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Heteronuclear Overhauser effects in carbohydrates

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Abstract

A theoretical full-relaxation matrix analysis of heteronuclear Overhauser effects in oligosaccharides is described. This analysis predicts that trans-glycosidic heteronuclear $^{1}H\{^{13}C\}$ NOEs should be measurable in a model disaccharide with appropriate enrichment with ^{13}C and ^{2}H. These predictions are confirmed experimentally, and the value of these measurements is discussed for conformational analysis.

Abbreviations: NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; HOESY, heteronuclear Overhauser effect spectroscopy; 2D, two-dimensional.

Introduction

A fundamental problem in the characterisation of the structure and dynamics in solution of oligosaccharides by NMR is the lack of structural restraints across glycosidic linkages (see Homans (1994) and van Halbeek (1994) for review). Usually one, and generally not more than three, trans-glycosidic ^{1}H-^{1}H nuclear Overhauser effects (NOEs) are detectable, and since torsional fluctuations about these linkages represent the major conformational determinants, the problem of structure determination is usually underconstrained. There is thus a requirement for additional conformation-dependent parameters with which to characterise the solution behaviour of oligosaccharides in more detail. Recent approaches to this problem include the measurement of additional ^{1}H-^{1}H NOE connectivities involving exchangeable (-OH and NH) protons in supercooled solutions (Poppe and van Halbeek, 1991a, 1994; Adams and Lerner, 1992; Harris et al., 1997) and measurement of trans-glycosidic ^{1}H-^{13}C (Poppe and van Halbeek, 1991b,c; Rutherford et al., 1993,1994; Xu et al., 1996) and ^{13}C-^{13}C (Milton et al., 1997) coupling constants. Here, we examine the feasibility of measurement of trans-glycosidic heteronuclear NOEs involving ^{13}C and ^{1}H in order further to increase the number of available structural restraints.

Materials and methods

U-^{13}C,^{2}H glucose was a generous gift from Martek Biosciences (Columbia, MD). The percentage enrichment with ^{2}H was determined by electrospray mass spectrometry to be 96.1% (data not shown). All other reagents were purchased from Sigma.

Preparation of Galβ1-4[U-^{13}C-^{2}H]Glc

Chemoenzymatic synthesis was performed in 2.5 ml solution containing 10 mM UDP-galactose and 10 mM U-^{13}C,^{2}H glucose in 50 mM phosphate buffer (pH 7.4). One unit of galactosyltransferase was added with 1 mM lactalbumin and Mn^{2+} to a concentration of 5 mM, and the mixture was incubated at 37 °C overnight. The disaccharide product was purified by Biogel P-2 column (2.5 cm × 100 cm) chromatography, with H_2O as eluant.

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Definitions
The torsion angles φ and ψ correspond with the official IUPAC definitions φ_H and ψ_H and are defined as H-1 – C-1 – O-1 – C-4 and C-1 – O-1 – C-4 – H-4 respectively, where C-4 and H-4 refer to the aglyconic atoms.

NMR measurements
Two-dimensional heteronuclear $^1H\{^{13}C\}$ nuclear Overhauser effect (HOESY) spectra were acquired at a proton frequency of 500 MHz and probe temperature of 303 K using the original sequence of Rinaldi (1983), except that the ^1H and ^{13}C channels were reversed, and broadband decoupling was not utilised during the acquisition period. Data were acquired with spectral widths of 6000 Hz and 1000 Hz in the F1 (^{13}C) and F2 (^1H) dimensions respectively, with 128 increments in the t_1 dimension and 64 transients per increment. A relaxation delay of 8 s was utilised between acquisitions, with a mixing time (τ_m) of 1 s. The total acquisition time was 45 h. Prior to two-dimensional Fourier transformation, data were apodised in the t_2 dimension by an exponential function corresponding to a line-broadening of 3 Hz, and in the t_1 dimension by a cosine-bell window function, followed by zero-filling once in the t_1 dimension.

