NMR spectroscopy of hydroxy protons of 3,4-disubstituted methyl α-D-galactopyranosides in aqueous solution

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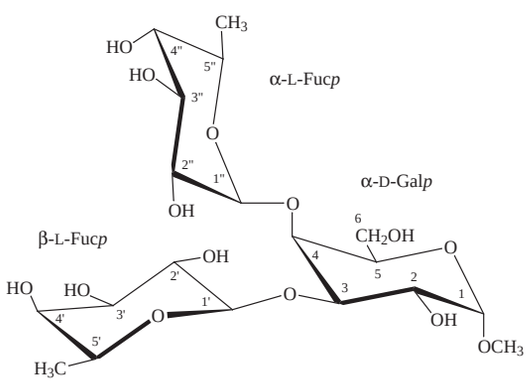
The ^{1}H NMR chemical shifts, coupling constants, temperature coefficients, exchange rates, inter-residue NOEs, and the deuterium isotope effects on ^{13}C chemical shifts have been measured, in aqueous solution, for the hydroxy protons of two 3,4-disubstituted galactopyranosides, β-L-Fuc*p*-(1⟶3)-[α-L-Fuc*p*-(1⟶4)]-α-D-Gal*p*-OMe and α-D-Glc*p*-(1⟶3)-[β-D-Glc*p*-(1⟶4)]-α-D-Gal*p*-OMe, and their constituent monomeric methyl glycosides. All the hydroxy proton resonances could be assigned and for both trisaccharides the data indicated hydrogen bonding interactions between the hydroxy groups at the C-2 position of the two non-reducing sugars. Using the hydroxy protons, the number of inter-residue NOEs for the two trisaccharides was increased from 4 to 6 and from 4 to 7, respectively, relative to those obtained in D_2O solutions. These results show that the NMR data obtained from hydroxy protons can provide important information in terms of hydrogen bonding interactions and inter-residue NOEs, which could be further used in structural and conformational analysis to improve the characterisation of oligosaccharides in aqueous solution.

Introduction

Due to the recognition role of oligosaccharides in biological systems, many studies are devoted to the correlation of the 3D solution structure with the biological function. But while the tertiary structure of quite large proteins (30 kDa) and nucleic acids (12 kDa) can be determined by NMR spectroscopy, only relatively smaller oligosaccharide fragments (2 kDa) have been investigated so far and their structures are not as well defined.

In oligosaccharides, the assignment of NMR resonances and the determination of tertiary solution structure is a difficult task due to several factors: (*i*) small dispersion of proton chemical shifts (δ: 3.3–4.2 ppm), (*ii*) a limited number of inter-residue distance constraints obtained from NOE-type experiments in D_2O, (*iii*) isotope labelling with ^{13}C is not yet as readily available as for proteins and nucleic acids; (*iv*) oligosaccharides are often flexible and exist in solution as an ensemble of conformers. Moreover, despite their abundance and potential importance in hydrogen bonding interactions, very little use has been made of the exchangeable hydroxy protons in aqueous solution. This lack of data has been due to difficulties in observing hydroxy protons in H_2O because of their rapid exchange with the bulk water, and to the dynamic problem of observation in H_2O. However, in the last few years, it has been shown[1–12] that by using low temperatures to reduce the rate of exchange with water, and pulse sequences that efficiently suppress the water signal without affecting the resonances of the exchangeable protons, it is possible to observe the hydroxy protons and to obtain structural information in terms of stereochemistry,[1] inter-proton distances[2–4,6] and hydrogen bonding.[2–10] DMSO has often been used as solvent to avoid the chemical exchange of OH protons with water, but in aprotic solvents, the possible influence of the solvent on the conformation must be considered. Thus, it has been shown that intramolecular hydrogen bonds observed in DMSO do not persist in water[4,6] or that different intramolecular hydrogen bonds can be formed.[9] As only few data are yet available, it is necessary that more NMR studies are carried out in order to estimate the importance of hydroxy protons in conformational analysis, and to address the controversial issue of their importance in stabilising the conformations of carbohydrates in aqueous solution.

In this work, we have studied two branched trisaccharides, methyl β-L-fucopyranosyl-(1⟶3)-[α-L-fucopyranosyl-(1⟶4)]-α-D-galactopyranoside, **6**, and methyl α-D-glucopyranosyl-(1⟶3)-[β-D-glucopyranosyl-(1⟶4)]-α-D-galactopyranoside, **7**. These compounds are good models for



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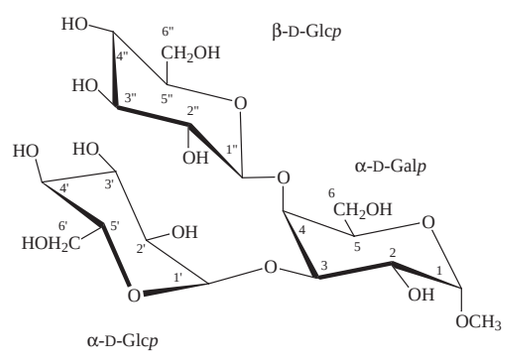

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a large proportion of naturally occurring carbohydrates. They are also good models for determining the use of hydroxy protons as conformational probes to identify the presence of hydrogen bonds by NMR. Their conformational analysis by HSEA and GESA approaches has been previously reported.[13]

Table 1 ^{1}H NMR chemical shifts (δ), temperature coefficients (κ/ppb deg^{-1}), $^3J_{HO,CH}$ coupling constants (J/Hz) and exchange rates (k_{ex}/s^{-1}) for the hydroxy protons of trisaccharide **6** and the constituent monosaccharide methyl glycosides **1–3**, measured at −8 °C in 85% H_2O–15% $(CD_3)_2CO$ [a]

