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High resolution 2D ^{1}H–^{13}C correlation of cholesterol in model membrane

Olivier Soubias, Valérie Réat, Olivier Saurel, and Alain Milon*

Institut de Pharmacologie et de Biologie Structurale, CNRS et Univ. P. Sabatier, 205 rte de Narbonne, Toulouse, France

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Abstract

High resolution 2D NMR MAS spectra of liposomes, in particular ^{1}H–^{13}C chemical shifts correlations have been obtained on fluid lipid bilayers made of pure phospholipids for several years. We have investigated herein the possibility to obtain high resolution 2D MAS spectra of cholesterol embedded in membranes, i.e. on a rigid molecule whose dynamics is characterized mainly by axial diffusion without internal segmental mobility. The efficiency of various pulse sequences for heteronuclear HETCOR has been compared in terms of resolution, sensitivity and selectivity, using either cross polarization or INEPT for coherence transfer, and with or without MREV-8 homonuclear decoupling during t1. At moderately high spinning speed (9 kHz), a similar resolution is obtained in all cases (0.2 ppm for $^{1}H_{3,4}$, 0.15 ppm for $^{13}C_{3,4}$ cholesterol resonances), while sensitivity increases in the order: INEPT $<$ CP($\times$4) $<$ CP + MREV. At reduced spinning speed (5 kHz), the homonuclear dipolar coupling between the two geminal protons attached to C_4 gives rise to spinning sidebands from which one can estimate a H–H dipolar coupling of 10 kHz which is in good agreement with the known dynamics of cholesterol in membranes.
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Keywords: HETCOR; Dipolar couplings; Order parameters; Lipid bilayer; Homonuclear decoupling; INEPT

1. Introduction

High resolution in both carbon and proton dimension is a major goal in solid state NMR. Over the last 20 years, pure lipids in liquid crystalline phase have been extensively studied by static [1,2] and magic angle spinning (MAS) solid state NMR [3,4]. In the fluid phase (i.e. above the gel to liquid crystalline phase transition temperature), large amplitude anisotropic molecular motions are fast relative to dipolar interaction time scales. Therefore, these interactions are significantly averaged relative to their rigid lattice values. Several experiments, using through space cross polarization (CP) and high power ^{1}H decoupling have been realized on such sample. It has been possible to obtain high resolution in the ^{13}C and ^{1}H dimensions when spinning at magic angle [5–8] and to perform ^{1}H–^{13}C 2D chemical shift correlation experiments which increase drastically the resolution and chemical information [9,10]. Any 2D technique implies a coherence transfer between pairs of nuclei which has often been based upon cross polarization. Indeed, many of the liquid state techniques based upon J couplings are difficult to implement in solids, because coherences decay faster than the delay required for efficient transfer. However, in purely lipidic bilayers in the fluid phase, various rapid motions occur (axial diffusion, wobbling, internal segmental motions), which increases ^{1}H and ^{13}C coherence T_2s, and it has been shown that common liquid state NMR techniques such as selective ^{1}J scalar coupling INEPT are efficient [11–13]. For pure solids, ^{1}H homonuclear decoupling schemes are required to obtain sufficient resolution in the proton dimension in a 2D ^{1}H–^{13}C correlation [14,15] and allow selective coherence transfer based on J-couplings [16,17].

Biological membranes are complex assemblies in which constituents such as cholesterol and proteins experience slower and reduced dynamics as compared with phospholipids. Cholesterol dynamics is well described by a fast axial diffusion (which provides axial symmetry to all anisotropic interactions), combined with a small amplitude

* Corresponding author. Fax: +33-5-61-17-54-24.
E-mail address: alain.milon@ipbs.fr (A. Milon).

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wobbling (the molecular order parameter of the diffusion axis is equal to 0.94 for DMPC/cholesterol in a 7/3 molar ratio at 310 K) [18–20]. But, contrary to DMPC protons, no internal motion within the four rings of cholesterol contributes further to the averaging of anisotropic interactions. Our purpose in the present work was therefore to compare various pulse sequences to obtain well resolved ^{1}H–^{13}C 2D correlation on the rigid part of cholesterol molecule, when it is inserted in a DMPC/cholesterol 7/3 lipid mixture, i.e. at a cholesterol molar ratio which is typically found in mammalian plasma membranes. In particular, we have compared dipolar (CP) and scalar coupling based (INEPT) transfers of coherences, and we have assessed the usefulness of homonuclear decoupling in the proton dimension. It is shown that 2D experiments with high resolution in both dimensions (0.1–0.2 ppm) can be obtained at moderately high spinning speed (9 kHz), and that, at a lower spinning (5 kHz), H–H dipolar couplings of methylenes can be determined.

