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Communication

A suite of 3D NMR methods for characterizing complex hydrocarbon structure fragments

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Abstract

A suite of triple resonance 3D NMR experiments is presented for the complete connectivity assignment of the hydrocarbon network in complex macromolecular and supramolecular organic structures. These new 3D NMR methods rely only on the presence of a unique set of ^{13}C resonances (from $^{13}C_X$) which are separated from the rest of the ^{13}C NMR spectrum. These experiments take the advantage of region selective excitation and selective inversion by composite pulses to provide correlations among H_A, $^{13}C_A$; H_B, $^{13}C_B$ and neighboring $^{13}C_X$ resonances along three frequency dimensions. These methods include: gHC_AC_X, gHC_AC_X-HH-TOCSY and gHC_AC_X-CC-TOCSY experiments. The utility of this approach is illustrated with spectra of selected structure fragments in poly(ethylene-*co*-*n*-butyl acrylate-*co*-carbon monoxide) (polyEBC) prepared from 1,2,3-$^{13}C_3$-*n*-butyl acrylate.

Keywords: 3D NMR; Polymer; Characterization; Hydrocarbon structure; TOCSY; gHC_AC_X

1. Introduction

The past half century has produced astounding advances in the preparation and characterization of complex organic structures. NMR methods have played a key role in these advances. NMR methods for characterizing increasingly complex macromolecular and supramolecular organic structures are vital for the future development of molecular-based science [1,2]. Triple resonance ($^1H/^{13}C/X$) 3D NMR has proven to be invaluable for dispersing and resolving the resonances of complex proteins and synthetic polymers containing a third NMR-active X nucleus (e.g., ^{15}N, ^{19}F, ^{29}Si, and ^{31}P) which is incorporated by isotopic labeling or is naturally present [3,4]. However, these methods are unsuitable for characterizing most structures which do not contain a suitable third NMR active X-nucleus. Here we describe the use of new 3D-NMR methods for characterizing such structures, which rely only on the presence of a unique set of ^{13}C resonances which are separated from the rest of the NMR spectrum.

Recently, a 3D NMR method was reported (Fig. 1A) to help disperse and identify the resonances from the complex mixture of hydrocarbon structures present in singly labeled terpolymer prepared from ethylene (E), carbon monoxide (C), and 1-^{13}C-*n*-butyl acrylate (B*) [5]. This method, which will be described as a gHC_AC_X experiment (where C_A and C_X form the AX part of a multispin system), is a modified version of the gHCACO experiment used to study protein structures [6,7]. The experiment takes advantage of region-selective excitation of C_X and aliphatic (C_A) ^{13}C resonances to provide correlations among H_A, C_A, and neighboring C_X resonances in the three dimensions of a spectrum from ^{13}C-labeled structure unit **1**. Ideally, C_X is ^{13}C enriched and has its resonance separated from the remaining ^{13}C resonances of the molecule by 10–20 ppm so that it can be selectively excited. Unfortunately, this experiment alone usually doesn't provide sufficient data to completely identify unknown structures.

