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Secondary H/D isotope effect on hydrogen-bonded hydroxyl groups as a tool for recognizing distance constraints in conformational analysis of oligosaccharides

Janusz Dabrowski[a,*], Horst Grosskurth[a], Carmen Baust[a] and Nicolay E. Nifant'ev[b]
[a]*Max Planck Institute for Medical Research, Jahnstrasse 29, D-69120 Heidelberg, Germany* [b]*Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospect 47, Moscow 117913, Russia*

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Abstract

An 'isotopomer-selected NOE' (ISNOE) method for the unequivocal identification of mutually hydrogen-bond-linked hydroxyl groups is described. It relies on the fact that the OH group's signal patterns obtained for a partially deuterated sample originate from both isotopomers of the 'partner' hydroxyl, whereas a NOE for this group can originate from cross-relaxation with the protio isotopomer of this hydroxyl only. Hence, the isotopically shifted component of this group's signal does not appear in a ROE difference spectrum obtained with selective excitation of the 'partner' hydroxyl. This method is also applicable in those cases when only one of two mutually hydrogen-bonded groups exhibits resolvable isotope shifts. Furthermore, it is shown that isotope shifts may occur even for pairs of OH groups that are not mutually hydrogen-bonded, if these participate in hydrogen bonds with other hydroxyls and thereby affect conformational equilibria. The ISNOE experiment enables one to distinguish between these two sources of isotope shifts. Since the O · · · O distance for hydrogen-bonded hydroxyls in sugars is known to lie between 2.7 and 3.0 Å, the hydrogen bonds established by ISNOE can be used in conformational analysis as reliable, motionally non-averaged distance constraints for the conformations containing these bonds.

Introduction

Interactions of the oligosaccharide moieties of glycolipids and glycoproteins with various receptors are fundamental processes in many biological events, particularly those related to immunology. For such interactions to be effective, it is essential that the three-dimensional (3D) structure (conformation) of the carbohydrate ligand fits the spatial requirements of the receptor's binding site. Hence, a knowledge of the conformational features of both entities is indispensable for understanding these processes on the molecular level. NMR is the main source of structural information for biomolecules in solution. However, a serious problem with conformational analysis based on NMR observables (NOEs, coupling constants, chemical shifts, relaxation times) is that their measured values represent averages in the case of rapidly equilibrating conformations. As a result, conformations derived from averaged parameters must be considered 'virtual' (Jardetzky, 1980; Cumming and Carver, 1987) and may, in fact, not exist. For this reason, the search for conformations that contribute to the equilibrium utilizes theoretical methods, e.g. molecular mechanics (MM) and dynamics (MD). However, the predictive capacity of these methods depends on the parametrization of the force fields used, which must be verified by experiment – a task for which conformationally averaged parameters are unsuitable in most cases.

One can escape this vicious circle if interresidue hydrogen bonds can be unequivocally established. These provide reliable, *non-averaged* structural constraints, with an O · · · O distance of ∼2.7–3.0 Å (Jeffrey and Saenger, 1991); hence, the validity of any

*To whom correspondence should be addressed.

force field can be tested by its ability to reproduce conformations containing hydrogen bonds in cases when these conformations are incompatible with the virtual one. The reliability of such a test depends on how convincingly a hydrogen bond can be defined experimentally. Although specific features of NMR spectra, such as reduced temperature dependence of OH chemical shifts or the deviation of $^3J_{HOCH}$ coupling constants from the free rotation value of 5.4 Hz, are often taken as indicators of hydrogen bonds, such evidence is equivocal because alternative interpretations are possible.

On the other hand, the deuterium isotope effect, manifested in partially deuterated samples by a doubling of the signals from two OH groups, has generally been accepted as proof of a hydrogen bond between these groups (Lemieux and Bock, 1979; Bock and Lemieux, 1982; Christofides and Davies, 1982; Poppe et al., 1990a,b; Dabrowski et al., 1995; Angyal and Christofides, 1996; Craig et al., 1996). However, the problem of the use of this information in conformational studies has turned out to be more complex than was originally thought. One important new observation is that a deuterium isotope effect may be resolvable for only one hydroxyl group in a molecule (Kozar et al., 1995). This means that the 'partner' OH group participating in the hydrogen bond must be identified before the isotope effect can be used in a conformational study. By the same token, even if two OH groups show isotope effect, it is possible that each of them is hydrogen-bonded to a partner which exhibits no resolvable isotope shift. Unless this possibility can be ruled out, the signal doubling observed for two OH groups cannot be considered as unequivocal evidence of hydrogen bonding between them. The knowledge of this ambiguity prompted us to scrutinize again the deuterium isotope effects on Gal OH2 and Glc OH4 in the disaccharide Galβ1-3Glcβ1-OMe. These effects were taken as proof for a hydrogen bond between these hydroxyl groups and, hence, for the existence of an *anti* conformation of the disaccharide (Dabrowski et al., 1995).

Another puzzling phenomenon was our observation of isotope effects for two OH groups which could not possibly form a hydrogen bond in any allowed conformation. Several examples of unexplained isotope patterns were also mentioned by Angyal and Christofides (1996).