2. Results and discussion

The pulse sequences used are shown in Fig. 1. During the evolution period, magnetization evolves under the ^{1}H chemical shift and the residual proton dipolar couplings. MREV-8 homonuclear dipolar decoupling sequence was optionally applied during the t1 dimension. ^{1}H to ^{13}C coherence transfer was then mediated either via ^{1}J isotropic scalar couplings (INEPT, Fig. 1A) or via dipolar couplings (CP, Fig. 1B). Carbon chemical shift is then measured during t2 under TPPM proton decoupling [21].

Fig. 2 shows that chemical shifts of protons directly bound to several sites of cholesterol can be determined with a good resolution and accuracy, as in solution NMR. This was achieved at a moderately high spinning speed (9 kHz) and without homonuclear ^{1}H decoupling during the evolution period. Interestingly, the linewidth obtained in the ^{1}H dimension was about 0.2 ppm for

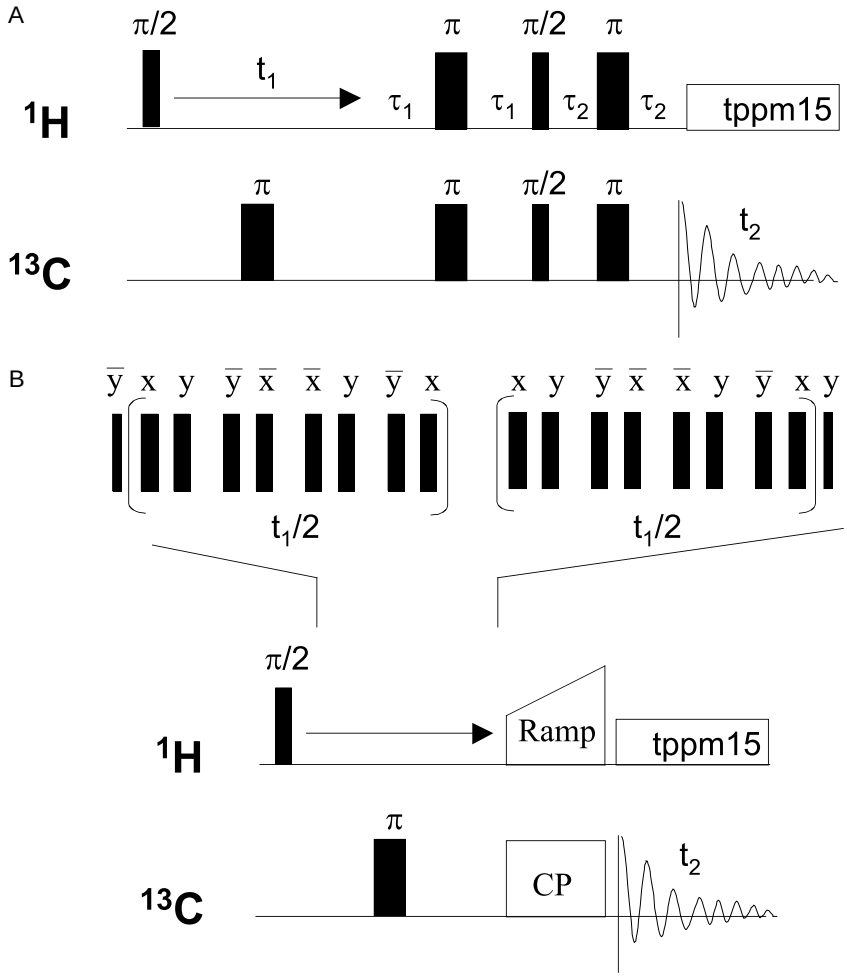

Fig. 1. (A) Pulse sequence for solid state HETCOR. A refocused INEPT is used for polarization transfer from ^{1}H to ^{13}C. Delays τ_1 and τ_2 are integer multiples of the rotor period and optimized empirically for the best transfer efficiency around $1/4J$ and $1/6J$, respectively ($J = 125$ Hz). (B) Pulse sequence for dipolar HETCOR (D-HETCOR). Magnetization is transferred from proton to carbon via cross polarization. The MREV-8 multiple pulse sequence was optionally inserted during the ^{1}H chemical shift evolution period in the dipolar HETCOR. Pulse sequence adapted from [15].

