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Accurate measurement of long-range heteronuclear coupling constants from undistorted multiplets of an enhanced CPMG-HSQMBC experiment

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Abstract

Here, we present a modified CPMG-HSQMBC experiment which is capable to reduce the detrimental phase twists in the ''long-range'' connectivity multiplets caused by proton–proton couplings. We demonstrate that concerted CPMG pulse trains applied on both nuclei in the starting CPMG-INEPT transfer step can considerably be improved by composite π pulses that compensate for pulse imperfections and off-resonance effects. Experimental optimization of the interpulse delay within the CPMG cycle was found to be crucial in order to achieve the best possible ''decoupling'' of homonuclear coupling modulation.

Keywords: Long-range heteronuclear couplings; HSQMBC; CPMG; Composite pulses; Carbohydrate; Glycopeptide

1. Introduction

Long-range heteronuclear coupling constants are extensively used for determination of constitution, configuration and conformation of molecules, and thus provide powerful tools in structural studies of small organic [1,2] and biomolecules [3] and structure elucidation of natural products [4]. As a result, numerous methods were developed for more accurate measurement of long-range heteronuclear coupling constants. These are summarized in detail by Marquez et al. [5]. The HSQMBC experiment [6] in particular has found widespread application since it allows extraction of heteronuclear coupling constants for both protonated and quaternary carbons via heteroatoms (e.g., O and N) in the coupling pathway. It also offers good sensitivity and straightforward implementation on most spectrometers. The long-range heteronuclear coupling constants for proton singlets can be directly measured from the antiphase splitting of HSQMBC multiplet in the acquisition (^{1}H) dimension; while for protons with complex multiplets usually fitting procedures must be performed to obtain the desired heteronuclear couplings.

Difficulties can arise, however, in case of complicated proton multiplets with large homonuclear coupling constants. The initial INEPT element of the HSQMBC sequence is tuned for the value of the long-range heteronuclear coupling constant, however, homonuclear couplings being of the same order of magnitude are also evolving and create multiple quantum coherences. These coherences become observable at a later stage of the pulse sequence, and contributing to the desired coherence distort the lineshape of the HSQMBC multiplet in the final spectrum. Koselka et al. suggested the use of CPMG pulse train simultaneously applied on both protons and heteronuclei. The proposed LR-CAHSQC experiment [7] ''decouples'' the homonuclear couplings while the evolution of heteronuclear couplings is

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allowed. Experimental implementation of the CPMG scheme in the initial INEPT element can be, however, seriously hampered by pulse imperfections of the repeated π pulses and off-resonance effects of the heteronucleus (e.g., ^{13}C) with large chemical shift range. In our proposed CPMG variant of the HSQMBC sequence all π pulses are therefore implemented as composite pulses resulting in significant improvement of the lineshape of HSQMBC multiplets. Furthermore, the interpulse delay of the CPMG cycle is experimentally optimized to achieve optimal suppression of homonuclear coupling evolution.

2. Methods

The CPMG sequence is most commonly applied for T_2 relaxation measurements [8]. The original repetitive π pulse train was improved to remove the effect of pulse imperfections by suitable phase cycling schemes [9,10].

The homonuclear proton–proton coupling evolution during the CPMG sequence can be suppressed [9,10] if the interpulse delay between the π pulses is shorter than $1/2\sqrt{(J^2+\Delta\nu^2)}$. In case of weak coupling the condition can be approximated as $1/(2\Delta\nu_{\max})$, where $\Delta\nu_{\max}$ is the largest chemical shift difference between the weakly coupled proton partners. The shortest duration of interpulse delay is limited by the maximum power the probehead can absorb, that is by its maximum duty cycle.

The CPMG-INEPT element consisting of simultaneous application of π pulse trains on both proton and heteronuclei during the INEPT period has initially been introduced for improving the polarization transfer between ^{1}H and ^{15}N for exchange-broadened amino protons in nucleic acids [11]. The concept has later been generalised by Mulder et al. [12] and utilised in ^{1}H–^{13}P CPMG-HSQC experiments as well to improve the efficiency of ^{1}H–^{31}P polarization transfer for nucleic acids [13]. Moreover the CPMG sequence was applied in TROSY transverse relaxation experiments for measuring chemical and/or conformational exchange rates with the use of the TROSY principle for sensitivity improvement [14]. A recent application of the CPMG sequence has been proposed by Koskela et al. [15] to improve the performance of the G-BIRD pulse for suppression of ^{12}C bound proton magnetization.

It is well known that π pulses replaced by composite pulses improve their performance [16–18]. In case of modest RF inhomogenities the 90_y–180_x–90_y composite pulse suggested by Levitt and Freeman [17] proved to be very efficient. CPMG sequences using composite pulses were shown to perform better in the presence of inhomogenities of the static B_0 field and that of the RF field strength, B_1 [19].

The proposed CPMG-HSQMBC experiment which is a straightforward amendment of the original HSQMBC sequence [6] starts with a composite pulse based CPMG-INEPT element as shown in Fig. 1.

