of hydroxy protons of oligosaccharides in aqueous solution is to remove all traces of metal ion impurities. This was achieved by purifying all trisaccharides on an Amberlite MB-3 mixed ion-exchange resin (see Experimental section). After this treatment, the rate of exchange of the hydroxy protons with the solvent was slow enough so that they could be assigned at −8 °C on the basis of scalar connectivities to the aliphatic protons from DQF–COSY and TOCSY experiments. The ^{1}H NMR chemical shifts and chemical shift differences $\Delta\delta$ (chemical shifts of the hydroxy proton signals in the trisaccharide minus that of the corresponding monosaccharide methyl glycoside) of the hydroxy protons of trisaccharides **1**–**15** (Scheme 1) at −8 °C are reported in Table 1. The NMR data for the constituent methyl monosaccharides have been previously reported.[7]

^{1}H NMR chemical shifts

Inspection of Table 1 shows that most of the hydroxy proton signals from **1**–**15** have chemical shifts which are very similar ($\Delta\delta \leq \pm 0.2$ ppm) to those in the corresponding monosaccharide methyl glycosides. This includes all O(3)H and O(4)H protons of the 3-*O*- and 4-*O*-glycosyls in **1**–**15**, and most of the O(6)H of the 3-*O*- and 4-*O*-glycosyls and α-D-Gal*p* moiety. Exceptions are found for the O(2)H signals of α-D-Gal*p* in compounds **6**, **10** and **14** which are deshielded by *ca.* 0.3 ppm relative to those of the monosaccharide. In these three compounds, the 3-O-linked sugar is a β-D-glucopyranosyl group. In **2**, where the 3-O-linked sugar is also a β-D-glucopyranosyl group, the O(2)H is also deshielded but to a lesser extent. All other $\Delta\delta$ larger than 0.2 ppm are negative (representing an upfield shift of the signals relative to those of the corresponding monosaccharide methyl glycoside). Thus, large upfield shifts are found for the O(2″)H signal of **6**, **7**, **9**, **10**, **11**, and **12**, for the O(2′)H signal of **1**, **3**, **4**, **7**, **13** and **15**, and for the O(6)H signal in **5**, **6** and **8**.

The effect of glycosylation on the aliphatic protons is typically a deshielding of the protons across the glycosidic bond as well as of the protons at the two neighbouring sites of the aglycon. The magnitude of the deshielding depends on the type of monosaccharide, anomeric linkage, and conformation around the glycosidic bond. The main causes for this deshielding are the steric repulsion between hydrogens and the fixation of oxygen lone pairs close in space to the hydrogens in question. Thus, the downfield shift of the O(2)H signals of α-D-Gal*p* in compounds **6**, **10** and **14** might be due to the proximity of the O(2)H proton to O(3) and the more directed lone pairs of this oxygen when it is involved in the linkage. Since the measured chemical shifts represent an average for all the conformations present in solution, the fact that such a downfield shift is not found for the other O(2)H of α-D-Gal*p* might be due to the greater conformational flexibility of the glycosidic linkage, resulting in a less restricted orientation of the lone pairs of O(3). That the conformational flexibility around the 3-O-linkage is more restricted in **6**, **10** and **14** is confirmed by the chemical exchange cross-peak observed between O(2)H of α-D-Gal*p* and O(2′)H of β-D-Glc*p* (Fig. 1) in the ROESY spectra.

To determine the possible origins of the large upfield shifts observed for some hydroxy protons, we have tried to correlate the chemical shifts of the hydroxy protons to the interatomic oxygen–oxygen distances measured in one minimum energy conformation obtained from HSEA and MM2 calculations (Fig. 1). We found that a relationship exists between the large upfield shifts measured for the hydroxy protons and the proximities to oxygen atoms. Fig. 2 shows a plot of the chemical shift differences ($\Delta\delta$) for the O(2)H hydroxy protons of the 3- and 4-linked sugars as a function of the distance between oxygen atoms. From this figure and from Fig. 1, it can be seen that:

(i) The oxygens of the OH groups always have a close contact to the oxygen of their own glycosidic linkage (*ca.* 2.8 Å). When only this close contact is present, the chemical shift difference $\Delta\delta$ for the corresponding hydroxy proton is smaller than −0.20 ppm.

(ii) The hydroxy groups which are close to both O(3) and O(4) have a $\Delta\delta$ larger than −0.25 ppm [O(2″)H in compounds **7**, **9** and **12**].

(iii) The largest upfield shifts are observed for hydroxy groups which are close to an O(5) oxygen [O(2′)H in **1**, **3**, **4** and **13** and O(2″)H in **6** and **10**].

(iv) The spatial proximity to another hydroxy group does not give any strong shielding effect [O(2′)H in **5**, **8**, **9**, **12** and O(2″)H in **5**, **8**].

(v) Upfield shifts are also observed for primary exocyclic hydroxy protons which are close to an O(5) oxygen [O(6′)H in **10**, O(6″)H in **2** and O(6)H in **5**, **6**, **7** and **8**]. The $\Delta\delta$ is smaller than for secondary hydroxy protons. This might be explained by changes in the conformational equilibrium for the hydroxymethyl groups which could contribute to the changes in hydroxy proton chemical shifts.