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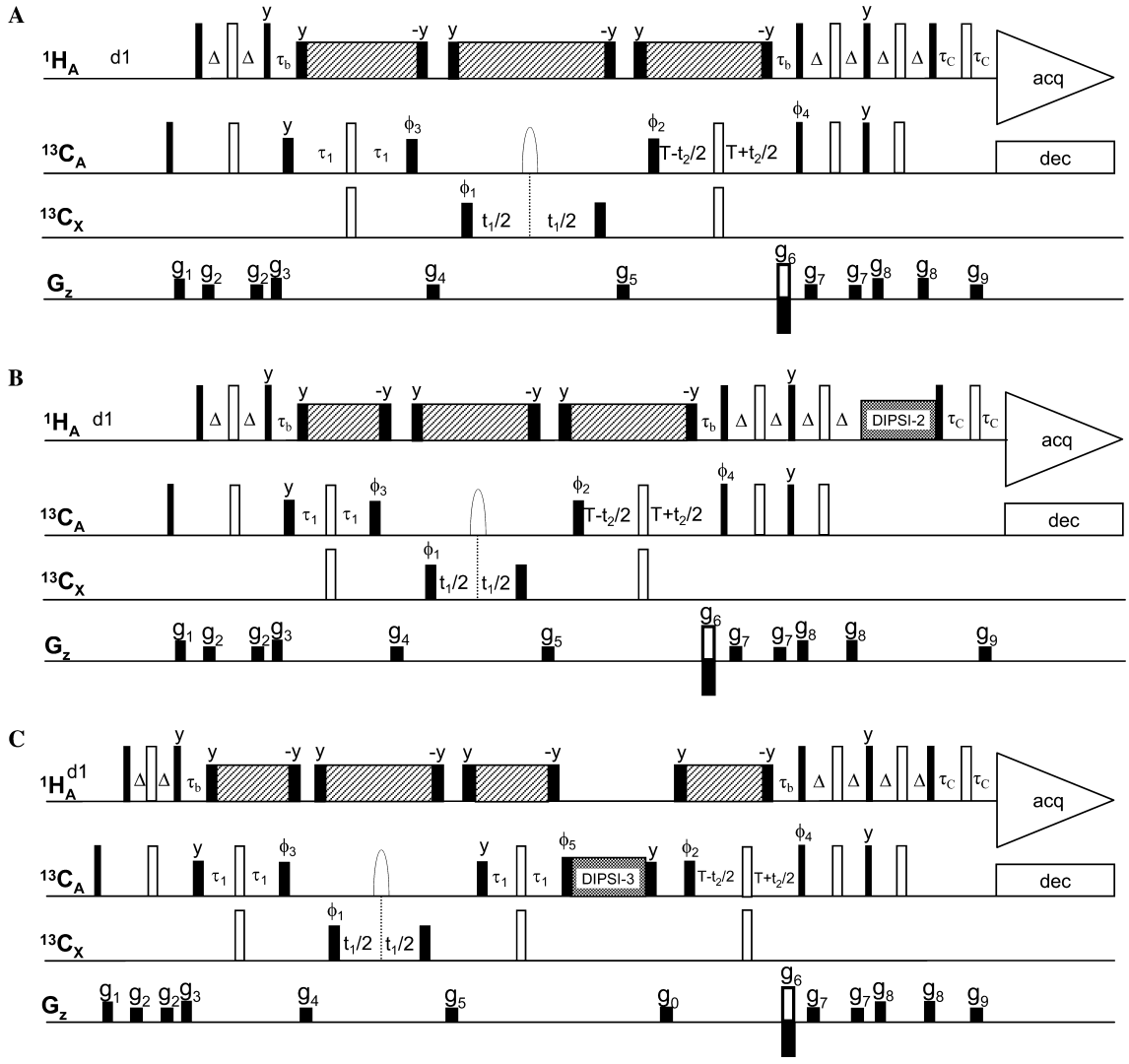
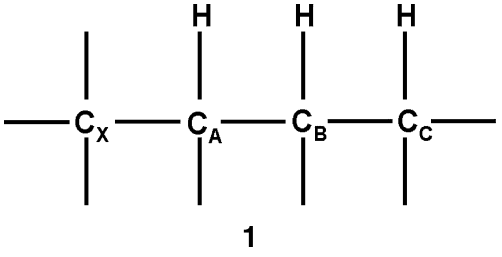
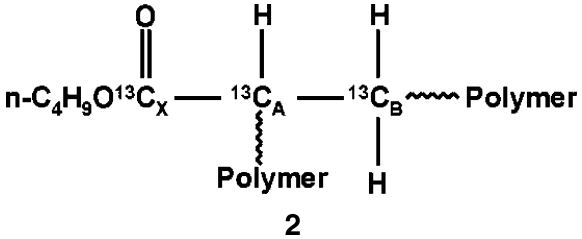


