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Accurate measurement of small heteronuclear coupling constants from pure-phase α/β HSQMBC cross-peaks

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ABSTRACT

A simple proton-selective α/β-HSQMBC experiment is proposed for the accurate measurement of long-range proton–carbon coupling constants ($^nJ_{CH}$) in small molecules without need for an individualized and time-consuming post-processing fitting procedure. The method acquires two pure-phase In-phase (IP) and Anti-phase (AP) multiplets completely free of any phase distortion due to the absence of J_{HH} evolution. Accurate $^nJ_{CH}$ values can be directly measured analyzing the relative displacement of the resulting IPAP cross-peaks. Discussion about signal intensity dependence and cross-talk is made for a range of experimental conditions. The robustness of the method is evaluated by comparing the $^nJ_{CH}$ value measured from the analysis of the three available IP, AP and IPAP multiplet patterns. Multiple-frequency and region-selective versions of the method can also be efficiently recorded provided that excited protons are not mutually coupled.

1. Introduction

For many years, it has been recognized that the use of long-range proton–carbon coupling constants ($^nJ_{CH}, n > 1$) is a very good complement to proton–proton coupling constants (J_{HH}) and/or NOE data for the structural and conformational analysis of natural-abundance molecules [1,2]. However, it is really surprising that the practical use of $^nJ_{CH}$ has been relatively scarce considering that most molecules are mainly composed by protons and carbons. It is known that these small $^nJ_{CH}$ coupling constants (ca. 0–10 Hz) present strong dependences with respect to coupling pathways, pattern substitutions and dihedral angles. However, the lack of extensive experimental data and trustworthy structural correlations often prevents its successful application to resolve routine problems.

The reason for this limited use of $^nJ_{CH}$ may be attributed to two main factors. First, there is no a single and general NMR method for their measurement [3,4] and, secondly, the accuracy of such measurements has always been a continuing source of discussion. Today is accepted that two general NMR strategies can be followed. On the one hand, HSQC-TOCSY pulse schemes [5–8] are the best approach to measure both the magnitude and the sign in protonated carbons but the method inevitably fails for non-protonated centers or when homonuclear TOCSY transfer is not efficient. Alternatively, long-range optimized correlation experiments (HMBC [9] and HSQMBC [10]) are highly suitable when quaternary carbons are involved. In these later experiments, the value of $^nJ_{CH}$ is usually extracted from an individualized and time-consuming post-processing fitting procedure of the resulting antiphase coupling pattern multiplets. Unfortunately, undesired mixed-phase multiplet distortions originated by the additional J_{HH}-coupling evolution during the long evolution INEPT-type periods introduce a common source of inaccuracy. The incorporation of G-BIRD and/or CPMG blocks into the INEPT periods has been proposed to minimize such modulations, but experimentally it is difficult to achieve this goal for all cross peaks into the same spectrum. In addition, the optimal setting of the inter-pulse delays [11] and pulse power levels [12] involved into the CPMG block is critical to minimize sample heating or heteroTOCSY/HEHAHA transfer whereas off-resonance effects and perfect suppression of J_{HH} modulation still remain a real challenge. Other related approaches have been also used, such as *J*-HMBC [13] or EXSIDE [14] experiments, which are based in the resolution and measurement of the $^nJ_{CH}$ value in the indirect carbon dimension.

In this work, a proton-selective version of the 2D IPAP-HSQMBC experiment [15] is proposed for the straightforward, direct and accurate measurement of $^nJ_{CH}$ in natural-abundance small molecules. The extraction of the $^nJ_{CH}$ value is realized without the need of the classical fitting procedure, simply by comparing the relative displacement of separate α- and β-cross-peaks that result of the time-domain addition/subtraction procedure of complementary In-Phase (IP) and Anti-phase (AP) HSQMBC data. In order to validate the accuracy of the measurement, the obtained $^nJ_{CH}$ value