NOE simulations
Heteronuclear NOEs were simulated using the in-house written program MDNOE2, which is a package for the full-relaxation matrix analysis of NOESY, ROESY, steady-state NOEs and transferred NOEs for an arbitrary heteronuclear spin system. In these simulations the influence on ^{13}C spins of scalar relaxation of the second kind (Abragam, 1961; London 1990) derived from ^2H is not included, since its influence on the longitudinal relaxation of ^{13}C under the described conditions is negligible. Simulations were performed on the disaccharide either in the global minimum energy configuration (φ,ψ + 60°, 0°), or from a 500 ps *in vacuo* molecular dynamics simulations performed as described (Homans and Forster, 1992) without experimental NOE restraints.

Theoretical

Full-relaxation matrix simulations of heteronuclear Overhauser effects
Heteronuclear Overhauser effects can in principle be measured in small molecules in a number of ways (Kover et al., 1980; Rinaldi, 1983; Yu and Levy, 1984; Kover and Batta, 1988; Neuhaus and Williamson, 1989; Neuhaus and van Mierlo, 1992; Stott and Keeler, 1996). In order to derive the most suitable experimental strategy, it is therefore convenient to examine a series of simulations under a variety of conditions. For this purpose we have chosen lactose (Galβ1-4Glc) uniformly ^{13}C enriched in the glucose moiety as a model compound.

The enhancement of ^{13}C resonance intensities in small molecules on saturation of ^1H is well known. In the case of directly bonded ^{13}C-^1H pairs, the enhancement is substantial and can approach 200%, resulting in a threefold increase in ^{13}C resonance intensity (Neuhaus and Williamson, 1989). In contrast, enhancements to more distant carbon resonances are generally small and negative, as shown in Figure 1A for the ^{13}C spins in the aglycon of Galβ1-4Glc on saturation of Gal H-1. The most intense NOEs are to Glc C-4, C-5 and C-6, with a weaker contribution to C-3 (not shown). The negative intensity arises from spin-diffusion via the relevant attached proton to the ^{13}C spin in question, a phenomenon historically termed the 'three-spin effect' originally observed in homonuclear ^1H spin systems (Noggle and Schirmer, 1971).

In the general case for larger molecules, selective saturation of proton resonances is inconvenient, especially in oligosaccharides where the majority of resonances experience severe overlap (Vliegenthart et al., 1983). An alternative strategy is therefore to employ the two-dimensional HOESY experiment (Rinaldi, 1983). Simulations of $^{13}C\{^1H\}$ HOESY intensities for various mixing times are shown in figure 1B. It is seen that HOESY intensities are substantially smaller than steady-state enhancements, in complete analogy with homonuclear ^1H systems (Neuhaus and Williamson, 1979), and reach maxima at times substantially shorter than steady-state measurements.

Conformational dependence of heteronuclear Overhauser effects: Since no direct trans-glycosidic $^{13}C^1H$ NOEs are observable in Galβ1-4Glc, it is instructive to consider whether the 'three-spin' NOEs are sufficiently sensitive to torsional variation about the glycosidic linkage to be of value as conformational restraints. As a simple test of this, both $^{13}C\{^1H\}$ and $^1H\{^1H\}$ NOEs were simulated for various geometries during complete rotation about the φ torsion angle of the disaccharide. Ignoring for illustrative purposes the fact that certain orientations about φ are energetically unfavourable, it is seen that only the NOE between