Fig. 1. Pulse sequence for the (A) gHC_AC_X, (B) gHC_AC_X-HH-TOCSY, and (C) gHC_AC_X-CC-TOCSY experiment. Narrow (black) and wide (white) bars indicate 90° and 180° pulses, respectively. Proton decoupling during the evolution periods was accomplished using WALTZ-16 modulation with an rf field strength of 7.5 kHz. Quadrature detection in t_1 was achieved using the States-TPPI method by incrementing the phase of the ϕ_1. Echo/anti-echo selection during t_2 was achieved by inverting the phase of ϕ_4 and inverting the sign of the g_6 gradient. ^1H and ^{13}C isotopic mixing were achieved by DIPSI-2 and DIPSI-3 schemes at rf field strength of 7 and 9 kHz, respectively. Delays Δ and τ are set to $(4 \times {}^1J_{CH})^{-1}$ and $(2 \times {}^1J_{CC})^{-1}$ or $(4 \times {}^1J_{CC})^{-1}$, respectively.



1

In this communication, two new 3D NMR pulse sequences (Figs. 1B and C) are described that can be used in combination with the parent gHC_AC_X experiment (Fig. 1A) for the complete assignment of ^1H and ^{13}C resonances in complex hydrocarbon structures. The methodology of using multiple 3D NMR pulse sequences for identifying different structure fragments is similar to that used for protein structure studies. The utility of this approach is illustrated with spectra of selected structure fragments in poly(ethylene-*co*-*n*-butyl acrylate-*co*-carbon monoxide) (polyEBC) prepared from 1,2,3-$^{13}C_3$-*n*-butyl acrylate. The labeled polymer fragment corresponding to general structure **1** can be represented by **2**.



2

Within a structure fragment **1**, the J coupling modulated magnetization transfer pathways for the gHC$_A$C$_X$-HH-TOCSY and gHC$_A$C$_X$-CC-TOCSY experiments are as follows:

$$H_A \xrightarrow{^1J_{CH}} C_A \xrightarrow{^1J_{CC}} C_X(t_1) \xrightarrow{^1J_{CC}} C_A(t_2) \xrightarrow{^1J_{CH}} H_A \xrightarrow[^nJ_{HH}]{\text{Spin-Lock}} H_A, H_n(t_3)$$

$$H_A \xrightarrow{^1J_{CH}} C_A \xrightarrow{^1J_{CC}} C_X(t_1) \xrightarrow{^1J_{CC}} C_A \xrightarrow[^nJ_{CC}]{\text{Spin-Lock}} C_A, C_n(t_2) \xrightarrow{^1J_{CH}} H_A, H_n(t_3)$$

Coherence in the gHC$_A$C$_X$-HH-TOCSY experiment (Fig. 1B) follows the same path as gHC$_A$C$_X$ experiment until the magnetization is transferred back to H$_A$, at which time, a DIPSI-2 isotropic mixing sequence [8] produces ^{1}H–^{1}H TOCSY transfer among protons that are part of the coupled spin system including H$_A$. A plane from the 3D spectrum at δC$_X$, will contain correlations between δC$_A$ and the δH's of all protons that are part of the proton spin system containing H$_A$. Ideally, this experiment should be performed with samples ^{13}C-enriched at minimum in the C$_X$ position.

In gHC$_A$C$_X$-CC-TOCSY 3D NMR experiment (Fig. 1C) ^{13}C spin-locking was performed before the t_2 evolution period using a DIPSI-3 isotropic mixing sequence [8]. A 5 ms gradient pulse, flanked by two selective 90° pulses, is applied between the CC isotropic mixing period and the constant time evolution period in the t_2 dimension to eliminate artifacts from unwanted transverse magnetization. The advantage of this method is that CC mixing is applied before the t_2 evolution period so that the H$_B$ resonance is dispersed in f_2 to δC$_B$, permitting unambiguous identification of the H$_B$ and C$_B$ resonances. This experiment requires a high abundance of ^{13}C–^{13}C spin pairs for reasonable sensitivity. All these experiments are extremely efficient at filtering the unwanted main-chain and solvent resonances from the spectrum.