		1–3			**6**				
		δ	J/Hz	k_{ex}/s^{-1}	δ	$\Delta\delta$ [b]	κ/ppb deg^{-1}	J/Hz	k_{ex}/s^{-1}
Me α-D-Gal*p*	O(2)H	6.154	4.8	11	6.028	−0.126	12.8	4.8	11
1	O(3)H	5.971	5.1	18					
	O(4)H	5.941	5.7	2					
	O(6)H	6.027	5.0	18	6.133	0.106	11.7	4.9	17
Me α-L-Fuc*p*	O(2″)H	6.117	5.5	15	5.559	−0.558	11.7	8.6	5
2	O(3″)H	5.914	5.7	11	5.976	0.062	10.5	5.9	17
	O(4″)H	5.961	5.5	24	6.000	0.039	11.5	5.7	16
Me β-L-Fuc*p*	O(2′)H	6.452	4.6	10	6.305	−0.147	9.1	4.8	4
3	O(3′)H	6.013	6.0	10	6.090	0.077	10.0	5.7	12
	O(4′)H	5.862	5.9	17	5.951	0.089	10.7	5.7	13

[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 monosaccharides **1–3** from **6**. A positive difference indicates a downfield shift.

It was shown that α-D/β-L- and α-L/β-D-glycosides have similar conformations around the glycosidic bond and that the OH groups at the C-2 position of the two non-reducing sugars are in close proximity. We have measured the NMR parameters of the hydroxy protons that can be used to study hydrogen bonding interactions, *i.e.* ^{1}H chemical shifts, coupling constants, temperature coefficients, exchange rates with the solvent, NOEs and deuterium isotope effects on ^{13}C chemical shifts. The NMR parameters of the constituent monosaccharides were also measured for comparison. We have also investigated the behaviour of the hydroxy protons of **6** and **7** in DMSO solutions. The goal of this work is to evaluate the use of NMR data obtained from the hydroxy protons to identify hydrogen bonds and to increase the number of inter-residue NOEs.

Experimental

The methyl glycosides Me α-D-Gal*p* **1**, Me α-L-Fuc*p* **2**, Me β-L-Fuc*p* **3**, Me β-D-Glc*p* **4** and Me α-D-Glc*p* **5** were obtained from Sigma and the trisaccharides **6** and **7** were available from a previous study.[13] All compounds were purified on an Amberlite MB-3 mixed ion-exchange resin. The pH values for the solutions were 7.0 ± 0.5. The NMR sample tubes were prepared to minimise adsorption of impurities from glass.[1] All NMR experiments were performed on a Bruker DRX-600 spectrometer operating at 600.13 MHz for proton and 150.90 MHz for carbon.

^{1}H NMR in H_2O

Compounds **1–7** 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 the lowering of 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 to 20 °C in steps of 5 °C. One-dimensional ^{1}H NMR spectra were acquired using the jump-return[14] and the WATERGATE[15] pulse sequences for water suppression. The 2D NMR spectra were recorded in the phase-sensitive mode using the TPPI method.[16] Solvent suppression was achieved by the WATERGATE[15] sequence. The DQF–COSY[17] and CLEAN–TOCSY[18] spectra were acquired with 2 K data points in t_2 and 256 points in t_1. For each FID, 2, 4 or 8 scans were averaged and a repetition delay of 1.5 s was used. The data were zero-filled to give a 2 K × 1 K 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[19] and ROESY[20] spectra were recorded with mixing times (τ_m) of 50, 100 and 200 ms with 256 spectra of 2 K data points. For each FID, 8 or 16 scans were recorded using a repetition delay of 1.5 s. The data were zero-filled to 2 K × 1 K before applying a π/2 shifted sine-square bell window function in both dimensions. The spectra with a 200 ms mixing time were not analysed further because of significant spin-diffusion.

Measurement of exchange rates

The rates of exchange of the hydroxy protons with water were calculated from 2D phase-sensitive chemical exchange experiments.[21] Mixing times of 3 to 24 ms in steps of 3 ms were used. 128 FIDs of 2 K 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 (see also text). Two different schemes for water suppression (WATERGATE[15] and two short spin-lock pulses[22]) were used for **6** in order to determine the possible influence of the water suppression technique on the calculated exchange rates. Both methods gave similar rates of exchange but a better water suppression and less spectral distortion were obtained with the WATERGATE[15] method.

^{13}C NMR in H_2O/D_2O

The compounds were dissolved in H_2O–D_2O, 1:1 v/v. The ^{13}C resonances were assigned from HMQC spectra. The ^{1}H and ^{13}C NMR spectra were referenced by setting the residual [$^2H_5$]acetone signal to δ_H = 2.204 ppm and to δ_C = 31.88.

Results and discussion

Hydroxy protons in 85% H_2O–15% $(CD_3)_2CO$

Assignment. The signals of the hydroxy protons show a relatively large dispersion of chemical shifts (δ 5.5–7, Tables 1 and 2). At −8 °C, the exchange rate of the OH protons with the solvent is slow enough so that they can be observed as relatively narrow lines, making their assignment on the basis of scalar connectivities to the aliphatic protons possible. The assignment of the hydroxy protons in **1–7** has been obtained from 2D DQF-COSY (Fig. 1) and TOCSY spectra. The chemical shifts and coupling constants of the hydroxy protons in monosaccharides **1**, **4** and **5** have been previously reported.[1] The slight differences in chemical shifts between those reported and those listed in Tables 1 and 2 are attributed to differences in temperature and the water : acetone ratio.