From Fig. 2, we can discuss some typical examples: In compounds **1**, **4** and **13**, the O(2′) of the 3-linked sugar is close to the O(5″) of the 4-linked sugar, and the O(2′)H proton signals show a large upfield shift. Similarly, in compounds **6** and **10**, the O(2″) of the 4-linked sugar is close to the O(5′) of the 3-linked sugar, and the O(2″)H proton signals show a large upfield shift. In trisaccharides **9** and **12**, the O(2″) of the 4-linked sugar is close to the O(3) glycosyl and the O(2″)H is also experiencing a

Table 1 ^{1}H NMR chemical shifts (δ/ppm) and chemical shifts differences ($\Delta\delta$ = chemical shift in the trisaccharide minus chemical shift in the corresponding monosaccharide methyl glycoside) at −8 °C in 85% H_2O–15% $(CD_3)_2CO$.[a] A positive difference indicates a downfield shift. The hydroxy protons with large $\Delta\delta$ (≥0.20 ppm) are indicated in bold

		4-*O*-Glycosyl				3-*O*-Glycosyl				α-D-Gal	
Compound		O(2″)H	O(3″)H	O(4″)H	O(6″)H	O(2′)H	O(3′)H	O(4′)H	O(6′)H	O(2)H	O(6)H
α-D-Glc*p* (1→3)-[α-D-Glc*p* (1→4)]-α-D-Gal*p* **1**	δ	6.400	6.303	6.365	5.945	**5.466**	6.404	6.365	5.874	6.213	6.079
	$\Delta\delta$	0.082	−0.047	−0.004	−0.013	**−0.852**	0.054	−0.004	−0.084	0.059	0.052
β-D-Glc*p* (1→3)-[α-D-Glc*p* (1→4)]-α-D-Gal*p* **2**	δ	6.270	6.374	6.425	**5.684**	6.479	6.541	6.425	5.990	6.300	6.141
	$\Delta\delta$	−0.048	0.024	0.056	**−0.274**	−0.173	−0.06	−0.028	−0.026	0.146	0.114
α-L-Fuc*p* (1→3)-[α-D-Glc*p* (1→4)]-α-D-Gal*p* **3**	δ	6.238	6.337	6.337	5.775	**5.582**	6.038	6.004		6.098	6.098
	$\Delta\delta$	−0.08	−0.013	−0.032	−0.183	**−0.535**	0.124	0.043		−0.056	0.071
β-L-Fuc*p* (1→3)-[α-D-Glc*p* (1→4)]-α-D-Gal*p* **4**	δ	6.354	6.331	6.339	6.106	**5.967**	6.096	5.917		6.056	6.108
	$\Delta\delta$	0.036	−0.019	−0.030	0.148	**−0.485**	0.083	0.055		−0.098	0.081
α-D-Glc*p* (1→3)-[β-D-Glc*p* (1→4)]-α-D-Gal*p* **5**	δ	6.467	6.570	6.415	5.984	6.120	6.415	6.372	5.871	6.206	**5.759**
	$\Delta\delta$	−0.185	0.059	−0.038	−0.032	−0.198	0.065	0.003	−0.087	0.052	**−0.268**
β-D-Glc*p* (1→3)-[β-D-Glc*p* (1→4)]-α-D-Gal*p* **6**	δ	**6.063**	6.436	6.538	5.985	6.663	6.436	6.538	5.957	**6.457**	**5.798**
	$\Delta\delta$	**−0.589**	−0.075	0.077	−0.032	0.011	−0.075	0.091	−0.062	**0.303**	**−0.230**
α-L-Fuc*p* (1→3)-[β-D-Glc*p* (1→4)]-α-D-Gal*p* **7**	δ	**6.395**	6.509	6.451	6.046	**5.780**	6.069	6.031		6.142	5.864
	$\Delta\delta$	**−0.257**	0.002	0.002	0.03	**−0.337**	0.155	0.07		−0.012	−0.163
β-L-Fuc*p* (1→3)-[β-D-Glc*p* (1→4)]-α-D-Gal*p* **8**	δ	6.575	6.575	6.409	6.004	6.319	6.042	5.943		6.083	**5.754**
	$\Delta\delta$	−0.077	0.064	−0.044	−0.012	−0.133	0.029	0.081		−0.071	**−0.273**
α-D-Glc*p* (1→3)-[α-L-Fuc*p* (1→4)]-α-D-Gal*p* **9**	δ	**5.815**	5.930	5.984		6.282	6.402	6.380	5.885	6.217	6.127
	$\Delta\delta$	**−0.307**	0.016	0.019		−0.038	0.05	0.011	−0.078	0.066	0.103
β-D-Glc*p* (1→3)-[α-L-Fuc*p* (1→4)]-α-D-Gal*p* **10**	δ	**5.324**	5.981	5.981		6.652	6.504	6.448	**5.772**	**6.504**	6.115
	$\Delta\delta$	**−0.793**	0.067	0.020		0.000	0.007	0.005	**−0.294**	**0.350**	0.088
α-L-Fuc*p* (1→3)-[α-L-Fuc*p* (1→4)]-α-D-Gal*p* **11**	δ	**5.795**	5.968	5.907		6.001	5.878	6.001		6.186	6.131
	$\Delta\delta$	**−0.322**	0.054	−0.054		−0.116	−0.036	0.04		0.032	0.104
β-L-Fuc*p* (1→3)-[α-L-Fuc*p* (1→4)]-α-D-Gal*p* **12**	δ	**5.559**	5.976	6.000		6.304	5.951	6.090		6.028	6.133
	$\Delta\delta$	**−0.558**	0.062	0.039		−0.148	0.089	0.077		−0.126	0.106
α-D-Glc*p* (1→3)-[β-L-Fuc*p* (1→4)]-α-D-Gal*p* **13**	δ	6.451	6.050	5.725		**4.880**	6.348	6.348	5.869	6.275	6.163
	$\Delta\delta$	0.001	0.037	−0.137		**−1.438**	0.002	−0.021	−0.089	0.121	0.136
β-D-Glc*p* (1→3)-[β-L-Fuc*p* (1→4)]-α-D-Gal*p* **14**	δ	6.357	6.028	5.840		6.552	6.395	6.552	6.028	**6.464**	6.028
	$\Delta\delta$	−0.095	0.015	−0.022		−0.100	−0.116	0.099	0.012	**0.310**	0.001
α-L-Fuc*p* (1→3)-[β-L-Fuc*p* (1→4)]-α-D-Gal*p* **15**	δ	6.540	6.046	5.704		**5.858**	6.097	5.993		6.251	6.150
	$\Delta\delta$	0.088	0.033	−0.158		**−0.259**	0.183	0.032		0.097	0.123

[a] Primed labels refer to the 3-*O*-glycosyl group and double-primed labels to the 4-*O*-glycosyl group.