Triply labeled poly(EB*C) is used here so that B* centered pentads could be easily identified. This terpolymer is chosen to demonstrate the utility of these pulse sequences because of its complexity. At minimum, the resonances are sensitive to structure differences between the 243 permutations of 3 monomers (ignoring stereochemistry) in pentad sequences. The number of signals concentrated in relatively small regions of the ^{1}H and ^{13}C NMR spectra has frustrated attempts to unambiguously assign the resonances from all the structures present using standard 1D and 2D NMR methods [9]. For complete characterization, two polymers selectively labeled in different monomer fragments were prepared so that C- and B-centered pentad structures could be studied separately. Even with this simplification 2D-NMR methods provided only a limited number of additional assignments [10].

Fig. 2A shows one of the f_2f_3 planes at $f_1 = 175.31$ ppm (ester ^{13}CO chemical shift) from the 750 MHz gHC$_A$C$_X$ 3D spectrum obtained at 120 °C. This plane shows a unique H$_A$–C$_A$ cross-peak (*c*) at $\delta_H = 2.35$ ppm and $\delta_C = 45.8$ ppm, from an EE<u>B</u>EE pentad structure. This is one of 36 unique correlations

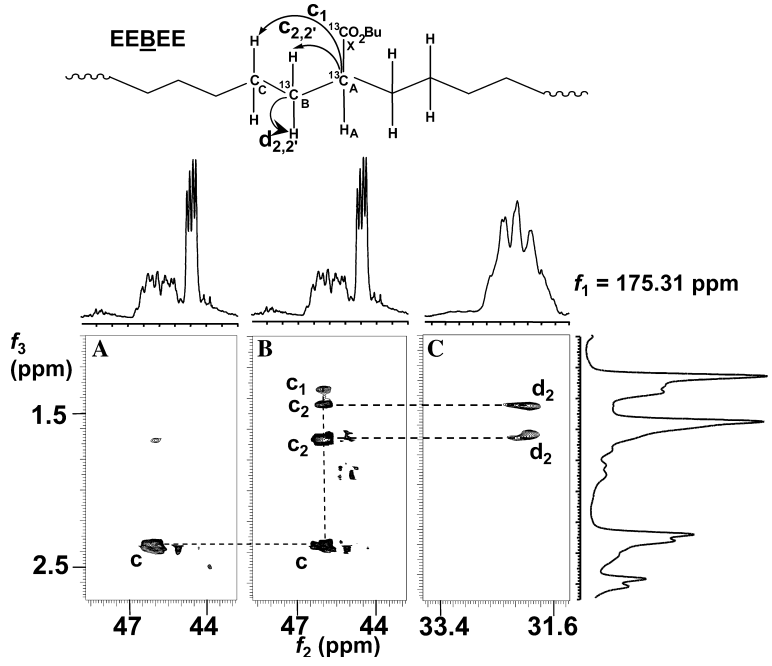


Fig. 2. f_2f_3 planes at one-^{13}CO$_2$R chemical shift ($f_1 = 175.31$ ppm) from the (A) gHC$_A$C$_X$, (B) gHC$_A$C$_X$-HH-TOCSY, and (C) gHC$_A$C$_X$-CC-TOCSY experiments. An EE<u>B</u>EE pentad structure fragment in poly(EBC) is displayed above the figure showing the correlation observed in the 3D NMR spectra (1D ^{13}C NMR spectra are displayed in f_2 and ^{1}H NMR spectrum in f_3).

resolved in different f_2f_3 planes from the 3D spectrum; however, it is not possible to identify the structure from the data in this spectrum alone. Since the resonances are sensitive to structure variations up to 5 bonds away, sorting of complete sets of ^1H and ^{13}C resonances from each of the 36 structures using additional 3D-NMR experiments permits their elucidation.