Chemical shifts and temperature coefficients. Most of the hydroxy protons in **6** and **7** have chemical shifts that are very

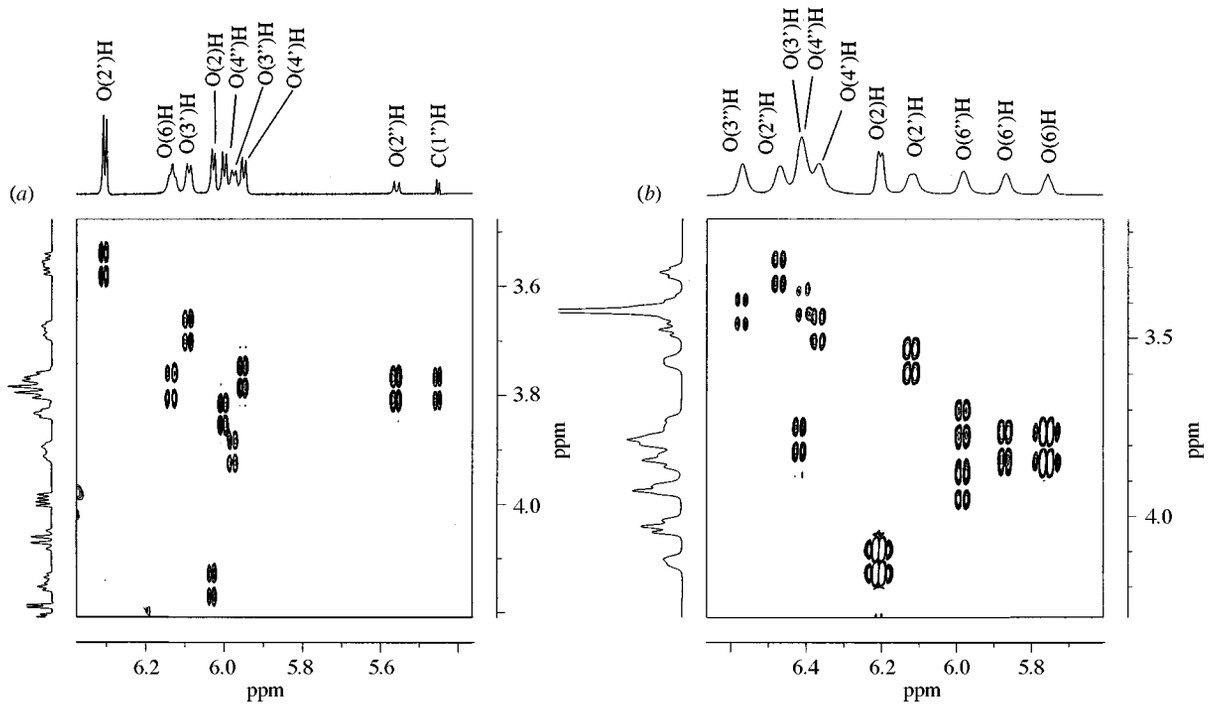

Fig. 1 Expanded region of the DQF-COSY spectra [85% H_2O–15% $(CD_3)_2CO$, −8 °C] of (*a*) trisaccharide **6** and (*b*) trisaccharide **7**, showing the scalar connectivities ($^3J_{HO,CH}$) between OH and CH protons

Table 2 ^{1}H NMR chemical shifts (δ), temperature coefficients (κ/ppb deg^{-1}), $^3J_{HO,CH}$ coupling constants (J/Hz) and exchange rates (k_{ex}/s^{-1}) for the hydroxy protons of trisaccharide **7** and the constituent monosaccharide methyl glycosides **1, 4** and **5**, measured at −8 °C in 85% H_2O–15% $(CD_3)_2CO$[a]

		1,**4**,**5**			**7**				
		δ	J/Hz	k_{ex}/s^{-1}	δ	$\Delta\delta$[b]	κ/ppb deg^{-1}	J/Hz	k_{ex}/s^{-1}
Me α-D-Gal*p*	O(2)H	6.154	4.8	11	6.206	0.052	10.7	6.9	7
1	O(3)H	5.971	5.1	18					
	O(4)H	5.941	5.7	2					
	O(6)H	6.027	5.0	18	5.759	−0.268	9.8	6.2	9
Me β-D-Glc*p*	O(2″)H	6.652	4.4	13	6.467	−0.185	9.7	6.0	13
4	O(3″)H	6.511	5.0	13	6.570	0.059	9.8	5.8	17
	O(4″)H	6.453	6.1	16	6.415	−0.038	9.0	6.3	17
	O(6″)H	6.016	5.3	31	5.984	−0.032	9.5	5.5	13
Me α-L-Glc*p*	O(2′)H	6.318	6.0	8	6.120	−0.198	6.2	9.2	6
5	O(3′)H	6.350	5.1	13	6.415	0.065	9.0	5.3	17
	O(4′)H	6.369	6.4	4	6.372	0.003	8.6	5.6	17
	O(6′)H	5.958	5.5	22	5.871	−0.087	9.2	5.0	14

[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 monosaccharides **1**, **4** and **5** from **7**. A positive difference indicates a downfield shift.

