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Measurement of one-bond heteronuclear dipolar coupling contributions for amine and diastereotopic methylene protons

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Abstract

One-bond heteronuclear and two-bond homonuclear residual dipolar couplings measured at methylene or amine sites can be utilized as long-range constraints in structure determination of molecules as well as to facilitate characterization of local conformation by stereospecific assignment of diastereotopic protons. We present two *J*-modulated HMQC type experiments to measure the one-bond heteronuclear dipolar coupling contributions of geminal protons individually. In addition two-bond homonuclear residual dipolar couplings between the diastereotopic protons are also obtained.

Keywords: Residual dipolar coupling constant; One-bond heteronuclear coupling constant; Homonuclear two-bond coupling constant; Amine/diastereotopic methylene protons; *J*-modulation; HMQC

1. Introduction

Residual dipolar couplings (RDC) provide powerful constraints for structure determination of molecules and have been extensively applied for structural studies of proteins, nucleic acids, and carbohydrates [1]. RDCs arise from the anisotropic tumbling of molecules resulting from their partial alignment in the applied medium. Intrinsic partial alignment is observed for molecules possessing sufficiently large magnetic susceptibility anisotropy in the presence of strong magnetic fields, whereas a tunable degree of alignment can be achieved by dissolving the molecule in partially oriented media such as a dilute liquid crystalline phase. RDCs manifest themselves in the NMR spectra as an increase or decrease of the splittings due to scalar couplings between nuclei, consequently their magnitude can be extracted by measuring changes of splittings in isotropic versus anisotropic sample conditions. Although in most applications RDCs are interpreted in the context of an average static structure, it has been shown that they are also sensitive to motions over a broad range of time scales, allowing characterization of molecular dynamics [2–5].

Structural studies on biomolecules relied so far primarily on the RDCs measured for methine or amide sites. XH_2 groups, like methylene or amine, are generally characterized by two different values of one-bond heteronuclear RDCs reflecting different orientation of the two X–H bond vectors with respect to the external magnetic field. Orientation of these groups can be further characterized by the homonuclear RDC of the geminal protons.

2. Measurement of one-bond heteronuclear RDCs at methylene or amine sites

Measurement of the one-bond heteronuclear residual dipolar contributions of individual protons in methylene or amine groups is not straightforward. Two approaches have been used so far: initial attempts aimed at the measurement of the sum of the two one-bond heteronuclear couplings; later efforts were focused on the separate measurement of the two couplings.

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The most straightforward way to extract the one-bond heteronuclear dipolar contributions is to use F2-coupled HSQC obtained by omitting the heteronuclear decoupling during acquisition [6,7]. For methylene sites this yields, for each of the diastereotopic protons, a doublet that is further split by the proton–proton couplings. Accurate measurement of the one-bond heteronuclear couplings from the F2 multiplets may, however, be hampered by several factors causing uncertainties in the definition of peak positions [8]. For example, the complexity and asymmetry of multiplets due to ^{1}H–^{1}H couplings and possible strong couplings may render the accurate characterization of peaks difficult. In oriented samples dipolar homonuclear couplings may also cause splittings in addition to scalar ones, and give rise to highly complex multiplets further complicating the one-bond heteronuclear RDC measurement. Similar observations were made by Yan et al. [9] who suggested a slightly modified version of 1D and 2D HMQC for acquiring and analyzing the ^{13}C-coupled ^{1}H multiplets.

In macromolecules, the measurement of the one-bond heteronuclear coupling constant in the indirect (F1) dimension becomes more attractive due to its favorable heteronuclear relaxation characteristics. F1-coupled HSQC spectrum [10] can be obtained by simply removing the ^{1}H 180° refocusing pulse from the heteronucleus frequency labeling period [11]. As a result, the heteronucleus magnetization will evolve under the effect of the one- and multiple-bond heteronuclear coupling interactions in addition to the heteronuclear chemical shift evolution. The splittings due to multiple-bond couplings can be removed with a G-BIRD$^{(r)}$ sequence inserted at midway of the t_1 period [8]. Note that the F1-multiplet corresponding to a CH_2 group is doublet rather than triplet (the central line of the triplet is missing) provided the one-bond heteronuclear couplings, $^1(J+D)^a$ and $^1(J+D)^b$ of the two diastereotopic protons, a and b, are similar. As a result, the measured splitting gives the sum of the coupling contributions from both protons. This was measured in uniformly ^{15}N/^{13}C labeled proteins by Chou and Bax [12] using a modified CB(CA)CONH pulse scheme. Recently, a quantitative *J*-correlation method, based on *J*-modulated 3D variant of the ^{1}H–^{13}C CT-HSQC experiment, was proposed by Ottiger et al. [13].