Fig. 2B shows the same f_2f_3 plane at $f_1 = 175.31$ ppm from the 750 MHz gHC$_A$C$_X$-HH-TOCSY 3D spectrum. This plane shows cross-peaks between the resonances of C_A and the first few mutually coupled protons (H_B and H_C, cross-peaks c_1 and $c_2/c_{2'}$) along the polymer chain in addition to the H_A–C_A cross-peak. The positions of these resonances are characteristic of the diastereotopic H_B (cross-peaks $c_2/c_{2'}$) and H_C (cross-peak c_1) protons in a long chain of methylene groups attached to the CH branch point. Since only one set of resonances is observed, a symmetrical EEBEE pentad structure is identified. The DIPSI-2 mixing time used here was 30 ms which is suitable to see correlations through 3–4 bonds; it is possible to increase the mixing time to observe longer range correlations.

Fig. 2C, shows the f_2f_3 plane at $f_1 = 175.31$ ppm from the 750 MHz gHC$_A$C$_X$-CC-TOCSY 3D spectrum. The CC isotropic mixing produces an additional set of cross-peaks between the resonances of $H_B/H_{B'}$ (at $\delta^1H = 1.45/1.65$ ppm) and C_B (at $\delta^{13}C = 32.0$ ppm) (cross-peaks $d_2/d_{2'}$), along with the cross-peaks along f_3 at δC_B in f_2 (not shown). This last experiment permits unambiguous identification of the resonances from the C_B–H_B atoms in the structure fragment. This experiment can only be performed if a sequence of carbons is ^{13}C labeled. In this spectrum, a cross-peak d_1 at the shift of C_C corresponding to c_1 is not observed because C_C is not labeled in this sample. However, if this position were labeled, this cross-peak would occur at a position along f_2 corresponding to δC_C.

The spectra obtained with these new pulse sequences are clean, permitting identification of structures representing less than 1% of the mixture. Typical experiment times are 15–30 h, and are determined by the need to obtain sufficient digital resolution in the indirectly detected dimensions and not by signal-to-noise requirements. When ^{13}C labeling is employed, signals can be detected in 1D and 2D versions of the experiments in less than 10–15 min. Thus the gHC$_A$C$_X$ and gHC$_A$C$_X$-HH-TOCSY experiments should be generally useful, and can provide valuable information about hydrocarbon structure fragments in complex molecules or in mixtures of small molecules when one group of ^{13}C resonances is isolated from the rest of the spectrum. The gHC$_A$C$_X$-CC-TOCSY experiment can provide additional structure information when two or more carbons can be ^{13}C labeled. Two channel versions of the experiment make it possible to do them on most modern instruments.

2. Experimental

The synthesis of the labeled polymer and thorough characterization of the sample have been reported elsewhere [10]. Crystals of 1,4-dichlorobenzene-d_4 were melted and 20 mg of the polymer were dissolved in 0.7 ml of the melt to produce 3% (w/v) polymer solutions. The samples were heated to 120 °C and rotated at 20 rpm in a Kugelrohr oven for 6 h to obtain a uniform dispersion of the polymer in solution and to minimize the formation of air bubbles. Hexamethyldisiloxane (HMDS) was added as an internal chemical shift standard ($\delta_H = 0.09$ ppm and $\delta_C = 2.00$ ppm).