We present here an 'isotopomer-selected NOE' method for the unequivocal identification of any two mutually hydrogen-bonded hydroxyl groups. This method enables one to resolve all of the ambiguities described above, so that hydrogen bonds can be unambiguously identified and used as distance constraints in conformational analysis.

Materials and Methods

Compounds investigated

Methyl 3-O-(β-L-rhamnopyranosyl)-β-D-talopyranoside (*1*) and methyl 3-O-(α-L-rhamnopyranosyl)-β-D-talopyranoside (*2*) (Nifant'ev et al., 1988), methyl 3-O-(β-D-galactopyranosyl)-β-D-glucopyranoside (*3*) (Nifant'ev et al., 1993), methyl 3-O-(β-D-fucopyranosyl)-β-D-quinovopyranoside (*4*) and methyl 3-O-(3-O-methyl-β-D-fucopyranosyl)-β-D-quinovopyranoside (*5*) (Khatuntseva et al., 1997) were obtained as described. Their structures are shown in Scheme 1.

Sample preparation

Samples of the compounds investigated (2–10 mg) were dried under high vacuum and dissolved in 0.4 mL Me_2SO-d_6 (99.95%; Merck, Darmstadt). Partially deuterated samples were obtained from these solutions by adding a calculated amount of '100%' D_2O (Merck, Sharp & Dohme).

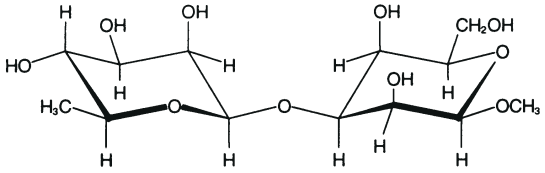
In order to obtain meaningful ROE spectra, the rate of proton exchange was reduced to satisfy the condition $k \ll (T_{1\rho})^{-1}$ by adding a tiny amount of 'Al_2O_3-90', activity II-III (Merck, Darmstadt), to the solution in the sample tube. In spite of the presence of undissolved particles, spectral resolution remained practically unchanged.

NMR measurements

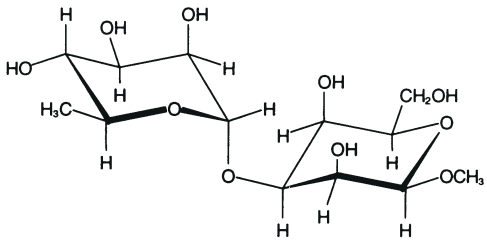
All spectra were recorded on a Bruker AM 500 spectrometer at a frequency of 500 MHz. Chemical shifts were referenced indirectly to Me_4Si by setting the ^1H signal of the residual Me_2SO-d_5 at 2.50 ppm. Assignments were obtained with the aid of two-dimensional (2D) COSY spectra (Bruker software).

Isotope effects were observed in conventional one-dimensional (1D) spectra recorded with a digital resolution of 0.18 Hz/pt. Transient 1D ROE experiments were performed with DANTE excitation, 300 ms mixing time, direct subtraction of reference FIDs every eight scans, and accumulation of a total of up to 5×10^4 scans, depending on the available amount of the substance.

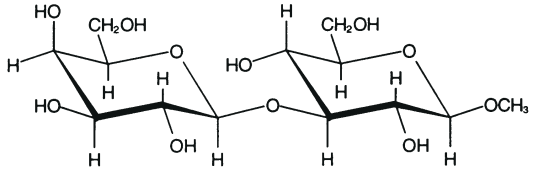
ROE rather than NOE spectra were recorded in order to distinguish ROE difference signals from signals due to proton–proton exchange. The latter are



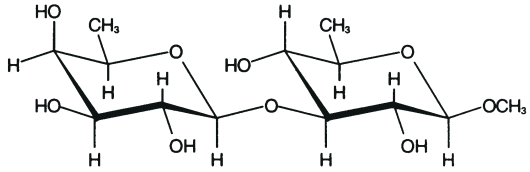
β-L-Rha(1-3)-β-D-Tal1-OMe (*1*)



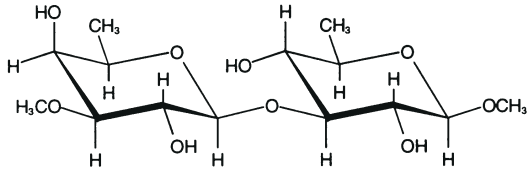
α-L-Rha(1-3)-β-D-Tal1-OMe (*2*)



β-D-Gal(1-3)-β-D-Glc1-OMe (*3*)



β-D-Fuc(1-3)-β-D-Qui1-OMe (*4*)



3-OMe-β-D-Fuc(1-3)-β-D-Qui1-OMe (*5*)

Scheme 1.

of opposite phase and can reduce or cancel the ROE signals. Examples of fully or only partially eliminated exchange will be discussed where appropriate.

Results

The ^{1}H chemical shifts for disaccharides *1*, *2*, *4* and *5* in DMSO-d_6 are gathered in Table 1, and those for *3* were published earlier (Dabrowski et al., 1995). The $^{3}J_{H,OH}$ coupling constants, temperature coefficients κ and isotope effects Δδ for hydroxyl protons of compounds *1–5* are presented in Table 2.

