J|A|C|S
ARTICLES
Published on Web 05/22/2002

# Determination of $^1$H Homonuclear Scalar Couplings in Unlabeled Carbohydrates

Catherine Zwahlen[†,‡] and Sébastien J. F. Vincent*[,§]

*Contribution from the Université de Lausanne, Institut de Chimie Organique, CH-1015 Lausanne, Switzerland, and Nestlé Research Center, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland*

Received October 23, 2001

***Abstract:*** The scarcity of structural information on carbohydrates results from combined difficulties to crystallize and the limitations in NMR analysis. Current methods for determining basic NMR parameters such as $^1$H homonuclear scalar couplings are very limited, especially for large molecules such as polysaccharides, oligonucleotides, and the carbohydrate part of glycoproteins. In this paper, a NMR experiment for the determination of endocyclic $^1$H homonuclear scalar couplings ($^3J_{HH}$) in unlabeled carbohydrates is presented. In addition to scalar couplings, cross-correlated dipole−dipole relaxation rates were measured for large polysaccharides. The measurement of all endocyclic homonuclear coupling constants within monosaccharide units is presented for lactose, a model disaccharide, and for a natural-abundance 2 MDa bacterial polysaccharide excreted by *Streptococcus thermophilus* Sfi39.

## Introduction

Carbohydrates are a group of molecules presenting a wide diversity of structures. Although several methods for structural studies of carbohydrates by NMR have been developed, these advances have been limited either to small molecules such as oligosaccharides[1,2] or to classes of molecules for which $^{13}$C-labeling was available, namely oligonucleotides[3] and oligosaccharides.[4] For polysaccharides and the carbohydrate part of glycoconjugates, the large variety of producing organisms and the diversity of carbon sources currently prevents general labeling strategies and only a small number of $^{13}$C-labeled samples have been described.[5] As a result, NMR-based structural analysis of large carbohydrates relies on two types of methods applicable to natural abundance samples, $^1$H−$^{13}$C HMBC and the $^1$H homonuclear two-dimensional experiments TOCSY and NOESY. Recent work has shown the usefulness of cross-correlated dipole-dipole relaxation in the determination of glycosidic linkages.[6]

In carbohydrates, $^1$H homonuclear scalar couplings are determined by direct measurement of peak separation for isolated signals either in 1D $^1$H spectra or 1D TOCSY spectra or in two-dimensional COSY spectra.[7,8] Experiments designed for small oligosaccharides have been used relying either on the use selective pulses[1] or on E.COSY.[9] The applicability of selective pulses for large carbohydrates is very limited mainly because of rapid relaxation, while partial signal cancellation inherent to E.COSY is a major difficulty in the case of natural abundance samples that are rapidly relaxing such as polysaccharides. Moreover, these methods are limited to well-separated multiplets with a line width smaller than the scalar couplings, a situation rare in carbohydrates and nonexistant in polysaccharides. Although recent advances in data analysis offer

* To whom correspondence should be addressed. E-mail: sebastien.vincent@rdls.nestle.com.
† Université de Lausanne.
‡ Present address: GeneProt, 2 Rue Pré-de-la-Fontaine, CH-1217 Meyrin, Switzerland.
§ Nestlé Research Center.

