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Communication

Detection of natural abundance ^{1}H–^{13}C correlations of cholesterol in its membrane environment using a gradient enhanced HSQC experiment under high resolution magic angle spinning

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Abstract

The quality and signal to noise ratio of a *J*-based HETCOR performed on a standard MAS probe have been compared with a gradient enhanced HSQC performed on a HR-MAS probe at 500 MHz. The sample selected was cholesterol, inserted at 30 mol% in acyl chain deuterated phospholipids (DMPC-d_{54}), at a temperature where the bilayer is in a liquid crystalline phase (310 K). It is representative of any rigid molecule undergoing fast axial diffusion in a bilayer as the main movement. After optimization of the spinning rate and carbon decoupling conditions, it is shown that the ge-HSQC/MAS approach is far superior to the more conventional *J*-HETCOR/MAS in terms of signal to noise ratio, and that it allows the detection of all the natural abundance cross peaks of cholesterol in a membrane environment. Clear differences between the ^{1}H and ^{13}C chemical shifts of cholesterol in a membrane and in chloroform solution were thus revealed.

Keywords: Solid state NMR; Lipids; Natural abundance; HETCOR; MAS

1. Introduction

Magic Angle Spinning experiments in solid state NMR of organic or biomolecular systems are conventionally carried out with low γ nuclei detection [1,2]. The large spectral range and the weak homonuclear dipolar couplings among them result in an inhomogeneous dipolar Hamiltonian that yields narrow lines under MAS conditions [3]. In contrast, indirect detection which enhances the signal intensity by detecting the high γ nucleus ^{1}H, while recording the heteronucleus in the indirect dimension, is commonly used in all structural studies of biological molecules in solution [4]. Recently, indirect detection was also introduced in solid-state ^{15}N experiments on polycrystalline peptide samples. The proton linewidth must be small enough to achieve effectively gains in sensitivity. Thus, significant sensitivity enhancement has been demonstrated with high speed MAS [5,6], i.e., spinning rate up to 30 kHz, or spin dilution by perdeuteration and back substitution of the exchangeable protons for powdered peptide samples at a lower MAS rate [7].

In contrast, it has been shown for molecules which experience rapid axial diffusion within the lipid bilayers that the dipolar broadening becomes inhomogeneous and that sharp resonances may be obtained if the sample is submitted to MAS at a rate faster than the intermediate time scale motions [8]. In this context, we have recently shown the relative uselessness of additional homonuclear decoupling pulse sequence in the proton dimension, at moderate spinning rate, i.e., 9 kHz, to obtain well resolved ^{1}H–^{13}C 2D chemical shift correlation on the rigid part of cholesterol molecule when it is inserted in a DMPC/cholesterol lipid mixture. Both dipolar couplings and scalar couplings can be used for coherence transfer, the latter making the experiment less sensitive but allowing a fully selective transfer between directly bound atoms [9]. Sensitivity of NMR experiments on membrane components in their lipid

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environment is usually low due to the limited amount of isotopically labeled samples available. So any improvement in sensitivity is important since it will facilitate the spectroscopy and widen its applicability.

Our purpose in the present work was therefore to compare the sensitivity of the liquid-like direct detected scheme (HETCOR) to the indirect detected scheme (ge-HSQC) of a ^{1}H–^{13}C 2D chemical shift correlation on the rigid part of a cholesterol molecule. We have analyzed the influence of the spinning rate and the heteronuclear decoupling strength on the sensitivity of the HSQC experiment. It appears that the HR-MAS probe provides enough resolution and sensitivity for detection of all the proton–carbon correlations of rigid cholesterol at natural abundance in a membrane.