As pointed out in the Introduction, the appearance of isotope-shifted signals for two hydroxyl groups after partial deuteriation of the sample does not necessarily mean that these groups are mutually hydrogen-bonded, because each of them may be bonded to hydroxyl groups which do not exhibit resolvable isotope shifts. Obviously, the assignment of hydrogen-bonded OH pairs will be even more complicated if more than two OH signals exhibit isotope shifts. We found a solution to this problem by measuring 1D ROE difference spectra under conditions where proton exchange rates were sufficiently lower than cross-relaxation rates. The rationale behind our method can be illustrated by the identification of the well-known hydrogen bond between the two axial hydroxyl groups, OH2 and OH4, of taloses (Reuben, 1984; Angyal and Christofides, 1996). In a partially deuterated sample there are four isotopomers, of which the first three are 'visible' in ^{1}H NMR (Scheme 2).

(i) OH2 OH4
(ii) OH2 OD4
(iii) OD2 OH4
(iv) OD2 OD4

Scheme 2.

Table 1. Chemical shifts (δ)[a] for C-linked protons of compounds *1*, *2*, *4* and *5* in DMSO-d_6 at 303 K

Proton	β-L-Rha(1-3)-β-D-Tal1-OMe (*1*)		α-L-Rha(1-3)-β-D-Tal1-OMe (*2*)		β-D-Fuc(1-3)-β-D-Qui1-OMe (*4*)		3-OMe-β-D-Fuc(1-3)-β-D-Qui1-OMe (*5*)	
	Rha	Tal	Rha	Tal	Fuc	Qui	3-OMeFuc	Qui
H1	4.63	4.29	4.78	4.26	4.27	4.15	4.30	4.14
H2	3.71	3.76	3.67	3.84	3.36	3.17	3.46	3.17
H3	3.23	3.64	3.54	3.50	3.34	3.29	3.06	3.30
H4	3.13	3.84	3.20	3.70	3.41	2.92	3.66	2.92
H5	3.11	3.29	3.56	3.32	3.63	3.28	3.61	3.27
H6	1.16	3.53	1.12	3.51	1.12	1.18	1.14	1.18
H6′		3.60		3.59				
OCH_3		3.40		3.40		3.36	3.33	3.36

[a]Chemical shifts are in ppm for DMSO-d_6 solution at 303 K, referred to DMSO-d_5 set at 2.50.

Table 2. ^{1}H NMR chemical shifts (δ)[a], coupling constants (J)[b], temperature coefficients (κ)[c] and isotope effects (Δδ)[d] for hydroxyl proton signals of the disaccharides *1–5*[e]

Compound	Residue	Proton	δ	J	κ	Δδ
1	β-Rha	OH2	4.32	4.3	−7.0	
	(B)	OH3	4.55	6.2	−7.4	+21.0
		OH4	4.75	5.1	−6.0	
	Tal	OH2	4.98	5.5	−6.3	+141.6
	(A)	OH4	4.43	8.3	−2.6	+71.3
		OH6	4.65	5.1	−5.4	
2	α-Rha	OH2	4.73	4.4	−5.8	
	(B)	OH3	4.55	5.9	−7.4	
		OH4	4.72	5.3	−6.8	
	Tal	OH2	5.02	6.2	−6.6	+155.8
	(A)	OH4	4.52	8.0	−2.2	+128.5
		OH6	4.64	6.3; 5.1	−5.3	
3[f]	Gal	OH2	4.89	3.3	−4.8	+28.4; −26.2
	(B)	OH3	4.75	5.6	−6.7	
		OH4	4.45	4.6	−5.6	
		OH6	4.62	5.3	−4.8	
	Glc	OH2	5.11	3.9	−5.5	
	(A)	OH4	4.68	2.0	−4.2	≈+33[g]
		OH6	4.53	5.9	−6.0	
4	Fuc	OH2	4.88	2.9	−5.2	+34.2; −31.8
	(B)	OH3	4.73	5.3	−6.8	
		OH4	4.51	4.8	−6.0	
	Qui	OH2	5.12	3.8	−6.0	+23.2[h]
	(A)	OH4	4.70	2.0	−4.7	+36.2; +17.0
5	3-OMeFuc	OH2	4.92	3.6	−5.4	+36.0; −36.0
	(B)	OH4	4.52	5.4	−6.2	
	Qui	OH2	5.12	4.0	−5.9	
	(A)	OH4	4.63	2.3	−4.5	+31.0; +16.6

[a]Chemical shifts are in ppm for DMSO-d_6 solutions at 303 K, referred to DMSO-d_5 set at 2.50 ppm.
[b]^{3}J values are in Hz at 303 K.
[c]Temperature coefficients are in ppm/K × 10.
[d]The values of the isotope effects are in $\Delta\delta \times 10^4$ ppm.
[e]The formulae are given in Scheme 1.
[f]Data from Dabrowski et al. (1995).
[g]A second isotope effect was not resolved.
[h]At 333 K.

