NMR Spectroscopy

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A Definitive NMR Solution for a Simple and Accurate Measurement of the Magnitude and the Sign of Small Heteronuclear Coupling Constants on Protonated and Non-Protonated Carbon Atoms**

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For many years the practical use and measurement of long-range proton–carbon coupling constants (^nJ(CH); $n>1$) in natural abundant molecules have been a timely topic in NMR spectroscopy and there are still a number of unresolved issues in this area.[1] There have always been doubts about the correct choice of the best NMR method to be used and many discussions have focused on the accuracy, reliability, and simplicity of the data analysis and determination of the ^nJ(CH) coupling constant. Despite the extensive number of NMR techniques that have been developed, there are still two unsolved experimental problems pertaining basically to non-protonated carbon atoms, namely, a) an accurate measurement of very small coupling constants (less than 2–3 Hz) and b) the absence of a general and robust approach to determine the sign of the coupling constant. Widely used pulse sequences like α/β-HSQC-TOCSY,[2] HETLOC,[3] or HECADE[4] that consist of a dual-step HSQC-type block followed by a TOCSY transfer (^1J(CH) + J(HH), Scheme 1A) provide the magnitude and the sign of $^nJ_{CH}$ coupling constants irrespective of their values (0–10 Hz) but only for protonated carbons. On the other hand, long-range correlation schemes such as HSQMBC,[5] EXSIDE,[6] or J-HMBC[7] experiments based on a direct ^nJ(CH) transfer (Scheme 1B), typically fail to measure very small coupling constants (0–3 Hz) as well as they do not provide information about the sign.

We show here that all these drawbacks can be solved by a slightly modified HSQC-TOCSY method in which the 180°(^{1}H) pulses in the INEPT blocks are applied selectively to one or several nonmutually coupled ^{1}H resonances and the delays are adjusted to the ^nJ(CH) values (Figure 1). The

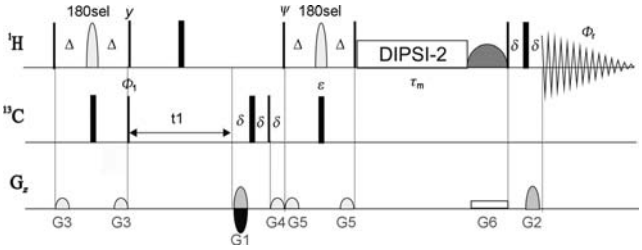
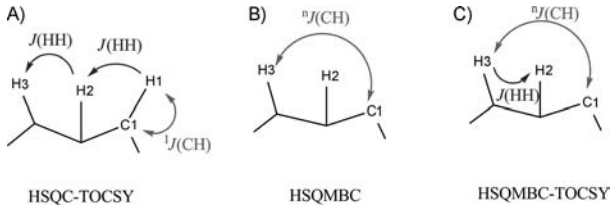


Figure 1. Pulse scheme of the 2D ^{1}H-selective HSQMBC-TOCSY experiment. Proton 180° pulses applied in the middle of the evolution periods ($(\Delta + p(180°sel)/2) = 1/(4\,^nJ(CH))$) can be selective for the frequency, region, or for multiple frequencies. Two independent IP ($\Psi = y$, $\varepsilon =$ on) and AP ($\Psi = x$, $\varepsilon =$ off) data sets are initially collected and further combined to provide complementary α and β data (IP ± AP) in separate spectra.



Scheme 1. The pathway transfer mechanisms involved in A) HSQC-TOCSY, B) HSQMBC, and C) HSQMBC-TOCSY experiments dedicated to the measurement of $^nJ_{CH}$.

selHSQMBC-TOCSY scheme generates α/β multiplets by recording two separate and complementary in-phase (IP) and anti-phase (AP) data sets followed by a basic addition/subtraction data processing.[8] The behavior of both J(HH) and ^nJ(CH) evolution throughout the entire pulse sequence yields pure-phase multiplets and, in addition, the TOCSY period preserves the α/β ^{13}C spin-state information. The method allows the efficient measurement of the magnitude and the sign of ^nJ(CH) by analyzing the relative displacement of α/β cross-peaks in the detected dimension with high resolution and accuracy even for very small values.