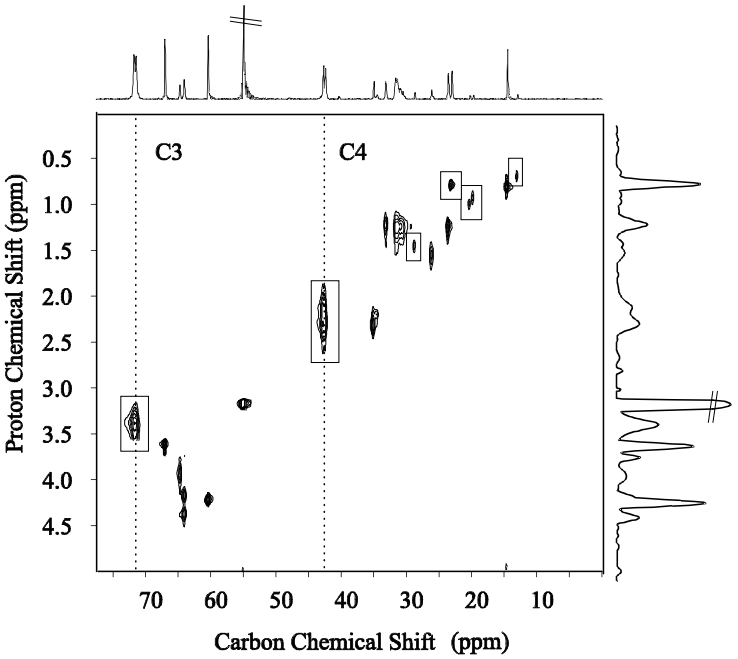

Fig. 2. 2D modified HETCOR spectrum on a sample of multilamellar vesicles of DMPC/cholesterol-$^{13}C_4$, $^{13}C_3$ (30 mol%, 3.5 mg), temperature 310 K, with ^1H and ^{13}C projections. The spectrum was obtained with the pulse sequence of Fig. 1A, on a Bruker DMX 500 MHz narrow bore spectrometer using a DOTY scientific XC5 5 mm doubly tuned probe. MAS spinning speed was adjusted to 9 kHz ± 1 Hz and the ^1H decoupling field was 66 kHz with a TPPM15 scheme [21]. Proton spectral width was 10 kHz. A total of 256 t1 increments with 64 scans each were collected. Cross peaks corresponding to cholesterol resonances are surrounded by square boxes. Other cross peaks correspond to lipid resonances. Proton and carbon chemical shifts were referenced relative to the internal choline resonances taken at 55 ppm (^{13}C) and 3.18 ppm (^1H).