(1) Uhrín, D.; Brisson, J. R.; Bundle, D. R. *J. Biomol. NMR* **1993**, *3*, 367−373. Kövér, K. E.; Jiao, D.; Uhrín, D.; Forgo, P.; Hruby, V. J. *J. Magn. Reson. Ser. A* **1994**, *106*, 119−122.
(2) Bolon, P. J.; Prestegard, J. H. *J. Am. Chem. Soc.* **1998**, *120*, 9366−9367. Tian, F.; Bolon, P. J.; Prestegard, J. H. *J. Am. Chem. Soc.* **1999**, *121*, 7712−7713.
(3) Nikonowicz, E. P.; Pardi, A. *Nature* **1992**, *355*, 184−186. Sklenár, V.; Peterson, R. D.; Rejante, M. R.; Wang, E.; Feigon, J. *J. Am. Chem. Soc.* **1993**, *115*, 12181−12182. Marino, J. P.; Schwalbe, H.; Anklin, C.; Bermel, W.; Crothers, D. M.; Griesinger, C. *J. Am. Chem. Soc.* **1994**, *116*, 6472−6473. Müller, L.; Legault, P.; Pardi, A. *J. Am. Chem. Soc.* **1995**, *117*, 11043−11048. Fiala, R.; Jiang, F.; Patel, D. J. *J. Am. Chem. Soc.* **1996**, *118*, 689−690. Zwahlen, C.; Legault, P.; Vincent, S. J. F.; Greenblatt, J.; Konrat, R.; Kay, L. E. *J. Am. Chem. Soc.* **1997**, *119*, 6711−6721. Brutscher, B.; Boisbouvier, J.; Pardi, A.; Marion, D.; Simorre, J.-P. *J. Am. Chem. Soc.* **1998**, *120*, 11845−11851. Dingley, A. J.; Masse, J. E.; Peterson, R. D.; Barfield, M.; Feigon, J.; Grzesiek, S. *J. Am. Chem. Soc.* **1999**, *121*, 6019−6027. Trantírek, L.; Urbásek, M.; Stefl, R.; Feigon, J.; Sklenár, V. *J. Am. Chem. Soc.* **2000**, *122*, 10454−10455.
(4) Yu, L.; Goldman, R.; Sullivan, P.; Walker, G. F.; Fesik, S. W. *J. Biomol. NMR* **1993**, *3*, 429−441. Harris, R.; Rutherford, T. J.; Milton, M. J.; Homans, S. W. *J. Biomol. NMR* **1997**, *9*, 47−54. Bose, B.; Zhao, S.; Stenutz, R.; Cloran, F.; Bondo, P. B.; Bondo, G.; Hertz, B.; Carmichael, I.; Serianni, A. S. *J. Am. Chem. Soc.* **1998**, *120*, 11158−11173. Yamaguchi, Y.; Kato, K.; Shindo, M.; Aoki, S.; Furusho, K.; Koga, K.; Takahashi, N.; Arata, Y.; Shimada, I. *J. Biomol. NMR* **1998**, *12*, 385−394. Harris, R.; Kiddle, G. R.; Field, R. A.; Milton, M. J.; Ernst, B.; Magnani, J. L.; Homans, S. W. *J. Am. Chem. Soc.* **1999**, *121*, 2546−2551. Yamaguchi, Y.; Takizawa, T.; Kato, K.; Arata, Y.; Shimada, I. *J. Biomol. NMR* **2000**, *18*, 357−360. Brun, E.; Brumer, H., III; MacKenzie, L. F.; Withers, S. G.; McIntosh, L. P. *J. Biomol. NMR* **2001**, *21*, 67−68.
(5) Gitti, R.; Long, G.; Bush, C. A. *Biopolymers* **1994**, *34*, 1327−1338. Wu, X.; Marino-Albernas, J. R.; Auzanneau, F. I.; Verez-Bencomo, V.; Pinto, M. *Carbohydr. Res.* **1998**, *306*, 493−503. Linnerborg, M.; Weintraub, A.; Widmalm, G. *Eur. J. Biochem.* **1999**, *266*, 246−251. Junicke, H.; Serianni, A. S.; Steinborn, D. *J. Org. Chem.* **2000**, *65*, 4153−4161. Vinogradov, E.; Petersen, B. O.; Duus, J. O. *Carbohydr. Res.* **2000**, *325*, 216−221.
(6) Vincent, S. J. F.; Zwahlen, C. *J. Am. Chem. Soc.* **2000**, *122*, 8307−8308.
(7) Otter, A.; Hindsgaul, O.; Bundle, D. R. *Carbohydr. Res.* **1995**, *275*, 381−389.
(8) Martin-Pastor, M.; Bush, C. A. *J. Biomol. NMR* **2001**, *19*, 125−139.
(9) Willker, W.; Leibfritz, D. *J. Magn. Reson.* **1992**, *99*, 421−425.

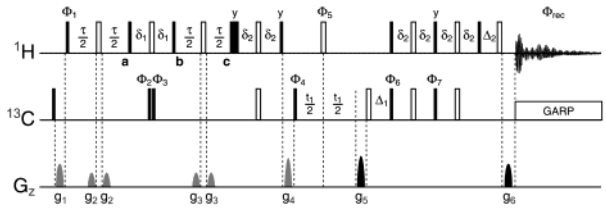


***Figure 1.*** $^{13}$C-COSMO-HSQC pulse sequence for the simultaneous determination of $^1$H homonuclear scalar couplings and $^1$H homonuclear cross-correlated dipole-dipole relaxation rates in natural abundance $^{13}$C carbohydrates. $2\tau$ is the total time for evolution of scalar couplings, $2\delta_1 \sim 1/^1J_{CH} = 6.25$ ms is the BIRD sandwich duration, $2\delta_2 \sim 1/(2 \times {}^1J_{CH}) = 6.9$ ms is the time for INEPT transfer, $\Delta_1$ is a compensation delay for pure phase in $t_1$, and $\Delta_2$ is a compensation delay for the decoding gradient. Narrow black rectangles indicate 90° pulses, open rectangles indicate 180°, while the large black rectangle was a 1 ms high-power purging pulse. All pulses without phase indication are along the $x$ axis. $\phi_1 = x,-x$; $\phi_2 = x$; $\phi_3 = x$ or $-x$; $\phi_4 = x,x,-x,-x$; $\phi_5 = x,x,x,x,-x,-x,-x,-x$; $\phi_6 = x,x,x,x,-x,-x,-x,-x$; $\phi_7 = y,y,y,y,-y,-y,-y,-y$; and $\phi_{rec} = x,-x,-x,x,-x,x,x,-x$. Phase sensitivity and sensitivity enhancement were achieved by echo-antiecho through cycling of $\phi_7$ in the reverse double-INEPT and alternation of the sign of g6. Gradients [G/cm] were either 500 $\mu$s (g2 and g3) or 1 ms (g1, g4, g5, and g6) long and of the following amplitudes: g1 = −25.5, g2 = 0.8, g3 = 1.1, g4 = 35.5, g5 = 40, and g6 = ±10.1.

