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Soft-triple resonance solid-state NMR experiments for assignments of U-^{13}C, ^{15}N labeled peptides and proteins

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Abstract

The process of obtaining sequential resonance assignments for heterogeneous polypeptides and large proteins by solid-state NMR (ssNMR) is impeded by extensive spectral degeneracy in these systems. Even in these challenging cases, the cross peaks are not distributed uniformly over the entire spectral width. Instead, there exist both well-resolved single resonances and distinct groups of resonances well separated from the most crowded region of the spectrum.

Here, we present a series of new triple resonance experiments that exploit the non-uniform clustering of resonances in heteronuclear correlation spectra to obtain additional resolution in the more crowded regions of a spectrum. Homonuclear and heteronuclear dipolar recoupling sequences are arranged to achieve directional transfer of coherence between neighboring residues in the peptide sequence. A frequency-selective (*soft*) pulse is applied to select initial polarization from a limited (and potentially) well-resolved region of the spectrum. The pre-existing resolution of one or more spins is thus utilized to obtain additional resolution in the more crowded regions of the spectrum. A new protocol to utilize these experiments for sequential resonance assignments in peptides and proteins is also demonstrated.

High molecular weight proteins and protein complexes which are not amenable to characterization by traditional solution NMR methods have emerged as the focus of structural investigation by magic angle spinning (MAS) solid-state NMR (ssNMR) [1]. Recent developments in dipolar recoupling schemes have facilitated the process of obtaining resonance assignments from uniformly labeled peptides and proteins in the solid state [2]. To date, complete (^{15}N, ^{13}C) backbone and side chain resonance assignments have been obtained for a number of small peptides [3–5] and (at least) one protein in the solid state, the SH3 domain from chicken α-spectrin [6]. In several additional systems partial assignments have been reported; these include both soluble proteins [7–9] and two integral membrane proteins: the light harvesting complex (LHII) from *Rhodopseudomonas acidophilia* [10] and the proton pump bacteriorhodopsin (bR) from *Halobacterium salinarum* [11].

In general, at least two experiments are required to assign a protein in the solid state. The NCA experiment is used to obtain the intra-residue backbone correlation between $^{15}N^{i}$ and $^{13}C\alpha^{i}$. The NCOCA is used to obtain the inter-residue $^{15}N^{i}$ to $^{13}CO^{i-1}$ and $^{13}C\alpha^{i-1}$ correlations. Sequential assignments are obtained by identifying contiguous $^{13}C\alpha^{i-1}$ and $^{13}C\alpha^{i}$ in the two experiments via the common $^{15}N^{i}$ chemical shift [5,7,8,10,11]. The process is aided by incorporating additional chemical shift information acquired by correlating the backbone to the side chain resonances. Backbone-side chain and side chain-side chain correlations are obtained by appending homonuclear transfer periods to the heteronuclear correlation experiments (i.e., NCACX and NCOCACX) [4–6,8,10–12] and/or using a ^{13}C–^{13}C homonuclear correlation scheme [4–6,8–10].

This approach is limited, however, in systems with extensive spectral degeneracy. Such systems include large proteins as well as small, heterogeneous polypeptides [5,13]. Nevertheless, even in these more challenging cases, the cross peaks are not distributed uniformly over the entire spectral width. Instead, there

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exist both well-resolved single resonances and distinct groups of resonances well separated from the most crowded region of the spectrum [7–11]. Characteristic ^{15}N or ^{13}Cα 'random-coil' chemical shifts of several amino acids such as glycine, threonine, and proline, as well as the primary, secondary, and tertiary structure all make significant contributions to the distribution of chemical shifts [14]. As such, adjacent residues in the peptide sequence often appear in distinct regions of even a poorly resolved multidimensional NMR spectrum.