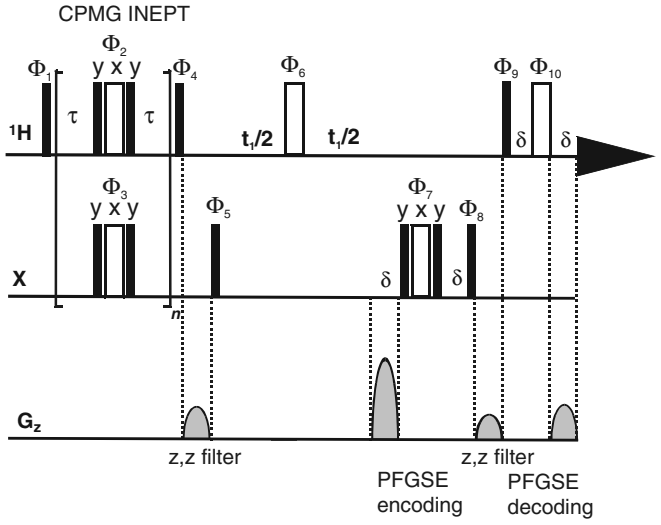


Fig. 1. Pulse sequence of the improved CPMG-HSQMBC experiment. Hard 90° and 180° pulses are marked by filled and open bars, respectively. Delay durations: $\tau = 120$ μs, 200 μs; $2n\tau = 70$ ms optimized for a ca. 7 Hz long-range heteronuclear coupling. Phase cycling: $\phi_1 = x$; ϕ_2 and ϕ_3 incremented according to XY-16 cycles within the CPMG sequence; $\phi_4 = y$; $\phi_5 = x\ -x$; $\phi_6 = x\ x\ -x\ -x$; $\phi_7 = x\ x\ x\ x\ -x\ -x\ -x\ -x$; $\phi_8 = x\ x\ x\ x\ -x\ -x\ -x\ -x$; $\phi_9 = x$; $\phi_{10} = x$; $\Phi = x\ -x\ x\ -x\ -x\ x\ -x\ x$. Sine bell shaped gradients of 1 ms duration (δ) are used, followed by a recovery delay of 200 μs. Gradient strengths for the z,z filters: 15% and 10%; for gradient encoding: 80%, for gradient decoding: 20% for ^{13}C and 8% for ^{15}N, given as percentage of the absolute gradient strength of 50 G/cm. The sign of the last decoding gradient is alternated for echo–antiecho coherence selection. To simplify the HSQMBC map the one-bond correlation peaks can be reduced or eliminated with the use of gradient enhanced second-order low-pass filter [24] applied in the preparation part of the sequence or with the inclusion of a G-BIRD^{r,X} pulse element at midway of the CPMG cycle [25].

The heteronuclear single quantum coherence (SQC) created during the initial INEPT step of the HSQMBC experiment can be described as follows:

$$\mathrm{H}_y\mathrm{C}_z \sin(\pi {}^{n}J_{\mathrm{CH}}\Delta)\cos(\pi J_{\mathrm{HH'}}\Delta), \tag{1}$$

where the INEPT delay, Δ is tuned for the magnitude of long-range heteronuclear coupling constant and calculated as $1/(2\,{}^{n}\hat{J}_{\mathrm{CH}})$, where ${}^{n}\hat{J}_{\mathrm{CH}}$ is the average of the expected heteronuclear long-range coupling constants. Evolution of homonuclear proton–proton couplings—which are of similar magnitude as the heteronuclear long-range couplings—can be efficiently suppressed by the CPMG scheme simultaneously applied for both nuclei with the XY-16 phase cycling. Since homonuclear Hartmann–Hahn transfer takes place between protons [20], the antiphase proton magnetization created during the CPMG-INEPT element can be described [7] by

$$1/2\mathrm{H}_y\mathrm{C}_z\{1+\cos(2\pi J_{\mathrm{HH'}}\Delta)\}\sin(\pi^{\mathrm{n}}J_{\mathrm{CH}}\Delta). \tag{2}$$

The lineshape of the CPMG-HSQMBC multiplet is determined only by this term, while the undesired, more complex coherences are efficiently suppressed.

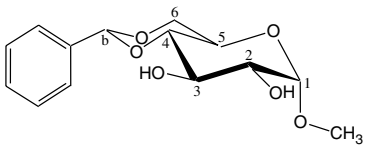
The performance of the CPMG-HSQMBC experiment is considerably improved when the π pulses of CPMG