2. Results and discussion

The major achievement of indirect detection schemes is certainly sensitivity enhancement. Apart from the obvious advantage of the high ^{1}H magnetogyric ratio, the resulting experimental sensitivity involves also the probe quality factor at the ^{1}H resonance frequency, the efficiency of the coherence transfer and the experimental ^{1}H linewidth. For real solids, homonuclear decoupling schemes are compulsory and are more efficient when placed in the indirect dimension, therefore proton detection is usually avoided. Only recently, very high spinning rates (30 kHz) have opened the way to proton detection in 2D NMR of real solids [5,6]. In liquid crystalline membrane samples however, we have recently observed that the proton resolution was not strongly influenced by an additional homonuclear decoupling pulse sequence: using an MREV-8 homonuclear decoupling, the length of the proton FID was doubled but rescaling of the proton dimension lead to the same effective resolution. It was therefore tempting to compare the efficiency of proton and carbon detection in the 2D NMR of this sort of samples. We used as previously a DMPC/cholesterol sample and focused our attention on cholesterol resonances. It should be stressed that pure phospholipid sample are highly dynamics, because of axial diffusion, large amplitude wobbling and internal motions and that they therefore lead to high quality 2D NMR experiments [8,10]. Cholesterol is a more difficult case, closer to real solids than phospholipids, since its dynamics is characterized by axial diffusion as a sole movement due to its rigid structure and to a very small amplitude of wobbling [11].

In the present work, we have thus compared a ^{13}C-detected HETCOR experiment performed on a standard MAS probe and a ^{1}H-detected HSQC experiment performed on a HR-MAS probe. A HR-MAS probe is designed to carry out liquid type experiments that do not involve cross polarization but require the highest resolution. Such probes have already shown their potential in the case of compounds attached to solid supports [12,13], combinatorial chemistry [14], cultured cells and tissues [15–17] and lipid bicelles [18].

We first compared experiments performed at 9 kHz. Besides the fully $^{13}C_{3,4}$ labeled carbons, the spectrum obtained with regular ^{13}C-detection displays several natural abundance cross peaks from cholesterol (data not shown). By analogy to liquid state assignments, these correlations could be attributed to the methyl resonances of the rigid rings (C_{18}: ^{13}C, 12.71 ppm, ^{1}H, 0.67 ppm; C_{19}: ^{13}C, 20.0 ppm, ^{1}H, 0.97 ppm) or the side chain (C_{26}, C_{27}: ^{13}C, 22.76 ppm, ^{1}H, 0.71 ppm; C_{21}: ^{13}C, 19.48 ppm, ^{1}H, 0.90 ppm). It should be noted that these natural abundance cross peaks correspond to the more mobile resonances. Interestingly, the ^{1}H-detected HSQC spectrum is not strictly identical to the ^{13}C-detected spectrum and displays also several methine resonance, which could be tentatively assigned to C_{14} or C_{17} (^{13}C, 57.53 ppm; ^{1}H, 1.02 ppm) and C_9 (^{13}C, 50.86 ppm; ^{1}H, 0.88 ppm). The signal-to-noise ratio (estimated from Table 1, taking into account the amount of sterol and the number of scans) in the indirectly detected spectrum is higher than that in the directly detected spectrum by a factor comprised between 2 and 5 depending on the considered carbon. For instance, S/N ratio is improved by a factor of 2 for the resonance corresponding to methylene $(CH_2)_4$, whereas a factor close to 5 is found for the methine $(CH)_3$. The sensitivity enhancement found here is not negligible but is clearly less than the one obtained classically for inverse detection experiment

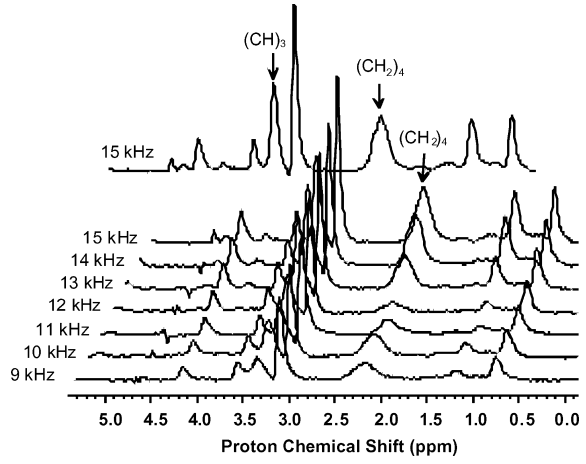

Fig. 1. Dependence on the spinning frequency of the ^{1}H MAS NMR spectrum of a sample of multilamellar vesicles of DMPC-d_{54}/cholesterol-$^{13}C_4$, $^{13}C_3$ (30 mol%, 3 mg), temperature 310 K. Signals were extracted from the first t1 increment of HSQC experiments. Note the oscillation in the H4 linewidth (CH_2 resonance at 2.2 ppm), which displays a maximum around 12 kHz and becomes optimal at 14–15 kHz. This phenomenon is not observed on the H3 (resonance at 3.4 ppm) and is therefore presumably due to an interference between the spinning rate and the CH_2 proton homonuclear dipolar couplings known to be of the same order of magnitude in this sample.