In the following we consider a resolved (^{15}N, ^{13}C) cross peak flanked in the peptide sequence by two potentially less resolved cross peaks. We refer to a group of three such resonances as a dipolar 'couplet.' In direct analogy to partially overlapped ^{1}H solution NMR multiplets, selective transfer from the well-resolved member(s) of a 'couplet' may be used to enhance the resolution of the more poorly resolved regions of the spectrum (*vide infra*). In a solution ^{1}H NMR experiment, this is achieved by replacing one or more non-selective 'hard' pulses with a '*soft*' or frequency-selective pulse and adjusting the receiver to follow the selected coherence pathways [15–24]. In ssNMR, the weak multiple bond couplings between members of a 'couplet' are typically obscured by the dominant single bond interactions. Triple resonance experiments with multiple single bond transfers must be specifically designed to establish the 'intra-couplet' correlation. Here, we present two triple resonance ssNMR experiments that utilize soft pulses to selectively transfer polarization from one member of a couplet to the next. The experiments may be used to obtain (^{15}N, ^{13}C) correlation spectra of resonances which are adjacent in the peptide sequence to those with chemical shifts which fall within the bandwidth of the selective pulse. The pulse sequences may also be implemented to give selective inter-residue homonuclear ^{13}C correlation spectra from which sequential resonance assignments may be directly obtained (*vide infra*).

Two experiments that meet these criteria are presented in Fig. 1. The *soft*-CACO NCA and *soft*-CA NCOCA experiments employ a '*soft*' filter for selectivity and sequential heteronuclear and homonuclear dipolar recoupling steps to achieve directed magnetization transfer between adjacent residues in the peptide sequence. The magnetization pathway for the CACO NCA is given by

$$^{13}\mathrm{C}\alpha^{i} \rightarrow {}^{13}\mathrm{CO}^{i} \rightarrow {}^{15}\mathrm{N}^{i+1} \rightarrow {}^{13}\mathrm{C}\alpha^{i+1} \quad (1)$$

An NCA spectrum, edited such that only resonances $(i+1)$ to those which fall within the bandwidth of the ^{13}C selective pulse is obtained in a *soft*-(CACO)NCA experiment (Fig. 1a). Initial ^{13}C magnetization is created by ramped cross polarization (CP) from ^{1}H [25–27]. In the (CACO)NCA experiment, the evolution period t_1^{b} is omitted, as is the corresponding 180° pulse on the ^{15}N channel. ^{13}C magnetization is stored longitudinally with a 90° pulse (residual transverse magnetization is removed using a *z*-filter). A selective pulse with