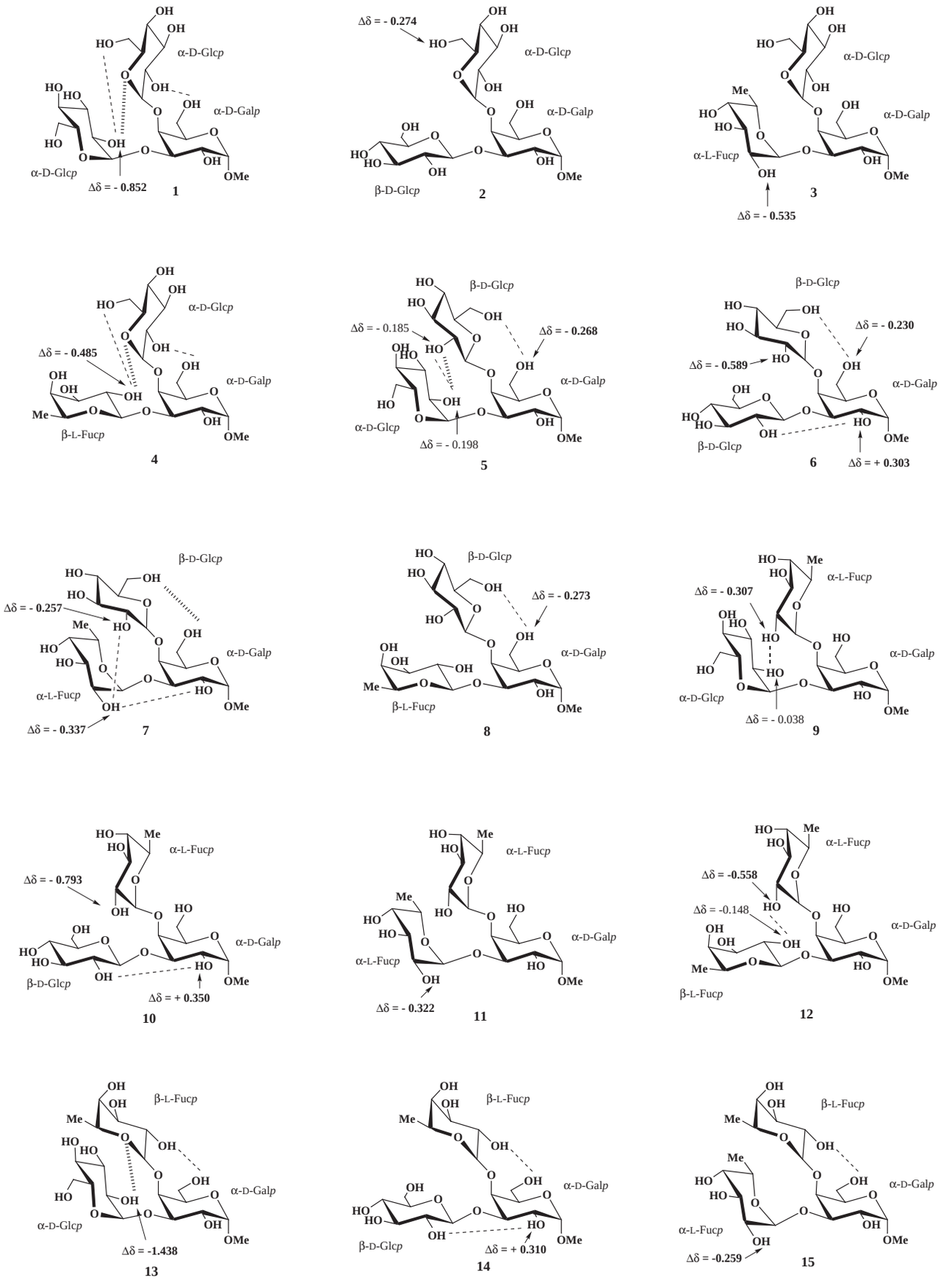

Fig. 1 Schematic representation of trisaccharides **1**–**15**, showing with dashed lines the inter-residue NOEs and with dotted lines the inter-residue hydrogen bonds. Only the NOEs between hydroxy protons are indicated. The hydroxy protons with large chemical shift differences $\Delta\delta > 0.20$ ppm (see text) are indicated in bold. The $\Delta\delta$ of the O(2′)H signal in **5**, **9** and **12**, and of the O(2″)H signal in **5**, are also indicated (see text).

large upfield shift. The O(2′)H protons of the 3-linked sugar which are close only to the oxygen of their own glycosidic linkage have a small $\Delta\delta$. In **5** and **8**, the O(2) of the 3- and 4-linked sugars are only close to each other and to the oxygen of their own glycosidic linkage and the O(2′)H and O(2″)H protons do not show a large upfield shift.