Individual one-bond heteronuclear dipolar couplings for the diastereotopic protons of methylene groups were obtained by Mittermaier and Kay [14] on ~50% fractionally deuterated proteins. This relied on a combination of two intensity based CT ^{1}H–^{13}C heteronuclear correlation spectra: one with selection of CHD methylene groups provides one of the couplings; the other selecting CH_2 isotopomers gives the sum of the two couplings. The SPITZE-HSQC suggested by Carlomagno et al. [15] allows the measurement of the two C,H and the H,H dipolar couplings at the same time using spin-state-selective transfers. Another recent method, NH_2-edited, spin-state-selective experiment (H_2N–HSQC–α/β-*J* sequence) was proposed by Permi [16] for measuring $^1J_{HN}$ couplings in Asn and Gln side chains.

3. *J*-modulated BIRD enhanced HMQC experiments for the individual measurement of one-bond heteronuclear RDCs for methylene and amine sites

The approach presented here is based on the coupling evolution properties of multiple quantum (MQ) coherences. It is known that an MQC is not modulated by the coupling between the active spins generating it, but evolves, on the other hand, under the coupling of any passive spin involved. The two geminal protons in methylene or amine sites will be notated as I^a and I^b, while other remote protons coupled to the SI^aI^b spin system (where $S = {}^{13}$C or ^{15}N) are labeled as I^r. The pulse sequence of the *J*-modulated HMQC experiment is depicted in Fig. 1. The experiment starts with a spin-echo sequence of $\tau = 1/(2^1J_{XH})$ duration to create antiphase proton magnetization which is then converted into heteronuclear multiple quantum coherence with the first 90° X-pulse. The multiple quantum coherence, $2I^a_xS_y$ created from I^a_z magnetization evolves under the effect of the passive spin I^b with $^1J_{X,Ib}$ heteronuclear and $^2J_{Ia,Ib}$ homonuclear coupling interactions. Furthermore, it evolves under the influence of all remote protons which are long-range coupled to S with $^nJ_{X,Ir}$ as well as to I^a with $^nJ_{Ia,Ir}$ couplings. This coupling network can be considerably simplified with a BIRD$^{(d,X)}$ pulse employed at midway of the MQC-evolution period (t_1). The evolution of couplings during t_1 is summarized in Fig. 2. The BIRD$^{(d,X)}$ pulse inverts the directly bonded protons (I^a, I^b) and the heteronucleus (S), but has no effect on the remote protons (I^r). As a result, the one-bond heteronuclear coupling between S and the passive spin I^b continues to evolve and yields the desired $^1J_{XIb}$ splitting in the indirect (F1) dimension. The geminal coupling between the protons I^a and I^b also evolves leading to further splitting in F1. On the other hand, all undesired homonuclear and heteronuclear long-range couplings from the remote protons I^r will be refocused at the end of t_1. As a consequence, the F1-multiplet appearing at the F2 frequency of I^a will be a doublet of doublet with $^1J_{X,Ib}$ and $^2J_{Ia,Ib}$ splittings allowing their individual and accurate measurement. Optional up- or down-scaling of the multiplets is also possible depending upon the applied scaling factor n.

After *J*-modulation of MQC during t_1 (*J*-HMQC) only the cosine-modulated component, $\cos(^1J_{XIb}\pi t_1)\cos(^2J_{Ia,Ib}\pi t_1)I^a_xS_y$, is retained for detection. To this end, the cosine-component is converted to two-spin order $I^a_zS_z$, using properly phased 90° proton and X pulses and left unaffected by the subsequent purging gradient. On