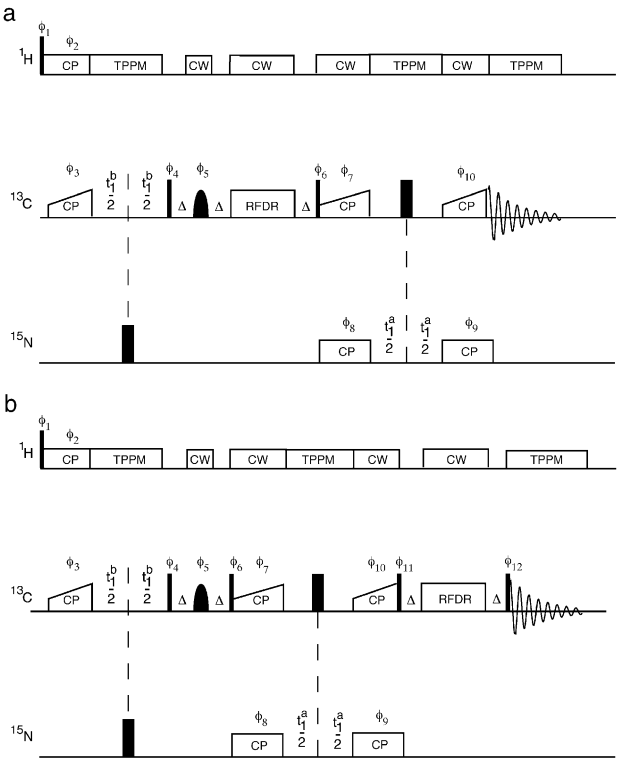

Fig. 1. Pulse sequences for (a) ^{13}C-filtered *soft*-CACO NCA, (b) ^{13}C-filtered *soft*-CA NCOCA. Narrow and wide boxes represent 90° and 180° pulses, respectively (100 kHz ^{1}H, 50 kHz ^{13}C, and 50 kHz ^{15}N). Pulses are applied at 62.5 ppm (^{13}C) and 110.3 ppm (^{15}N). During the initial ^{1}H $\rightarrow$ ^{13}C CP [25], the ^{1}H field is fixed at 50 kHz and the ^{13}C field is linearly ramped from 50 to 60 kHz through the $n = 1$ Hartman–Hahn CP/MAS match condition in 0.2 ms [26]. TPPM [29] (83 kHz, 12° phase modulation) and CW decoupling (100 kHz) are applied during evolution and mixing periods, respectively. Residual transverse magnetization is removed during the *z*-filter periods in the absence of decoupling ($\Delta = 112.28\,\mu$s). The selective pulse is applied on alternating scans with a Gaussian profile, cosine modulated by 20.5° in 128 points to obtain the necessary frequency offset [30]. Typical inversion efficiency is 85%. Homonuclear ^{13}C–^{13}C polarization transfer is accomplished using radio frequency driven recoupling (RFDR) (50 kHz ^{13}C field) [27]. Heteronuclear CP transfers are obtained using a constant (30 kHz) ^{15}N field, applying a linear power ramp through the $n = 1$ SPECIFIC match condition on the ^{13}C channel in 3.0 ms [28]. The phase cycle employed is: (a) $\phi_1 = x$; $\phi_2 = y$; $\phi_3 = 2[8(x), 8(-x)]$; $\phi_4 = y$; $\phi_5 = y$; $\phi_6 = y$; $\phi_7 = x$; $\phi_8 = 4[x,x,-x,-x], 4[y,y,-y,-y]$; $\phi_9 = 16(x), 16(y)$; $\phi_{10} = 4[4(x), 4(-x)]$; and $\phi_{\mathrm{rec}} = 2[(x,-x,-x,x), (-x,x,x,-x), (-x,x,x,-x), (x,-x,-x,x)]$. The phase of all 180° pulses is set to *y*. Quadrature detection is obtained for (CACO)NCA by alternating $\phi_9 = [16(x), 16(y)], [16(y), 16(-x)]$ [31]. For CA(CON)CA quadrature detection is obtained alternating $\phi_3 = 2[8(x), 8(-x)], 2[8(y), 8(-y)]$ [31]. (b) $\phi_1 = 2[8(x), 8(-x)]$; $\phi_2 = y$; $\phi_3 = x$; $\phi_4 = 2[8(y), 8(-y)]$; $\phi_5 = y$; $\phi_6 = 2[8(-y), 8(y)]$; $\phi_7 = x$; $\phi_8 = 4[x,x,-x,-x], 4[y,y,-y,-y]$; $\phi_9 = [16(x)16(y)]$; $\phi_{10} = 4[4(x), 4(-x)]\phi_{11} = y$; $\phi_{12} = -y$; and $\phi_{\mathrm{rec}} = 2[(x, -x,-x,x), (-x,x,x,-x), (-x,x,x,-x), (x,-x,-x,x)]$. Quadrature detection is obtained for (CA)NCOCA alternating $\phi_9 = [16(x), 16(y)]$, $[16(y), 16(-x)]$ [31]. For CA(N)COCA (Fig. 3) quadrature detection is obtained alternating $\phi_3 = (x, y)$ [31].

bandwidth $\Delta\Omega$ is applied on alternating scans. Thus, the magnetization is described by three terms, two for resonances within $\Delta\Omega$:

$$\sigma(\mathbf{I}) = {}^{13}\mathrm{C}\alpha_z^i \quad \text{(odd scans)} \tag{2a}$$

$$\sigma(\mathbf{II}) = -{}^{13}\mathrm{C}\alpha_z^i \quad \text{(even scans)} \tag{2b}$$

and one for all resonances outside of $\Delta\Omega$:

$$\sigma(\mathbf{III}) = {}^{13}\mathrm{C}\alpha_z^i \quad \text{(all scans)} \tag{2c}$$

Next, magnetization exchange is obtained via radio frequency driven recoupling (RFDR) [27]. The magnetization is subsequently returned to the transverse plane by a second 90° pulse. At this point

$$\sigma(\mathbf{I}) = ({}^{13}\mathrm{C}\alpha_x^i + {}^{13}\mathrm{CO}_x^i) \tag{3a}$$

$$\sigma(\mathbf{II}) = -({}^{13}\mathrm{C}\alpha_x^i + {}^{13}\mathrm{CO}_x^i) \tag{3b}$$

$$\sigma(\mathbf{III}) = ({}^{13}\mathrm{C}\alpha_x^i + {}^{13}\mathrm{CO}_x^i) \tag{3c}$$