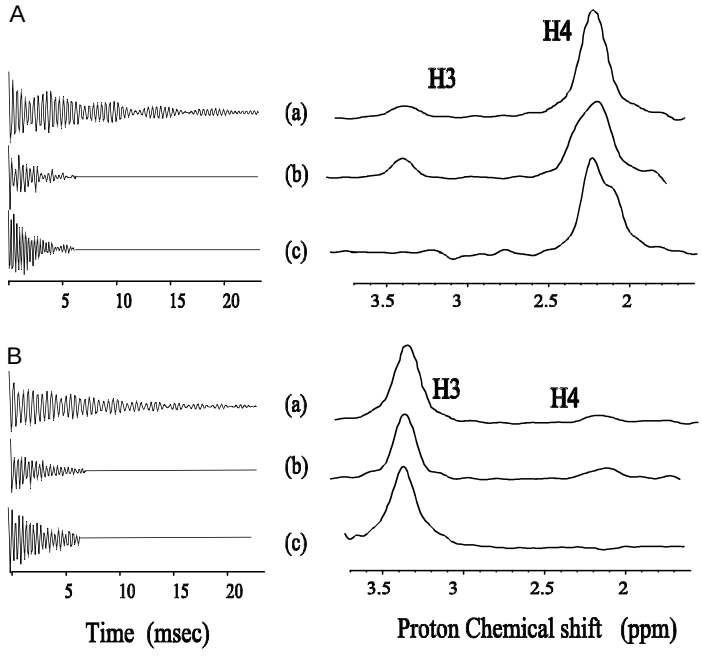

Fig. 3. Evolution along the t1 dimension (left) and column extracted from the 2D experiment along the proton dimension for the two labeled carbon resonances, $(CH_2)_4$ (A) and $(CH)_3$ (B). Three different pulse sequences are compared: (a) D-HETCOR, based on CP transfer, with MREV-8 decoupling during t1 (sequence of Fig. 1B, 32 scans, 256 t1 increments of 86.9 μs). (b) D-HETCOR, based on CP transfer, without MREV-8 decoupling, 32 scans, 128 t1 increments of 50 μs. (c) Liquid state HETCOR, based on refocused INEPT transfer (sequence of Fig. 1A, 256 scans, 128 t1 increments of 50 μs.) The real experimental time evolution is shown for the various FIDs. The proton chemical shift scales in both (a) spectra were corrected with a 0.54 ± 0.02 experimental MREV-8 scaling factor (selected to obtain the same chemical shift difference between H_3 and H_4 protons as in the HETCOR experiment without homonuclear decoupling, and fairly close to the theoretical scaling factor 0.536 expected for a semi-windowless MREV-8). CP contact time was 2 ms. The proton radiofrequency (rf) field was set to 66 kHz during both the evolution period (MREV-8 decoupling) and the acquisition period (TPPM decoupling). All other experimental parameters are identical to those of Fig. 2, in particular the spinning rate was set to 9 kHz.

cholesterol resonances and slightly less (0.1–0.2 ppm) for the more mobile lipid resonances. $(CH_2)_4$ (^{13}C: 42.5 ppm, ^{1}H: 2.22 ppm) and $(CH)_3$ (^{13}C: 71.8 ppm, ^{1}H: 3.39 ppm) correlations were clearly resolved and correspond to solution state assignments (see Fig. 4 for atom numbering in cholesterol). It should be noticed that beyond the fully $^{13}C_{3,4}$ labeled carbons, several natural abundance cross peaks from cholesterol were observed (surrounded by square boxes). All the other peaks have been identified as DMPC resonances by comparison with the pure DMPC spectrum [6].

Fig. 3 illustrates the comparison between the various pulse sequences employed: two resonances have been analyzed, corresponding either to a CH group (position 3, Fig. 3B) or to a CH_2 group (position 4, Fig. 3A) for which H–H dipolar couplings are stronger. As expected, coherence transfer was selective for the *J*-based INEPT pulse sequence, and only cross peaks corresponding to the directly attached protons were observed (Figs. 3A-c and B-c). In the case of $(CH_2)_4$ group the resolution was sufficient to reveal the non equivalence of axial and equatorial H4 protons (using linear prediction method for processing, spectrum not shown). For the CP based experiment, cross peaks arising from remote protons were also observed such as between C_4 and H_3 (Fig. 3A-a and b) and between C_3 and H_4 (Fig. 3B-a and b). At this spinning speed they were of relatively weak intensity as compared with the experiments performed at 5 kHz (see below). The introduction of an MREV-8 homonuclear decoupling scheme during t1 drastically increased the length of the FIDs, specially for the CH_2 protons (compare for instance Fig. 3A-a and b). However, after Fourier transform, and rescaling of the chemical shifts to take into account the MREV-8 scaling factor of the chemical shift interaction (equal to 0.54 in this case), it appeared that the gain in linewidth was only marginal for molecules having the dynamics of cholesterol in a lipid bilayer (undetectable for the H3 proton; 30% gain for the two H4 protons). It did nevertheless improve the peak to peak signal to noise ratio, by a factor of