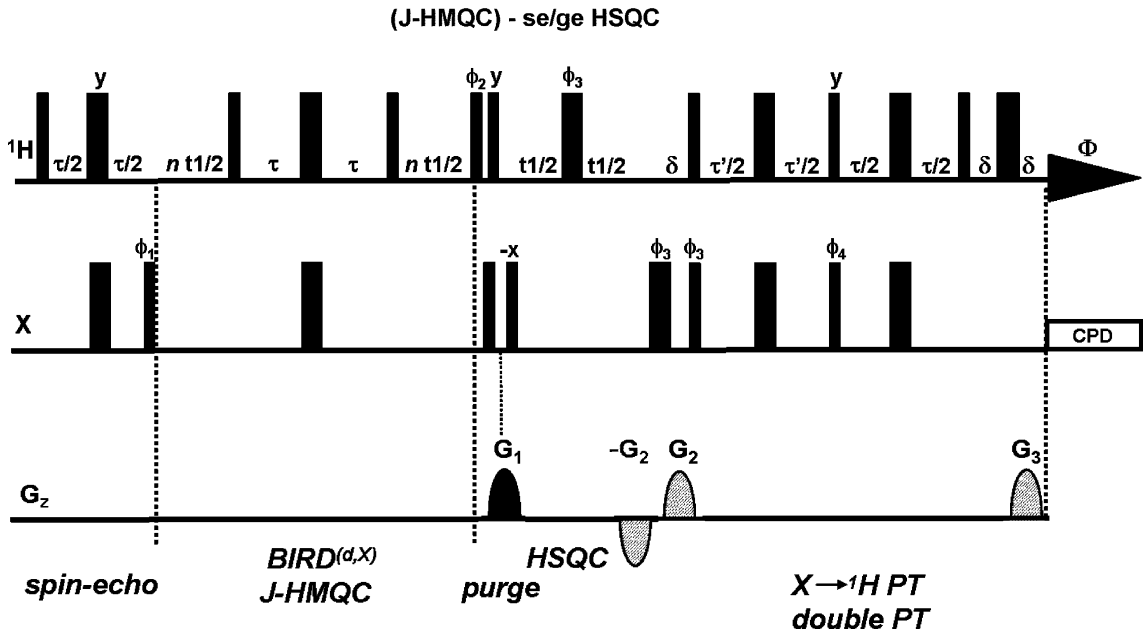


Fig. 1. Pulse sequence of the *J*-HMQC and *J*-HMQC-ge/se-HSQC experiments for the simultaneous and individual measurement of the one-bond heteronuclear and the two-bond homonuclear couplings. Hard 90° and 180° pulses are marked by narrow and wide bars, respectively, with the phase *x*, unless indicated otherwise. In the *J*-HMQC experiment, the heteronuclear SQ-evolution period (HSQC) is set to a constant duration of 3 μs. Delay durations: $\tau = 1/(2^1J_{XH})$ and $\tau' = 1/(4^1J_{XH})$. Phase cycling: $\phi_1 = x, -x; \phi_2 = 4(x), 4(-x); \phi_3 = 2(x), 2(-x); \phi_4 = 2(y), 2(-y); \Phi = x, -x, -x, x$. Shaded gradients of 1 ms duration (δ) are used for echo-antiecho coherence selection: $G_2 = 40$ G/cm and $G_3 = 20$ G/cm for ^{13}C. Purging gradient: $G_1 = 7.5$ G/cm of 1 ms duration. The phase ϕ_4 is in incremented in echo-antiecho manner. Scaling (*n*) of the *J*-evolution is optional.