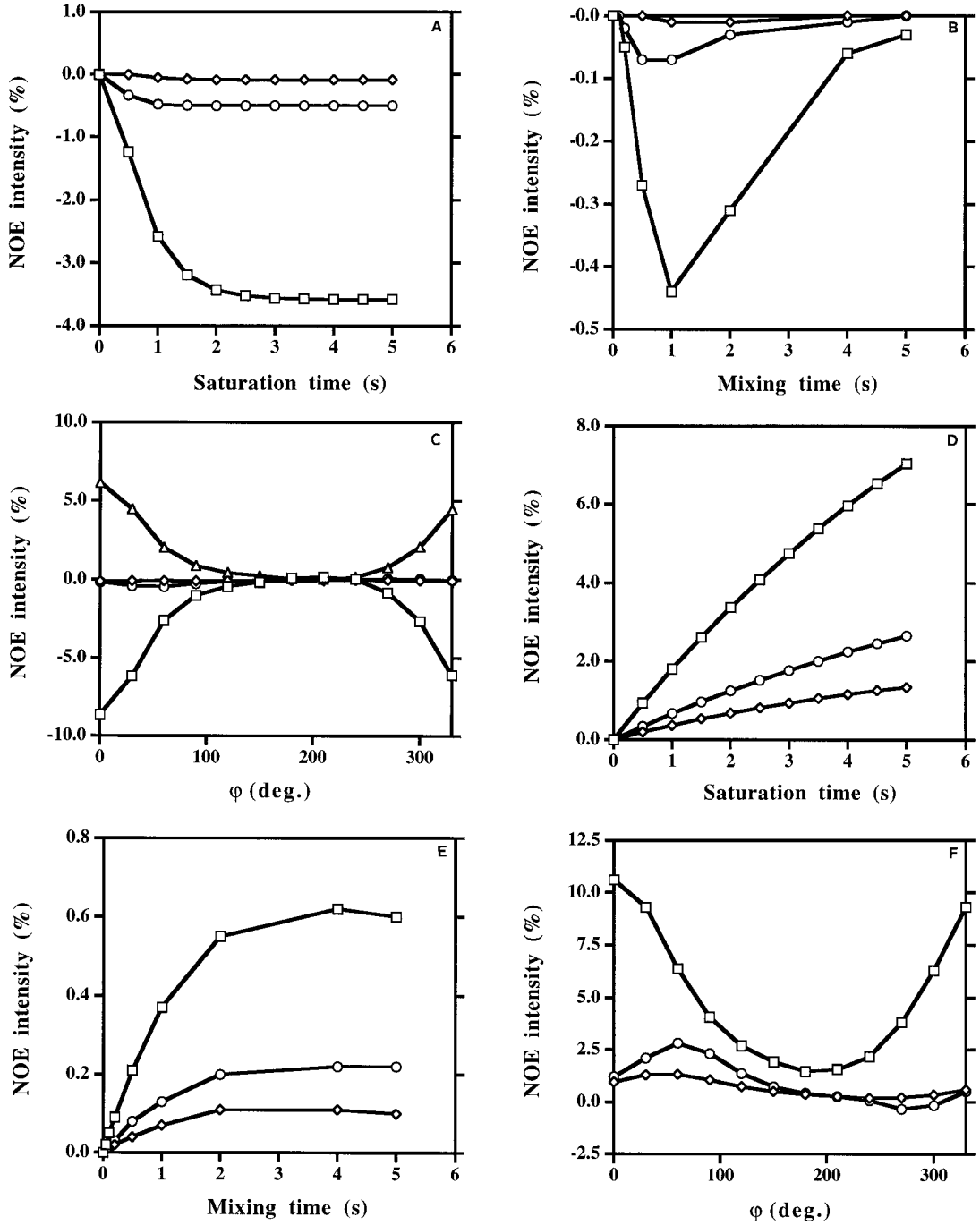

Figure 1. Full-relaxation matrix simulations of trans-glycosidic heteronuclear NOEs in Galβ1-4[U-^{13}C-Glc] (panels A-C) and Galβ1-4[U-^{13}C,^{2}H-Glc] (panels D-F). (A,D) Time dependence of the steady-state NOE intensities experienced by Glc C-4 (□), C-5 (◊) and C-6 (○) on saturation of Gal H-1; (B,E) time dependence of the transient (HOESY) cross-peak intensities experienced by Glc C-4 (□), C-5 (◊) and C-6 (○) on saturation of Gal H-1; (C,F) Conformational dependence of steady-state NOE intensities experienced by Glc C-4 (□), C-5 (◊) and C-6 (○) and H-4 (Δ, panel C only) on saturation of Gal H-1 during rotation of the glycosidic torsion angle φ. A rotational correlation time of 0.15 ns was used in all simulations using a proton resonance frequency of 500 MHz.