In the talopyranose-containing disaccharide *1* (Figure 1b) the hydrogen-bonded hydroxyl Tal OH4 exhibits two doublets due to the two isotopomers (i) and (iii) arising from partial deuteration (30%) of its partner, Tal OH2, whereas an Overhauser effect for OH4 due to cross-relaxation with OH2 can originate from the protio isotopomer (i) only. As a consequence, the downfield shifted OH4 doublet from isotopomer (iii) does not appear in the ROE difference spectrum obtained with selective excitation of the OH2 proton (Figure 1c). In an analogous manner the OH2 signal for only isotopomer (i) appears when OH4 is excited (Figure 1d). We designate this procedure as 'isotopomer-selected NOE' (ISNOE). Virtually identical results were obtained for the disaccharide *2* (not shown; cf. Table 2).

In general, the signal pattern for a hydroxyl group exhibits a maximum of $N = n \times 2^m$ components, where n is the number of signal components for the all-protio isotopomer and m is the number of OH(D)

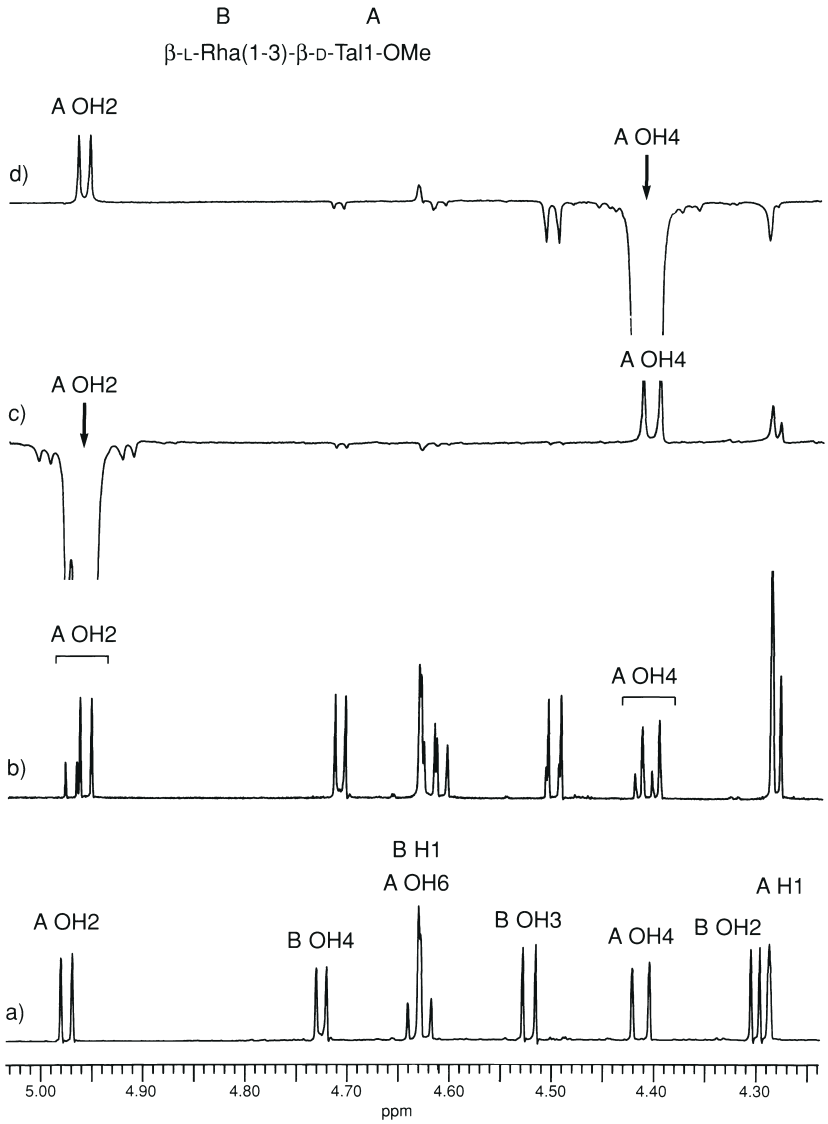


Figure 1. (a) Hydroxyl and anomeric proton region of a 500 MHz ^{1}H NMR spectrum of the disaccharide *1* in Me_2SO-d_6 at 303 K. (b) As in (a), with partially deuterated hydroxyl groups (H:D ≈ 70:30). (c,d) ROESY difference spectra of the deuterated sample, obtained with selective excitation (marked with arrows) of the A OH2 and A OH4 protons, respectively.

groups exerting the isotope effects. The number of resolved hydroxyl signal components is often less than $n \times 2^m$, due to limitations in resolution, the small magnitude of the isotope effect, and/or overlap of some of the components. If a signal pattern cannot be interpreted by inspection, it can usually be analysed by simulation. The variables required are the coupling constant $^{3}J_{H,OH}$, the number, magnitude and sign of the isotope shifts, the degree of deuteration, and the line width. For example, the Gal OH2 signal of the disaccharide *3* (Figure 2b) consists of five components, and the simulation revealed that these resulted from the overlap of several components due to the HH, HD, DH and DD isotopomeric combinations for the Glc-2 and Glc-4 hydroxyl groups, which produced the isotope shift for the Gal OH2 group. This is illustrated by Scheme 3.