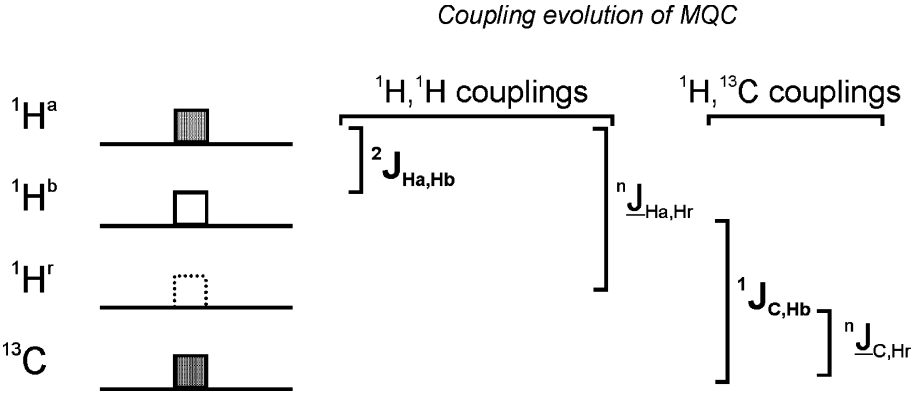


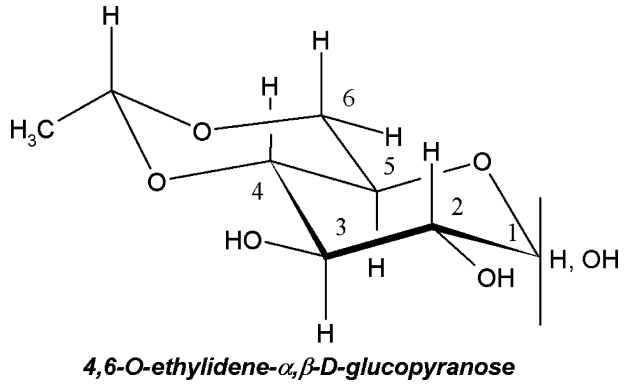
Fig. 2. Effect of the BIRD$^{(d,X)}$ pulse on the coupling evolution of the multiple quantum coherence generated from H^a. (H^a and ^{13}C are the active spins of MQC, while H^b and H^r are the passive spins.) Couplings indicated by underlined *J* ($\underline{J}$) are not active, while that noted by ***J*** are evolving during t_1.

contrary, the sine-modulated components ($\sin(^1J_{XIb}\pi t_1)\cos(^2J_{Ia,Ib}\pi t_1)I_x^aS_xI_z^b$, $\cos(^1J_{XIb}\pi t_1)\sin(^2J_{Ia,Ib}\pi t_1)I_y^aS_yI_z^b$, and $\sin(^1J_{XIb}\pi t_1)\sin(^2J_{Ia,Ib}\pi t_1)I_y^aS_xI_z^b$) conserved as multiple quantum coherence are eliminated by the purging gradient. Afterward the 90° X-pulse converts the two-spin order into antiphase *S* magnetization, which at this point can be labeled by the chemical shift of the heteronucleus as in conventional HSQC sequence. In the end, the enhanced sensitivity protocol (PEP) [17] is employed for X → ^{1}H back transfer.

It is important to note that introduction of the heteronuclear chemical shift information is optional. If the duration of SQ-evolution is set to a constant value of ~3 μs (instead of the incremented t_1 evolution) only the coupling information encoded during the *J*-HMQC module is retained in the F1 dimension, resulting in a *J*-spectrum with its reduced frequency window as an inherent advantage. Applying the resolving power of the heteronuclear chemical shift can be still useful in case of severe overlap between the ^{1}H resonances of CH_2 groups. The *J*-resolved type experiment is called as *J*-HMQC, while the other with the heteronuclear chemical shift labeling is referred to as *J*-HMQC-ge/se-HSQC experiment.

In summary, the *J*-HMQC type *J*-resolved and correlation spectra of methylene or amine moieties display

F1-multiplets with large splitting due to the one-bond heteronuclear coupling. The one-bond coupling of the heteronucleus with the passive spin I^b can be separately measured at the chemical shift of the active spin I^a and vice versa. In addition, the large doublet is further split by the two-bond homonuclear coupling between the geminal protons yielding a doublet of doublet. Therefore, the geminal coupling between the diastereotopic protons and subsequently its dipolar contribution can also be readily determined. Although the proposed pulse sequences are probably more suitable for small to medium sized molecules due to overlap and relaxation considerations, but the basic principle can be adapted for designing new experiments applicable for larger molecules as well.



Scheme 1. Structure of 4,6-ethylidene-α, β-D-glucopyranose.

An application of the proposed experiments is demonstrated using the model compound, 4,6-ethylidene-α, β-D-glucopyranose (**1**) (Scheme 1), of Yan et al. [8,9] under isotropic and partially oriented sample conditions. The sample consists of a 2:1 mixture of the α- and β-anomeric form resulting in two sets of signals in the NMR spectra. The ^{1}H multiplets of the two diastereotopic methylene protons ($H6_{a,b}$) are separated by ca. 0.5 ppm in both anomers. The signals at higher chemical shift will be noted as H_a. The H_b signals of the two anomers strongly overlap, while the corresponding H_a resonances are separated by 0.1 ppm in the ^{1}H NMR spectra. The ^{13}C resonances of the methylene carbons (C6) in the anomers display 0.5 ppm chemical shift difference. The spectral characteristics of the model compound chosen therefore represent unfavorable spectral situations frequently encountered in real cases.