similar ($\Delta\delta \leqslant \pm 0.1$ ppm) to those in the corresponding monosaccharides (Tables 1 and 2). In **6**, exceptions are found for O(2′)H and O(2″)H which are shielded by 0.147 and 0.558 ppm, respectively (Fig. 2). In **7**, O(6)H, O(2′)H and O(2″)H are shielded by 0.268, 0.198 and 0.185 ppm, respectively. Hydroxy protons involved in hydrogen bonds tend to be deshielded but examples of shielding have also been reported.[5,8] Since chemical shifts are subject to several effects which are difficult to predict, the use of them alone to determine the involvement of a particular hydroxy proton in a hydrogen bond is not recommended. Large shifts, as observed for O(2″)H in **6**, should however be taken into account since they must reflect a particular environment for the proton under consideration. The temperature coefficients (κ) calculated for the different hydroxy protons in **6** and **7** are in the range 10.0 to 12.8 ppb deg^{-1} in **6** and 8.6 to 10.7 ppb deg^{-1} in **7** (Tables 1 and 2). Only O(2″)H shows a slightly smaller temperature coefficient (9.1 ppb deg^{-1} in **6** and 6.2 ppb deg^{-1} in **7**). These temperature coefficients are however larger than the κ values of ≈3 ppb deg^{-1} which have been reported in DMSO solution for hydroxy protons involved in strong hydrogen bonds.[2,5]

Vicinal coupling constants ($^3J_{HO,CH}$). According to the Karplus equation derived for hydroxy protons,[23] vicinal coupling constants ($^3J_{HO,CH}$) of the order of 5.5 ± 0.5 Hz indicate a free rotation of the hydroxy group around the C–O bond. Tables 1 and 2 show that all hydroxy protons in the monosaccharides **1**–**5** and all hydroxy protons with the exception of O(2″)H in **6** and O(2′)H in **7** have $^3J_{HO,CH}$ values of 5.5 ± 1 Hz representing rotational averaging of the hydroxy groups. In **6**, the O(2″)H has a $^3J_{HO,CH}$ value of 8.6 Hz and in **7**, the O(2′)H has a value of 9.2 Hz. These large coupling constants indicate that the rotation around the H–C–O–H fragment is relatively restricted. According to the Karplus equation,[23] a coupling constant of ≈ 9 Hz indicates a predominant (≈ 70%) conformation in which the O–H bond is almost *anti* to the C–H bond. It is also possible that the hydroxy proton is locked in an orientation around the C–O bond which gives such a value of the coupling constant. This rotational restriction around the C–O bond may indicate that O(2″)H in **6** and O(2′)H in **7** are involved in hydrogen bonding. A space filling model, built for the minimum energy conformation calculated from the HSEA program,[13] shows that in **6**, the O(2′)–H(2′) bond is *syn* to

the C(2′)–H(2′) bond. This *syn* orientation agrees well with a $^3J_{HO,CH}$ for O(2′)H of 4.8 Hz and places O(2′)H in close proximity to O(2″).

NOESY and ROESY experiments. Both NOESY and ROESY spectra were acquired to discriminate between cross-peaks due to dipolar relaxation and cross-peaks due to chemical exchange. In NOESY both exchange and dipolar relaxation contributions 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 proton exchange are positive and of the same sign as the diagonal peaks.

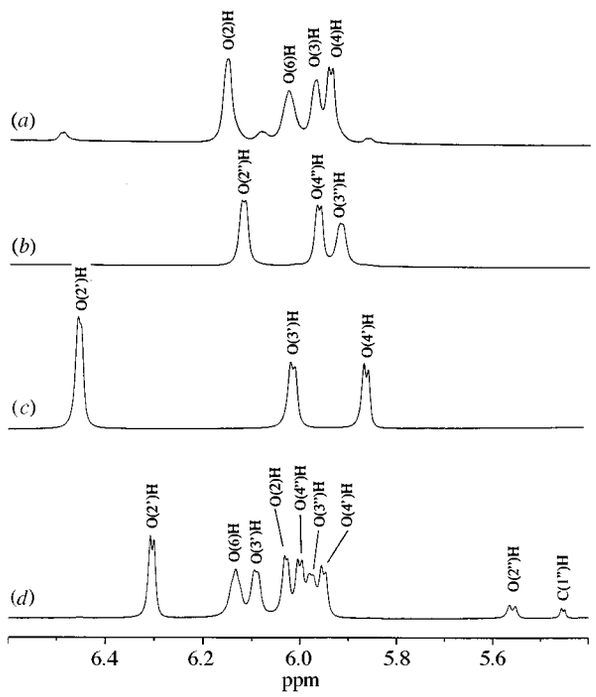

Fig. 2 Hydroxy region of the ^{1}H NMR spectra [85% H_2O–15% $(CD_3)_2CO$, −8 °C] of (*a*) Me α-D-Gal*p*, (*b*) Me α-L-Fuc*p*, (*c*) Me β-L-Fuc*p* and (*d*) trisaccharide **6**

Trisaccharide **6**.—NOESY, recorded at −8 °C and with a mixing time of 100 ms, shows NOEs between the O(2″)H and the O(2′)H [Fig. 3(a)]. In the ROESY spectrum, the O(2″)H–O(2′)H cross-peak has the same sign as the diagonal peaks indicating that the contribution of chemical exchange dominates. The NOESY and ROESY spectra additionally revealed spatial proximity between O(2′)H and C(1″)H, C(1″)H and C(1′)H, C(1″)H and C(4)H, C(1′)H and C(3)H and between C(1′)H and C(4)H [Fig. 4(*a*)].

Trisaccharide **7**.—Using the same experimental conditions as for **6**, we found a chemical exchange cross-peak between O(2″)H and O(2′)H as well as a weaker chemical exchange cross-peak between O(6)H and O(6″)H [Fig. 3(*b*)]. NOE cross-peaks are also present between O(2′)H and C(1″)H, C(1″)H and C(1′)H, C(1″)H and C(4)H, C(1′)H and C(4)H and between C(1′)H and C(3)H [Fig. 4(*b*)].