The proposed selHSQMBC-TOCSY experiment consists of a two-step procedure based on a sequential ^nJ(CH) + J(HH) transfer mechanism (Scheme 1C). The success of the method relies on the presence of a large coupling constant between the initial H3 and the intermediate C1 spins and, secondly, on the effective magnetization transfer to the third H2 spin through a TOCSY process. Thus, starting from a selected H3 proton, the signal intensity for a relayed H2-C1

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α/β cross-peak will depend on a $\sin(2\pi^{n}J(\mathrm{C1H3})\Delta)\cos(\pi J(\mathrm{H2H3})\tau_{m})[1+\sin(2\pi^{n}J(\mathrm{C1H3})\Delta)]$ function as a result of an additive $^{n}J(\mathrm{H3\text{-}C1})+J(\mathrm{H2\text{-}H3})$ transfer mechanism, so it is completely independent of the ^{n}J(H2-C1) value and also of the C1 carbon multiplicity.

The benefits to append a TOCSY transfer in selHSQMBC experiments[8] are evident comparing the spectra of Figure 2A vs. 2B. The number of cross-peaks is notoriously

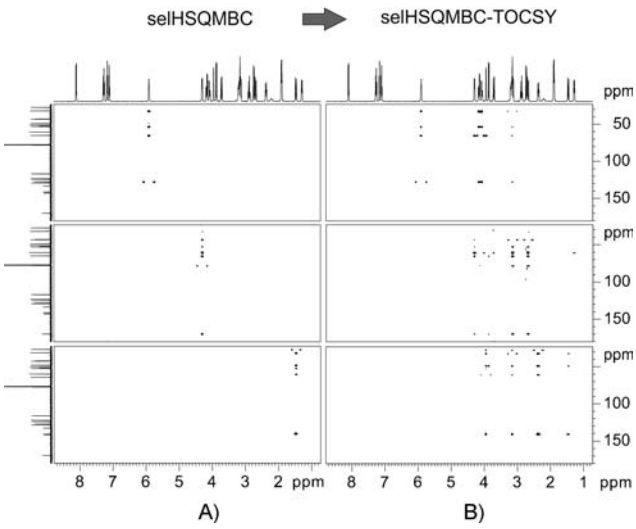

Figure 2. In-phase A) selHSQMBC and B) selHSQMBC-TOCSY spectra after selective refocusing of H22, H12, and H15a protons in strychnine (**1**).

increased thanks to the combination of selective proton excitation and TOCSY transfer highlighting very much its superior information content. While in the selHSQMBC experiment only the largest coupling constants of the excited proton can be determined, the additional TOCSY cross-peaks allow the measurement between these carbon atoms and all other protons belonging to the same spin system of the excited proton. The magnitude and the sign of ^{n}J(CH) in selHSQMBC-TOCSY experiments are easily determined by analyzing the relative sense of the displacement of the α/β components, which are generated by a linear combination of the IP ± AP data. For protonated carbon atoms, the sense of the corresponding direct $^{1}J_{CH}$ correlation (positive sign) can be used as the reference. In the absence of this direct correlation or for non-protonated centers, the comparison of the relative sense between cross-peaks is necessary. Assuming that a large three-bond correlation will have a positive value, such direction of the displacement can be taken as the reference for sign determination of other cross-peaks in the same row. Thus, if a cross-peak defined as positive shows a left/right pattern, other cross-peaks having the same left/right pattern will be positive whereas those peaks having an opposite right/left pattern will be negative. For instance, the simple analysis of the relative displacement for the carbonyl C10 carbon cross-peaks at 169.3 ppm (Figure 3) after simultaneous excitation of the three overlapped, nonmutually coupled H14, H11, and H18a protons of the alkaloid