solutions in the absence of overlap,[10] few $^1$H homonuclear scalar coupling constants for polysaccharides were measured and published. Methods developed in protein NMR[11] such as quantitative $J$ were designed for the nonoverlapping amide protons $H^N$ and aliphatic protons $H^\alpha$. In carbohydrates, extensive $^1$H overlap prevents the use of these experiments.

A strategy is presented for the determination of endocyclic three-bond $^1$H homonuclear $^3J_{HiHi+1}$ scalar couplings in natural abundance carbohydrates. The $^{13}$C-COSMO-HSQC experiment relies on a $^1$H magnetization *cos*ine *mo*dulation of all active $^1$H homonuclear scalar couplings as a preparation before a *HSQC*. The experiment can be applied to all types of unlabeled carbohydrates. An application is shown for lactose, a small disaccharide for which several scalar coupling values were available, and for a natural-abundance 2 MDa polysaccharide secreted by a lactic acid bacteria, *Streptococcus thermophilus* Sfi39. In both cases, all endocyclic three-bond $^1$H homonuclear scalar couplings were determined using the $^{13}$C-COSMO-HSQC pulse sequence.

## Theory

Within each monosaccharide ring, the anomeric proton H1 is coupled only to its direct neighbor, H2, through $^3J_{H1H2}$. The other endocyclic protons, namely H2 ($^3J_{H1H2}$ and $^3J_{H2H3}$), H3 ($^3J_{H2H3}$ and $^3J_{H3H4}$), and H4 ($^3J_{H3H4}$ and $^3J_{H4H5}$), are all coupled to both their $i-1$ and $i+1$ neighbors.

The $^{13}$C-COSMO-HSQC experiment (Figure 1) is a 2D HSQC experiment where $^1$H in-phase magnetization is either labeled by a cosine function of the $^1$H homonuclear scalar couplings or left unperturbed. Modulations resulting from $^1$H homonuclear cross-correlated dipole-dipole relaxation rates $\Gamma^{DD/DD}_{Hi-1Hi/HiHi+1}$ also occur for large molecules. The delay $2\tau$ during which magnetization is labeled by the scalar couplings is separated in two identical halves by a BIRD sandwich[12] that either selectively inverts protons attached to a $^{13}$C (180° ($^{13}$C), $\phi_3 = \phi_2$) or leaves all spins unperturbed (0° ($^{13}$C), $\phi_3 = -\phi_2$). As a result of the low natural $^{13}$C abundance, each $^{13}$C-labeled position is isolated from other $^{13}$C and the inversion by the BIRD sandwich is selective for $^1$H directly attached to a $^{13}$C. Since in carbohydrates the one-bond scalar couplings $^1J_{CH}$ are relatively uniform around 145 Hz for positions C-2 to C-6 and 165 to 175 Hz for the anomeric position C-1, a BIRD sandwich tuned to an average value of 160 Hz is an efficient way to invert all $^{13}$C-bound protons. It was estimated by numerical simulations that the error induced by an incorrectly tuned BIRD sandwich would result in a negligibly small error (<1%) on the determined $^1$H homonuclear scalar couplings.

Initially neglecting cross-correlated dipole-dipole relaxation, the evolution of magnetization can be described as follows: after initial purging of residual $^{13}$C magnetization, proton magnetization is excited by a 90° ($^1$H) pulse and let evolve for a time $\tau$ under the effect of $^1$H homonuclear scalar couplings. The 180° ($^1$H) proton pulse applied at $\tau/2$ refocuses evolution of both chemical shifts and one-bond heteronuclear scalar couplings $^1J_{CH}$. At the end of the delay $\tau$ (point ***a*** in the sequence), the density matrix contains the four terms $-H^i_y$, $2H^{i-1}_z H^i_x$, $2H^i_x H^{i+1}_z$, and $4H^{i-1}_z H^i_y H^{i+1}_z$, where $H^i$ is the product operator term for $i = 1$, 2, 3, and 4, modulated by cosine and sine functions of the respective scalar couplings (See Supporting Information for details).