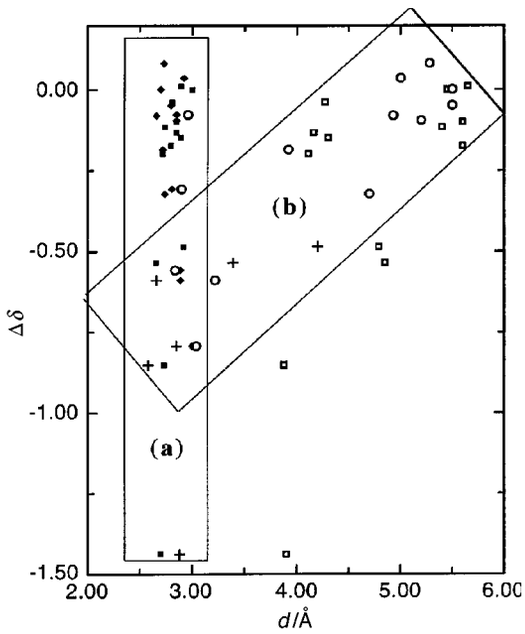

Fig. 2 Plot of the chemical shift differences ($\Delta\delta$, ppm) for the O(2)H hydroxy protons of the 3- and 4-linked glycosides as a function of the distance between oxygen atoms (*d*, Å). ■ O(2′)–O(3), ◆ O(2″)–O(4), □ O(2′)–O(4), ○ O(2″)–O(3), + O(2′),O(2″)–O(5″),O(5′). The OH groups close to only the oxygen of their glycosidic linkage have $\Delta\delta \leq 0.20$ ppm. The OH groups which are also close to the other *O*-glycosyl [O(3), O(4)] or to an O(5) oxygen have $\Delta\delta > 0.20$ ppm. This graph can be roughly divided into two regions: (a) one region which shows a correlation between the hydroxy proton shifts $\Delta\delta$ and the orientation of the oxygen atom lone pairs; (b) one region which shows a correlation between the hydroxy proton shifts $\Delta\delta$ and the distance to oxygen atoms.

Upfield shifts generally occur when the electron density around the proton is increased in some way. In trisaccharides **1**–**15**, the large upfield shifts are observed for hydroxy protons which are close to non protonated oxygen atoms with a more defined orientation such as the O(3), O(4) of α-D-Gal*p* or an O(5) oxygen. The minimum energy conformation shows that these hydroxy protons are pointing (located) between the two lone pairs of the oxygen atoms with a more defined orientation (Fig. 3). It also appears that one of the lone pair of the hydroxy groups, for which the proton signals show a large upfield shift, has a distance and orientation favorable for overlap and interaction with one lone pair of the oxygen of their glycosidic linkage and one lone pair of another non protonated oxygen. For example, the minimum energy conformation of **1** shows that the O(2′) oxygen is close to both the O(3′) and O(5″) oxygens. The axial lone pairs of these three oxygen atoms are oriented in such a way that they can overlap and interact through space. This through-space delocalization could increase in some way the electron density at the O(2′)H hydrogen atom leading to its shift to lower frequency. *Ab initio* calculations are now in progress to investigate the charge distributions in different conformations of the hydroxy groups, and address the above hypothesis.

There is no perfect correlation between $\Delta\delta$ and the oxygen–oxygen distances (Fig. 2), but this is not surprising since only one energy minimum conformation is considered, while the trisaccharides are not rigid molecules but instead have conformational flexibility. Very small variations in distances to oxygen atoms, lone pair orientations and conformations might have a pronounced effect on the chemical shifts. Also, the possibility of hydrogen bond interaction must be considered. It is generally accepted that protons involved in hydrogen bonds are deshielded.[18] Thus, for hydrogen bonded hydroxy protons the deshielding due to the hydrogen bond will oppose the shielding due to stereoelectronic effects, and the chemical shifts measured will be a balance between the two opposite contributions.

The $^3J_{H,OH}$ coupling constants, temperature coefficients, exchange rates with water and NOEs were also measured for the hydroxy protons of the trisaccharides **1**–**15** in order to determine possible hydrogen bond interactions. The NOEs between exchangeable hydroxy protons are shown in Fig. 1. The other NMR data are reported only for compounds **1**, **4** and **13** which have NMR parameters suggesting the presence of hydrogen bonding. (The NMR data for all trisaccharides can be obtained on request from the authors).

Conformational analysis of compounds 1, 4 and 13

Table 2 shows that O(2′)H in **1**, **4** and **13** has values which differ significantly from those of the other hydroxy groups. Thus, all hydroxy protons in **1**, **4** and **13** have chemical shifts that are similar ($\Delta\delta \leq \pm 0.15$ ppm) to those in the corresponding monosaccharide methyl glycoside with the exception of O(2′)H which is shielded by 0.852, 0.485 and 1.438 ppm, respectively. All hydroxy protons with the exception of O(2′)H have $^3J_{H,OH}$ values of 5.5 ± 1.5 Hz representing rotational averaging of the hydroxy groups in terms of the Karplus equation.[19] The O(2′)H in **1**, **4** and **13** have $^3J_{H,OH}$ coupling constants which do not represent conformational averaging but which instead indicate a restricted rotation around the H–O–C–H bond. In **1**, the very large $^3J_{H,OH}$ of 10.5 Hz shows that the O(2′)H proton might adopt a locked *trans* conformation with respect to the C(2′)H proton. It is noteworthy that while the 3- and 4-linked sugars in **1** are both α-D-glucopyranoside, they have very different $^3J_{H,OH}$ values for O(2′)H and O(2″)H. The O(2″)H of the 4-linked sugar has a $^3J_{H,OH}$ which is practically identical to that measured[7] in the corresponding methyl α-D-glucopyranoside (6.4 Hz). On the contrary, the $^3J_{H,OH}$ value measured for the O(2′)H of the 3-linked sugar (10.5 Hz) indicates little rotational freedom of the hydroxy group, and suggests its involvement in intramolecular interaction. In **4**, the small $^3J_{H,OH}$ coupling of 1.8 Hz indicates a strong preference for the *syn* conformation. This value should also be compared to that of 6.5 Hz obtained[7] for methyl β-L-fucopyranoside. The $^3J_{H,OH}$ of 3 Hz measured for O(2′)H in **13** indicates also a preferred *syn* orientation.

All hydroxy protons in **1**, **4** and **13** have temperature coefficients $d\delta/dT$ around 10 ppb deg^{-1}, with the exception of O(2′)H which have much smaller values. The small temperature coefficients $d\delta/dT$ measured for O(2′)H in **1**, **4** and **13** (4.8, 4.4 and 5.5 ppb deg^{-1}, respectively) indicate that this proton is strongly protected from exchange with the solvent, probably due to hydrogen bond interactions in which they must act as hydrogen bond donor. Since it is often believed that strong hydrogen bonds do not persist in aqueous solution, it is important to note that these temperature coefficients are close to the temperature coefficient of *ca.* 3 ppb deg^{-1} reported for hydroxy protons involved in strong hydrogen bonds in DMSO solutions.