scheme are implemented as 90_y–180_x–90_y composite pulses and additionally the power levels for proton and heteronucleus are adjusted to give pulses of equal duration, allowing perfectly simultaneous effects on the two channels. The interpulse delay, τ within the CPMG cycle will determine the proton bandwidth over the homonuclear couplings can be decoupled. A shorter interpulse delay will increase this bandwidth: for example a delay between 200 and 120 μs provides decoupling over ca. 2500 and 4150 Hz frequency range, respectively. We found that 120 μs delay provides efficient homonuclear decoupling over the whole proton spectral range at 500 MHz without any detrimental heating effect. However, because of the large chemical shift difference between carbons and protons $\Delta\nu_{\mathrm{maxH,C}}$, the heteronuclear coupling will evolve. Note that heteronuclear magnetization arising from HEHAHA [21] transfer and evolving during t_1 has no contribution to the detected proton magnetization due to the lack of carbon-proton back transfer. Following the CPMG-INEPT element a gradient-enhanced heteronuclear z,z filter is used to destroy the remaining homonuclear multiple quantum coherence that may not be decoupled due to the large shift separation of the coupling partners. Coherence selection is achieved by encoding–decoding pulsed field gradients implemented in spin-echos after the polarization transfer steps. A composite pulse is used to compensate for the off-resonance effects of heteronuclei in the first, encoding gradient spin-echo. The sign of the last coherence selection gradient is alternated to obtain phase sensitive spectra according to the echo–antiecho protocol.

The coherence detected during acquisition is proton magnetization antiphase with respect to the active heteronuclear coupling and frequency labelled with the chemical shift of the heteronucleus in the indirect dimension

$$1/2\mathrm{H}_y\mathrm{C}_z \sin(\pi^{\mathrm{n}}J_{\mathrm{CH}}\Delta)\{1+\cos(2\pi J_{\mathrm{HH'}}\Delta)\}\cos(\Omega_{\mathrm{C}}t_1). \quad (3)$$

During acquisition this coherence evolves according to the long-range heteronuclear coupling constant together with homonuclear couplings as function of t_2. The first term produces antiphase while the latter in-phase splitting of the HSQMBC multiplets. For a singlet proton signal this yields simple antiphase splitting from which the heteronuclear long-range coupling constant can be directly read as the frequency difference between the antiphase multiplet components. In case of complex ^{1}H pattern, the HSQMBC multiplet extracted at the frequency of the heteronucleus of interest is subsequently compared with a multiplet that is simulated as a sum of the parent ^{1}H multiplets, but one of them is inverted and frequency shifted by a trial value of the heteronuclear long-range coupling, or optionally matched with computer fitting.



Scheme 1. Structure of methyl-4,6-*O*-benzylidene-α-D-glucopyranoside (**1**).

3. Results and discussion

The performance of the proposed CPMG-HSQMBC experiment is analysed here using suitable model

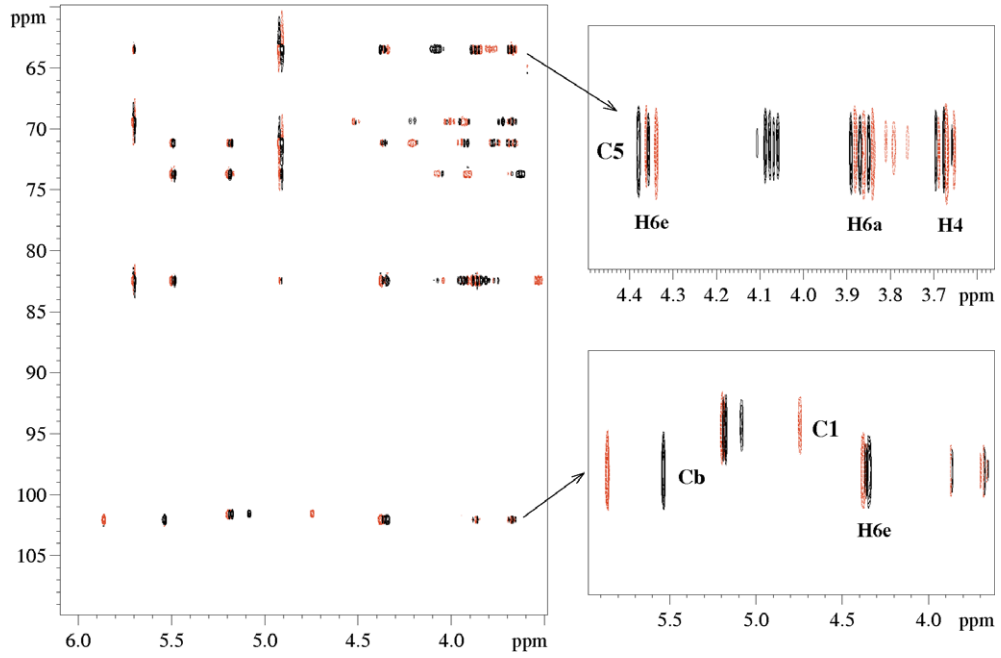


Fig. 2. Partial contour plots of the CPMG-HSQMBC spectrum of **1** recorded with pulse scheme shown in Fig. 1. Experimental details of the measurement are summarized in the text.

compounds. Methyl-4,6-*O*-benzylidene-α-D-glucopyranoside (Scheme 1, the sugar ring is labelled with G), displays an ^{1}H NMR spectrum of simple and complex proton multiplets. For comparison, 2D long-range heteronuclear correlation spectra with the original HSQMBC [6] sequence, with the CPMG-HSQMBC experiment using simple hard π versus composite π pulses and using different CPMG interpulse delays were recorded otherwise under the same experimental conditions. A contour plot of the CPMG-HSQMBC spectrum and two excerpts recorded with the proposed sequence of Fig. 1 are presented in Fig. 2. To compare the phase quality of signals, selected multiplets extracted from spectra of the different variants of HSQMBC experiment are also shown in Figs. 3–5.