At point ***a*** in the sequence, a BIRD sandwich of duration $2\delta_1 = 1/^1J_{CH}$ is applied with either a 180° ($^{13}$C) or a 0° ($^{13}$C) pulse. A 180° ($^{13}$C) pulse inverts the two singly antiphase terms resulting in an effective decoupling of $^3J_{HH}$ scalar couplings for the period $2\tau$, while a 0° ($^{13}$C) pulse leaves these terms unperturbed. After the BIRD sandwich (point ***b*** in the sequence), a second evolution time $\tau$ enables evolution under the effect of three-bond $^1$H homonuclear $^3J_{HiHi+1}$ scalar couplings yielding either $-H^i_y$ unmodulated for 180° ($^{13}$C), or $-H^i_y$, $2H^{i-1}_z H^i_x$, $2H^i_x H^{i+1}_z$, and $4H^{i-1}_z H^i_y H^{i+1}_z$ terms still modulated by the cosine and sine functions of the respective scalar couplings for 0° ($^{13}$C). The antiphase terms are then all purged by the trim pulse applied at the end of the delay $2\tau$ (point ***c*** in the sequence), which prevents the conversion of antiphase magnetization $2H^i_x H^{i+1}_z$ (or $4H^{i-1}_z H^i_y H^{i+1}_z$) by the INEPT into higher order heteronuclear coherences ($4C^i_y H^i_y H^{i+1}_x$ or $8C^i_y H^{i-1}_x H^i_z H^{i+1}_x$) that could lead to observable magnetization distorting the resulting signal intensities. After evolution of carbon chemical shifts during $t_1$ and encoding of the coherence by the pulsed field gradient g5, the sensitivity-enhancement scheme[13] was applied with signal decoding by the alternating pulsed field gradient g6.

In practice, two experiments are recorded in an interleaved manner. After separation of the datasets with standard processing software, the ratio of peak intensities gives a direct access to the coupling constants:

$$\frac{I^{mod}(2\tau)}{I^{dec}(2\tau)} = \cos(\pi\, {}^3J_{Hi-1Hi}\, 2\tau)\cos(\pi\, {}^3J_{HiHi+1}\, 2\tau) \qquad (1)$$

where $I^{mod}$ is the intensity measured from the cosine modulated $^{13}$C-COSMO-HSQC spectrum recorded with a 0° ($^{13}$C) pulse and $I^{dec}$ the intensity measured from the decoupled $^{13}$C-COSMO-HSQC spectrum recorded with a 180° ($^{13}$C) pulse.

The anomeric H1 protons are coupled to only one single proton H2 through $^3J_{H1H2}$. As a result, the ratio simplifies to:

$$\frac{I^{mod}(2\tau)}{I^{dec}(2\tau)} = \cos(\pi\, {}^3J_{H1H2}\, \tau) \qquad (2)$$

Cross-correlated dipole-dipole relaxation rates $\Gamma^{DD/DD}_{Hi-1Hi/HiHi+1}$ transfer magnetization within the same subspace as $^1$H homonuclear scalar couplings $^3J_{HH}$. Since the cross-correlated dipole-dipole relaxation rates

(10) Delaglio, F.; Wu, Z.; Bax, A. *J. Magn. Reson.* **2001**, *149*, 276−281.
(11) Billeter, M.; Neri, D.; Otting, G.; Qian, Y. Q.; Wüthrich, K. *J. Biomol. NMR* **1992**, *2*, 257−274. Vuister, G. W.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 7772−7777. Case, D. A.; Dyson, H. J.; Wright, P. E. *Methods Enzymol.* **1994**, *239*, 392−416. Vuister, G. W.; Bax, A. *J. Biomol. NMR*, **1994**, *4*, 193−200. Ponstingl, H.; Otting, G. *J. Biomol. NMR* **1998**, *12*, 319−324. Permi, P.; Kilpelainen, I.; Annila, A.; Heikkinen, S. *J. Biomol. NMR* **2000**, *16*, 29−37.
(12) Garbow, J. R.; Weitekamp, D. P.; Pines, A. *Chem. Phys. Lett.* **1982**, *93*, 504−509. Bax, A. *J. Magn. Reson.* **1983**, *53*, 517−520.
(13) Kay, L. E.; Keifer, P.; Saarinen, T. *J. Am. Chem. Soc.* **1992**, *114*, 10663−10665.