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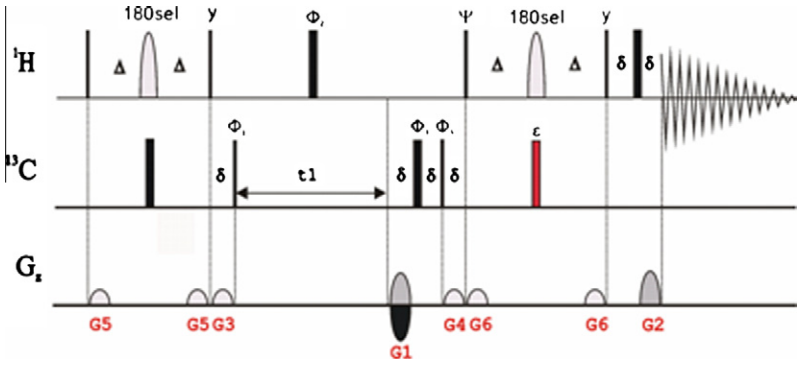

Fig. 1. Pulse scheme of the 2D proton-selective α/β-HSQMBC experiment. Proton 180° pulses applied in the middle of the evolution Δ (=1/(4*$^{n}J_{XH}$)) period can be frequency-selective, region-selection or a multiple-frequency. Two independent IP ($\Psi = y$, ε = on) and AP ($\Psi = x$, ε = off) data are initially collected and further combined to provide complementary α and β data (IP ± AP) in separate spectra.

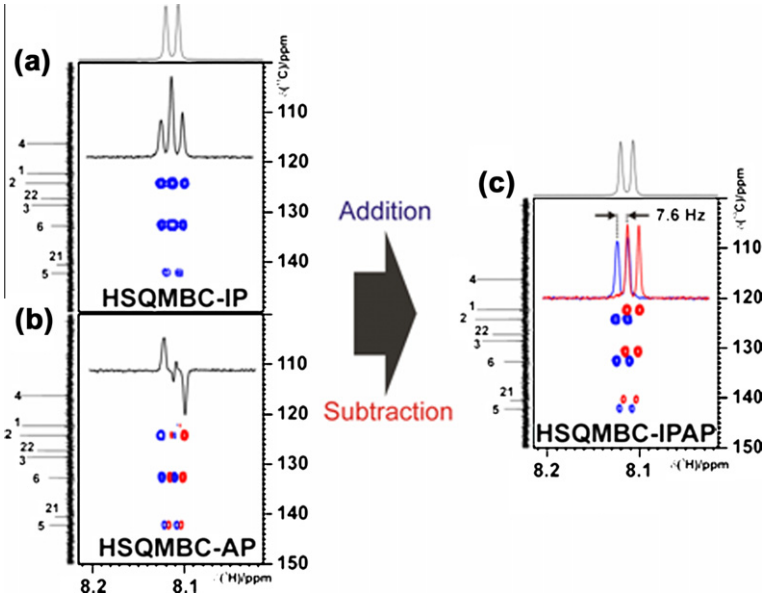

Fig. 2. Basic protocol to achieve α/β-HSQMBC spectra: 2D expanded areas corresponding to the H4 proton of strychnine in (a) HSQMBC-IP; (b) HSQMBC-AP and (c) spin-state-selective α/β-HSQMBC spectra are shown. Well separated α (blue) and β (red) cross-peaks permit an easy, direct and accurate measurement of $^{n}J_{CH}$ by simple measurement of their relative displacements in the acquisition dimension.