Gal H-1 and Glc C-4 displays substantial variation (Figure 1C). However, this is principally as a result of the fact that the NOE between Gal H-1 and Glc H-4 is sensitive to conformation, and by virtue of the fact that the Glc H-4 – Glc C-4 bond distance is invariant to conformation to first order, the 'three-spin' NOE between Gal H-1 and Glc C-4 shows an essentially identical functional dependence to the homonuclear NOE (Figure 1C). It can therefore be concluded that trans-glycosidic 'three-spin' $^{13}C\{^1H\}$ NOEs are not useful parameters for conformational analysis in this disaccharide.

Effects of specific ^2H enrichment on NOE intensities: A possible mechanism whereby the conformational sensitivity of trans-glycosidic heteronuclear NOEs could be improved is by perdeuteration of the aglycon. The relevant simulations for this scenario are shown in Figures 1D and 1E. It is seen that both steady-state and HOESY intensities are now positive, as a result of the attenuation of the trans-glycosidic pathway for indirect enhancement. In addition, the longitudinal relaxation rate of the deuterated ^{13}C spin is considerably reduced, with benefits for the maximum observable enhancement. This phenomenon forms the basis of early work indicating that $^{13}C\{^1H\}$ NOEs are readily observable at natural abundance to quaternary carbons (Kover et al., 1980). Unlike the 'three-spin' trans-glycosidic NOEs, the direct NOEs simulated in Figures 1D,E are much more sensitive to torsional variations across the glycosidic linkage (Figure 1F), and at least three such NOEs are predicted to be measurable.

In principle, it would be more appropriate to measure trans-glycosidic NOEs in glycans which are uniformly deuterated to a level of 50%, since this would permit a variety of other NMR techniques involving ^1H to be applied using a single sample. However, under most experimental conditions the magnitude of the trans-glycosidic heteronuclear NOE from deuterated carbon sites is predicted to be opposite in sign and of similar magnitude to the heteronuclear NOE observed from protonated carbon sites (Figures 1A,D and B,E). Although a significant isotope shift is experienced by deuterated ^{13}C sites ($\sim$ 40 Hz), in the 2D HOESY the digital resolution in the ^{13}C dimension is typically insufficient to resolve the ^{13}C resonances from each isotopomer, resulting in substantial cancellation. We note, however, that the transient NOE from protonated ^{13}C sites decays much more rapidly (Figure 1B) than that from deuterated ^{13}C sites (Figure 1E), suggesting that the latter can be selectively observed at long mixing times. Efforts are underway to validate this prediction.

Choice of detected nucleus: Comparison of the NOE buildup rates in Figure 1D and 1E shows that the steady-state NOEs reach a value more than tenfold greater than the transient NOE, suggesting that the former would be the experiment of choice on grounds of sensitivity. However, there are additional factors which need to be considered for practical applications. First, application of steady-state NOE measurements requires the application of a selective, saturating radio-frequency field on protons. In the case of oligosaccharides, the resonance overlap in the proton NMR spectrum is generally so severe that steady-state measurements are of limited applicability. Second, steady-state $^{13}C\{^1H\}$ NOE measurements by definition involve detection of ^{13}C, whereas in contrast, transient HOESY experiments can be performed with detection of either nucleus. The symmetry of the relaxation matrix in homonuclear systems also holds for heteronuclear systems, hence the transient simulations in Figure 1 are relevant to either detection of ^{13}C or ^1H. Since it can be demonstrated that ^1H detection offers approximately eightfold enhancement of signal-to-noise in comparison with ^{13}C detection (Stott and Keeler, 1996), the sensitivity disadvantages of measurement of transient NOEs become less severe with proton detection. It is also necessary to consider the sensitivity advantage per unit time of each approach. In the case of steady-state experiments, the repetition rate is determined not only by the build-up rates of the NOE enhancements (Figure 1D), but also by the need for the ^{13}C atoms to recover their equilibrium magnetisation between experiments. In the transient $^1H\{^{13}C\}$ experiment, the repetition rate is again determined by the need for ^{13}C atoms to recover since the initial polarisation derives from them, but the mixing time can be relatively short since the maximum enhancement is obtained in a relatively short time (Figure 1E). In summary, therefore, transient experiments with ^1H detection offer comparable sensitivity per unit time with steady-state experiments with ^{13}C detection, and indeed should offer greater sensitivity with conventional 'inverse' probes which are not optimised for ^{13}C detection, since these offer $\sim$ 50% of the sensitivity offered by their ^{13}C-optimised counterparts.