increase with the correlation time $\tau_c$,[14] their effect which can be neglected for small oligosaccharides needs to be included for large molecules such as polysaccharides. For a $^{13}$C-COSMO-HSQC experiment with 180° ($^{13}$C) pulse in the BIRD sandwich, the modulation of the in-phase term $-H_y^i$ is expressed as an hyperbolic cosine function $\cosh(\Gamma^{DD/DD}_{Hi-1Hi/HiHi+1}\ 2\tau)$. In the case of a $^{13}$C-COSMO-HSQC experiment with 0° ($^{13}$C) pulse in the BIRD sandwich, the in-phase coherence is modulated by the following function: $\cosh(\Gamma^{DD/DD}_{Hi-1Hi/HiHi+1}\ 2\tau)\cos(\pi\ ^3J_{Hi-1Hi}\ 2\tau)\cos(\pi\ ^3J_{HiHi+1}\ 2\tau) + \sinh(\Gamma^{DD/DD}_{Hi-1Hi/HiHi+1}\ 2\tau)\sin(\pi\ ^3J_{Hi-1Hi}\ 2\tau)\sin(\pi\ ^3J_{HiHi+1}\ 2\tau)$. The intensity ratio is then given by:

$$\frac{I^{mod}(2\tau)}{I^{dec}(2\tau)} = \cos(\pi\ ^3J_{Hi-1Hi}\ 2\tau)\cos(\pi\ ^3J_{HiHi+1}\ 2\tau) + \tanh(2\tau\ \Gamma^{DD/DD}_{Hi-1Hi/HiHi+1})\sin(\pi\ ^3J_{Hi-1Hi}\ 2\tau)\sin(\pi\ ^3J_{HiHi+1}\ 2\tau) \quad (3)$$

Since the anomeric H1 protons are coupled only to H2, $^1$H homonuclear cross-correlated dipole-dipole relaxation does not affect the peak ratio and eq 2 remains valid for H1 protons in large carbohydrates.

Scalar couplings and cross-correlated dipole-dipole relaxation rates can be obtained from the peak intensity ratio, using eq 3. One consequence of having to take cross-correlated dipole-dipole relaxation rates into account is that it becomes difficult to evaluate the couplings and rates for protons H5 and H6 which are either coupled to three coupling partners (H5) or strongly coupled (H6). We therefore limited our analysis to the endocyclic protons H1 to H4.

## Material and Methods

Spectra were recorded on a 600 MHz AVANCE spectrometer equipped with a shielded single axis pulsed field gradient inverse probe. The temperature was set to 63 °C and the samples were prepared with either 20 mg of lactose (**1**) or 4 mg of Sfi39 polysaccharide[15] (**2**) dissolved in 400 $\mu$L of 99.96% $^2H_2O$ (Euriso-Top, France): $\beta$-D-Gal*p*-(1→4)-D-Glc*p* [lactose (**1**)] and →3)-α-D-Glc*p*-(1→3)-[$\beta$-D-Gal*p*-(1→6)]-$\beta$-D-Glc*p*-(1→3)-$\beta$-D-Gal*f*-(1→ [Sfi39 polysaccharide repeating unit (**2**)].

Data matrices consisted of 64 × 512 complex points, the spectral widths in $\omega_1$ and $\omega_2$ were 3773 and 4195 Hz and were processed with the NMRPipe/NMRDraw package.[16] A relaxation delay of 3 (for **1**) or 1 s (for **2**) and 8 (for **1**) or 64 scans (for **2**) were accumulated. For a given $2\tau$ period, the coupled and decoupled experiments were acquired in an interleaved manner. Total acquisition times for a pair of $^{13}$C-COSMO-HSQC spectra were 1h45 for lactose and 5h45 for Sfi39 polysaccharide. In both cases, evolution times $2\tau$ between 10 and 200 ms were recorded. The indirect dimension was linear predicted up to 128 complex points and each dimension was apodized with a sine square window function and zero-filled once prior to Fourier transformation. The volumes were fitted with NMRPipe/NMRDraw.

The scalar couplings were then obtained by fitting the curves representing the $I^{mod}(2\tau)/I^{dec}(2\tau)$ ratio (eqs 2 and 3) as a function of the time $2\tau$, using the Levenberg-Marquart algorithm implemented in Matlab.[17] Two procedures were compared: First, a sequential fitting was developed in which a first evaluation for the anomeric signals yielded $^3J_{H1H2}$ from eq 2. This coupling was used as an initial value for the fitting of the curve for H2 according to eq 3, the curve for H2 containing the two scalar couplings $^3J_{H1H2}$ and $^3J_{H2H3}$, and the cross-correlated dipole-dipole relaxation rate $\Gamma^{DD/DD}_{H1H2/H2H3}$. During the fitting procedure, the $^3J_{H1H2}$ scalar coupling was allowed to vary within 10% of the previously determined value, while the other scalar coupling $^3J_{H2H3}$ and the cross-correlated dipole-dipole relaxation rate were not constrained. This yields values for $\Gamma^{DD/DD}_{H1H2/H2H3}$, a second determination of the scalar coupling $^3J_{H1H2}$ and a value for $^3J_{H2H3}$ that was then used, again within ±10% boundaries, as an initial value for the fit of the H3 curve. This procedure was then repeated up to H4, the last proton for which eq 3 could be used. The second procedure was to fit simultaneously all four curves recorded for H1, H2, H3, and H4. The coupling constants are expected to be more constrained since they are simultaneously used on two curves. Due to the large number of parameters and to the fact that the convergence of the Levenberg-Marquart algorithm was sensitive to initial values of the parameters, a systematic search through possible starting $^1$H homonuclear scalar coupling values (−15 to 15 Hz by 1 Hz steps) was implemented.