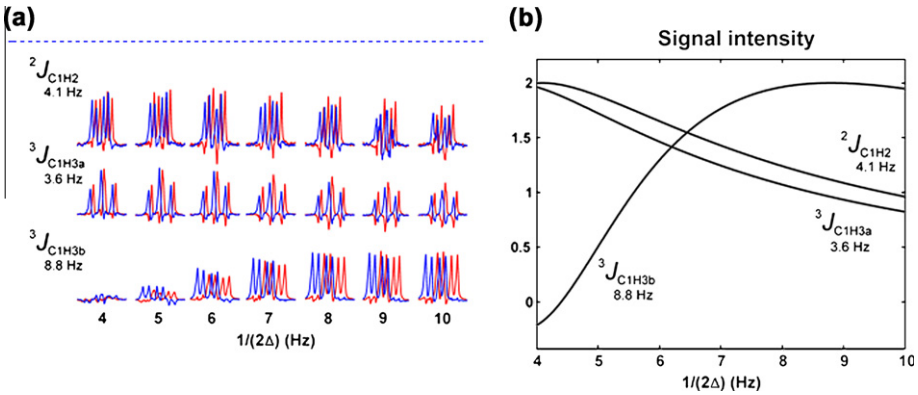

Fig. 3. (a) Experimental and (b) theoretical signal intensity dependences for several α/β-HSQMBC cross-peaks, involving the quaternary C1 carbon of DBPA, as a function of the optimized Δ period. Note clean IPAP editing for all range values that allow a general accurate measurement of $^{n}J_{CH}$ irrespective of Δ optimization.

has been compared with those obtained from the acquired IP and AP multiplet patterns. A detailed discussion about the unwanted effects of cross-talk is also provided on a range of experimental conditions.

2. Results and discussion

The most novel aspect of the new proposed α/β-HSQMBC (Fig. 1) lies in the application of frequency-selective 180° proton pulses into the classical INEPT blocks in order to achieve selective heteronuclear polarization transfer and to efficiently prevent the typical and undesired J_{HH} coupling evolution [14,16–18]. Our proposal could be applied in the original HSQMBC experiment where the resulting pure absorption cross-peaks would present pure AP pattern with respect to the active $^nJ_{CH}$ coupling and pure IP pattern for all passive J_{HH} couplings. However, all typical problems associated to accidental line cancelation or complex analysis of AP multiplets could remain and additional fitting procedures would still be required. To avoid this, the use of the IPAP approach is recommended. Two independent but complementary pure-phased IP and AP HSQMBC data are separately collected using the pulse sequence of Fig. 1. The IP data, generated using $\Psi = y$ (Fig. 2B), present a $\sin^2(\pi^nJ_{CH}\Delta)$ intensity dependence whereas the AP data, obtained using $\Psi = x$ and omitting the last 180° ^{13}C inversion pulse to avoid J_{XH} refocusing (Fig. 2A), present a simple $\sin(\pi^nJ_{CH}\Delta)$ intensity dependence. Then, these time-domain data are added/subtracted to afford pure-phase α/β-HSQMBC spectra (Fig. 2C). Although IP and/or AP data could be individually used for measurement of $^nJ_{CH}$, this may require a fitting procedure using reference multiplets. We show here how the $^nJ_{CH}$ value can be extracted by direct analysis of the relative frequency displacement between these α/β cross peaks with high accuracy.

The complete absence of J_{HH} evolution in the proposed HSQMBC experiment permits a much better control on the IPAP cross-peak intensity behavior due to a pure $\sin^2(\pi^nJ_{CH}\Delta) + \sin(\pi^nJ_{CH}\Delta)$ dependence. Fig. 3A shows that $^nJ_{CH}$ can be easily measured even for a non-perfect matching between the Δ optimization and the active $^nJ_{CH}$ value, as theoretically predicted for DBPA (compare with Fig. 3B). In general, the proposed IPAP approach works for a wide