Protons involved in hydrogen bonds should also exchange more slowly with the solvent. Since exchange rates are very sensitive to pH, solvent composition and to catalysis by small traces of impurities, a comparison of exchange rates should only be done for hydroxy protons within one compound and comparison between different compounds should be avoided. Table 2 shows that in each trisaccharide, the rate of exchange of O(2′)H with water is much slower than for the other hydroxy protons. However, very large differences in exchange rates are found between the three compounds ($k_{ex} = 0.6$ s^{-1} in **1**, 59 s^{-1} in **4** and 5 s^{-1} in **13**). The causes leading to these large differences are not yet clear to us, and are under investigation.

From HSEA and MM2 calculations, the most probable hydrogen bonding partner which could be identified for O(2′)H in **1**, **4** and **13** is the O(5″) of the 4-linked sugar. Hydrogen bonding is considered to exist[20] if the distance between the hydrogen atom and the acceptor oxygen is <2.5 Å, the angle about the O–H···O is larger than 120°, and these con-

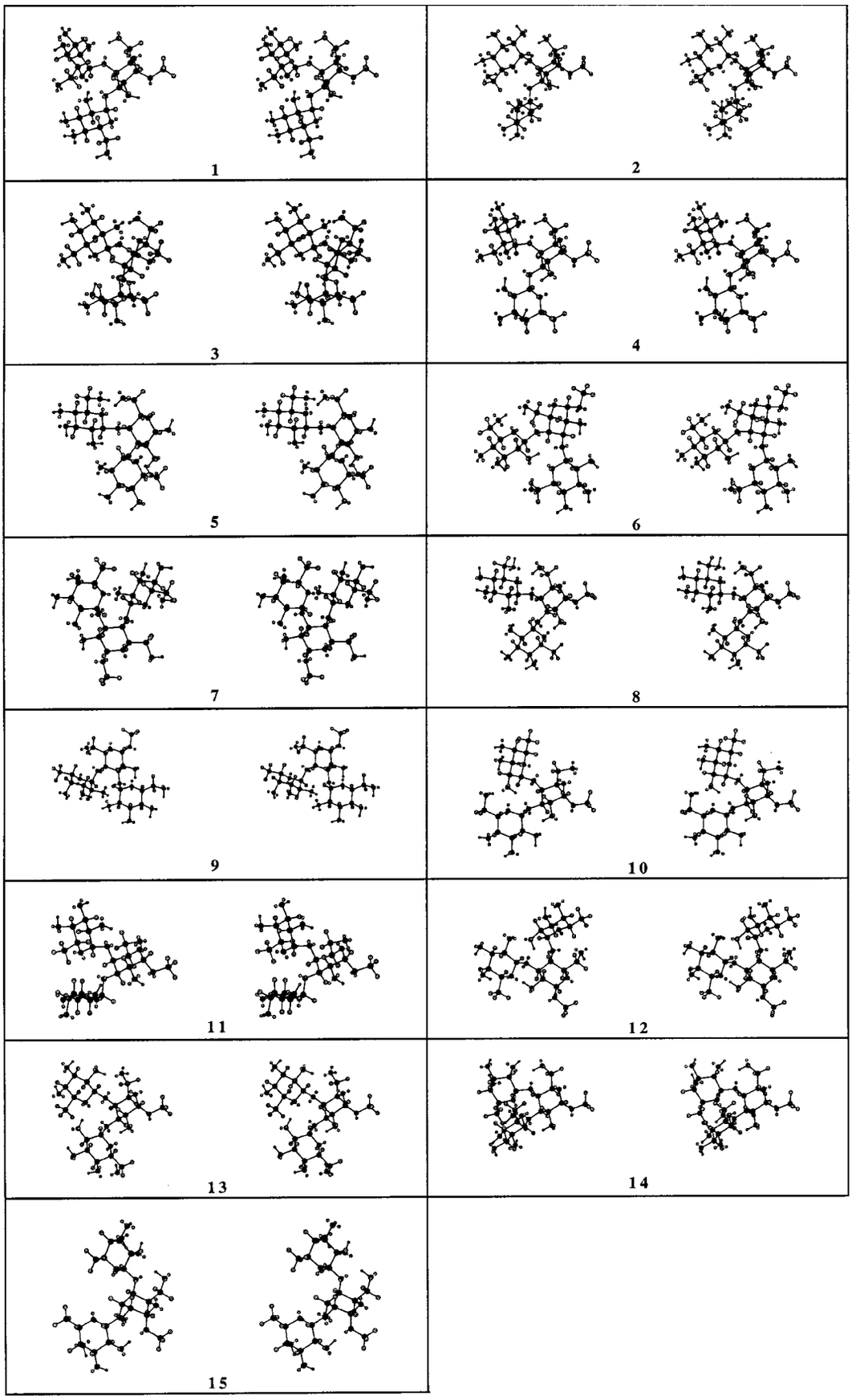

Fig. 3 Stereorepresentations of one energy minimum conformation of trisaccharides **1**–**15**. The hydroxy protons and oxygen atoms involved in hydrogen bonds are represented as filled black circles [in **1**, **4** and **13**, O(2′)H–O(5″) hydrogen bond and in **5** and **12**, O(2′)H–O(2″) hydrogen bond].

ditions persist for at least 0.5 ps. From MM2 calculations, the lowest energy structure shows for trisaccharide **1** an O(2′)H · · · O(5″) distance of 1.7 Å and an angle about the O–H · · · O of 151°. In **4** and **13**, O(2′)H · · · O(5″) distances of 2.3 Å and 1.9 Å and angles of 160° and 161° are measured, respectively.