It has been shown that exchange cross-peaks can be diagnostic of spatial proximity and hydrogen bonds.[24,25] It is thus reasonable to assume that, in both **6** and **7**, a hydrogen bond between O(2″)H and O(2′)H exists in aqueous solution. In **6**, the O(2′)H–C(1″)H and O(2′)H–O(2″)H connectivities indicate an O(2′)H–O(2″) hydrogen bond direction. That the O(2′)H of β-L-Fuc*p* is the donor is consistent with its slower rate of exchange with water (*vide infra*). In **7**, the O(2′)H–C(1″)H and O(2′)H–O(2″)H connectivities are consistent with an O(2′)H–O(2″) hydrogen bond direction. This hydrogen bond direction is also supported by the large $^3J_{HO,CH}$ of O(2′)H and by its slower exchange rate with water (*vide infra*).

Rate of exchange with water. Protons involved in strong hydrogen bonds exchange more slowly with the solvent and the measurement of the rate of exchange of hydroxy protons with water should provide direct evidence of their existence. The rates of exchange (k_{ex}) for the hydroxy protons in **1**–**7** with the bulk water were measured at −8 °C from a series of eight chemical exchange experiments recorded with mixing times in the range 3–24 ms (see Experimental). At short mixing times

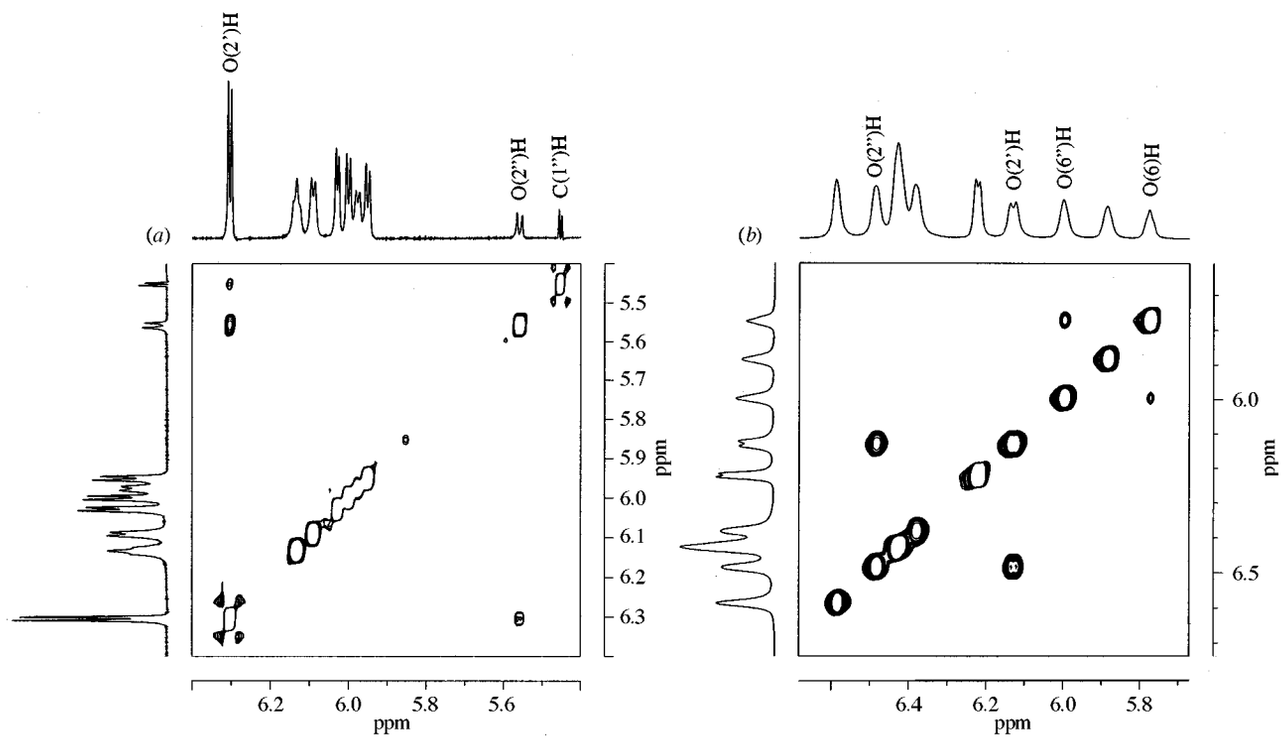

Fig. 3 Expanded hydroxy proton region of the NOESY spectra [85% H_2O–15% $(CD_3)_2CO$, −8 °C] of (*a*) trisaccharide **6**, showing the O(2′)H–O(2″)H and O(2′)H–C(1″)H cross-peaks. (*b*) Trisaccharide **7**, showing the O(2′)H–O(2″)H and O(6)H–O(6″)H cross-peaks. From ROESY experiments, the cross-peaks between hydroxy protons in **6** and **7** were found to be dominated by chemical exchange.

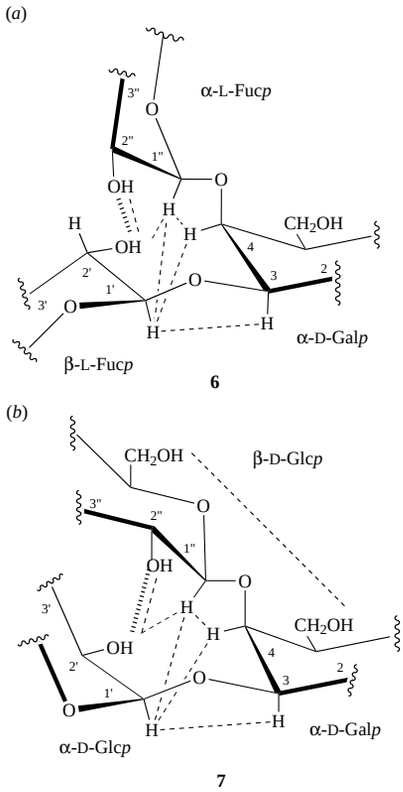

Fig. 4 Schematic representation of (*a*) trisaccharide **6** and (*b*) trisaccharide **7**, showing with dashed lines the inter-residue NOEs and with dotted lines the inter-residue hydrogen bond between the O(2′)H and O(2″) atoms

(3–18 ms), only cross-peaks between hydroxy protons and water were observed and no chemical exchange cross-peaks were observed between hydroxy protons.