The *J*-HMQC spectrum of **1** acquired under isotropic sample condition and overlays of F1-multiplets from the corresponding "isotropic" and "anisotropic" spectra are shown in Fig. 3. The crosspeaks for the methylene groups appear as doublet of doublet at the corresponding proton frequencies, while that for methine groups display unsplit peaks (singlets) in the middle of the spectra (at 0 Hz F1

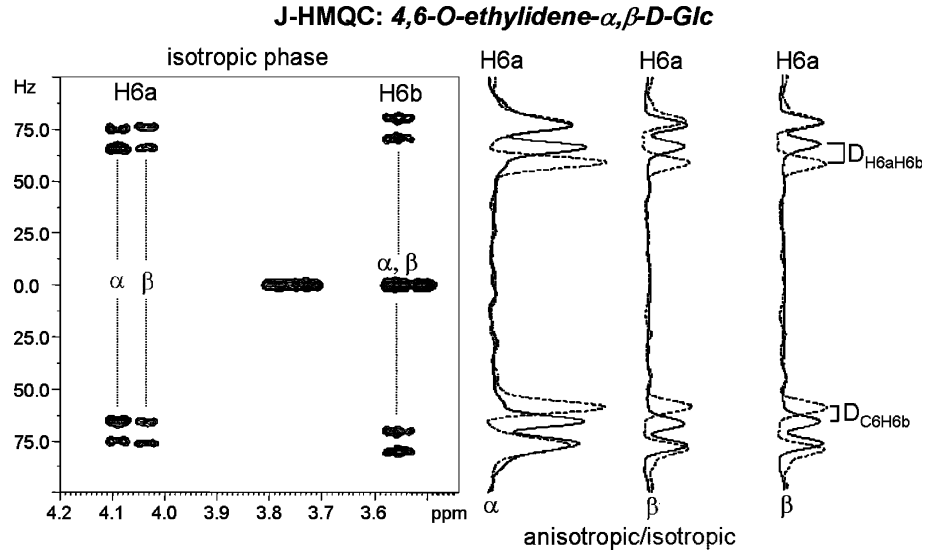


Fig. 3. Methylene region of the *J*-HMQC spectrum of **1** recorded with the pulse sequence in Fig. 1 under isotropic sample condition. Overlays of the F1-multiplets extracted at H6a frequencies of both isomers (α, β) from the corresponding "isotropic" and "anisotropic" spectra are also depicted. Traces with solid line correspond to isotropic phase, while that with dashed line are obtained under anisotropic condition. The rightmost spectrum displays the same traces as the second one, however, the traces are vertically shifted (the peaks on the top are overlapped) to display the difference associated to the residual dipolar coupling constant. Experimental parameters: proton 90° pulse of 10.6 μs, carbon 90° pulse of 13.0 μs and carbon 90° pulse of 80.0 μs for GARP decoupling. Spectral widths in F1 (F2) dimension = 440 (2440) Hz, number of t_1 increments = 150, number of scans = 32, number of data points in $F2 = 1$ K, relaxation delay = 1.8 s, scaling factor $n = 1$. Data were zero-filled to 1 K in F1 dimension; squared cosine window functions were applied in both dimensions. Measured coupling constants in isotropic phase: $^1J_{C6H6b}$(α-anomer) = 141.0 ± 0.4 Hz; $^2J_{H6aH6b}$(α-anomer) = 10.7 ± 0.4 Hz; $^1J_{C6H6b}$(β-anomer) = 142.7 ± 0.4 Hz; $^2J_{H6aH6b}$(β-anomer) = 10.3 ± 0.4 Hz. Coupling constants $(J + D)$ measured in anisotropic phase: $^1(J + D)_{C6H6b}$(α-anomer) = 134.6 ± 0.4 Hz; $^2(J + D)_{H6aH6b}$(α-anomer) = 18.2 ± 0.4 Hz; $^1(J + D)_{C6H6b}$(β-anomer) = 135.8 ± 0.4 Hz; $^2(J + D)_{H6aH6b}$(β-anomer) = 19.8 ± 0.4 Hz.