Table 1
Summary of experimental parameters for the main experiments described in this paper: chol., cholesterol; t.d., time domain; d.t., dwell time; Acq. time, acquisition time; n.t., number of transients; TPPM decoupling [21]; e.m., exponential multiplication; s.s.q., 90° shifted sinus square; l.p. 1k, linear prediction to get 1k (i.e., 1024) experimental + calculated points; S/N C4, signal to noise ratio of the C4 resonance

Experiment	HETCOR	HSQC	HSQC
Probes/samples	MAS 5 mm DOTY, 4 mg chol. ^{13}C detection	HR-MAS 4 mm BRUKER, 2.5 mg chol. ^{1}H detection	HR-MAS 4 mm BRUKER, 2 mg chol. ^{1}H detection
Spinning rate	9 kHz	9 kHz	15 kHz
90° ^{1}H	6 μs	8.2 μs	8.2 μs
90° ^{13}C	6 μs	6.4 μs	6.4 μs
decoupling	^{1}H, TPPM 66 kHz	^{13}C, GARP 2.5 kHz	^{13}C, GARP 2.5 kHz
Lock ^{2}H	No	Yes	Yes
F2: t.d./d.t./acq.time	8k/5 μs/40 ms	2k/83 μs/170 ms	2k/83 μs/170 ms
F1: t.d./d.t./acq.time	256/50 s/12.8 ms	256/20 μs/5.1 ms	1024/20 μs/20.5 ms
Data size after zero filling: F2/F1	8k/1k	8k/1k	8k/8k
n.t./exp. time	256/36 h 30 min	16/2 h 15 min	64/36 h 30 min
Data treatment F1	e.m. 5 Hz	l.p. 1k, e.m. 5 Hz	l.p. 2k, e.m. 5 Hz
Data treatment F2	e.m. 5 Hz	s.s.q.	s.s.q.
S/N C4	89	80	700
S/N C3	105	220	850
S/N C19	20	30	160

All the HSQC experiments performed at spinning rates from 9 to 15 kHz used identical experimental parameters. The temperature was 310 K. The relaxation delay was 2 s for all the experiments.

in liquid state NMR. Moreover, at this spinning rate, none of the natural abundance methylene cross peaks could be detected. This could be attributed to the fact that, at this fairly low spinning speed, the residual ^{1}H homonuclear dipolar couplings were still sufficiently strong to severely restrict the efficiency of *J*-coupling transfer of polarization in these strongly coupled spins pairs. Indeed, values of H-H homonuclear dipolar couplings between protons of methylene group calculated from the average orientation and dynamics of cholesterol in DMPC membrane range from 10 to 12 kHz [11]. Fig. 1 shows ^{1}H MAS spectra extracted from the first t1 increment of the HSQC experiment acquired with spinning frequencies ν_R ranging from 9 to 15 kHz. It can be easily seen that MAS spinning rates higher than 12 kHz produce a dramatic enhancement in resolution and sensitivity, especially for methylene protons (see in particular the $(CH_2)_4$ at 2.2 ppm). Carbon decoupling in this HR-MAS probe presented a technical difficulty. Indeed, since this probe was developed specifically for liquids and "soft solids" and has the same electronics than a standard liquid state probe, high power decoupling (decoupling fields superior to 15–20 kHz) can not be achieved. On the other hand, since decoupling fields should not be in the same range of values as the spinning rate in order to prevent unwanted dipolar recoupling, they should be kept to low values.