Trisaccharide **6**.—The O(2′)H has the slowest rate of exchange with water corroborating its participation in hydrogen bonding [Table 1 and Fig. 5(*a*)]. The exchange rate measured for O(2″)H in **6** is not reliable, because, being close to the water resonance, it can be affected by the solvent suppression scheme. The k_{ex} values for the other hydroxy protons in the mono- and tri-saccharide are very similar. Such a comparison must however be done with caution because, while chemical shifts and coupling constants are quite insensitive to the solvent composition and to the presence of impurities, exchange rates are sensitive to solvent composition and to catalysis by small traces of impurities.

Trisaccharide **7**.—O(2′)H shows the slowest exchange rate [Table 2 and Fig. 5(*b*)]. O(2)H and O(6)H have similar and intermediate exchange rates, while the other hydroxy protons exchange faster with the solvent. The slower k_{ex} of O(2′)H and O(6)H is in agreement with their involvement in hydrogen bond formation, but the participation of O(2)H in an inter-residue hydrogen bond is not anticipated.

Deuterium isotope effects on ^{13}C chemical shifts. To obtain additional evidence on the existence of hydrogen bonding between O(2) of α-L-Fuc*p* and O(2)H of β-L-Fuc*p* in **6** and between O(2) of β-D-Glc*p* and O(2)H of α-D-Glc*p* in **7**, the ^{1}H/^{2}H isotope effects of hydroxy protons on the ^{13}C chemical shifts[5,26,27] were investigated. If the exchange of the hydroxy protons with water is slow enough, the ^{1}H/^{2}H isotope effects of hydroxy protons on the ^{13}C resonances can be observed as broadened or split resonances. The effect is more pronounced for carbons bearing hydroxy groups involved in stable hydrogen bonds. The ^{13}C NMR spectra of **6** and **7** in H_2O–D_2O, 1 : 1, solution are shown in Fig. 6 for different temperatures.

Trisaccharide **6**.—As the temperature is decreased, the signals of the carbons bearing OH groups begin to broaden [Fig. 6(*a*)]. At 1 °C, four resonances, namely for C(2′), C(4″), C(2)

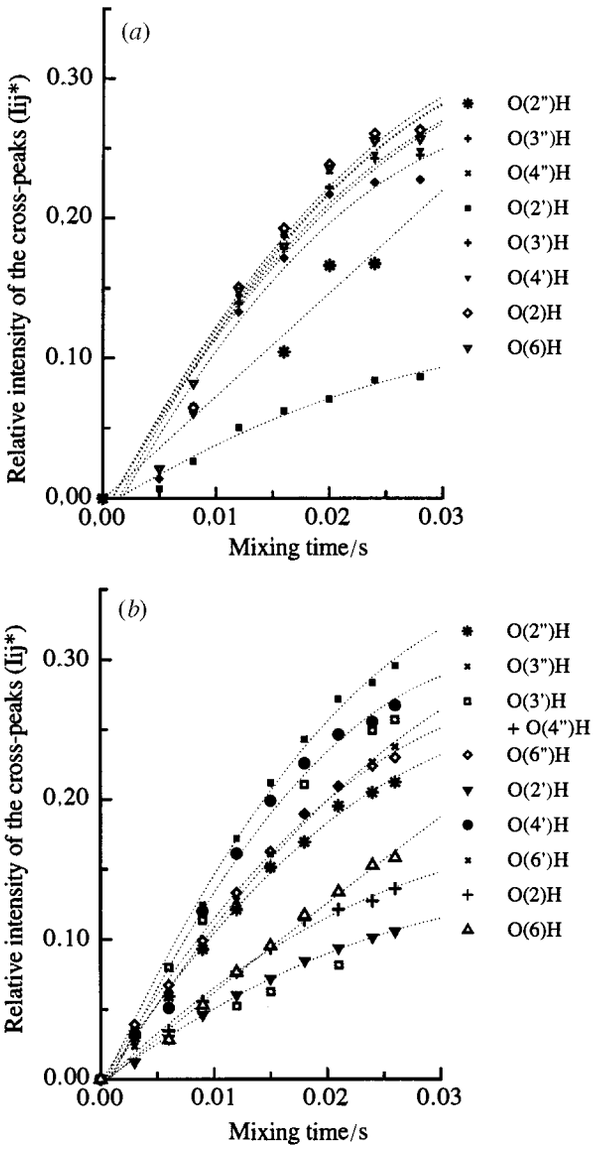

Fig. 5 Build-up curves for the chemical exchange between hydroxy protons and water of (*a*) trisaccharide **6** and (*b*) trisaccharide **7**. The cross-peaks I_{ij}* have been normalized to the diagonal intensity at $\tau_m = 0$ ms. The diagonal intensity at zero mixing time was obtained by extrapolation of the diagonal peaks at a particular mixing time to zero mixing time. The build-up curve for O(2″)H in **6** is not reliable due to the proximity of O(2″)H to the water resonance.