For the fitting of couplings involving H4 and H5 protons, particular care was required to evaluate the reliability of the fitted values since only one curve was available. Values were judged unreliable when the fit did not converge, i.e., the Levenberg-Marquart algorithm never reached a minimum even by multiplying several times the number of fitting steps.

Only absolute values of the scalar couplings are determined by this method.

***Table 1.*** Three-Bond $^1$H Homonuclear Scalar Couplings $^3J_{HiHi+1}$ Determined for Lactose from a Series of $^{13}$C-COSMO-HSQC Spectra[a]

| | $\beta$-D-Gal*p*-(1→ | →4)-α-Glc*p* | →4)-$\beta$-Glc*p* |
|---|---|---|---|
| $^3J_{H1H2}$ | 7.8 (7.8) | 3.8 (3.7) | 8.4 (7.9) |
| $^3J_{H2H3}$ | 10.7 (10.0) | 9.7 (9.8) | 9.1 (9.2) |
| $^3J_{H3H4}$ | 3.3 (3.4) | 9.5 (8.9) | 9.7 (na)[b] |
| $^3J_{H4H5}$[a] | 1.6 (na)[b] | 10.2 (9.9) | 9.2 (na)[b] |

[a] Literature values given in parentheses are from ref 18. [b] na = not available.

## Results and Discussion

As a test case, lactose (**1**) was chosen as a nonlabeled carbohydrate of small size for which several $^1$H homonuclear coupling constants were available from the literature.[18] For this small molecule, cross-correlated dipole-dipole effects are negligible and eqs 1 and 2 were used for the fitting. The endocyclic $^1$H homonuclear scalar couplings are shown in Table 1 and Figure 2. The agreement between the values from $^{13}$C-COSMO-HSQC and literature is excellent (Figure 2).

Figure 3 shows two spectra obtained from a $^{13}$C-COSMO-HSQC experiment recorded with an evolution time of $2\tau = 90$ ms for the Sfi39 polysaccharide (**2**). In the spectrum in Figure 3a, evolution of $^1$H homonuclear scalar couplings was refocused with a 180° ($^{13}$C) pulse within the BIRD sandwich and the spectrum is similar to an HSQC of **2**. The anomeric peaks were folded once as a result of a diminished spectral window in the indirect carbon dimension. All peaks (except for **C6**, the cross-peaks between the carbon 6 and geminal protons H6a and H6b of the $\beta$-D-Glc*p* unit **C**) are positive. On the other hand, $^1$H homonuclear scalar couplings modulate the spectrum in Figure 3b and many peaks have changed sign. Figure 4 shows the $I^{mod}(2\tau)/I^{dec}(2\tau)$ intensity ratios for the four $^1$H of unit **A** (→3)-α-D-Glc*p*-(1→) of the Sfi39 polysaccharide repeating unit (**2**). The $I^{mod}(2\tau)/I^{dec}(2\tau)$ ratios were fitted by using the two procedures described in Materials and Methods. The results were very similar and the resulting coupling constants and cross-

(14) Reif, B.; Henning, M.; Griesinger, C. *Science* **1997**, *276*, 1230−1233. Felli, I. C.; Richter, C.; Griesinger, C.; Schwalbe, H. *J. Am. Chem. Soc.* **1999**, *121*, 1956−1957.
(15) Germond, J.-E.; Delley, M.; D'Amico, N.; Vincent, S. J. F. *Eur J. Biochem.* **2001**, *268*, 5149−5156.
(16) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277−293.
(17) MATLAB (c), 1984−2002.
(18) Dorland, L.; van Halbeek, H.; Vliegenthart, J. F.; Schauer, R.; Wiegandt, H. *Carbohydr. Res.* **1986**, *151*, 233−245. Platzer, N.; Davoust, D.; Lhermitte, M.; Bauvy, C.; Meyer, D. M.; Derappe, C. *Carbohydr. Res.* **1989**, *191*, 191−207.