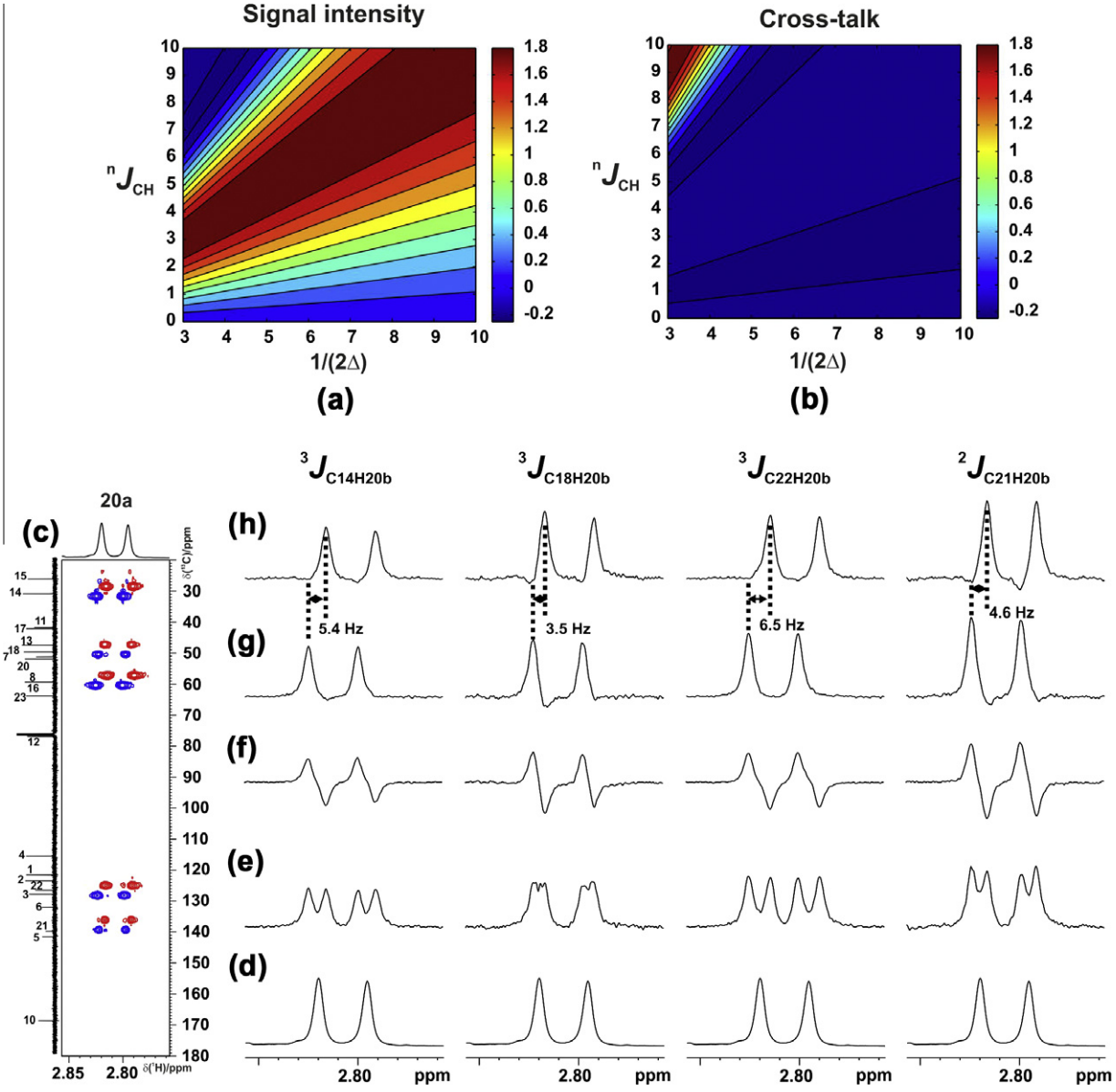

Fig. 4. (a and b) Theoretical signal intensity dependence and cross-talk effect, respectively, for a given IPAP multiplet as a function of the interpulse Δ delay optimization and $^nJ_{CH}$ value. (c) 2D HSQMBC-IPAP spectrum (optimized to 8 Hz) after selective inversion of H20a proton of strychnine. (d–h) 1D multiplets of some cross-peaks involving the H20a proton corresponding to the conventional proton spectrum (d), IP-HSQMBC (e) AP-HSQMBC (f), α-multiplet (g) and β-multiplet (h). Measurement of $^nJ_{CH}$ values can be performed by analysis of IP, AP and/or IPAP multiplet patterns.