frequency). Since there is no chemical shift evolution of the heteronuclei, the spectral window in F1 corresponds to the magnitude of one-bond heteronuclear coupling, requiring fewer t_1 increments and shorter experimental time for the coupling constant measurement. With the lack of the heteronucleus chemical shift evolution, the methylene signals with overlapping ^{1}H signals (H_{6b} in α- and β-anomers) give rise to overlapping crosspeaks in Fig. 3. Hence, the one-bond heteronuclear coupling constants for the H_{6b} protons can readily be determined at the well resolved H_{6a} resonances, while that for the H_{6a} protons cannot be simply measured in Fig. 3. However, it is possible to calculate them from the sum of the heteronuclear one-bond couplings ($^1J_{CH6a} + {}^1J_{CH6b}$) and the above determined $^1J_{CH6b}$. The sum of the couplings can be obtained from the F1-coupled HSQC spectrum (Fig. 4), which is generally acquired for determining the one-bond heteronuclear RDCs for CH groups. The spectra overlaid in Fig. 4 were obtained under isotropic and anisotropic conditions by the F1-coupled G-BIRD$^{(r)}$-HSQC sequence [8] to decouple the undesired heteronuclear long-range couplings, while retaining the evolution of the one-bond ^{1}H–^{13}C couplings.

The chemical shift evolution of the heteronucleus can be reintroduced with *J*-HMQC-ge/se-HSQC experiment. An expansion of the relevant spectrum displaying the CH_2-region of **1** and the corresponding F1-traces can be seen in Fig. 5. Due to the 0.5 ppm ^{13}C chemical shift difference between the methylene carbons of the α- and β-anomers, now the crosspeaks for all methylene protons are resolved, allowing separate and accurate measurement of the desired coupling constants. This experiment however needs longer measurement time due to the large number of t_1 increments required by the chemical shift range of the heteronucleus. This requirement can be somewhat relaxed by folding the spectra which, on the other hand, can introduce complications in the course of the analysis.

4. An overview of applications and potential utilizations of RDCs measured for methylene or amine sites

Stereospecific assignment of the diastereotopic methylene protons is often a prerequisite for accurate structure determination. One-bond heteronuclear RDCs were successfully utilized to obtain stereospecific assignments for methylene protons by comparing the measured RDCs with those expected/calculated for possible epimers [7,9,18,19]. The main advantage of this method is based on the fact that RDCs provide long-range information, therefore the relative stereochemistry