All the 3D NMR spectra were collected on a Varian Inova 750 MHz spectrometer, equipped with ProteinPack pulse sequence software module, a Nalorac $^1H/^2H/^{13}C/^{15}N$ 5 mm probe and z-axis pulse field gradient (PFG) accessory, operating at 120 °C using the pulse programs shown in Fig. 1. In this figure solid black and wide white bars represent 90° and 180° hard rectangular pulses, respectively. Spectra were collected with 90° pulse widths of 10 and 15 μs on the ^1H and ^{13}C channels, respectively. The width of the selective pulses to $^{13}C_A$ or $^{13}C_X$ was set based on the difference in the frequency offsets between the two regions, such that selective $^{13}C_A$ pulse did not excite $^{13}C_X$ nuclei, and vice versa. The selective 90° pulse width on either $^{13}C_A$ or $^{13}C_X$ was 38.9 μs and the selective 180° pulse width was 34.7 μs based on the difference in offsets of 24,890 Hz between the $^{13}C_A$ and $^{13}C_X$ resonances. In this way, a pulse with maximum rf field strength at one offset produced a null in the rf field strength at the second offset. The selective 180° (34.7 μs) pulse to $^{13}C_A$ in middle of the t_1 evolution period was a shifted laminar pulse [11] because the ^{13}C transmitter at this point was placed in the $^{13}C_X$ region. All the pulses have x-phase unless otherwise indicated. The pulses on the first channel (^1H observe transmitter) were applied at a transmitter offset corresponding to 2 ppm in the ^1H spectral window. The pulses to $^{13}C_A$ and $^{13}C_X$ were applied at offsets corresponding to 42 and 174 ppm, respectively, in the ^{13}C spectral window. Carbon decoupling during the acquisition time was achieved using GARP modulated decoupling with a 17 kHz rf field. The phase cycles employed were: $\phi_1 = 2(x), 2(-x)$; $\phi_2 = x, -x$; $\phi_3 = 4(y), 4(-y)$; $\phi_4 = x/-x$; $\phi_r = x, -x, -x, x, -x, x, x, -x$.

Gradient pulses g_1–g_9, along the z-axis were applied with amplitudes and durations: $g_1 = 0.3$ T/m, 1.0 ms; $g_2 = 0.15$ T/m, 0.75 ms; $g_3 = 0.3$ T/m, 1.0 ms; $g_4 = 0.2$ T/m, 0.3 ms; $g_5 = 0.15$ T/m, 0.2 ms; $g_6 = \mp 0.25$ T/m, 1.6 ms; $g_7 = 0.15$ T/m, 1.0 ms; $g_8 = 0.25$ T/m, 0.8 ms; and $g_9 = 0.25$ T/m, 0.4 ms. The z-gradient pulses g_6 and g_9 were used for ^1H–^{13}C coherence selection and must have areas in a ratio of 4:1. Spectral windows in the f_1, f_2, and f_3 dimensions were 760, 1500, and 6000 Hz, respectively. WALTZ-16 decoupling with field strength of

7.5 kHz was employed during delays τ_1, t_1, and T; to remove the ^{1}H couplings. Quadrature detection in the t_1 ($^{13}C_X$ chemical shift) dimension was achieved by alternating the phase of ϕ_1 in a States-TPPI manner [12]. Echo/anti-echo selection in the t_2 ($^{13}C_A$ chemical shift) dimension was achieved by inverting the amplitude of the g_6 gradient pulse and the phase ϕ_4 [13]. Axial peak displacement in the f_2 dimension was obtained by inverting the phase of ϕ_2 and the receiver (ϕ_r) on every second increment [7]. For the spectra from all three sequences, the delays were: 0.8 s relaxation delay, 0.25 s acquisition time, constant evolution delay $2T = 27$ ms (based on $(n \times J_{CC})^{-1}$, where $n =$ integer), $\Delta = 1.9$ ms (based on $(4 \times J_{CH})^{-1}$, where $J_{CH} = 127$ Hz), $\tau_b = 2.3$ ms (to accommodate the g_6 gradient pulse), and $\tau_c = 1.5$ ms (to accommodate recovery from the g_9 gradient pulse before data acquisition). In the data presented here, the delay τ_1 was set to $(n \times J_{CACX})^{-1}$, where $n = 4$ or 6. In structures where only methine C_A–H fragments are labeled, the delay τ_1 was 5.0 ms (based on $(4 \times J_{CACX})^{-1}$, where $J_{CACX} = 50$ Hz). In structures where both methine and methylene C_A–H fragments are labeled, a shorter delay $\tau_1 = 3.5$ ms (based on $(6 \times J_{CACX})^{-1}$) produced optimal results. The spectra were collected with 2048 points in each free induction decay (FID) with ^{13}C-GARP decoupling; 16 transients were averaged for each of 2×32 and 2×32 FIDs in t_1 and t_2 dimensions, respectively.