A doublet of the glucose H1 (G-H1) proton coupled to G-H2 with small 3.5 Hz coupling is shown in Fig. 3A. The HSQMBC multiplet can be simulated by adding the proton multiplet to its inverted image with a frequency shift corresponding to the value of the long-range heteronuclear coupling constant, $^3J_{\text{G-H1,C5}} = 6.5$ Hz, as shown in Fig. 3B. Note that in the case of simple proton multiplet of G-H1 there is a good agreement between the simulated (Fig. 3B) and the experimental multiplet measured with the original HSQMBC sequence (Fig. 3F). The reason is the following. The multiplet detected with the original HSQMBC experiment is in fact a sum of the desired anti-phase proton magnetization

$$-\mathrm{H}_y^1\mathrm{C}_z^5 \sin(\pi J_{\mathrm{C5H1}}\Delta)\cos(\pi J_{\mathrm{H1H2}}\Delta)\cos(\Omega_{\mathrm{C}}t_1) \qquad (4)$$

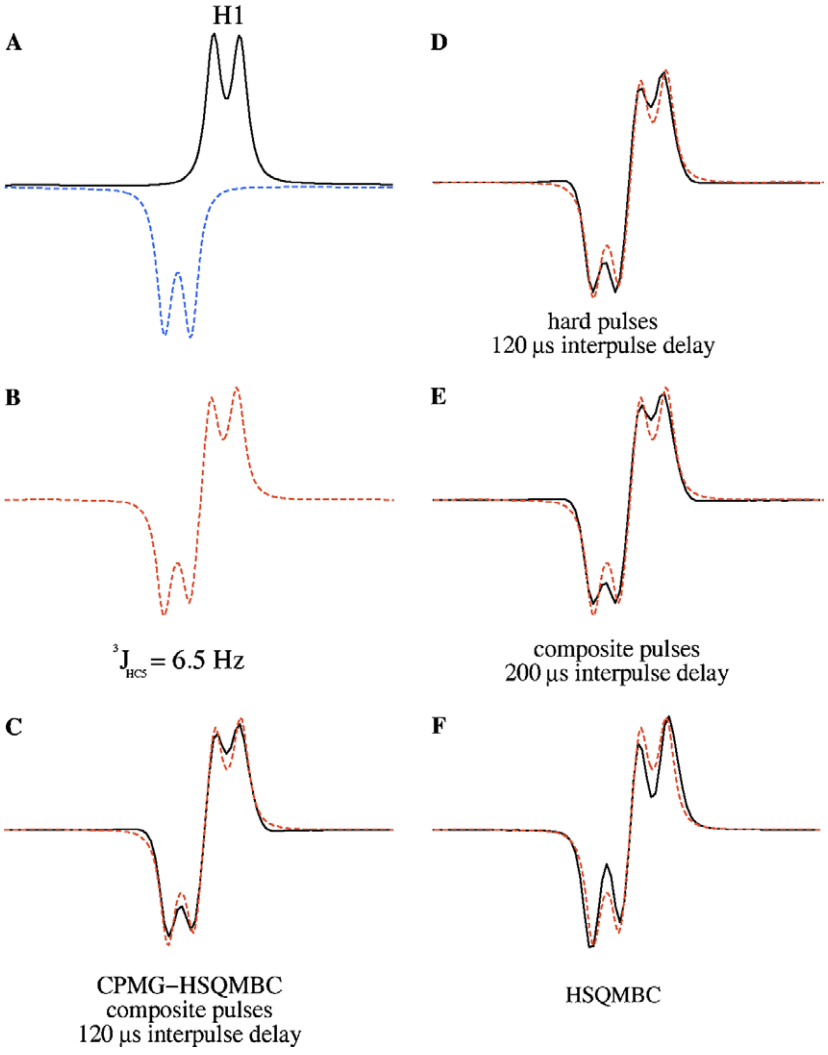


Fig. 3. Comparison of simulated and experimental HSQMBC multiplets for the correlation between G-H1 to G-C5. (A) G1-H1 proton doublet and its inverted image shifted with 6.5 Hz, corresponding to the magnitude of the long-range heteronuclear coupling constant; (B) simulated HSQMBC multiplet generated by the addition of the two ^{1}H multiplets. F2 multiplets extracted from the crosspeak between G-H1 to G-C5 in the CPMG-HSQMBC spectra recorded by the sequence of Fig. 1 using composite π pulses and 120 μs interpulse delay (C), using simple π pulses and 120 μs interpulse delay (D), using composite π pulses and 200 μs interpulse delay (E), and in the HSQMBC spectrum acquired with the original HSQMBC experiment (F). Note that the rapid pulsing within the CPMG cycle caused only slight decrease (10–15%) of signal intensity. The heteronuclear coupling constant estimated from the original HSQMBC multiplet (F) was 7.5 Hz (±0.3 Hz) in contrast to 6.5 Hz measured from the other multiplets, which indicates that minor phase distortion can lead to erroneous value of the coupling constant.