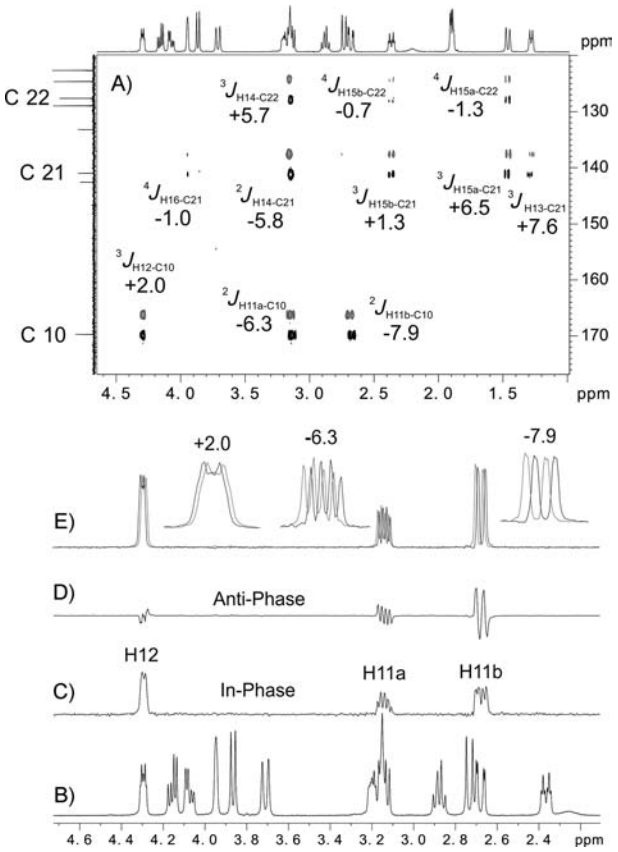

Figure 3. A) Expanded area showing the overlayed α/β selHSQMBC-TOCSY spectra (visualized here as vertically shifted red/black peaks) after selective refocusing of the overlapped H14, H11, and H18a protons resonating close to the 3.15 ppm signal of strychnine. B) Conventional 1D ^{1}H NMR spectrum and C–E) 1D slices taken at the carbonyl C10 chemical shift corresponding to the C) IP, D) AP and E) α/β spectra. The determination of the magnitude and the sign of the coupling value are made in a very straightforward way by analyzing the relative displacement between relayed α/β cross-peaks.

strychnine, **1**, all of them resonating around 3.15 ppm, clearly shows large negative values for H11a and H11b (−6.3 and −7.9 Hz, respectively) and a small positive value for H12 (+2.0 Hz). On the other hand, the olefinic C21 carbon at 140.5 ppm (see Figure 3A) presents positive values for H13 (+7.6 Hz), H15a (+6.5 Hz), and H15b (+1.3 Hz) and negative values for the two-bond H14 (−5.8 Hz) and even for the tiny four-bond H16 (−1.0 Hz) correlations.

The proposed IPAP methodology successfully works even for nonresolved, broad, or complex resonances and the accuracy of the measurement can be further validated by comparing the ^{n}J(CH) values obtained from the α/β data analysis with those of the recorded IP and AP data. On the other hand, the complementarity between selHSQMBC-TOCSY experiments with different proton excitations is displayed in Figure 4. Thus, whereas the selHSQMBC experiment on the H13 proton only shows some correlations corresponding to the most intense coupling values (Figure 4A), the complementary selHSQMBC-TOCSY experiment on the adjacent H8 proton provides up to six additional cross-peaks for this relayed H13 proton from which coupling