range of $^nJ_{CH}$ values and Δ optimization and coupling values ranging from 3 to 9 Hz can be easily measured in experiments optimized to 6–8 Hz. A crucial point is how complementary are IP and AP data. The percentage of undesired cross-talk generated in such addition/subtraction (IP ± AP) procedures should be proportional to a $\sin^2(\pi^nJ_{CH}\Delta) - \sin(\pi^nJ_{CH}\Delta)$ factor and the percentage of cross-talk with respect to the overall multiplet sensitivity will be determined by a $[\sin(\pi^nJ_{CH}\Delta) - 1]/[\sin(\pi^nJ_{CH}\Delta) + 1]$ factor. In order to avoid inaccuracy in the measurement due to strong unbalanced IPAP cross-peaks, an individualized scaling factor (IP ± k ∗ AP) could correct such uncertainties. On the other hand, cross-peak intensities can found a critical minimum in experiments optimized close to $\Delta = {}^nJ_{CH}/2$ as observed for the low sensitivity of the C1-H3b cross-peak in DBPA ($^3J_{C1H3b}$ = 8.8 Hz) in experiments optimized to 4–5 Hz (Fig. 3). In these cases, repeating the experiments with another Δ optimization could resolve such inconvenience.

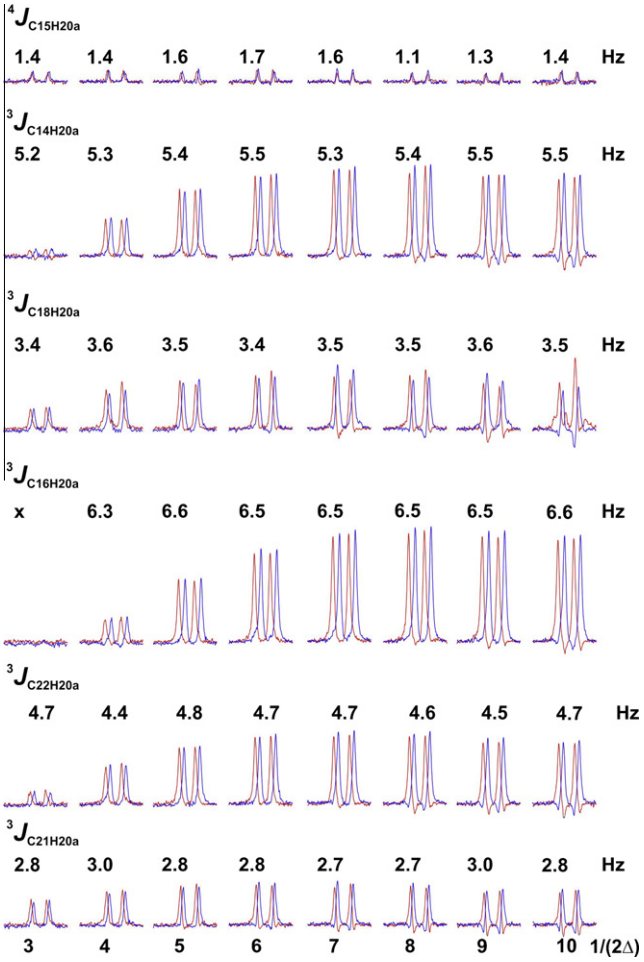

Fig. 5. Experimental 1D α/β multiplets of some cross peaks involving the H20a proton as a function of interpulse Δ delay.

We have chosen the resulting two-dimensional IPAP-HSQMBC spectrum optimized to 8 Hz for the H20a proton of strychnine as a practical example to illustrate such theoretical effects (Fig. 4). Up to five cross-peaks are clearly observed with $^nJ_{CH}$ values ranging from 3 to 7 Hz whereas cross-talk is kept at a minimum value for all these observed cross-peaks, as predicted theoretically. Note that although $^nJ_{CH}$ could be determined form high-resolved IP and AP multiplets, it is more simple and independent to multiplet complexity to measure them from relative signal displacement in IPAP multiplets.