It is noteworthy that the DD shift is practically equal to the algebraic sum of the HD and DH shifts, although, theoretically, these are not exactly additive. It should also be remarked that DD components will never appear as positive signals in any ISNOE exper-

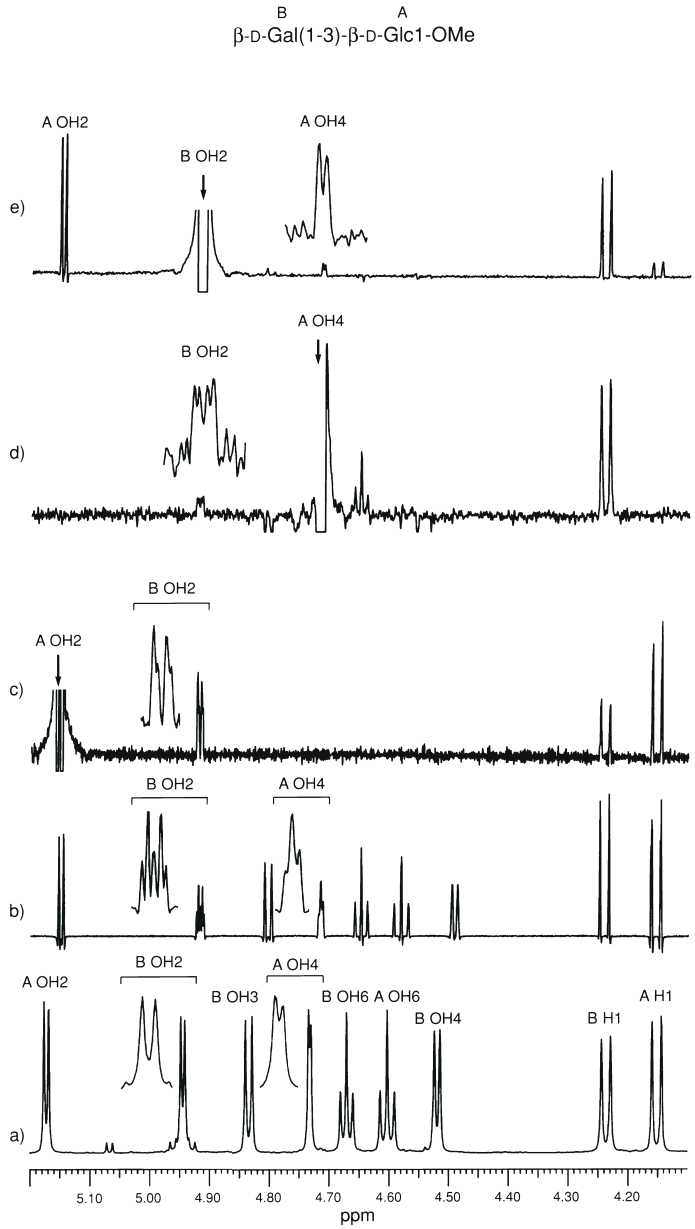


Figure 2. (a) Hydroxyl and anomeric proton region of a 500 MHz ^{1}H NMR spectrum of the disaccharide *3* in Me_2SO-d_6 at 293 K. (b) As in (a), with partially deuterated hydroxyl groups (H:D ≈ 63:37); inserts show expansions of the B OH2 and A OH4 signals. (c,d,e) ROESY difference spectra of the deuterated sample, obtained with selective excitation (marked with arrows) of the A OH2, A OH4 and B OH2 protons, respectively.

iment. As will be seen (Figure 3 and Scheme 4), the D components can contribute negative intensity due to undesirable exchange.

The ISNOE procedure is particularly useful for assigning hydrogen-bonded partners if only one of the hydroxyl groups shows an isotope effect. This is the case with the Gal OH2 group just discussed, which shows two isotope effects (Figure 2b), and Figure 2c proves that one of these effects, which induced a low-field shift of the signal, is due to hydrogen bonding with the Glc OH2 group, whose signal shows no resolved isotope effects in Figure 2b. Consequently, the question arises as to whether the isotope effect observed for the Glc OH4 group (Figure 2b) is due to