In the gHC$_A$C$_X$-HH-TOCSY experiment (Fig. 1B), spin-locking in ^{1}H dimension was achieved by applying a DIPSI-2 [8] isotropic mixing sequence for 30 ms with $\gamma B_1 = 7$ kHz. Other parameters of the sequence remain the same as in the gHC$_A$C$_X$ experiment described above.

In gHC$_A$C$_X$-CC-TOCSY NMR experiment (Fig. 1C), C_A–C_B spin-locking was achieved by applying a DIPSI-3 [8] isotropic mixing sequence for 18 ms (corresponding to three DIPSI-3 cycles) with $\gamma B_1 = 9$ kHz. The phase cycling of $\phi_5 = 4(x), 4(-x)$. Before CC isotropic mixing, the anti-phase transverse C_A magnetization was refocused. After the mixing period, a 5 ms gradient pulse (g_0)

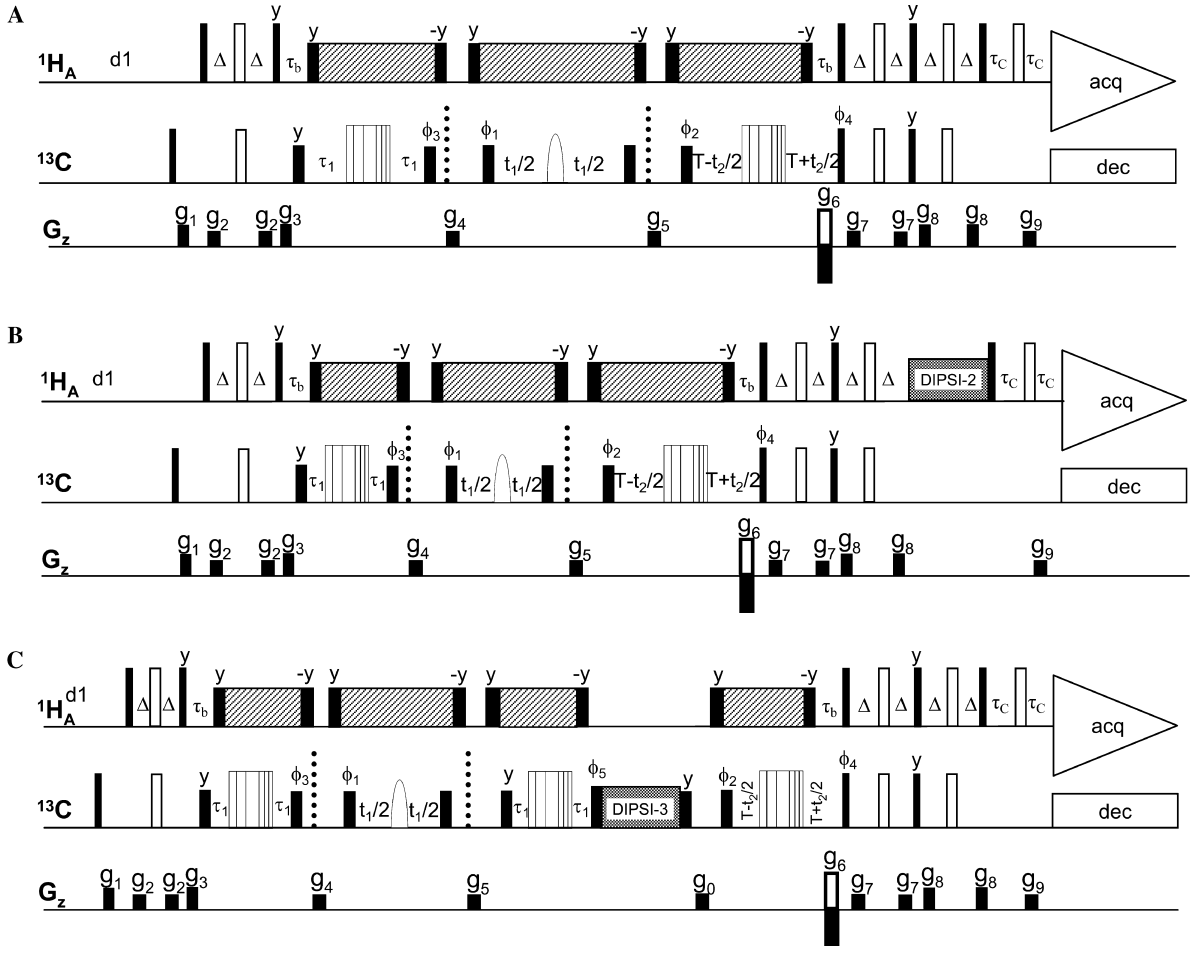


Fig. 3. Two channel versions of pulse sequences for the (A) gHC$_A$C$_X$, (B) gHC$_A$C$_X$-HH-TOCSY, and (C) gHC$_A$C$_X$-CC-TOCSY experiments. The nomenclature is the same as described in Fig. 1. Shaka composite pulses (six consecutively placed pulses with different widths) were used to simultaneously invert both $^{13}C_A$ and $^{13}C_X$ carbons during τ_1, t_1, and T periods. The carbon transmitter was initially placed at 42 ppm (38 ppm in gHC$_A$C$_X$-CC-TOCSY experiment), corresponding to the middle of the $^{13}C_A$ region. It was moved to 174 ppm (middle of the $^{13}C_X$ region) before the g_4 gradient pulse (marked as -----) and moved back to the $^{13}C_A$ region before g_5 gradient pulse and after the t_1 evolution period.

was applied flanked by two selective 90° pulses (to $^{13}C_A$ and $^{13}C_B$, with a null at $^{13}C_X$) to eliminate unwanted residual transverse magnetization before t_2 evolution. The spectral window in the f_2 dimension was 4525 Hz and the $^{13}C_A$ transmitter was at 38 ppm so that the difference between the two ($^{13}C_A$ and $^{13}C_X$) carrier frequencies was 136 ppm. The selective 90° and 180° pulse powers (widths) for this experiment were adjusted to 6.6 kHz (37.8 μs) and 14.8 kHz (33.6 μs), respectively. Other parameters for collection of the spectrum with this sequence were the same as in the gHC_AC_X experiment described above.