Several cross-peaks involving the exchangeable hydroxy protons could be observed in the NOESY spectra of **1**, **4** and **13**

Table 2 ^{1}H NMR chemical shifts (δ/ppm), temperature coefficients (dδ/dT/ppb deg^{-1}), $^3J_{OH,CH}$ coupling constants (J/Hz) and exchange rates (k_{ex}/s^{-1}) for the hydroxy protons of trisaccharides **1**, **4** and **13** measured at −8 °C in 85% H_2O–15% $(CD_3)_2CO$ [a]

	4-*O*-Glycosyl				3-*O*-Glycosyl					
1	O(2″)H	O(3″)H	O(4″)H	O(6″)H	O(2′)H	O(3′)H	O(4′)H	O(6′)H	O(2)H	O(6)H
δ	6.440	6.303	6,365	5.945	5.466	6.404	6.365	5.874	6.213	6.079
$\Delta\delta$ [b]	0.082	−0.047	−0.004	−0.013	−0.852	0.054	−0.004	−0.084	0.059	0.052
J	6	5.1	[c]	9.5/2	10.3	[c]	[c]	10.5/2	6.2	9.7/2
dδ/dT	10.3	10.8	[c]	11.1	4.8	[c]	[c]	10.8	12.9	10.2
k_{ex}	19	21	24	21	0.6	20	23	19	8	16
4	O(2″)H	O(3″)H	O(4″)H	O(6″)H	O(2′)H	O(3′)H	O(4′)H	O(6′)H	O(2)H	O(6)H
δ	6.354	6.331	6.339	6.106	5.967	6.096	5.917		6.056	6.108
$\Delta\delta$ [b]	0.036	−0.019	−0.030	0.148	−0.485	0.083	0.055		−0.098	0.081
J	[d]	[d]	[d]	[d]	1.8	[d]	[d]		[d]	[d]
dδ/dT	10.7	10.7	10.7	9.2	4.4	11.6	[d]		13.9	11.6
k_{ex}	[d]	[d]	[d]	102	59	474	102		98	474
13	O(2″)H	O(3″)H	O(4″)H	O(6″)H	O(2′)H	O(3′)H	O(4′)H	O(6′)H	O(2)H	O(6)H
δ	6.451	6.05	5.725		4.880	6.348	6.348	−5.869	6.275	6.163
$\Delta\delta$ [b]	0.001	0.037	−0.137		−1.438	0.002	−0.021	−0.089	0.121	0.136
J	4	4	4.6		3.5	5.5	7	9.7/2	4.8	10.3/2
dδ/dT	9.8	10.6	10.3		5.5	11.4	11.4	11.5	14.3	12.3
k_{ex}	26	34	12		5	36b	36b	30	18	41

[a] Primed labels refer to the 3-*O*-glycosyl group and double-primed labels to the 4-*O*-glycosyl group. [b] $\Delta\delta$: Chemical shift differences calculated by subtraction of chemical shifts of the corresponding monosaccharide methyl glycoside. A positive difference indicates a downfield shift. [c] Could not be measured due to spectral overlap. [d] Broad signals and spectral overlap.

Table 3 Inter-residue NOEs observed between exchangeable hydroxy protons and non-exchangeable aliphatic protons in the NOESY spectra of trisaccharides **1**, **4** and **13** (−8 °C, 85% H_2O–15% $(CD_3)_2CO$, mixing time 100 ms) [a]

	O(2)H	O(6)H	O(2′)H	O(6′)H	O(2″)H	O(6″)H
1	C(1′)H C(5′)H C(6′)H	C(1″)H C(2″)H C(3″)H C(5″)H	C(3)H C(4)H C(1″)H C(6″)H			C(3′)H
4	C(1′)H	C(1″)H C(2″)H C(3″)H C(5″)H	C(4)H C(1″)H C(6″)H			C(2′)H
13	C(5′)H	C(4)H C(1)OMe C(1″)H	C(1″)H C(2″)H C(5″)H C(5″)Me	C(2)H C(4)H	C(6)H	

[a] Primed labels refer to the 3-*O*-glycosyl group and double-primed labels to the 4-*O*-glycosyl group.

recorded at −8 °C with a mixing time of 100 ms (Table 3). ROESY spectra were also acquired to be able to discriminate between chemical exchange and dipolar relaxation. In NOESY, both exchange and dipolar relaxation have the same sign and their relative contributions to a given cross-peak cannot be distinguished. In ROESY, cross-peaks due to dipolar relaxation are negative, while cross-peaks coming from chemical exchange are positive and of the same sign as the diagonal peaks. In both trisaccharides **1** and **4**, chemical exchange exists between O(2′)H and O(6″)H and between O(6)H and O(2″)H. In **13**, a chemical exchange interaction is found between O(2′)H and O(4″)H and between O(6)H and O(2″)H. Exchange cross-peaks can be diagnostic of spatial proximity and also of hydrogen bond interaction,[21,22] but the presence of hydrogen bonds should be confirmed by other techniques. The coupling constants, temperature coefficients and exchange rates discussed previously strongly support the involvement of O(2′)H in a hydrogen bond interaction, but the participation of O(6″)H, O(6)H and O(2″)H in **1** and **4**, and of O(4″)H, O(6)H and O(2″)H in **13** is not anticipated from these data (*vide infra* and Table 2). The NOEs due to dipolar relaxation between exchangeable hydroxy protons and non-exchangeable aliphatic protons are listed in Table 3. It can be seen that 12, 9 and 11 additional inter-residue NOEs could be obtained in **1**, **4** and **13**, respectively. These additional NOEs corroborate well a conformation in which O(2′)H and O(5″) are involved in hydrogen bonding.

The NOEs between the exocyclic hydroxy protons and the other exchangeable or non-exchangeable protons also allow the determination of the favoured conformation around the C(5)–C(6) bond [due to spectral overlap, the conformation around the C(5)–C(6) bond could not be obtained from the $^3J_{H5,H6a}$ and $^3J_{H5,H6b}$ coupling constants]. In **1** and **4**, the presence of a chemical exchange cross-peak between O(6)H and O(2″)H together with the NOE between O(6)H and C(5″)H indicate that the *gauche–gauche* conformation is favoured over the *gauche–trans* conformation (different to what was obtained from HSEA calculations where the *gauche–trans* conformation is preferred[16,17]). The chemical exchange between O(2′)H and O(6″)H together with the absence of NOE between O(6″)H and C(2)H suggest a preferred *gauche–gauche* conformation for the 4-linked residue. In **13**, the NOE between O(6′)H and C(2)H indicates a preferred *gauche–trans* conformation for the 3-linked glucose. For the galactose residue, NOEs are found between O(6)H and C(1″)H, C(4)H and the 1-*O*-methyl. The NOEs between O(6)H and C(1″)H and between O(6)H and C(4)H indicate the presence of *gauche–gauche* conformation, while the NOE between O(6)H and the 1-*O*-methyl of Gal*p* indicates that the *gauche–trans* conformation is also present.