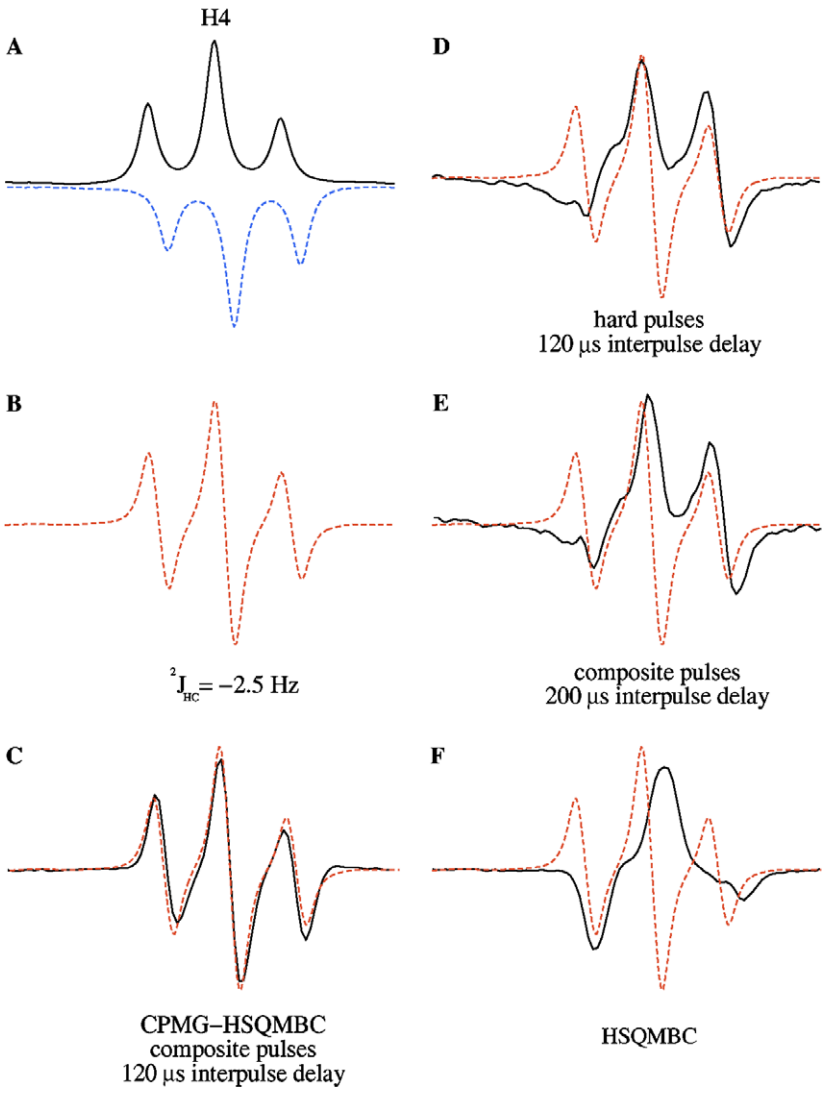


Fig. 4. Comparison of simulated and experimental HSQMBC multiplets for the correlation between G-H4 to G-C5. (A) G-H4 proton multiplet and its inverse image shifted with the value of the corresponding long-range heteronuclear coupling constant of −2.5 Hz; (B) simulated HSQMBC multiplet generated by the addition of the two ^{1}H multiplets. F2 multiplets extracted from the crosspeak between G-H4 to G-C5 in the CPMG-HSQMBC spectra recorded by the sequence of Fig. 1 using composite π pulses and 120 μs interpulse delay (C), using simple π pulses and 120 μs interpulse delay (D), using composite π pulses and 200 μs interpulse delay (E), and in the HSQMBC spectrum acquired with the original HSQMBC experiment (F). The coupling constant of −2.5 Hz (±0.3 Hz) was determined from direct measurement and subsequent manual peak fitting analysis of the (C) multiplet. The detrimental phase twist of the multiplets in (D–F) resulted in significant sensitivity loss and underestimation of the coupling constant of interest.

and the proton magnetization antiphase both to the heteronucleus and the proton coupling partner

$$H_x^1 H_z^2 C_z^5 \sin(\pi J_{C5H1} \Delta) \sin(\pi J_{H1H2} \Delta) \cos(\Omega_C t_1). \quad (5)$$

The former coherence yields antiphase splitting according to the long-range heteronuclear coupling with G-C5 and in-phase splitting with G-H2; while the latter double antiphase coherence gives antiphase splittings with respect to both hetero- and homonuclear couplings. Nevertheless, the desired coherence is dominant if the homonuclear coupling (3.5 Hz) is smaller than the heteronuclear coupling (6.5 Hz). Since the disturbing coherence does not contribute significantly to the lineshape, the multiplets obtained with the CPMG-HSQMBC using simple π pulses (Fig. 3D), with composite π pulses allowing 200 μs (Fig. 3E) and 120 μs (Fig. 3C) CPMG interpulse delay show only slightly improved intensity match with the simulated multiplet. Consequently, the coupling constant $^3J_{\text{G-H1,C5}}$ measured with different methods agree within the experimental error of ±0.3 Hz except the original HSQMBC multiplet (Fig. 3F) where 7.5 Hz was measured. Note that rapid pulsing in the CPMG-INEPT block decreased the intensity of G-H1 multiplet by only 10–15%.