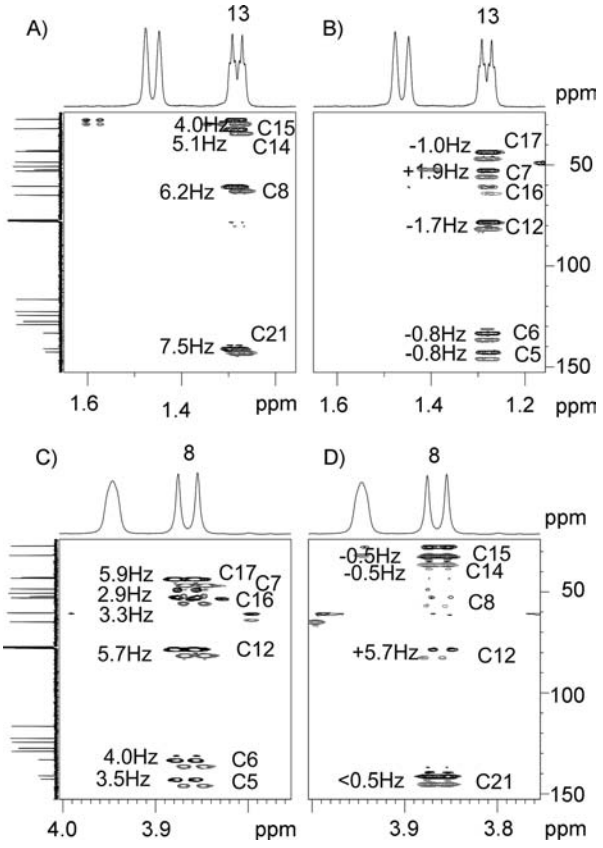

Figure 4. 2D expanded plots showing the complementarity between the selHSQMBC experiments of A) H13 and C) H8 and between selHSQMBC-TOCSY experiments of B) H8 and D) H13. Note that relayed cross-peaks in selHSQMBC-TOCSY spectra make it possible to measure the very small coupling values that are missing in the original selHSQMBC spectra.

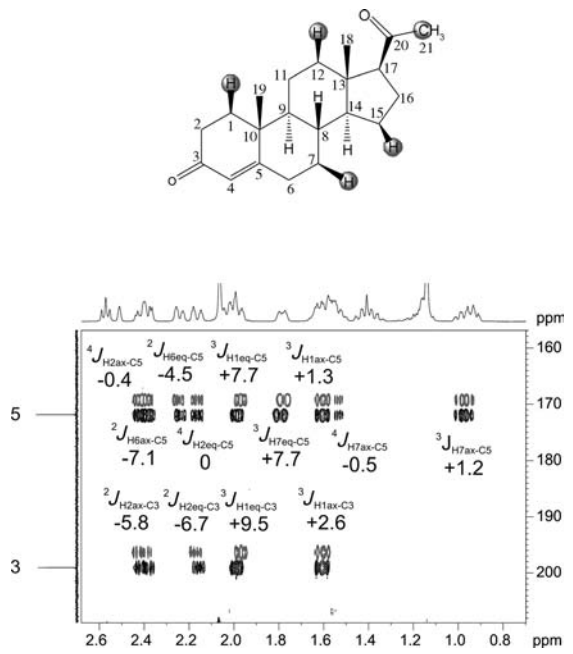

Figure 5. Expanded area of the α/β-selHSQMBC-TOCSY spectra of progesterone after selective inversion of all protons resonating in the region of 1.75–2.1 ppm.

values smaller than 2 Hz can be determined (Figure 4B). The opposite reasoning can also be done for the H8 proton (compare Figure 4C vs. 4D).

The enormous versatility of the method can be further enhanced by simultaneously selection of multiple, nonmutually coupled protons located in different parts of the molecular skeleton by region- or multiple-site selective excitation (see the Supporting Information for examples). This can represent a good strategy to simultaneously map out independent coupling topologies and different structural environments.