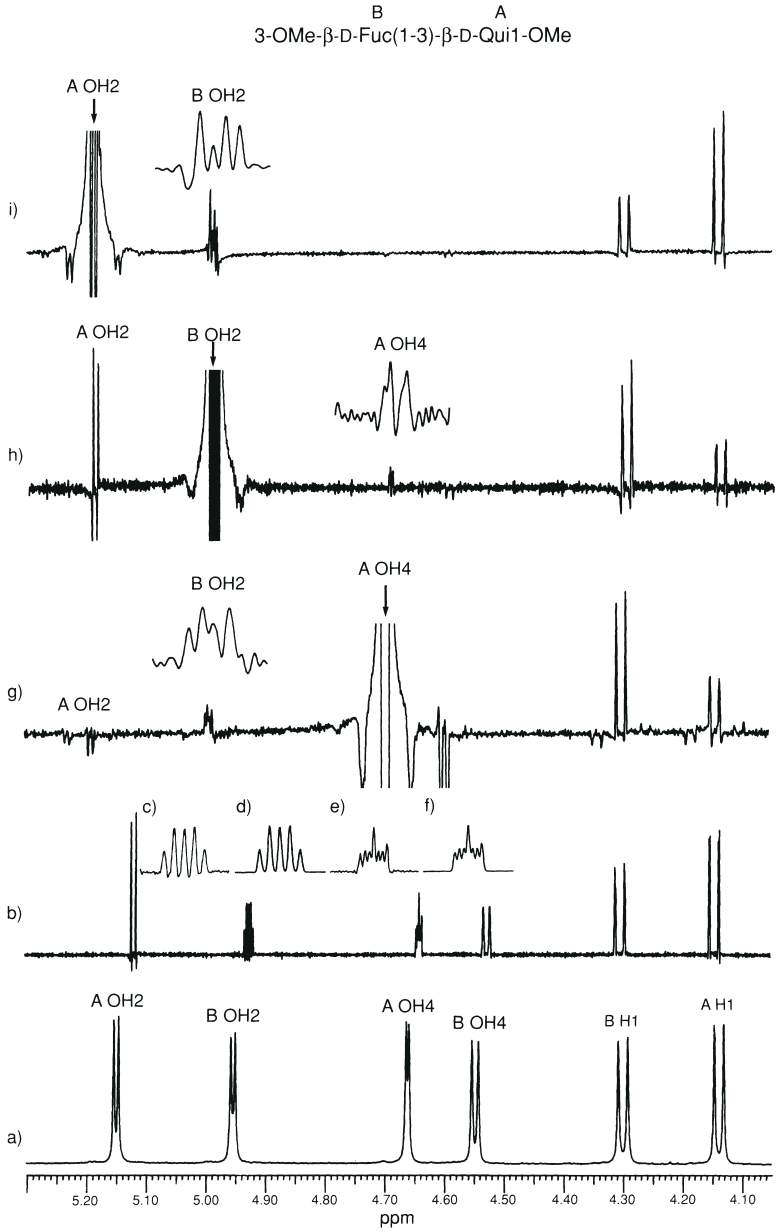
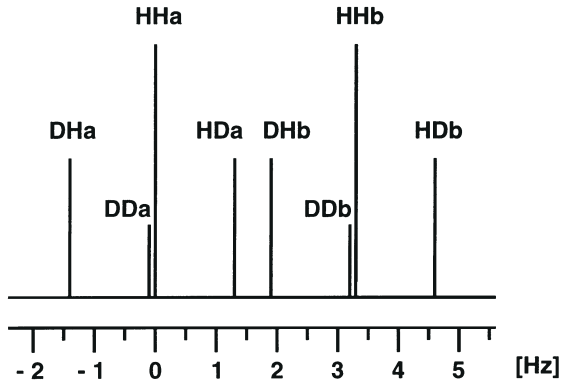


Figure 3. (a,b) As in Figures 2a and b, for disaccharide *5* (H:D ≈ 55:45). (c,d) Expanded and simulated B OH2 signal, respectively. (e,f) Expanded and simulated A OH4 signal, respectively. (g,h,i) ROESY difference spectra of the deuterated sample and expansions of the relevant signals; selective irradiation is indicated by arrows.

hydrogen bonding with the Gal OH2 group and can be taken as evidence for the existence of an *anti* conformation of *3* (Dabrowski et al., 1995), or due to hydrogen bonding with the nearby Glc OH6 and Gal OH6 groups in a *syn* conformation. However, the ISNOE experiment (Figure 2d) shows that the isotopically shifted high-field components of the Gal OH2 signal disappear from the spectrum on irradiation of Glc OH4, thus proving the existence of hydrogen bonding between these groups.

Most interestingly, the 6, 6′-dideoxy derivative of *3*, β-D-Fuc(1-3)-β-D-Qui1-OMe (*4*), exhibits two downfield isotope effects for the Qui OH4 group, one of them being due to the hydrogen bond with the Fuc

(i)	Gal OH2	Glc OH2	Glc OH4	(HH)
(ii)	Gal OH2	Glc OH2	Glc OD4	(HD)
(iii)	Gal OH2	Glc OD2	Glc OH4	(DH)
(iv)	Gal OH2	Glc OD2	Glc OD4	(DD)



Scheme 3.

OH2 group according to the ISNOE experiment (not shown; cf. Table 2). Since no other hydroxyl group can approach within hydrogen bonding distance in any conformation, one might conclude that the second isotope effect is transmitted through two hydrogen bonds from Fuc OH3/D3 via Fuc OH2/D2. Transmission of deuterium isotope effects through two hydrogen bonds was postulated recently by Angyal and Christofides (1996). However, the Qui OH4 signal pattern for the derivative lacking the Fuc OH3 group, 3-OMe-β-D-Fuc(1-3)-β-D-Qui1-OMe (*5*), also shows two isotope effects (eight partially overlapping lines, Figures 3b, e and f). One of these effects originates from hydrogen bonding with Fuc OH2 (Figure 3h), as was the case with *4*, but since Fuc OH3 is lacking in *5*, the source of the second isotope effect at Qui OH4 must be sought elsewhere.

Before pursuing this issue, let us first analyse the particularly instructive results of ISNOE experiments concerning the Fuc OH2 group with 45% deuteration (Figure 3). For $n = 2$, $m = 2$ (a doublet, two isotope effects), eight signal components are expected, some of which overlap (Figure 3c), as confirmed by the simulated signal pattern in Figure 3d. ISNOE experiments not only enable one to establish the hydrogen bond 'connectivities' between OH groups, but also facilitate the determination of the sign of the isotope shift, a low-field shift being positive by convention. For example, the isotope effect of Qui OD4 on Fuc OH2 is negative (Figure 3g versus 3c) and the isotope effect exerted by Fuc OD2 on Qui OH4 is positive (Figure 3h versus 3e).