The situation is markedly different for a more complex proton multiplet. The G-H4 proton multiplet is a triplet displaying two large couplings of 9.2 Hz with respect to the axially oriented protons, G-H3 and G-H5, as shown in Fig. 4A. The simulated HSQMBC multiplet obtained with $^2J_{\text{G-H4,C5}} = -2.5$ Hz and shown in Fig. 4B is noticeably different from the F2 multiplet in Fig. 4F

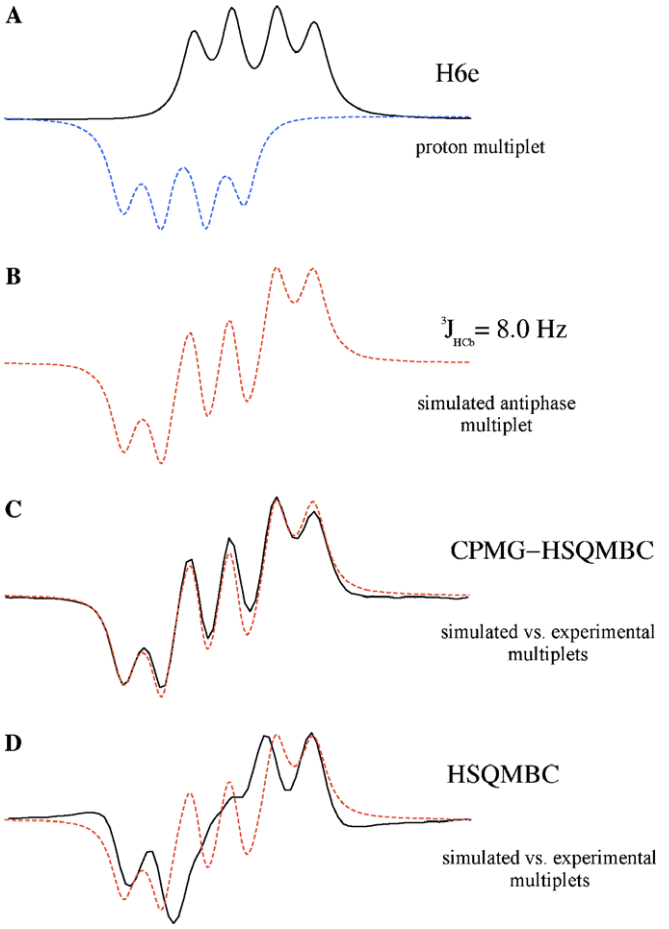


Fig. 5. Comparison of simulated and experimental HSQMBC multiplets for the correlation between G-H6e to Cb. (A) G-H6e proton multiplet and its inverse image shifted with 8.0 Hz corresponding to the value of the long-range heteronuclear coupling constant; (B) simulated HSQMBC multiplet generated by the addition of the two ^{1}H multiplets. F2 multiplets extracted from the crosspeak between G-H6e to Cb in the improved CPMG-HSQMBC (C) and in the original HSQMBC (D) spectra overlaid with the simulated HSQMBC multiplet. The coupling constant of 8.0 Hz (±0.3 Hz) was determined from direct measurement and subsequent manual peak fitting analysis of the (C) multiplet. The detrimental phase twist of the original HSQMBC multiplet (D) resulted in significant intensity loss and underestimation of the coupling constant.

corresponding to the correlation peak of the original HSQMBC spectrum. This is due to the fact that the coherence evolving under the effect of a large homonuclear coupling has significant contribution to the lineshape. In this case, the detected multiplet consists of a sum of the desired proton magnetization in which G-H4 is antiphase to G-C5 and in-phase to G-H5 and G-H3, respectively

$$-H_y^4 C_z^5 \sin(\pi J_{C5H4}\Delta)\cos(\pi J_{H4H5}\Delta)\cos(\pi J_{H4H3}\Delta)\cos(\Omega_C t_1) \quad (6)$$

and the proton magnetization created from the homonuclear multiple quantum coherence in which G-H4 is antiphase to both G-H5 and G-H3 as well as to G-C5

$$H_y^4 H_z^5 H_z^3 C_z^5 \sin(\pi J_{C5H4}\Delta)\sin(\pi J_{H4H3}\Delta)\sin(\pi J_{H4H5}\Delta) \times \cos(\Omega_C t_1). \quad (7)$$

The former coherence will yield antiphase splitting according to the long-range heteronuclear coupling with G-C5 and in-phase splittings with respect to the homonuclear couplings to G-H5 and G-H3; while the latter coherence will yield antiphase splittings with respect to all couplings. Since the homonuclear couplings in this case are quite large (9.2 Hz) compared with the heteronuclear coupling of −2.5 Hz, the contribution of the desired coherence is small and the multiplet exhibits a triple antiphase structure. As a result, the match with the simulated multiplet is poor (Fig. 4F), and due to the detrimental phase twist the long-range heteronuclear coupling cannot be determined by any method, including manual peak matching. Moreover, the phase distortion destroyed the requested antiphase pattern of the multiplet and led to significant intensity loss.