The data presented here were collected using the two channel versions of the three pulse sequences as shown in Fig. 3. As commonly practiced in protein NMR pulse sequences, Shaka [14] composite pulses were used to simultaneously invert both $^{13}C_A$ and $^{13}C_X$ magnetization between the two τ_1 periods, even though the carrier was centered in the $^{13}C_A$ region. Two selective 90° pulses on $^{13}C_X$ before and after t_1 evolution period were applied by shifting the carrier from center of the $^{13}C_A$ region to center of the $^{13}C_X$ region and brought back to $^{13}C_A$ region before the g_5 gradient pulse in Fig. 3. A selective phase shifted 180° pulse (shifted laminar pulse) was applied in the middle of the t_1 evolution period to invert the $^{13}C_A$ magnetization without affecting $^{13}C_X$ nuclei to refocus $^1J_{CACX}$ coupling. These two-channel versions of the experiment can be performed on any commercial NMR spectrometer, equipped with two channels and a gradient accessory as there is no requirement for the special hardware used in most heteronuclear triple resonance 3D experiments. The fixed pulse power (width) needed to selectively excite one part of the carbon spectrum without perturbing the resonances in other parts of the spectrum imposes the restriction that the $^{13}C_X$ resonances be separated from the $^{13}C_A$ and $^{13}C_B$ resonances by at least 10–20 ppm depending on the instruments field strength.

The data were processed using Varian's VNMR software with $f1_{coef} = 1, 0, 0, 0, 0, 0, -1, 0$ and $f2_{coef} = 1, 0, -1, 0, 0, -1, 0, -1$. The time domain data were forward linear predicted to four times of the original number of data points in both the t_1 and t_2 dimensions, zero filled to $512 \times 512 \times 4096$ points and weighted with a shifted sinebell function before Fourier transformation.

Acknowledgments

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