The steroid progesterone is a challenging example demostrating that the method succesfully works on complicated NMR spectra with an elevated degree of signal overlap including highly complex multiplets. Thus, many different long-range correlations are obtained when all protons resonating at 1.75–2.1 ppm (H21, H15, H12eq, H1eq, and H7eq protons) are simultaneously inverted. For instance, whereas the H1eq proton shows strong correlations with C2 (4.0 Hz), C3 (9.5 Hz), C10(4.0 Hz), C5(7.7 Hz), and C9(4 Hz) carbon atoms in the selHSQMBC spectrum, the TOCSY transfer allows the additional measurement of ^nJ(CH) for all these carbon atoms and the relayed H1ax, H2eq, and H2ax protons (Figure 5). For instance, coupling values of +1.3 and +2.6 Hz are measured for the three-bond correlation between H1ax, C5, and C3, respectively, as well as a very small and negative −0.5 Hz for the four-bond H11eq-C5 correlation. Such measurements are practically impossible to be done using conventional HSQMBC and HSQC-TOCSY experiments.

In conclusion, a robust NMR method for the measurement of ^nJ(CH) of general applicability and high versatility solves the major problems encountered in the original HSQC-TOCSY and HSQMBC experiments while the most important advantages are retained: a) both protonated and non-protonated carbon atoms can be observed, b) the magnitude and the sign of $^nJ_{CH}$ are directly determined from the analysis of the relative displacement of α/β multiplets avoiding the need of complex and time-consuming analysis and individualized fitting procedures, c) the measurement is made in the detected dimension and therefore a great number of t_1 increments is not a requisite, d) pure-phase multiplets are obtained that allow the easy and accurate extraction of ^nJ(CH) even for broad, unresolved or highly complex multiplets, e) coupling values smaller than the line width can be determined, and f) the combination of different forms of selective proton excitation and TOCSY editing offers an excellent versatility to clean overlapped regions and make reliable measurements even in congested areas or with the presence of mixtures of compounds.

The method can be understood as a way to obtain a ^{1}H TOCSY spectrum from a quaternary carbon chemical shift and this can also be interesting for structural elucidation and chemical assignment purposes. The method is equally applicable for the measurement of other heteronuclear coupling constants and, in particular, can be of enormeous interest in the measurement of residual dipolar coupling constants where the sign information is mandatory. The relative

simplicity in the experimental setup, the multiplet analysis, and the data interpretation will make this versatil HSQMBC-TOCSY experiment an essential tool for a reliable determination of $^{n}J_{CH}$ in the structural and conformational studies of organic and natural products, even for inexperienced NMR users.

Experimental Section

All NMR experiments were recorded on a BRUKER DRX-500 spectrometer equiped with a three-channel 5 mm cryoprobe incorporating a *z*-gradient coil. The test samples were strychnine (**1**, 25 mg) in $CDCl_3$ (0.6 mL) and progesterone (**4**, 25 mg) in [D_6]DMSO (0.6 mL). ^{1}H–^{13}C IP and AP-HSQMBC experiments were separately recorded as described in Figure 1 using a recycle delay of 1 s, the interpulse delay ($\Delta + p(180°sel)/2 = 1/4\,^{n}J_{CH}$) was optimized to 8 Hz, and the duration and shape of the selective proton 180° ^{1}H pulse (p(180°sel)) was set accordingly to the required selectivity in each case. A basic two-step phase cycle was applied: $\phi_1 = x,-x$ and $\phi_{rec} = x,-x$. Gradients G1 and G2 are used for coherence selection using echo-antiecho, G4 acts as a *zz*-filter, G3 and G5 flank the inversion proton pulses to generate pure refocusing elements, and G6 is applied simultaneously to a chirp pulse (30 ms) to remove undesired zero-quantum (ZQ) contributions.[9] This last element can be optionally incorporated into the TOCSY building block of 20–40 ms (τ_m) of duration consisting of a *z*-filtered DIPSI-2 scheme. The duration of each gradient was $\delta = 1$ ms and the proportionality between gradients G1:G2:G3:G4:G5:G6 were set to 80:20.1:33:50:17:3. The IP and AP data were acquired with 128 t_1 increments of 4056 data points for each one, and then added/subtracted in the time domain. Prior to Fourier transformation of the data, zero filling to 1024 points in F1, 8192 points in F2, and a sine-squared function in both dimensions were applied. The data were finally obtained without any scaling factor to provide two separate α/β data sets.

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