The match with the simulated multiplet could be improved by applying the CPMG pulse train with simple π pulses and using 120 μs interpulse delay (Fig. 4D). The 120 μs delay allows decoupling of homonuclear couplings over a ca. 4150 Hz frequency range. The simple π pulses, however, neither compensate for the imperfection of the π pulses nor provide homogenous refocusing over the whole bandwidth of the heteronucleus during the CPMG cycle or the encoding pulsed field gradient spin-echo sequence. Similar result could be achieved using composite pulses that compensate for imperfect pulses, but allowing long, 200 μs interpulse delays with a homonuclear decoupling bandwidth of ca. 2500 Hz (Fig. 4E). Combining composite π pulses with short, 120 μs interpulse delays within the CPMG cycle results in a perfect match with the simulated HSQMBC multiplet as shown in Fig. 4C that allows accurate measurement of the heteronuclear coupling constant.

As a further example, the complex multiplet of H6e (G-H6e) is shown in Fig. 5. This multiplet is a double of doublet due to the geminal coupling of −9.8 Hz to G-H6a and an equatorial-axial coupling of 4.5 Hz to G-H5. The simulated multiplet in Fig. 5B is obtained with $^3J_{\text{G-H6e,Cb}} = 8.0$ Hz. The multiplet in Fig. 5D obtained with the original HSQMBC experiment is seriously distorted, while the CPMG-HSQMBC sequence using composite π pulses and 120 μs interpulse delay more adequately reproduces (Fig. 5C) the simulated pattern in Fig. 5B. A further example demonstrates (Fig. 6) that the proposed CPMG-HSQMBC method works for medium sized molecules as shown for the ^{1}H–^{15}N long-range coupling measurement of the heptapeptide aglycon of the antibiotic Eremomycin (MW ~1108 Da) [22] (Scheme 2). As in our case, ^{15}N labelling [23] helps a lot for moderate size biomolecules, and provides excellent signal to noise ratio, but for smaller molecules with good solubility, natural abundance experiments are also feasible. When the ^{1}H linewidth is similar or bigger than the coupling constants, concurrent, "quantitative" methods are preferred.

The examples shown in this work demonstrate that the improved CPMG-HSQMBC sequence with composite

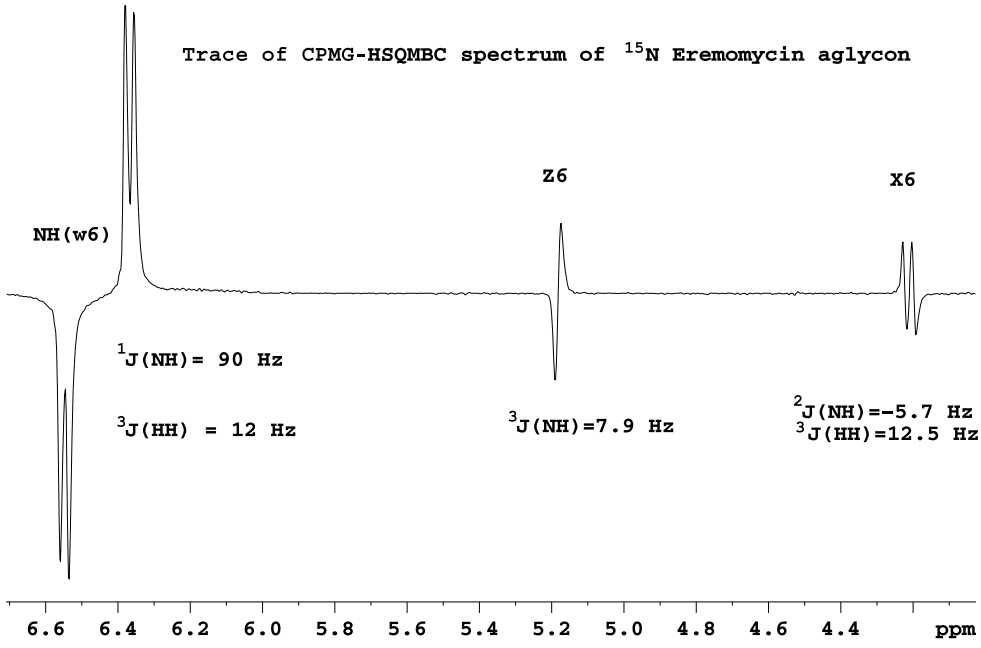
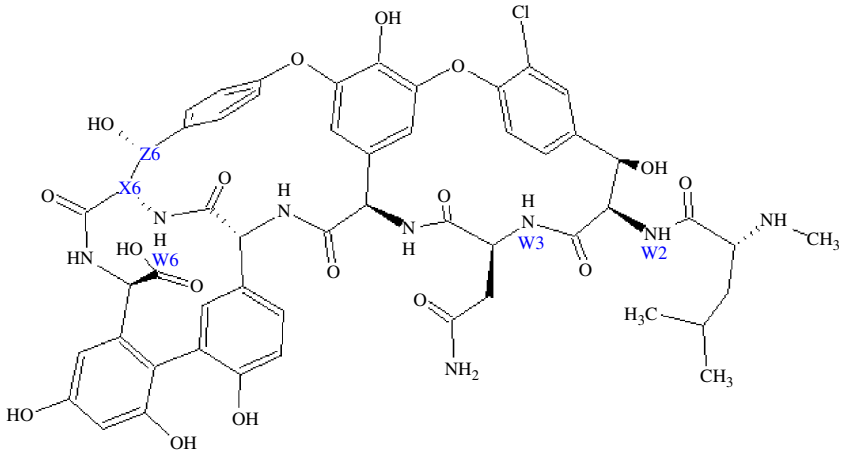