The dependence of signal intensity, the presence of unwanted cross-talk and the accuracy of $^nJ_{CH}$ measurement as a function of experimental interpulse Δ delay optimization have been also evaluated. Fig. 5 shows some α/β multiplets involving the H20a proton for experiments recorded between 3 and 10 Hz. In all cases the measurement is highly reliable, providing deviations smaller than 0.3 Hz. The simplicity of the experiment and the lack of any J_{HH} dependence afford a predictable behavior of the cross-peak intensity dependence irrespective of multiplet pattern. Thus, a general delay optimization between 6 and 8 Hz affords good compromise to obtain good sensitivity ratios and keep possible relaxation losses at minimum. It is also observed that the unwanted effects of cross-talk distortions are not critical. Only in the case of a great deviation between the experimental Δ optimization and $^nJ_{CH}$ values can produce some considerable cross talk effects that must be taken in account. Fig. 6 shows the effect to apply a scaling k factor where cross-talking produce distorted cross-peaks. It is observed in these cases that the presence of cross-talk does not strongly affect the measurement. In general, for an experiment optimized to 7 Hz, cross-talk is keep below 20% for all couplings ranging from 3 to 10 Hz.

In order to validate the measurement performed with the proposed IPAP approach, our proposed method allows the measurement of the $^nJ_{CH}$ value by analysis of the acquired IP and AP data.

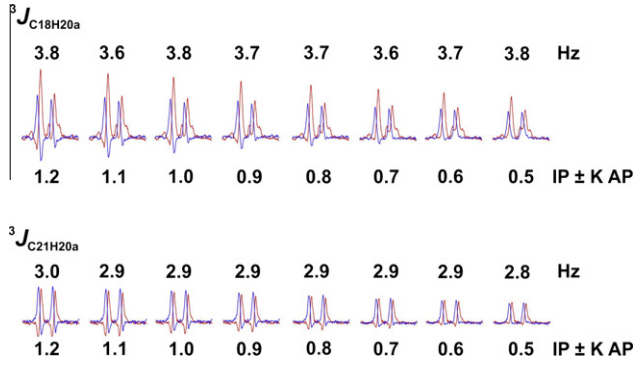

Fig. 6. Effect of cross-talk and scaling correction factor in the $^nJ_{CH}$ measurement of some IPAP multiplets.

Table 1
Comparison of proton–carbon coupling constants values measured by different 2D NMR methods involving the H-20a proton fo strychnine.

NMR method	C15	C14	C18	C16	C22	C21
Selective HSQMBC-IPAP[a]	1.1	5.4	3.5	6.5	4.6	2.7
Selective HSQMBC-IP[b]	<2	5.3	2.8	6.7	4.5	2.4
Selective HSQMBC-AP[b]	2.6	5.5	3.5	6.5	4.7	3.2
HSQC-TOCSY-IPAP[c]	–	5.4	–	6.4	5.0	–
HSQMBC using CPMG[d]	–	5.7	3.6	6.5	4.7	2.9
HSQMBC-IPAP[e]	–	5.7	3.2	6.8	4.7	2.9
Selective J-Resolved[f]	<2	5.3	2.9	6.3	4.4	1.8
Phase sensitive HMBC[g]		5.4	3.5	6.9	4.5	2.4

[a] Optimized to 8 Hz. Directly measured from the relative displacement of signals resulting of the IP/AP time-domain addition/subtraction procedure.
[b] Measured after applying a fitting procedure on the IP or AP data acquired with the pulse sequence of Fig. 1.
[c] Measured in this work using experiment from Ref. [7] with a mixing time of 60 ms.
[d] Measured in this work using experiment from Ref. [10] after applying a fitting procedure.
[e] Measured in this work using experiment from Ref. [15].
[f] Measured in this work using the J-Resolved selective INEPT experiment from Ref. [19].
[g] Taken from Ref. [9].