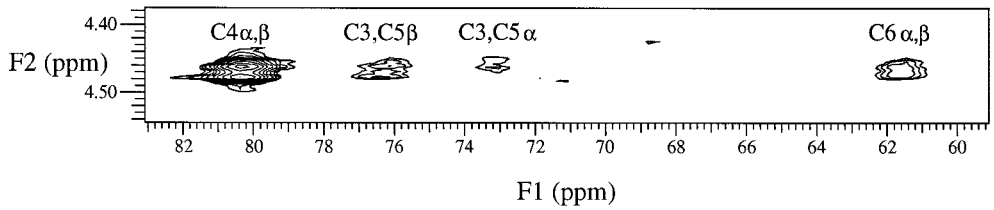

Figure 2. Section from $^{1}H^{13}C$ HOESY spectrum of Galβ1-4[U-^{13}C,^{2}H-Glc], illustrating trans-glycosidic NOEs to Gal H-1.

Results and discussion

$^{1}H\{^{13}C\}$ HOESY: The validity of the transient NOE simulations in Figure 1 was assessed by application to a sample of Galβ1-4[U-^{13}C,^{2}H]Glc, prepared by chemoenzymatic synthesis (see experimental section). Two-dimensional HOESY data were acquired using the conventional HOESY pulse sequence (Rinaldi, 1983), but with the ^{13}C and ^{1}H channels reversed and with no broadband decoupling during the acquisition period. Broad-band deuterium decoupling was not utilised since the effective resolution in the F_1 (^{13}C) dimension is very low, and the contribution of scalar relaxation of the second kind (Abragam, 1961; London, 1990) to the ^{13}C linewidth is very small for molecules with small rotational correlation times. Shown in Figure 2 is a section from the $^{1}H\{^{13}C\}$ HOESY spectrum of Galβ1-4[U-^{13}C,^{2}H]Glc illustrating connectivities from Glc C-4, C-5/C-3 and C-6 to Gal H-1. The NOE from Glc C-6 is particularly useful as a conformational restraint since, as an exocyclic atom, the distance to Gal H-1 is very sensitive to torsional fluctuations about the glycosidic linkage. In conventional homonuclear NOE measurements, NOEs are observed from Gal H-1 – Glc H-6(H-6′), and to Glc OH-6 in studies in H_2O at low temperature (Harris et al., 1997), but their use as conformational restraints is limited by the fact that they are dependent upon the orientation of the hydroxymethyl group of the aglycon. In contrast, the heteronuclear NOE is, to first order, independent of the orientation of pendant groups.