and C(6), show a clear splitting of *ca.* 0.09 to 0.12 ppm. This spacing correlates well with the deuterium isotope effect observed for carbons directly bonded to hydroxy groups.[26] The other ^{13}C–OH resonances are broad with no clear splitting. The signal of C(2′) is split because of slower exchange due to a hydrogen bond with O(2″). Since the ^{1}H NMR data indicated that the hydroxy protons on C(4″), C(2) and C(6) are not involved in hydrogen bonds, the ^{13}C NMR spectra of the monosaccharides **1**–**5** were also measured. We found that at 1 °C, the C(2) and C(6) signals of Me α-D-Gal*p* are split by 0.11 ppm. Beside the influence of temperature, the splitting is also dependent on the concentration and on the solvent composition. At concentration above 100 mM, C(2) and C(6) are split at 5 °C, while at 50 mM, only broadening of the carbon resonances is observed. The addition of 10% $(CD_3)_2CO$ to the 50 mM solution results in a clear splitting already at 5 °C. All ^{13}C–OH resonances in Me α-D-Glc*p* appear as doublets while in Me α-L-Fuc*p*, Me β-L-Fuc*p* and Me β-D-Glc*p*, no splitting is observed and the ^{13}C resonances are still sharp at 1 °C.

In **6**, the isotope effect observed for C(2) and C(6) of Me α-D-Gal*p* might be explained by a slower exchange due to intra-

molecular hydrogen bonding between O(2)H and O(3) and between O(6)H and O(5). The splitting observed for C(4″) of α-L-Fuc*p* in **6** can be explained by an intramolecular hydrogen bonding between O(4)H and O(3). To the solution, 10% $(CD_3)_2CO$ was added in order to be able to decrease the temperature to −5 °C. Fig. 6(*a*) shows that at −5 °C, all ^{13}C–OH resonances are split into more or less resolved multiplets indicative of short and long range isotope effects. For example, the signal of C(2″) is split by 0.12 ppm due to C(2″)–OH and C(2″)–OD components. An extra splitting of 0.05 ppm is also observed on C(2″) due to the long range C(3″)–OH − C(3″)–OD effect.

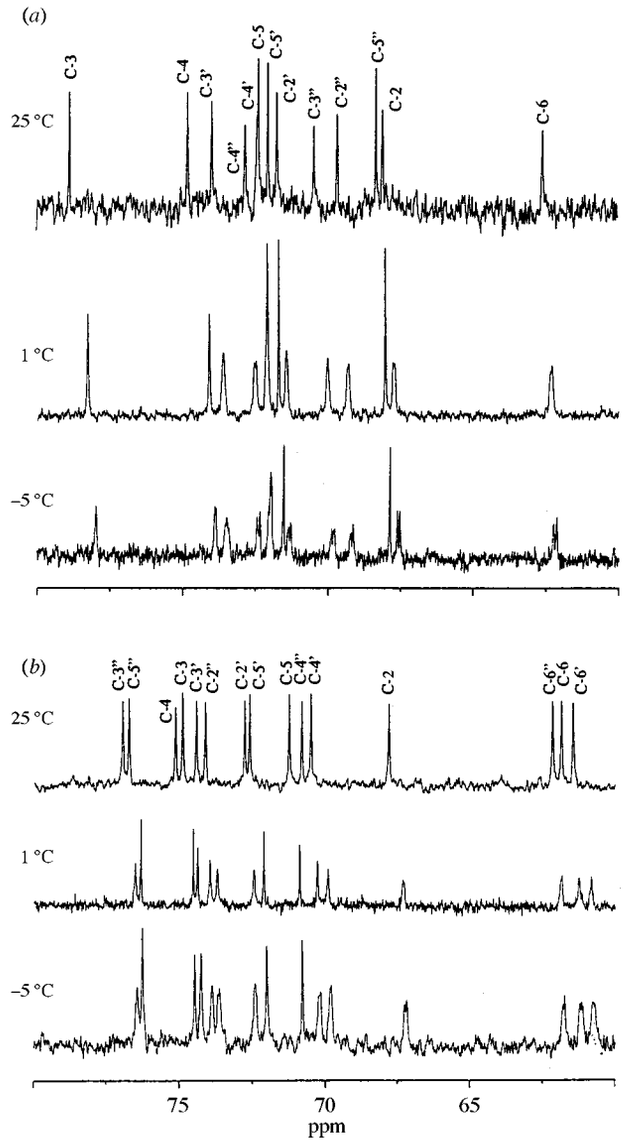

Fig. 6 One-dimensional $^{13}C\{^1H\}$ NMR spectra in 50% H_2O–50% D_2O of (*a*) trisaccharide **6** and (*b*) trisaccharide **7**

Trisaccharide **7**.—As the temperature is decreased, the ^{13}C–OH resonances begin to broaden [Fig. 6(*b*)] and at 1 °C, C(2′) and C(2″) are the most broadened signals. A further decrease in temperature to −5 °C [10% $(CD_3)_2CO$ added to the solution] results in a splitting of the ^{13}C–OH resonances, but as in **6**, no clear evidence upon the involvement of a particular hydroxy group in an inter-residue hydrogen bonding can be obtained.

Hydroxy protons in $[^2H_6]Me_2SO$ solution (Table 3)

Trisaccharide **6**.—As expected, all hydroxy proton resonances experience an upfield shift in DMSO if compared to those obtained in H_2O, but as in H_2O, the O(2″)H is very shielded. The $^3J_{HO,CH}$ coupling constants are very similar to those measured in water except for O(2″)H which shows a coupling constant in DMSO of 5.6 Hz (8.6 Hz in H_2O). The temperature coefficients calculated from the values of the chemical shifts from 10 to 30 °C are smaller (5 to 9 ppb deg^{-1}) than in water, but no major differences between the different OH protons are found.