Fig. 6. Eremomycin aglycone (MW = 1108, 60% ^{15}N labelled) 5 mg dissolved in 500 μl DMSO-d_6 and measured at 327 K. In an overnight experiment 352 scans were acquired in each of the 64 t_1 increments for ^{15}N domain. Relaxation delay of 1.5 and 0.45 s acquisition time was used, for the evolution of long-range couplings 65 ms was allowed. The excerpt of the 2D spectrum obtained with the proposed CPMG-HSQMBC experiment of Fig. 1 shows the one-bond heteronuclear coupling of w6 amide nitrogen as well as its two- and three-bond couplings to x6 α-CH and z6 protons. No comparison is shown with more conventional sequences.



Scheme 2. Structure of eremomycin aglycon (**2**).

π pulses performs superior to the original HSQMBC experiment in case of complex ^{1}H multiplets with large homonuclear splittings, or provides similar result for proton multiplets where evolution of homonuclear couplings is insignificant. Moreover, the HSQMBC spectrum can be simplified with removing or reducing the intense one-bond correlation peaks with the use of gradient enhanced second-order low-pass filter [24] applied in the preparation part of the sequence or with the inclusion of a G-BIRD^{r,X} pulse element at halfway of the CPMG sequence [25].

4. Conclusion

We have analysed the contributions of the desired and unwanted coherences to the HSQMBC multiplets with different homonuclear coupling patterns. For protons with no or small homonuclear coupling constant the pattern is determined by the desired antiphase proton coherence, and the long-range heteronuclear coupling constant can be readily determined. For proton signals with large homonuclear coupling constants the HSQMBC multiplet is distorted by unwanted multiple antiphase coherences

originating from the evolution of homonuclear couplings. Consequently, under such circumstances, the determination of long-range heteronuclear coupling constants becomes doubtful or even impossible.

The evolution of homonuclear couplings, however, can be suppressed by the use of CPMG cycle during the initial INEPT element. We have demonstrated that the performance of the CPMG-HSQMBC experiment can be significantly improved using composite π pulses and optimized interpulse delays. The composite pulses compensate for the cumulative effect of the π pulse imperfections and heteronuclear off-resonance effects. Interpulse delay of 120 μs within the CPMG cycle was shown to improve significantly the lineshape of the multiplets by ''decoupling'' over a larger proton frequency range. Such short interpulse delays are well within the power limits of modern probeheads and RF amplifiers, and do not cause significant sensitivity loss due to sample heating effects.

In summary, the proposed CPMG-HSQMBC experiment performs better than the original HSQMBC sequence and can be recommended for accurate measurement of heteronuclear long-range coupling constants.

5. Experimental

All NMR experiments were performed on a Bruker Avance DRX 500 spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm *z*-gradient multinuclear proton detected (bbi) probehead. All spectra were processed with XWINNMR 2.6. The sample of the model compound (**1**) was prepared by dissolving ca. 70 mg substance in 500 μl 99.98% deuterated DMSO-d_6. The temperature was set to 298 K.

The HSQMBC spectra were acquired with 32 scans per t_1 increment and 200×2 hypercomplex points in F_1. In the F_2 dimension 4 K data points were collected. In order to provide simultaneous composite π pulses on the ^{1}H and ^{13}C or ^{15}N channel the power levels were carefully calibrated to give equal duration (13 μs for ^{1}H, ^{13}C or 23 μs for ^{1}H, ^{15}N) of proton and heteronucleus pulses. Multiplication with shifted squared sine bell function and zero filling was performed in both dimensions prior to processing in accordance with the echo–antiecho protocol resulting in a 4×1 K complex dataset. For determination of the heteronuclear coupling constants, the selected F_2 slices were inverse Fourier transformed, zero-filled to 16 K and then back-transformed, allowing the coupling constant measurement with ±0.3 Hz uncertainty.

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