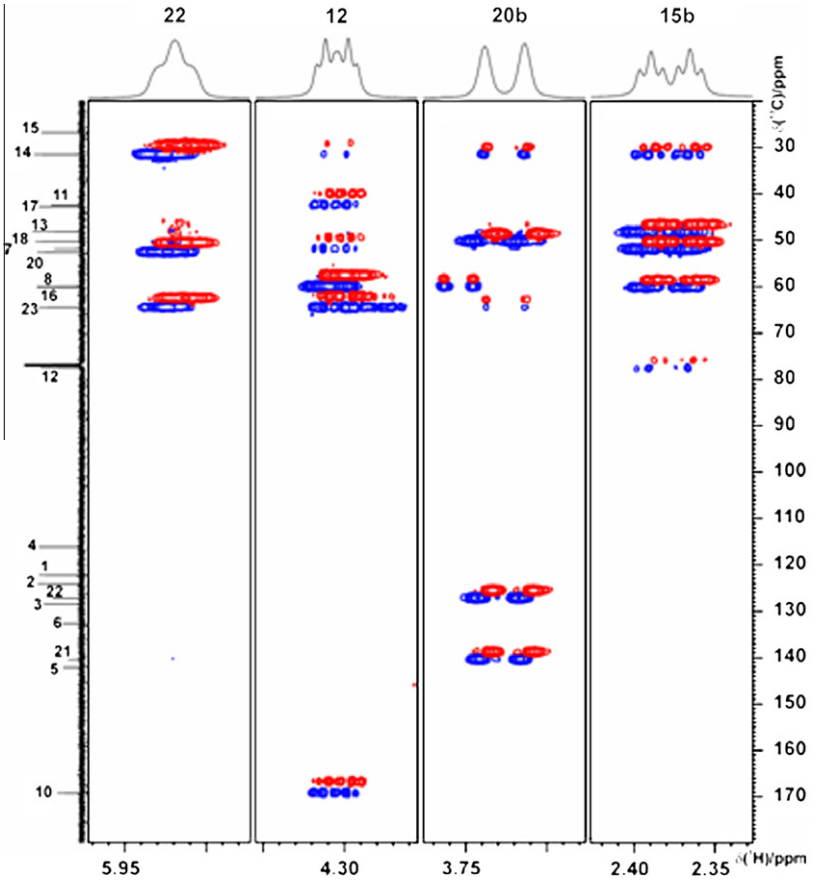

Fig. 7. Expanded regions of the 2D α/β-HSQMBC spectra after simultaneous selective inversion of H22, H12, H20b and H15b protons in strychnine.

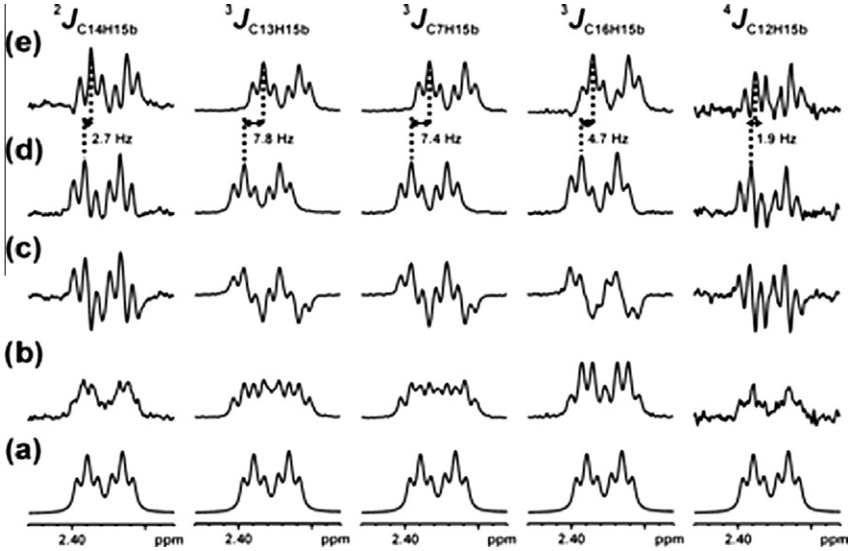

Fig. 8. Experimental 1D slices (a) reference ^{1}H multiplet; (b) IP; (c) AP; (d) α = IP + AP and (e) β = IP–AP) showing the perfect phase properties and the absence of considerable cross-talk effects for the same H15b proton in different cross peaks showing different $^nJ_{CH}$ values. No scaling factor has been applied in any case.

Table 1 summarizes the values obtained for the couplings involving the H20a proton. In addition, we have performed the same measurement by other published methods and all results are in strong agreement among them within the experimental error.