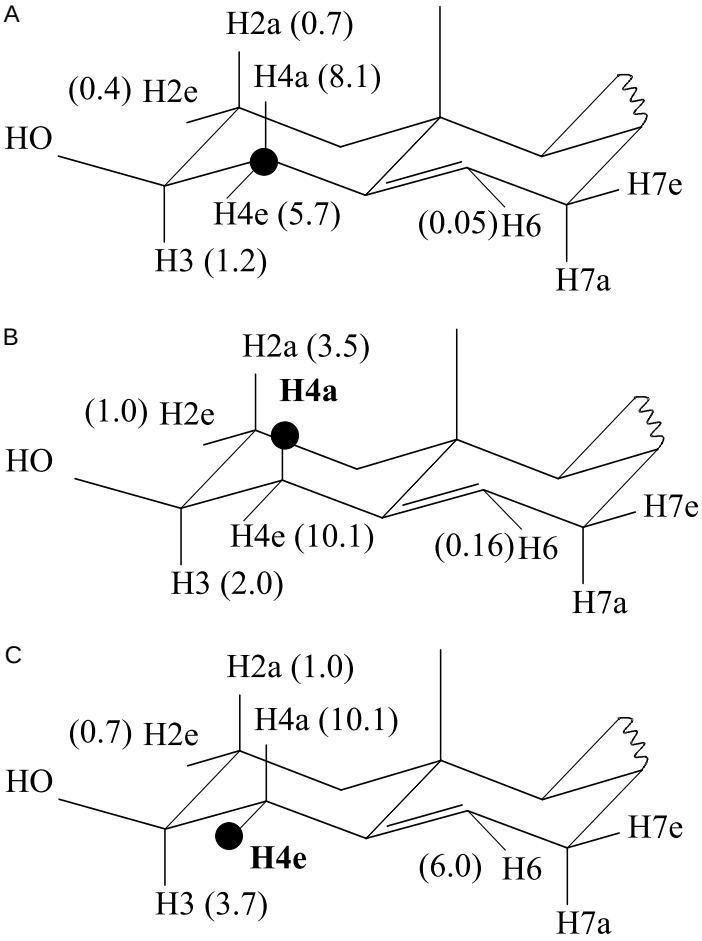

Fig. 4. Calculated values of dipolar couplings within cholesterol in fluid bilayers (these couplings, in kHz, are those expected for bilayer normal oriented at 90° with respect to the magnetic field). (A) heteronuclear dipolar coupling between carbon C_4 and the closest protons, (B) homonuclear dipolar couplings between H_{4a} and the closest protons, (C) homonuclear dipolar couplings between H_{4e} and the closest protons. These couplings were calculated from the average orientation and dynamics of cholesterol in DMPC/cholesterol 7:3 bilayers at 310 K, which have been previously determined accurately using deuterium NMR [20].

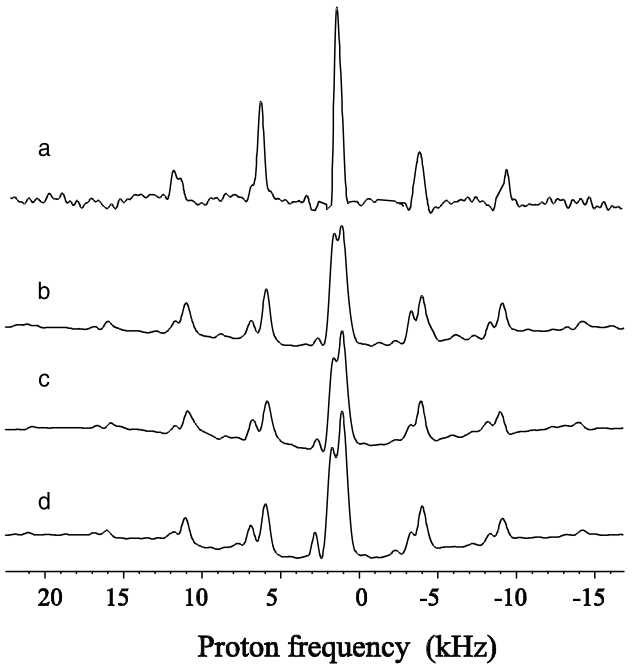

Fig. 5. Stack of F1 columns corresponding to the isotropic chemical shift of the C_4 at a spinning speed of 5 kHz. Proton spectral width was 25 kHz for all the experiments. A total of 128 t1 increments with 32 scans each were collected and all the other parameters were the same as in Fig. 2. (a) 2D modified HETCOR (b–d) 2D dipolar HETCOR without MREV-8 with CP contact times of 200 (b), 500, (c) and 2000 μs (d). These spectra were acquired with liposomes made of DMPC/cholesterol-$^{13}C_4$, in a 7/3 molar ratio at 310 K.

approximately 1.5 when one compares spectra a and b. Another reduction of signal by a factor 4 was observed for spectra A-c and B-c (note that for the experiment of Figs. 3A and B-c 256 scans per t1 increments were acquired) to the less efficient coherence transfer via the INEPT sequence, as shown in Figs. 3A and B-c.