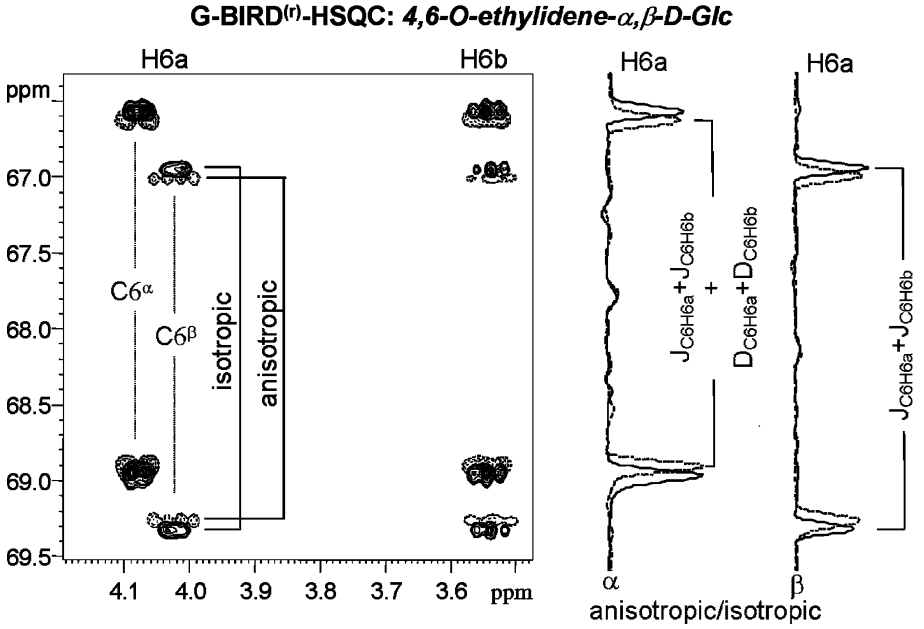


Fig. 4. Overlay of the F1-coupled G-BIRD$^{(r)}$-HSQC [8] spectra of **1** recorded under isotropic and anisotropic conditions showing the sum of the individual one-bond heteronuclear couplings of the C6-methylene group. F1-multiplets extracted at H6a frequencies of both isomers (α, β) are depicted: traces with solid line correspond to isotropic phase, while that with dashed line are obtained under anisotropic condition. Experimental parameters: spectral widths in F1 (F2) dimension = 3018 (2440) Hz, number of t_1 increments = 320, number of scans = 32, number of data points in $F2 = 1$ K, relaxation delay = 1.8 s, and scaling factor $n = 1$. Data were zero-filled to 2 K in F1 dimension; squared cosine window functions were applied in both dimensions. Measured coupling constants in isotropic phase: $({}^1J_{C6H6a} + {}^1J_{C6H6b})$(α-anomer) = 294.8 ± 0.5 Hz; $({}^1J_{C6H6a} + {}^1J_{C6H6b})$(β-anomer) = 294.4 ± 0.5 Hz; Coupling constants in anisotropic phase: $({}^1J + {}^1D)_{C6H6a} + ({}^1J + {}^1D)_{C6H6b}$ = 281.5 ± 0.5 Hz (in α-anomer); $({}^1J + {}^1D)_{C6H6a} + ({}^1J + {}^1D)_{C6H6b}$ = 279.3 Hz ± 0.5 Hz (in β-anomer). Calculated coupling constants: $^1J_{C6H6a}$ (α-anomer) = 153.8 ± 0.4 Hz and $^1J_{C6H6a}$(β-anomer) = 151.7 ± 0.4 Hz in isotropic phase; $^1(J + D)_{C6H6a}$(α-anomer) = 146.9 ± 0.4 Hz and $^1(J + D)_{C6H6a}$(β-anomer) = 143.5 ± 0.4 Hz in anisotropic phase.