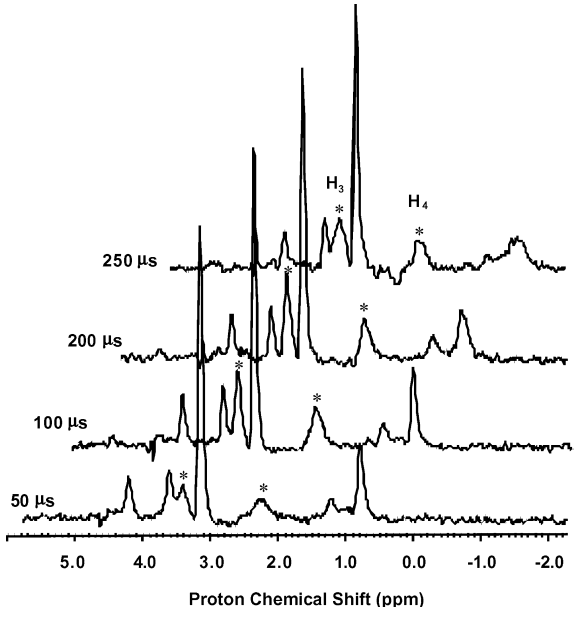

Fig. 2. Evolution of ^{1}H spectrum resolution with respect to the GARP carbon decoupling strength during the acquisition time. The ^{13}C $\pi/2$ pulse lengths corresponding to the decoupling strengths are indicated, and the MAS spinning rate was 12 kHz. An asterisk marks resonances corresponding to H3 (3.4 ppm) and H4 (2.2 ppm). The decoupling efficiency degrades when the decoupling field is either too small (^{13}C $\pi/2 = 250$ μs) or too close to the spinning rate (^{13}C $\pi/2 = 50$ μs).

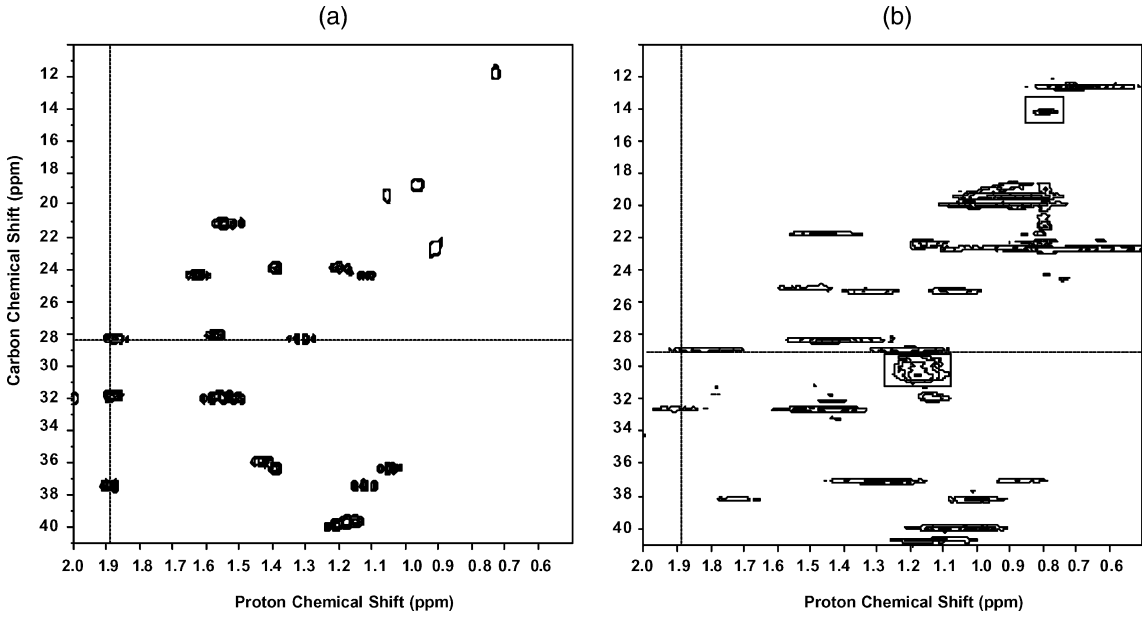

Fig. 3. (a) Typical 2D ^{13}C–^{1}H HSQC spectrum of cholesterol in $CDCl_3$ centered around the aliphatic region and referenced to TMS; Temp. 310 K. (b) Same region of a 2D ^{13}C–^{1}H HSQC spectrum of cholesterol in DMPC-d_{54}, at the same temperature and a MAS spinning rate of 15 kHz. Carbon spectral width 25 kHz. GARP carbon decoupling with a ^{13}C $\pi/2$ of 100 μs. A total of 1024 t1 increments with 64 scans each were collected. Square boxes surround cross peaks corresponding to the lipids. All the other cross peaks correspond to cholesterol resonances. Proton and carbon chemical shifts were internally referenced relative to the choline γ-CH_3 resonance taken at 55 ppm (^{13}C) and 3.18 ppm (^{1}H) [9]. The linewidths were equal to 0.1 ppm (CH, CH_3) and 0.2 ppm (CH_2) in the proton dimension and 0.2 ppm in the carbon dimension to be compared with 0.005 ppm for the corresponding liquid state spectrum.