Since most of the H–H dipolar couplings in cholesterol are smaller than 10 kHz (Fig. 4), they are effectively averaged out by MAS at 9 kHz spinning speed and virtually no rotational sideband were observed along the proton dimension. This was not the case at 5 kHz spinning speed, and these sideband patterns could be observed with a larger spectral width in the proton dimension (25 kHz instead of 10 kHz). In Fig. 5, the columns extracted at the C_4 carbon chemical shift are shown for the modified HETCOR (a) and for the dipolar HETCOR without homonuclear decoupling, at several mixing times (b–d). The spinning sideband pattern is clearly resolved in all cases. In the CP based experiments transfer between not directly bound protons and carbons were more efficient at 5 kHz spinning speed: the centerband in spectrum (d) contains three resonances assigned to the directly bound H_4 protons (at 1.1 kHz), the H_3 resonance (at 1.7 kHz) and the H_6 resonance (small peak at 2.8 kHz). These long range H–C transfers may be due to the corresponding heteronuclear couplings, or rather to a combination of homonuclear and heteronuclear transfers: thus, H_6–C_4 coherence transfer would rather go through H_6–H_{4e} homonuclear coupling (6.0 kHz) and H_{4e}–C_4 heteronuclear coupling (5.7 kHz) than through the H_6–C_4 heteronuclear coupling which is less than 0.1 kHz (Fig. 4). Long range transfer may be minimized using shorter mixing times (as seen in Fig. 5b), or using LG-CP experiments in order to prevent proton spin diffusion [22,23], but the most selective experiment is obviously the coherence transfer based on *J*-couplings shown in Fig. 5a. There, only cross peak with directly attached protons were observed, and the spinning sideband pattern of the C_4 methylene could be analyzed unambiguously. Since it was predicted, from our previous work on cholesterol dynamics in this model system [20], that the intra-methylene H_{4a}–H_{4e} homonuclear dipolar coupling was the strongest coupling these two protons were experiencing (Figs. 4B and C), we tested the analysis of the sideband pattern observed in Fig. 5a with the isolated spin pair approximation. We found a dipolar coupling of 10.3 ± 1 kHz, in good agreement with the expected value.

3. Conclusion

We have shown in the present work that high resolution 2D HETCOR experiments of cholesterol, with a good resolution in both dimension (around 0.2 ppm), may be obtained by MAS experiments on liposomes. The cholesterol dynamics, characterized by a fast axial diffusion but with only a small wobbling amplitude and no internal segmental motion is suitable for this experiment. Contrary to some peptides such as gramicidin [8,24], no low frequency motion deteriorates the resolution obtainable at moderate spinning speed. Again due to the specific dynamics of cholesterol on model membranes, it was shown that homonuclear decoupling during the proton evolution is not an absolute requirement and that when a MREV-8 sequence is employed it improves the effective resolution and sensitivity only marginally. Both cross polarization and INEPT can be used for coherence transfer, the former making the experiment more sensitive by a factor 4, the latter allowing for a fully selective transfer between directly bound atoms. Since the four rings of cholesterol form a rigid part of the molecule and thus present the same dynamical properties [20], these conclusions, based mainly on the analyses of correlation peaks in position 3 (CH) and 4 (CH_2), may safely extended to other positions in the rings and indeed various correlations on natural abundance cholesterol carbons have been observed in Fig. 2. Finally, since methylenes possess internal H–H dipolar couplings around 10 kHz, which are significantly higher than any other coupling within the cholesterol molecule, MAS experiments at 5 kHz spinning speed can be used

to measure and analyze the spinning sidebands in order to yield the strength of the underlying dipolar pair coupling. This approach provides a way to determine order parameters for each methylene in the molecule using the resolution brought by the carbon dimension.

Acknowledgments

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