Quantitation of heteronuclear NOEs: Determination of the absolute magnitudes of heteronuclear NOEs in Galβ1-4[U-^{13}C,^{2}H]Glc is not trivial since there is no intra-residue ^{13}C-^{1}H vector in a given molecule that can be used as a reference distance. One approach to this problem is to utilise the fact that ^{2}H isotopic enrichment of the commercially available U-^{13}C,^{2}H glucose utilised in this study is not 100%. While the percentage of protons in the aglycon of Galβ1-4[U-^{13}C,^{2}H]Glc is low, this is nevertheless sufficient to generate cross peaks (not shown) in the 2D HOESY spectrum arising from one-bond ^{13}C-^{1}H heteronuclear NOEs in protonated isotopomers. Since the magnitudes of these NOEs are independent of the conformation about the glycosidic linkage to first order, theoretical simulations of their magnitudes can be used to calibrate the observed heteronuclear trans-glycosidic NOEs. For this purpose, it is necessary in principle to correct the measured intensities for the longer T_1 relaxation times of the ^{13}C spins that are directly bonded to ^{2}H compared with ^{1}H, which will result in differential recovery of z magnetisation during the relaxation delay. However, the T_1 values of the ^{13}C spins directly bonded to ^{2}H in the disaccharide, measured by conventional inversion-recovery experiments, are all less than 3.2 s. Hence the z magnetisations of deuterated carbons under the experimental conditions employed here have relaxed to >95% of their equilibrium values between successive transients, and no correction is required within experimental error. The resulting $^{1}H\{^{13}C\}$ intensities are shown in Table 1. These are all of lower intensity than predicted from the relevant simulation (Figure 1E and Table 1). However, these simulations were computed with the geometry about the glycosidic linkage fixed in a single orientation corresponding to the global minimum energy conformation of the glycan ($\varphi,\psi \sim +60°, 0°$). Simulations obtained from a 500 ps *in vacuo* free dynamics simulation of the glycan (Table 1), which is more representative of the dynamic behaviour of oligosaccharides in solution (Rutherford et al., 1993), are in better agreement with experimental values (Table 1).

Conclusions

It has been demonstrated that trans-glycosidic heteronuclear NOEs can be observed in oligosaccharides appropriately enriched with ^{13}C and ^{2}H. Heteronuclear full-relaxation matrix calculations of steady-state $^{13}C\{^{1}H\}$ and transient $^{1}H\{^{13}C\}$ NOEs indicate that

Table 1. Experimental $^1H\{^{13}C\}$ heteronuclear Overhauser effect (HOESY) intensities in Galβ1-4[U-^{13}C,^2H-Glc] versus theoretical values computed from the global minimum energy conformation (global) and from a 500 ps *in vacuo* free dynamics simulation (dynamic) of the disaccharide.

Connectivity	$^1H\{^{13}C\}$ NOE (%)[a]		
	Experimental	Theoretical (global)	Theoretical (dynamic)
Glcα H-1 - Glcα C-1[b]	0.21	–	–
Gal H-1 - Glc C-4	0.28	0.37	0.26
Gal H-1 - Glc C-5[c]	0.06	0.07	0.04
Gal H-1 - Glc C-3[c]		0.03	0.04
Gal H-1 - Glc C-6	0.05	0.13	0.05

[a] Trans-glycosidic NOEs were quantified indirectly with respect to the $^1H\{^{13}C\}$ NOE between Glcα H-1 and Glcα C-1, and represent the sum of the NOE intensities in each anomer of the disaccharide

[b] The reported value is the theoretical intensity of the $^1H\{^{13}C\}$ NOE between Glcα H-1 and Glcα C-1 (10.87%) using a rotational correlation time of the molecule of 0.15 ns, followed by correction for the percentage of isotopomers bearing Glcα H-1 (3.9%) and the anomeric ratio in the disaccharide.

[c] Resonance overlap of C-3 and C-5 for both anomers prevents independent experimental measurement of these NOEs.

while the latter have smaller maximum intensities, proton detection results in a similar absolute sensitivity. Although the observed trans-glycosidic NOEs are of low intensity (< 1%), they can readily be detected with conventional 2D HOESY experiments. Three trans-glycosidic heteronuclear NOEs were detectable experimentally with certainty in Galβ1-4[U-^{13}C,^2H]Glc. With measurement of NOEs involving exchangeable protons and trans-glycosidic heteronuclear long-range ^{13}C-^1H and ^{13}C–^{13}C coupling constants, at least twelve conformational determinants should be available. The value of these parameters in combination for the refinement of current models of oligosaccharide structure and dynamics is currently under investigation. Clearly, a limitation of these approaches is the requirement for isotopically enriched material. However, given that efficient chemoenzymatic strategies are now available for the synthesis of oligosaccharides in isotopically enriched form (see e.g. Ichikawa et al., 1992; Probert et al., 1997), this limitation is unlikely to be prohibitive.

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