The Fuc OH2{Qui OH2} signal (Figure 3i) requires comment because of some contribution of proton-proton exchange during the mixing time.

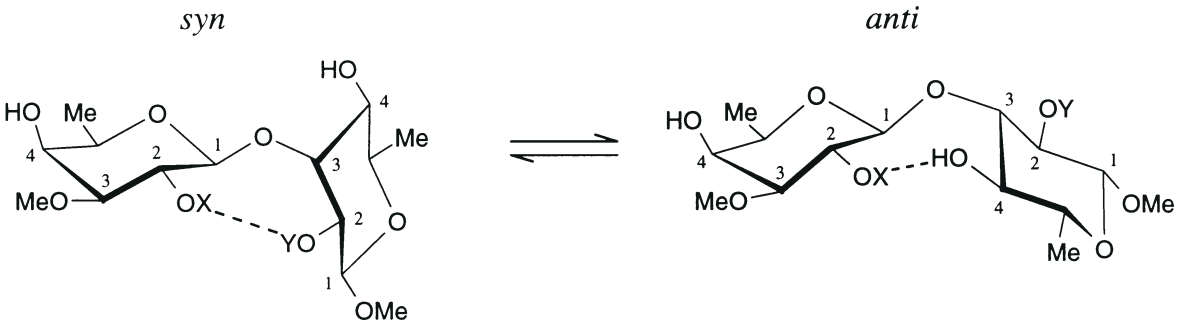
The exchange rate is smaller than the cross-relaxation rate, so that net positive Fuc OH2 doublets for isotopomers (i) and (ii) (Scheme 4) are observed. However, the low-field component of the high-field shifted doublet for (ii) is reduced in intensity because it overlaps with the high-field component of the low-field shifted doublet for (iii), which cannot contribute an Overhauser effect, but, on the contrary, contributes a negative intensity due to exchange from Qui OH2 to Fuc OH2. Correspondingly, the low-field component of the low-field shifted doublet for (iii) is of negative intensity. Isotopomer (iv) also contributes negative intensity due to the same exchange process, thus slightly reducing the almost exactly overlapping Fuc OH2 signals of the all-protio isotopomer (i) (cf. the similar Scheme 3).

(i)	Fuc OH2	Qui OH2	Qui OH4	(HH)
(ii)	Fuc OH2	Qui OH2	Qui OD4	(HD)
(iii)	Fuc OH2	Qui OD2	Qui OH4	(DH)
(iv)	Fuc OH2	Qui OD2	Qui OD4	(DD)

Scheme 4.

The above analysis has shown that the multiplet pattern of the Fuc OH2 signal has its origin in hydrogen bonds with the Qui OH2 and Qui OH4 groups. Since these groups lie on opposite sides of the glycosidic bond; the hydrogen bonding of FucOH2 with the former leads to *syn* and with the latter to *anti* conformations. Since the energy of hydrogen bond formation is different for OH and OD groups (Buckingham and Fan-Chen, 1981; Janoschek, 1996; Scheiner and Cuma, 1996), the *syn* $\rightleftharpoons$ *anti* equilibrium will be different for each of the four isotopomers in Scheme 5, and so will be the ensemble-averaged chemical shifts for Qui OH4.

Thus, it is the *isotopic perturbation of the conformational equilibrium* which explains how deuterium substitution at Qui OH2 can produce an isotope shift effect at Qui OH4, even though O2 and O4 are 5 Å apart, far beyond the hydrogen bond distance. Based on the arguments concerning the conformational ensemble averaging one might expect that perturbations of conformational equilibria could produce observable isotope-induced shifts for Fuc OH4 or for any



the four isotopomers are: (i) X = H; Y = H; (ii) X = D, Y = H; (iii) X = H, Y = D; (iv) X = D, Y = D

Scheme 5.

of the C-linked protons. However, the actual perturbations of equilibria induced by substituting OD for OH are minute, and correspondingly, measurable chemical shift changes can only be expected for hydroxyl protons that participate in hydrogen bonds in only one of two conformers (e.g. Qui OH4 but not Fuc OH4), and/or change the 'in' for 'out' (or vice versa) position in the course of of the syn $\rightleftharpoons$ anti transition. The isotope effects observed the saccharides *1–5* are presented in Table 2.

Discussion

The isotope effects on OH chemical shifts described in a series of papers by Christofides and Davies, and referred to in the article by Angyal and Christofides (1996), were interpreted in the frame of a static model of fixed hydrogen bonds, with positive (low-field) shifts assigned to OH acceptor groups and negative (high-field) shifts ascribed to OH proton donor groups. Reuben (1984) contradicted this model with the argument that *hydrogen bond* lifetimes would have to be of the order of hundreds of milliseconds for OH groups to show separate, isotopically shifted signals together with the parent, non-shifted ones. This argument is not valid since it is the lifetimes of the *isotopomers* that matter in this respect (Dabrowski et al., 1995). However, Reuben's model of an isotopically perturbed 'flip-flop' equilibrium (OH $\cdots$ OD $\rightleftharpoons$ HO $\cdots$ DO) correctly accounts for the main features of the observed positive and negative isotope shifts.