The NMR data ($^3J_{H,OH}$, dδ/dT and k_{ex}) indicate that the hydrogen bond between O(2′)H and O(5″) is stronger in **1** and **4** than in **13**. In **1** and **4**, there is an additional chemical exchange cross-peak between O(2′)H and O(6″)H. This exchange cross-peak might indicate a close proximity and a weak hydrogen bond between O(2′)H and O(6″)H. This interaction could further stabilize the conformation in which O(2′)H and O(5″) are hydrogen bonded. The O(2′)H in **1** and **4** is less shielded ($\Delta\delta = -0.852$ in **1** and −0.485 ppm in **4**) than the O(2′)H proton in **13** ($\Delta\delta = -1.438$ ppm). As mentioned above, for hydrogen bonded hydroxy protons the deshielding due to the hydrogen bond will oppose the shielding due to stereoelectronic effects, and the chemical shifts measured will be a balance

between the two opposite contributions. Thus the difference in chemical shifts measured for **1**, **4** and **13** could be explained in two ways: (i) In **1** and **4**, a stronger hydrogen bond exists and the contribution of the deshielding effect due to this hydrogen bond to the measured chemical shift is more important than in **13** where the hydrogen bond interaction is weaker. (ii) The lone pairs of O(5″), O(3) and (O2′) in **13** are oriented in such a way that they can better overlap than in **1** and **4**, and this stereoelectronic effect is reflected in the much larger upfield shift measured for O(2′)H in **13**.

Conclusion

The vicinal $^3J_{H,OH}$ coupling constants and NOEs, together with HSEA and MM2 calculations indicate that O(2′)H in trisaccharides **1**, **4** and **13** is well positioned to form a hydrogen bond with O(5″). The low rate of exchange with water and the small temperature coefficient of the O(2′)H hydroxy proton further indicate structures which are stabilized by strong hydrogen bonding in which O(2′)H is the hydrogen bond donor. Thus, persistent hydrogen bond interactions can also exist in aqueous solution, and not only in DMSO solutions as is often believed. Even if the trisaccharides are not rigid molecules, and exist in solution as an ensemble of several conformers, the detection of strong hydrogen bonds suggests also that the corresponding conformation is present in solution during a significant amount of time. The observation of hydroxy protons also allows us to observe an increased number of inter-residue NOEs and to determine the conformation around the C(5)–C(6) bond. These additional NOEs are very important for conformational analysis since often only few inter-residue NOEs involving non-exchangeable protons are observed. Such NOEs often involve protons located very close to the glycosidic linkage which are not very sensitive to torsional fluctuations of the glycosidic linkage. On the other hand, the NOEs involving hydroxy protons are not always close to the glycosidic linkage, and are thereby much more sensitive to conformational changes. The additional distance constraints obtained from NOEs (even if only qualitative) together with the additional structural information obtained from the detection of hydrogen bonds should with molecular mechanics and dynamics calculations allow us to better define the conformation(s) of oligosaccharides in solution.

The chemical shifts of hydroxy protons are very sensitive to the electronic environment. A correlation is found between the chemical shifts and the proximity and orientation of oxygen atom lone pairs. When the hydroxy protons are involved in hydrogen bond interactions, the measured chemical shift is a balance between two opposite effects, a deshielding effect due to hydrogen bonding and a shielding effect due to interactions with the lone pairs of the oxygen atoms (stereoelectronics effects). This study is obviously only a starting point for the investigation of correlations between chemical shifts of hydroxy protons and conformation of oligosaccharides. More data together with complete *ab initio* calculations need to be collected to determine the origin of the upfield shifts observed and to determine how we can use the information obtained from hydroxy proton chemical shifts in conformational analysis.

Experimental

Sample preparation

The trisaccharides **1**–**15** were available from previous studies.[16,17] The NMR sample tubes were soaked for a minimum of 1 h in a 50 mM solution of phosphate buffer, pH 7, to minimise adsorption of impurities from glass.[10] All compounds were purified on an Amberlite MB-3 mixed ion-exchange resin. In some cases, broad hydroxy signals were still obtained after running the compounds through the column. We found that to obtain sharp hydroxy resonances, it was necessary to leave the compounds in a bath of the Amberlite MB-3 mixed ion-exchange resin with smooth mixing. Typically, the mixture was left overnight at room temperature, filtered and freeze-dried. Under these conditions all hydroxy protons for all trisaccharides could be observed as relatively narrow lines.