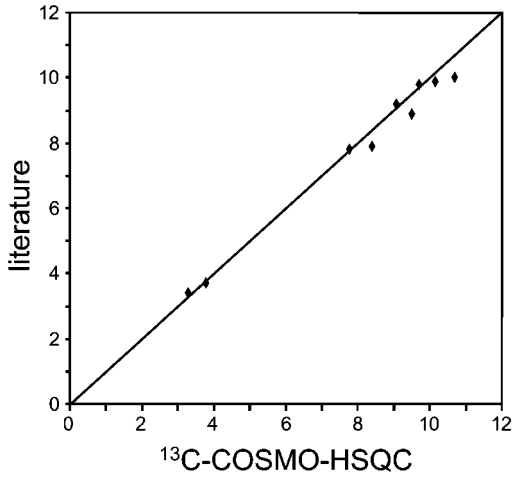


***Figure 2.*** Comparison of $^{1}$H homonuclear three-bond scalar couplings $^{3}J_{HH}$ from literature and from $^{13}$C-COSMO-HSQC measured for lactose (**1**). The correlation coefficient is $R^2 = 0.986$.

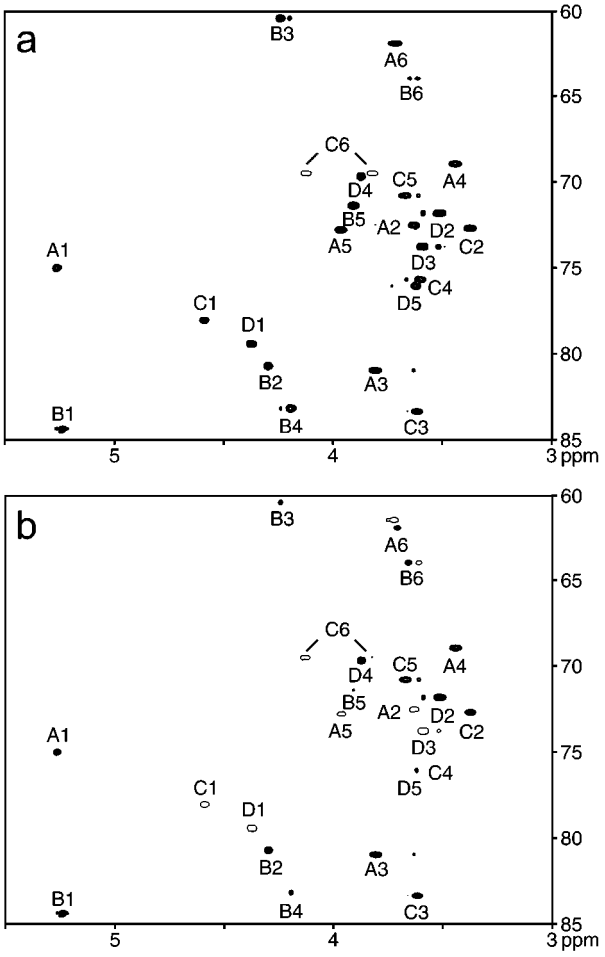


***Figure 3.*** Two $^{13}$C-COSMO-HSQC spectra obtained with the pulse sequence of Figure 1 on the Sfi39 polysaccharide with an evolution time $2\tau = 90$ ms. (a) Decoupled spectrum obtained with a 180° ($^{13}$C) pulse in the BIRD sandwich and (b) modulated spectrum obtained with a 0° ($^{13}$C) pulse within the BIRD sandwich. All parameters were as in Figure 1. The peaks are labeled with a letter for monosaccharide assignment (**A** = α-D-Glc*p*, **B** = β-D-Gal*f*, **C** = β-D-Glc*p*, and **D** = β-D-Gal*p*) and a number for the position (**1** to **6**). Positive peaks: black circles with several contour lines. Negative peaks: one single contour line.

correlated dipole-dipole relaxation rates are given in Table 2. The scalar couplings are in good agreement with couplings

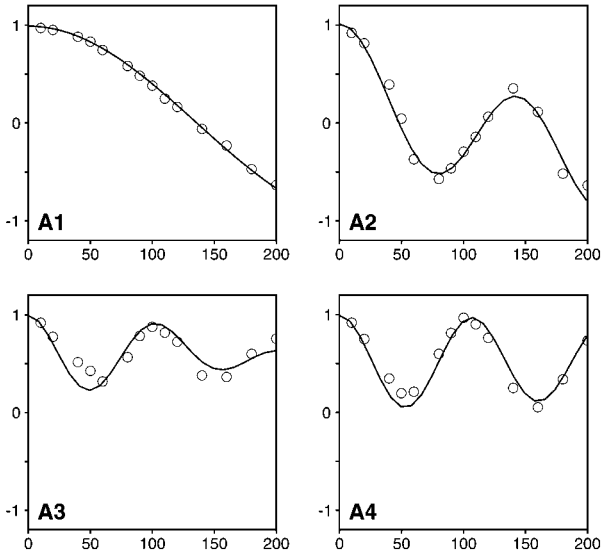


***Figure 4.*** Experimental points of $I^{mod}(2\tau)/I^{dec}(2\tau)$ in Sfi39 polysaccharide as a function of the evolution time $2\tau$. $I^{mod}$ and $I^{dec}$ were obtained from integration of peak intensities in $^{13}$C-COSMO-HSQC spectra recorded with evolution time $2\tau$ varying between 5 and 200 ms. The monosaccharide unit chosen was **A**, the backbone Glc*p* unit.