Craig et al. (1996) applied the same perturbed equilibrium scheme, using a more informative 'in-out' description, which distinguishes between interior and exterior positions of the protio hydroxyl group. However, since the H/D isotope effects originate from differences in the vibrational characteristics of the fragment directly involved in hydrogen bonding (Buckingham and Fan-Chen, 1981), the use of the $^{n}\Delta$ symbol, to refer to the number of covalent bonds n separating the protons at issue is misleading, as it suggests that the effect of isotopic substitution is transmitted through n bonds. This is obviously impossible for large n, as for example, in the case of a branched trisaccharide where n = 11 (Kozar et al., 1995) or in an extreme (hypothetical) case of a hydrogen bond between OH groups of distant serine residues in a globular protein, where n could mean several hundred bonds.

Referring to Scheme 5, it would certainly be wrong to interpret the effect of replacing Qui OH2 with Qui OD2 as an isotope effect transmitted through six bonds to Qui OH4. Instead, the effect observed at Qui OH4 is readily explained by the fact that Qui OD2 results in two additional hydrogen bond combinations in the *syn* conformation, (iii) and (iv), each of which is isotopically different from (i) and (ii) giving rise to a second isotope effect at the Qui OH4 signal through perturbation of the conformational equilibria. The in-out mechanism cannot be applied to the Qui OH2 and OH4 hydroxyl groups as a pair since the O2 $\cdots$ O4 distance is 5 Å. However, the Fuc OH2 (or OD2) group is hydrogen-bonded to Qui OH2/OD2 in the *syn* conformers or to Qui OH4 in the *anti* conformers. Hence the in-out mechanism can be operative for each of the isotopomers (i)–(iv), thereby affecting the conformational equilibrium and, consequently, the magnitude of the isotope-induced chemical shift, without changing the number of observable isotope effects.

The temperature dependence of the isotope shift measured for some of the hydrogen-bonded protons

confirms that equilibrium perturbation is operating (Siehl, 1987; Hansen, 1988). For example, the shift for *1* Tal OH2 increased linearly from 110.1×10^{-4} ppm at 333 K to 157.5×10^{-4} ppm at 288 K, i.e. by 43%, and that for *1* Tal OH4 increased in the same temperature interval from 47.0×10^{-4} ppm to 83.1×10^{-4} ppm, i.e. by 77%. This is comparable with a ca. 50% increase in an 80 K interval observed by Craig et al. (1996) for two $\mathbf{C}_s$-symmetric OH groups of a rigid *myo*-inositol derivative, where the only dynamic process possible was the in-out (flip-flop) isomerization. While an increase of any equilibrium effect with decreasing temperature is expected on thermodynamical grounds, it is interesting to note that the small isotope shift for the Rha OH3 resonance (Figure 1b) decreased linearly from 38×10^{-4} ppm at 333 K to 18×10^{-4} ppm at 293 K. This can probably be attributed to the contributions to this group's chemical shift from other equilibrating processes that the molecule is undergoing.

An interesting issue is the reason why some hydrogen bonding partners exhibit no resolvable isotope shift effects, as was the case, for example, with Qui OH2 in *5* just discussed. Reuben has shown that the chemical shift manifestation of isotopic perturbation on the equilibrium is more probable when the equilibrium constant (K) is close to unity. It follows that the lack of isotope effect on Qui OH2, and a simultaneous distinct effect on Fuc OH2 and Qui OH4, cannot be explained by the perturbation of the *syn* $\rightleftharpoons$ *anti* equilibrium alone, because any deviation from $K = 1$ will be the same for each of these groups. One could speculate that Qui OH2 is predominantly a proton donor, with the result that the isotope effect exerted by the partner Fuc OD2 is negligible, because deuterium in the 'out' position has only a negligible effect on the vibrational characteristics of the hydrogen bond, which are responsible for the shielding effects. Concerning the marked isotope effect exerted by FucOH/D2 on Qui OH/D4 (and vice versa), this requires that both the 'in' and 'out' positions be markedly populated by both of these groups in the *anti* conformation. Another likely cause for the lack of isotope-induced splitting of the signals of hydrogen-bonded OH groups is the nature of conformationally ensemble-averaged values, which can involve both positive and negative shifts so that these cancel each other and a 'null' effect is observed.

Conclusions

Splitting of ^{1}H NMR signals of hydrogen-bonded hydroxyl groups, which reflects the isotope effect on chemical shifts in partially deuterated samples, stems from three sources: (a) perturbation of the flip-flop (in-out) equilibrium; (b) perturbation of the conformational equilibrium; and (c) intrinsic effect, the last mentioned being likely to cause noticeable shifts only for proton acceptor groups ($HO \cdots DO$). Mutually hydrogen-bonded hydroxyls (cases (a) and (c)) can unambiguously be discovered by measuring isotopomer-selected ROE difference spectra (the 'IS-NOE' method). Interresidue hydrogen bonds found in this way can serve as reliable, motionally non-averaged distance constraints of ~2.7–3.0 Å length in conformational analysis of oligosaccharides.

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