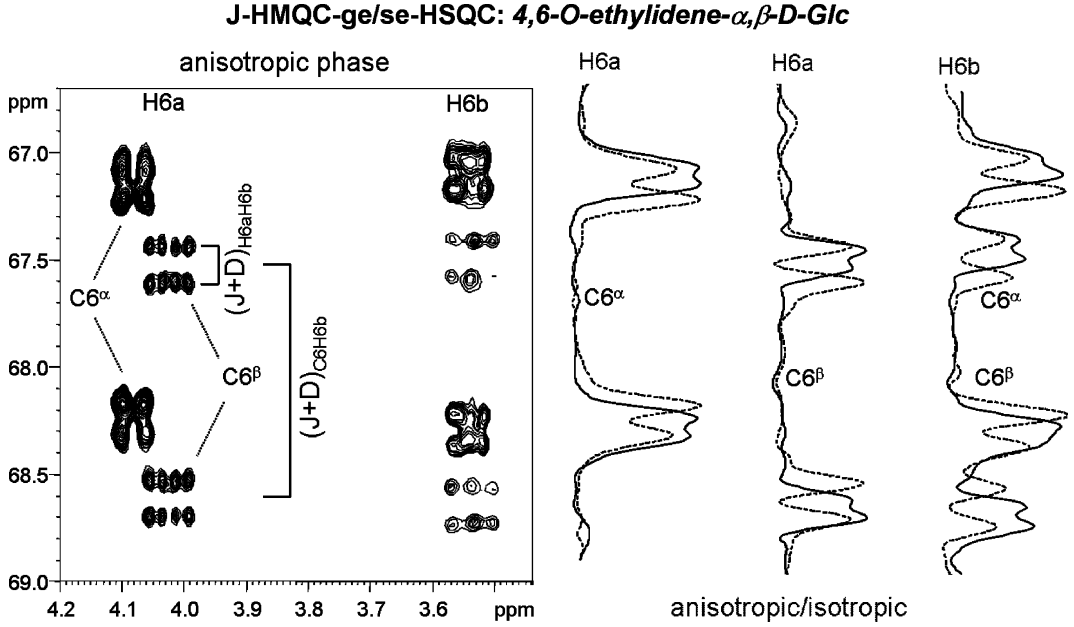


Fig. 5. Methylene region of the *J*-HMQC-ge/se-HSQC spectrum of **1** recorded with the pulse sequence in Fig. 1 under anisotropic sample condition. Overlays of the F1-multiplets extracted at H6a and H6b frequencies of both isomers (α, β) from the corresponding "isotropic" and "anisotropic" spectra are also depicted. Traces with solid line correspond to isotropic phase, while that with dashed line are obtained under anisotropic condition. Experimental parameters: spectral widths in F1 (F2) dimension = 3018 (2440) Hz, number of t_1 increments = 320, number of scans = 32, number of data points in $F2 = 1$ K, relaxation delay = 1.8 s, and scaling factor $n = 1$. Data were zero-filled to 2 K in F1 dimension; squared cosine window functions were applied in both dimensions. Coupling constants measured from the F1-multiplets are in good agreement with those given in the captions of Figs. 3 and 4.

of two stereocenters can be assessed regardless of their relative distance. This is not the case for NOE and *J* coupling methods.

In compounds with six-membered chair conformation only two orientations are possible for each of the C–H/C–X bonds, therefore similarity of the one-bond C–H RDCs implies the same orientation of the corresponding C–H vectors [9].

By determining the individual values of one-bond heteronuclear RDCs and the dipolar contributions between geminal protons in pendant methylene groups one can supplement the RDC data for a particular ring or rigid domain. Thus the measurement of RDCs for 2-methylene groups might play important role in structural studies of deoxy ribonucleic acids.

In carbohydrates the rotamer populations of exocyclic CH_2OH groups and orientation of (1–6) interglycosidic linkages can also be characterized by utilizing the RDCs of methylene sites [20,21].

In a recent study [13], the sum of the two individual one-bond heteronuclear RDCs measured for methylene sites has been utilized for structure refinement of a protein.

It has been shown [22] that the RDCs measured in methylene and amine groups of Asn and Gln have a significant impact on the accurate characterization of the orientation of carboxamide groups, which being both H-bond donor and acceptor play important role in determining the protein structure. Recently, Mittermaier and Kay [14] reported one-bond heteronuclear RDCs for protein sidechains (C_β-methylene) and interpreted them in the context of side chain dynamics.

5. Conclusion

The proposed *J*-HMQC type experiments allow accurate and individual measurement of the one-bond heteronuclear coupling constants and their dipolar contributions in methylene and amine moieties. The *J*-HMQC experiment is applicable in any case where at least one of the two diastereotopic proton signals is not overlapped by another (arbitrary) methylene proton signal. If this condition is met, one of the coupling constants can be determined by measuring the splitting at the non-overlapped proton resonance in the resulting *J*-HMQC spectrum, while the other coupling can be obtained from the difference between this value and the sum of the two couplings available from the F1 coupled G-BIRD$^{(r)}$-HSQC experiment. Note that overlap with methine and methyl proton signals is allowed, since the corresponding F1 crosspeaks appear symmetrical at the 0 Hz line with different multiplicity—singlet for CH, doublet for CH_2 and triplet for CH_3—allowing accurate measurement of the desired couplings. Alternatively, the resolution power of the heteronuclear chemical shift can be utilized with the

application of the *J*-HMQC-ge/se-HSQC pulse scheme. This experiment though requires longer experimental time, but may resolve crosspeaks overlapped in the ^{1}H dimension. A useful feature of these experiments is that they provide also the homonuclear geminal coupling constants and their dipolar contributions.

6. Experimental

All NMR experiments were performed on Bruker DRX-500 spectrometer (Bruker AG, Rheinstetten, Germany) equipped with a 5 mm *z*-gradient multinuclear proton detected (bbi) probe. All spectra were processed with XWINNMR 3.1 (Bruker AG, Karlsruhe, Germany). The use of the proposed experiments for the extraction of RDCs was demonstrated on liquid crystal sample of 4,6-ethylidene-α, β-D-glucopyranose which was prepared by dissolving 24 mg substance in 700 μl media. As a result, the concentration of the α- and β-anomers was 110 and 55 mM, respectively. For the alignment, bicelles were formed from CHAPSO and DMPC in the ratio of 1:3.5 with 3.25% of total concentration. CHAPSO and DMPC were purchased from Sigma–Aldrich and used without further purification. The temperature for measurements was set to 295 K for isotropic conditions and to 299 K to achieve anisotropic conditions.

All spectra were acquired with high spectral resolution of ca. 0.3–0.5 Hz for accurate measurement of small residual dipolar couplings.

Acknowledgments

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