NMR spectroscopy

All NMR experiments were performed on a BRUKER DRX-600 spectrometer operating at 600.13 MHz for proton observation. Compounds **1**–**15** were dissolved in a mixture of 85% H_2O–15% $(CD_3)_2CO$ to give a sample concentration of *ca.* 50 mM. The addition of acetone to the samples allowed us to lower the sample temperature to −15 °C without freezing. All spectra unless specified were recorded at −8 °C except for the temperature coefficients, which were measured by variation of the temperature from −15 °C to 20 °C in steps of 5 °C. The ^{1}H NMR spectra were referenced by setting the residual [$^2H_5$]-acetone signal to $\delta_H = 2.204$ ppm. One- and two-dimensional ^{1}H NMR spectra were acquired using the WATERGATE pulse sequence[23] for water suppression. The 2D NMR spectra were recorded in the phase-sensitive mode using the TPPI method.[24] The DQF-COSY[25] and CLEAN-TOCSY[26] spectra were acquired with 2K data points in t_2 and 256 points in t_1. For each FID, 8 scans were averaged and a repetition delay of 1.5 s was used. The data were zero-filled to give a 2K × 1K matrix, and a π/4 shifted sine-square bell window was applied in both dimensions prior to Fourier transformation. In TOCSY, mixing times of 20 and 80 ms were used and the MLEV-17 sequence was applied for mixing using an extra delay of 65 μs for compensation of NOE. NOESY[27] and ROESY[28] spectra were recorded with mixing times (τ_m) of 50 and 100 ms with 256 spectra of 2K data points. For each FID, 16 scans were recorded using a repetition delay of 1.5 s. The data were zero-filled to 2K × 1K before applying a π/2 shifted sine-square bell window function in both dimensions. The rates of exchange of the hydroxy protons with water were calculated from 2D phase-sensitive chemical exchange experiments.[29] Mixing times of 3 to 24 ms in steps of 3 ms were used. 128 FIDs of 2K data points were acquired and a recycle delay of 1.5 s was used. A polynomial baseline correction was applied in both dimensions. The volumes of the NOE cross-peaks and diagonal peaks were measured using the program AURELIA (Bruker, Germany). The initial build-up rates of the exchange cross-peak volumes were determined from the spectra, and the volumes of the hydroxy proton diagonal peaks at zero mixing time were obtained by extrapolation from the volumes of the diagonal peaks in the spectra. The exchange rate constants were then calculated as the ratio of the initial build-up rates of the exchange peak over the volume of the diagonal peaks at zero mixing time.

MM2 calculations

Chem3D plus version 3.5 for Macintosh was used. Minimization was performed with the "MM2" force field. The default convergence criterion was used (rms [root mean square] force 0.1 kcal mol^{-1} A^{-1}). The starting structures were the published[16,17] minimum energy conformations calculated using the HSEA and GSEA methods.

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References

1 L. Poppe and H. van Halbeek, *J. Am. Chem. Soc.*, 1991, **113**, 363.
2 H. van Halbeek and L. Poppe, *Magn. Reson. Chem.*, 1992, **30**, S74.
3 L. Poppe, R. Stuike-Prill, B. Meyer and H. van Halbeek, *J. Biomol. NMR*, 1992, **2**, 109.

4 L. Poppe and H. van Halbeek, *J. Am. Chem. Soc.*, 1992, **114**, 1092.
5 J.-R. Brisson, S. Uhrinova, R. J. Woods, M. van der Zwan, H. C. Jarrell, L. C. Paoletti, D. L. Kasper and H. J. Jennings, *Biochemistry*, 1997, **36**, 3278.
6 R. Harris, T. J. Rutherford, M. J. Milton and S. W. Homans, *J. Biomol. NMR*, 1997, **9**, 47.
7 C. Sandström, H. Baumann and L. Kenne, *J. Chem. Soc.*, *Perkin Trans. 2*, 1998, 809.
8 L. Poppe and H. van Halbeek, *Struct. Biol.*, 1994, **1**, 215.
9 S. Sheng and H. van Halbeek, *Biochem. Biophys. Res. Commun.*, 1995, **215**, 504.
10 B. Adams and L. Lerner, *J. Am. Chem. Soc.*, 1992, **114**, 4827.
11 B. R. Leeflang, J. F. G. Vliegenthart, L. M. J. Kroon-Batenburg, B. P. van Eijck and J. Kroon, *Carbohydr. Res.*, 1992, **230**, 41.
12 K. Bock, T. Frejd, J. Kihlberg and G. Magnusson, *Carbohydr. Res.*, 1988, **176**, 253.
13 D. R. Bundle, H. Baumann, J.-R. Brisson, S. M. Gagné, A. Zdanov and M. Cygler, *Biochemistry*, 1994, **33**, 5183.
14 B. Adams and L. E. Lerner, *Magn. Reson. Chem.*, 1994, **32**, 225.
15 S. Sheng, R. Cherniak and H. van Halbeek, *Anal. Biochem.*, 1998, **256**, 63.
16 H. Baumann, B. Erbing, P.-E. Jansson and L. Kenne, *J. Chem. Soc.*, *Perkin Trans. 1*, 1989, 2153.
17 H. Baumann, B. Erbing, P.-E. Jansson and L. Kenne, *J. Chem. Soc.*, *Perkin Trans. 1*, 1989, 2167.
18 J. A. Pople, W. G. Schneider and H. J. Bernstein, *High Resolution Nuclear Magnetic Resonance*, McGraw Hill, New York, 1959.
19 R. T. Fraser, M. Kaufman, P. Morand and G. Govil, *Can. J. Chem.*, 1969, **47**, 403.
20 G. A. Jeffrey and W. Saenger, *Hydrogen Bonding In Biological Structures*, Springer, Berlin, 1991.
21 L. Poppe, J. Dabrowski, C.-W. von der Lieth, K. Koike and T. Ogawa, *Eur. J. Biochem.*, 1990, **189**, 313.
22 J. Dabrowski and L. Poppe, *J. Am. Chem. Soc.*, 1989, **111**, 1510.
23 M. Piotto, V. Saudek and V. Sklenár, *J. Biomol. NMR*, 1992, **2**, 661.
24 D. Marion and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 1983, **113**, 967.
25 A. Derome and M. Williamson, *J. Magn. Reson.*, 1990, **88**, 177.
26 C. Griesinger, G. Otting, K. Wüthrich and R. R. Ernst, *J. Am. Chem. Soc.*, 1988, **110**, 7870.
27 J. Jeener, B. H. Meier, P. Bachmann and R. R. Ernst, *J. Chem. Phys.*, 1979, **71**, 4546.
28 A. Bax and D. G. Davis, *J. Magn. Reson.*, 1985, **63**, 207.
29 C. M. Dobson, L.-Y. Lian, C. Redfield and K. D. Topping, *J. Magn. Reson.*, 1986, **69**, 201.

Paper 8/05678A