We compared several heteronuclear scalar composite pulse decoupling schemes such as MLEV, WALTZ, GARP and several field strengths between 1 and 10 kHz and found that GARP was the most efficient technique. The best compromise in terms of field was around 2.5 kHz for a 12 kHz spinning rate (Fig. 2).

At 15 kHz, the HSQC experiment is far less sensitive to heteronuclear carbon decoupling field strength. Furthermore, as shown in Fig. 1 both CH and CH_2 resonances sharpen drastically at spinning rates higher than 13 kHz. As a result, the signal to noise ratio of all the cross peaks measurable in both experiments (CH, CH_2 and CH_3) increase by a factor 8, in the same experimental time, although using two times less sample (compare column 2 and 4 in Table 1).

A quantitative discussion of this sensitivity enhancement can not be performed since it is a result of a number of parameters: benefit of indirect detection, higher spinning rate, but also probe with a higher quality factor, smaller diameter coil, presence of a deuterium lock and of gradient pulses, different data processing. Gradients are essential if high quality HSQC's are to be obtained for natural abundance samples. The high rotation speeds will unavoidably create some instability in the system making the suppression of ^{12}C-bound protons more problematical. The number of increments in the indirect ^{13}C dimension deserves a special comment: the T_2 of the carbon resonances is equal to 16 ms (20 Hz linewidth) in this sample. This requires about 1k t_1 increments in order to get the maximum carbon resolution. Experiments have been performed using only 256 increments and linear prediction being applied to restore a reasonable resolution, thus saving time at the expense of a slightly degraded resolution (for instance the J coupling doublet fine structure of carbons 3 and 4 is not resolved). This is one drawback of inverse detection in a sample were the carbon linewidth are significantly smaller than the proton linewidth (~50 Hz). Nevertheless it clearly appears that in the same amount of experimental time and of sample, the HSQC/HR-MAS probe approach is far superior to the more conventional HETCOR/MAS approach. When comparing both strategies, the question arises of the relative sensitivities of a HRMAS probe and a conventional CPMAS probe. Measurements performed on the same sample, same coil diameter and same spectrometer demonstrated a gain of 11 and 8% for proton (on ethyl benzene) and carbon (on ASTM) detection, respectively, in the HRMAS probe with respect to a CPMAS probe. This modest increase in sensitivity with the HRMAS probe clearly shows that the proton detection plays the major role in our approximately 10-fold increase in sensitivity.

This gain in sensitivity did allow for the observation of all the natural abundance cross peaks of cholesterol protons. A comparison between an HSQC of pure cholesterol typically obtained in organic solvent and the HSQC presently obtained, with cholesterol inserted in lipid membranes at a ratio typically found in mamma-

lian plasma membranes, is represented in Fig. 3. Fig. 3b shows that, at 15 kHz spinning speed, several natural abundance cross peaks between directly bound carbon and protons can be observed with a good resolution and accuracy. Linewidth obtained in the proton dimension were close to 0.1 ppm. In the carbon dimension, T_2 relaxation time restricts the linewidth to 0.2 ppm. In this experiment, there was sufficient sensitivity to detect all the 24 expected natural abundance cross peaks, and the resolution was sufficient to resolve 20 out of them. Interestingly, clear differences occur between Figs. 3a and 3b, i.e., between corresponding cholesterol chemical shifts in organic solvent and in membranes. For instance, at a ^{1}H chemical shift corresponding to 1.9 ppm three cross peaks can be detected in $CDCl_3$, whereas the same column of the HSQC in membrane display only one cross peak. The same observation can be also made in the carbon dimension for example around 28–30 ppm. These significant differences may reveal important features in the conformations of cholesterol and its hydrogen bonding characteristics and quantitative analysis of these differences using quantum mechanics calculations is currently under investigation.