Trisaccharide **7**.—O(2′)H in **7** has, as in H_2O, a large $^3J_{HO,CH}$ of 9.4 Hz indicating the same restricted rotation around the H–C–O–H fragment. The temperature coefficient for O(2′)H is 2.6 ppb deg^{-1}, suggesting its involvement in a strong hydrogen bond.

The conformation around the C(5)–C(6) bond, which was not determined in aqueous solution due to spectral overlap, could be estimated for **7** in DMSO solution from the $^3J_{H5,H6a}$ and $^3J_{H5,H6b}$ measured in DQF–COSY spectra. The α-D-Glc*p* and α-D-Gal*p* residues have $^3J_{H5,H6a}$ of respectively 2.4 and 2.0 Hz and $^3J_{H5,H6b}$ of respectively 5.9 and 5.2 Hz indicating a slight preference for the *gg* conformation. For the β-D-Glc*p* residue, a $^3J_{H5,H6a}$ of 2.7 Hz and a $^3J_{H5,H6b}$ of 8.7 Hz indicate a preferred *gt* conformation. The *gg* conformation of α-D-Gal*p* and *gt* conformation of β-D-Glc*p* are favourable for hydrogen bonding between O(6) and O(6″)H and is in agreement with the NOE observed between O(6)H and O(6″)H in both aqueous and DMSO solutions. The assignment of the prochiral protons H(6a) and H(6b) for the α-D- and β-D-Glc*p* moieties was assumed to be analogous to D-glucose, as is usually done in the literature.[28,29] The same rules were applied for α-D-Gal*p*. If the assignment for α-D-Gal*p* is reversed, the *gg* conformation is still favoured, but the equilibrium is between the *gg* and *tg* conformation instead of between the *gt* and *gg* conformation.

All NOEs found in aqueous solution are also present in

Table 3 ^{1}H NMR chemical shifts (δ), $^3J_{HO,CH}$ coupling constants (J/Hz) and temperature coefficients (κ/ppb deg^{-1}) for the hydroxy protons of trisaccharides **6** and **7** measured in $[^2H_6]Me_2SO$

		6			**7**		
		δ^a	J/Hz	κ/ppb deg^{-1}	δ	J/Hz	κ/ppb deg^{-1}
Me α-D-Gal*p*	O(2)H	4.454	3.8	6.5	4.102	6.4	6.3
	O(3)H						
	O(4)H						
	O(6)H	4.596	5.7	5.9	4.420	5.7	4.3
4-*O*-glycosyl	O(2″)H	3.845	4.6	5.8	5.081	5.6	9.5
	O(3″)H	4.551	5.5	7.6	4.920	4.9	7.6
	O(4″)H	4.389	4.5	6.9	4.950	5.3	6.3
	O(6″)H				4.650	5.1	5.9
3-*O*-glycosyl	O(2′)H	4.793	3.9	5.5	4.571	9.3	2.6
	O(3′)H	4.727	5.8	7.5	4.720	4.6	6.9
	O(4′)H	4.519	4.7	6.7	4.740	5.3	6.0
	O(6′)H				4.312	5.6	5.2

[a] Chemical shifts at 25 °C. Primed labels refer to the 3-*O*-glycosyl group and double-primed labels to the 4-*O*-glycosyl group.

DMSO for both trisaccharides. A major difference comes from the cross-peaks between hydroxy protons. In water, chemical exchange is dominant. In DMSO, the ROESY cross-peaks have an opposite sign from the diagonal indicating that the relative contribution of dipolar relaxation is predominant.

Conclusion

The chemical shifts, coupling constants and rates of exchange with water of the hydroxy protons together with the NOESY and ROESY experiments strongly suggest the existence of a hydrogen bond between O(2) of α-L-Fuc*p* and O(2)H of β-L-Fuc*p* in **6** [Fig. 4(*a*)] and between O(2) of β-D-Glc*p* and O(2)H of α-D-Glc*p* in **7** [Fig. 4(*b*)]. A weak and transient hydrogen bond may also exist between O(6) of α-D-Gal*p* and O(6)H of β-D-Glc*p* in **7**. It is probable that hydrogen bonds involving the latter two hydroxy groups form and break during the conformational transitions of the exocyclic groups. The NOEs and hydrogen bond interactions observed for **6** and **7** in aqueous solution are the same as those observed in DMSO and are in good agreement with the interatomic distances obtained using HSEA/GESA methods.[13]

The NOEs between hydroxy and aliphatic protons provide an additional set of data for the determination of 3D structures in a similar way as is being done for proteins and nucleic acids. Even if, due to exchange with water, only a qualitative interpretation can be done, the measurement of NMR constraints with hydroxy protons should improve the characterisation of oligosaccharides in aqueous solution.

The data obtained from the deuterium isotope effects on the ^{13}C chemical shifts are difficult to interpret since other carbons than the one substituted with a hydroxy group involved in interresidue hydrogen bonds show splitting due to deuterium isotope effects. The measurement of ^{13}C NMR spectra with a sufficient signal to noise ratio is anyhow limited to relatively concentrated solutions and is not today applicable to dilute solutions of larger carbohydrates.

We are aware that the NMR data might represent conformational averaging and that the hydrogen bond interactions might be mediated by complexed water molecules. From molecular dynamics simulations[30–32] that include the solvent molecules, information about the hydration, the persistence of the hydrogen bonds and their importance in determining and stabilising a particular conformation could be obtained.

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