The method allows a set of variants as a function of proton selectivity. For instance, the use of multiple-frequency excitation of protons not mutually coupled can also be used. Fig. 7 shows the respective columns after selective excitation at four different proton sites in strychnine. Note that excellent measurements can be made for couplings ranging from 2 to 8 Hz in this experiment when optimized to 8 Hz (Fig. 8).

Region-selective proton pulses can be applied in areas that contain protons not mutually coupled. This can be interesting, for instance, in the case of NH and H_α regions in peptides and

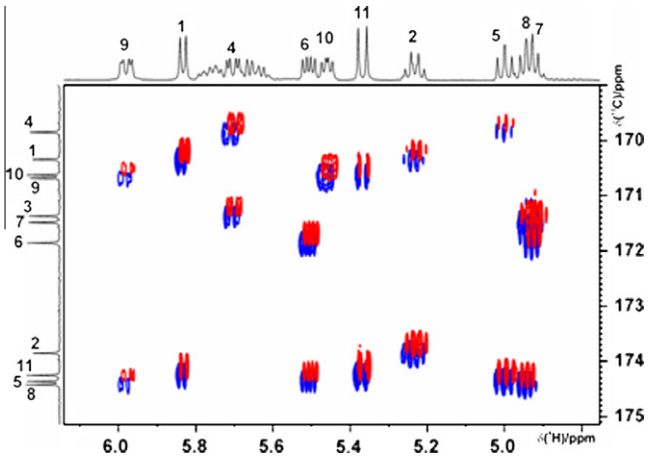

Fig. 9. 2D ^{13}CO-band-selective $\alpha\beta$-HSQMBC spectra of cyclosporine resulting of apply a region-selective 180° 7.5 ms REBURP pulse on the H_α region.

proteins. Fig. 9 shows that both intra-residue $^2J_{H\alpha-CO}$ and inter-residue $^3J_{H\alpha-CO}$ coupling constants can be measured with high precision in a doubly-selective H_α and CO α/β-HSQMBC experiment. An equivalent experiment exciting only the NH region would permit to simultaneous measure the intra-residue $^2J_{NH-C\alpha}$, $^3J_{NH-C\beta}$ and the inter-residue $^3J_{NH-C\alpha}$ and $^2J_{NH-CO}$ coupling values (see Supporting Information for more experimental details).

3. Methods and materials

Samples of 430 mM 2,3-dibromopropionic acid in $CDCl_3$ (**1**), 75 mM Strychnine in 0.6 ml of $CDCl_3$ (**2**) and 25 mM cyclosporine in C_6D_6 (**3**), were chosen as model samples. NMR experiments have been recorded on a BRUKER 600 Avance II + with a 5-mm broadband TXI inverse probehead incorporating a *z*-gradient coil and on a BRUKER DRX-500 spectrometer equiped with a 3-channel 5-mm cryoprobe incorporating a *z*-gradient coil. All data were acquired and processed with TOPSPIN v2.1. All ^{1}H–^{13}C IP and AP-HSQMBC experiments were separately recorded at 600 MHz for **1** and **2**, and at 500 MHz for **3**. The recycle delay was set to 1 s and the interpulse Δ delay (=1/4$^*{}^nJ_{CH}$) was set to 8 Hz. IP and AP data were added/subtracted in the time-domain without any scaling factor to provide spin-state selective data. Prior to Fourier-transformation of each data, zero filling to 1024 in F1, 8192 points in F2 and a sine squared function in both dimensions were applied. See more experimental details in Supporting Information.

4. Conclusions

In summary, a straightforward modification of the HSQMBC experiment has been proposed to measure $^nJ_{CH}$ with high accuracy thanks to the excellent pure phase properties of the resulting cross-peaks. The measurement is made using the IPAP approach where two separate spin-state-selective α/β components are generated from two independently acquired IP and AP HSQMBC spectra. The simplicity and general applicability of the method relies in the easy of the measurement, irrespective of the multiplet complexity and experiment parameters while covering a wide range of $^nJ_{CH}$ values. However, the measurement of very small coupling values (<1.5–2 Hz) can remain a challenge because these cross peaks will usually present very small intensities due to the direct sine intensity dependence with respect to the duration of the interpulse delay.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmr.2011.09.036.

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