***Table 2.*** Three-Bond $^{1}$H Homonuclear Scalar Couplings $^{3}J_{HiHi+1}$ and Cross-Correlated Dipole-Dipole Relaxation Rates $\Gamma^{DD/DD}_{Hi-1Hi/HiHi+1}$ Determined for the Sfi39 Polysaccharide from a Series of $^{13}$C-COSMO-HSQC Spectra Recorded with Varying Evolution Times $2\tau$

| | α-D-Glc*p* (**A**) | β-D-Gal*f* (**B**) | β-D-Glc*p* (**C**) | β-D-Gal*p* (**D**) |
|---|---|---|---|---|
| $^{3}J_{H1H2}$ | 3.8 | 1.4 | 8.1 | 8.0 |
| $^{3}J_{H2H3}$ | 10.3 | 3.1 | 9.1 | 10.9 |
| $^{3}J_{H3H4}$ | 9.2 | 5.6 | 8.6 | 3.6 |
| $^{3}J_{H4H5}$[a] | 9.6 | 3.6 | −[b] | 1.0 |
| $\Gamma^{DD/DD}_{H1H2/H2H3}$ | −1.8 | 0.6 | 0.1 | 2.7 |
| $\Gamma^{DD/DD}_{H2H3/H3H4}$ | 5.0 | 1.5 | −0.7 | 0.1 |
| $\Gamma^{DD/DD}_{H3H4/H4H5}$ | −[c] | 1.2 | 1.2 | 2.0 |

[a] The $^{3}J_{H4H5}$ scalar couplings were fitted from only one curve. [b] $^{3}J_{H4H5}$ was unreliable for unit **C**. [c] $\Gamma^{DD/DD}_{H3H4/H4H5}$ was unreliable for unit **A**.

measured for the corresponding monosaccharide moieties in small molecules. These represent the first scalar coupling values available for a polysaccharide.

The determination of scalar couplings by $^{13}$C-COSMO-HSQC did not pose any particular problem for furanose moieties (such as residue **B** of **2**), as well as for other moieties such as *N*-acetylglucosamine and *N*-acetylgalactosamine.

The cross-correlated dipole-dipole relaxation rates determined by this procedure are related to the dynamical properties of the corresponding pairs of dipolar vectors. The major difficulty in interpreting these values is the lack of molecular model describing accurately molecular tumbling of a polymer.

## Conclusions

A two-dimensional $^{13}$C-COSMO-HSQC experiment was presented which enables the determination of all endocyclic $^{1}$H homonuclear scalar couplings within unlabeled carbohydrates of any molecular size. All couplings are obtained from a series of $^{13}$C-COSMO-HSQC spectra recorded with varying evolution times. The results were demonstrated for lactose (**1**), a model disaccharide, and for a biopolymer composed of several thousand repeating units, an exopolysaccharide produced by

milk fermentation from the lactic acid bacteria *Streptococcus thermophilus* Sfi39 (**2**). Scalar couplings smaller than the line widths can and have been determined in the presence of extensive $^1$H signal overlap.

The $^{13}$C-COSMO-HSQC experiment provides the first method for determining all endocyclic $^1$H homonuclear scalar couplings in natural abundance carbohydrates independently of $^1$H resonance overlap and line widths. These important structural constraints should enable a faster development in the study of carbohydrate structures. Although presently $^1$H homonuclear scalar couplings are scarcely used in the structural analysis of carbohydrates, the introduction of a general method for their measurement is likely to increase the usefulness. Furthermore, both in the context of polysaccharides dynamics, as approached for example by molecular modeling, and for the measurement of residual dipolar couplings, the $^{13}$C-COSMO-HSQC method opens new fields of investigation. Finally, the determination of $^1$H homonuclear cross-correlated dipole-dipole relaxation rates in carbohydrates also opens new perspectives concerning the study of carbohydrates' tertiary structures and dynamics.

**Acknowledgment.** C.Z. acknowledges funding by the FNRS (project No. 3100-056951.99) while S.J.F.V. thanks Jean-Richard Neeser for continuous support.

**Supporting Information Available:** A product operator treatment of the $^{13}$C-COSMO-HSQC sequence and the determination of eqs 1−3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA017358V