3. Conclusions

We have shown in the present work that HSQC experiments of cholesterol in liquid crystalline membranes, with a good resolution in both dimensions, can be obtained by HR-MAS experiments. Inverse detection scheme is very suitable to enhance sensitivity on such samples and has allowed the detection of all the expected cross peaks. Obviously, spinning rate is a key factor for an efficient transfer of coherence during the overall pulse sequence. Although a definitive assignment cannot be achieved with this experiment alone, detection of all heteronuclei and directly bound protons is usually the first step in this process. Since the relatively fast relaxation of coherences involved in the polarization transfer will probably prevent the use of a liquid-like HMBC experiment, CP based HETCOR experiment with long contact time will be required to establish connectivity between unbound atoms. It can be foreseen that the gain in sensitivity brought by the use of a gradient HR-MAS probe (or a CPMAS probe with lock and gradients) and inverse detection in the HSQC experiment will play a key role in assignment efforts and analysis of fluid membrane components.

4. Experimental

Samples of multilamellar vesicles made of chain deuterated DMPC-d_{54} (Avanti Polar Lipids, Alabaster, AL)/cholesterol-$^{13}C_4$,$^{13}C_3$ (Cambridge Isotope Laboratories, Andover, MA), 30 mol% of cholesterol in the fluid phase were prepared (cf. Table 1). Multilamellar vesicles were made by hydrating a film of dry DMPC/Cholesterol mixture. After lyophilisation the lipids were hydrated in the rotor with the same weight amount of D_2O. It was checked by deuterium NMR that the lipid quadrupolar splittings had standard values for such an MLV sample.

The NMR experiments were performed with a Bruker DMX narrow bore spectrometer operating at a ^{1}H Larmor frequency of 500.13 MHz. The pulse sequences are described in Fig. 4. HSQC spectra were acquired using a Bruker 4 mm ^{1}H/^{13}C/^{2}H gradient HR-MAS probe. This probe contains a single radio-frequency solenoid coil as well as a gradient coil designed to generate a linear field gradient along the magic angle [19]. The sample was placed in a 4 mm rotor fitted with an upper teflon insert delimiting a total volume of 50 μl corresponding to the active region of the solenoid coil of the probe. In order to compensate for temperature increases in the rotor due to high speed MAS, the driving air was pre-cooled to a temperature allowing for the desired temperature in the rotor. This temperature was checked from the known temperature dependence of the water chemical shift. Desiccation of the sample during the experimental time was not observed. A conventional echo–antiecho phase sensitive gradient HSQC experiment [20], using a double INEPT polarization transfer, was used to obtain the ^{1}H–^{13}C correlations. Two 800 μs sine-shaped gradient pulses of strength 40 and 10.05 G cm^{-1} were used in the experiment to detect only the protons attached to a ^{13}C nucleus. HETCOR spectra were obtained with a 5 mm DOTY scientific XC-5 MAS probe designed for ^{13}C detection and were performed as described previously [9]. Refocused INEPT was used for polarization transfer from ^{1}H to ^{13}C. Other NMR experimental parameters are given in Table 1.

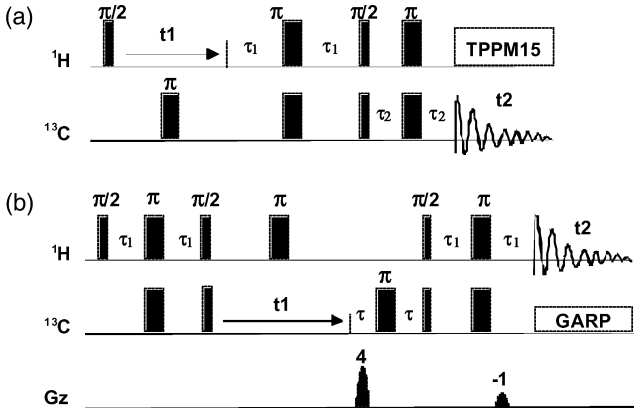

Fig. 4. Pulse sequences applied in the present study. (a) Carbon detected HETCOR; $\tau1 = 2$ ms, $\tau2 = 1.33$ ms; other parameters are given in Table 1 and in Section 4. (b) Proton detected gradient enhanced HSQC; $\tau1 = 1.75$ ms, $\tau = 1$ ms.

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