Subsequently, band-selective CP from ${}^{13}\mathrm{CO}^i$ to ${}^{15}\mathrm{N}^{i+1}$ is obtained by setting the appropriate spin lock field strength on the ^{15}N channel and ramping through the SPECIFIC match condition on the ^{13}C channel [28]. Transferred magnetization evolves at $(\Omega^{15}\mathrm{N}^{i+1})$ during t_1^{a} (heteronuclear ^{15}N–^{13}C scalar couplings are refocused by the ^{13}C 180° pulse applied in the center of the evolution period.) The magnetization is transferred to ${}^{13}\mathrm{C}\alpha^{i+1}$ via a second SPECIFIC CP period. The detected signal is described by

$$\sigma(\mathbf{I}) = \exp[\mathrm{i}\Omega({}^{13}\mathrm{C}\alpha^{i+1})t_2]\cos[\Omega({}^{15}\mathrm{N}^{i+1})t_1^{\mathrm{a}}] \tag{4a}$$

$$\sigma(\mathbf{II}) = -\exp[\mathrm{i}\Omega({}^{13}\mathrm{C}\alpha^{i+1})t_2]\cos[\Omega({}^{15}\mathrm{N}^{i+1})t_1^{\mathrm{a}}] \tag{4b}$$

$$\sigma(\mathbf{III}) = \exp[\mathrm{i}\Omega({}^{13}\mathrm{C}\alpha^{i+1})t_2]\cos[\Omega({}^{15}\mathrm{N}^{i+1})t_1^{\mathrm{a}}] \tag{4c}$$

If the receiver is phase cycled $\pm$ on alternate scans, magnetization from pathways **I** and **II** add while magnetization from pathway **III** (outside the bandwidth of the selective pulse) is eliminated. Thus, the final spectrum is described by

$$\sigma(\mathbf{I}+\mathbf{II}) = 2\exp[\mathrm{i}\Omega({}^{13}\mathrm{C}\alpha^{i+1})t_2]\cos[\Omega({}^{15}\mathrm{N}^{i+1})t_1^{\mathrm{a}}] \tag{5}$$

Resonances which are $(i+1)$ in the peptide sequence to those falling within the $\Delta\Omega$ of the selective pulse are resolved and identified using the *soft*-(CACO)NCA experiment. An analogous (CA)NCOCA experiment may be performed to obtain the identical resolution and identification of the $(i-1)$ residues. The details of the coherence transfer mechanism are similar to the (CACO)NCA experiment. The magnetization pathway of this experiment is given by

$${}^{13}\mathrm{C}\alpha^i \rightarrow {}^{15}\mathrm{N}^i \rightarrow {}^{13}\mathrm{CO}^{i-1} \rightarrow {}^{13}\mathrm{C}\alpha^{i-1} \tag{6}$$

and the final signal, after phase cycling of the receiver $\pm$, is given by

$$\sigma(\mathbf{I}+\mathbf{II}) = 2\{\exp[\mathrm{i}\Omega({}^{13}\mathrm{C}\alpha^{i-1})t_2] + \exp[\mathrm{i}\Omega({}^{13}\mathrm{CO}^{i-1})t_2]\}\cos[\Omega({}^{15}\mathrm{N}^i)t_1^{\mathrm{a}}] \tag{7}$$

The net signal is an $\Delta\Omega$-edited NCOCA spectrum from which the correlations ${}^{15}\mathrm{N}^i \rightarrow {}^{13}\mathrm{C}\alpha^{i-1}$ and ${}^{15}\mathrm{N}^i \rightarrow {}^{13}\mathrm{CO}^{i-1}$ may be obtained. In both experiments, magnetization originating on this central spin is transferred to (potentially) less well-resolved regions in the spectrum with the direction of transfer depending upon the coherence pathway of the experiment. One potential application of these experiments is the selective identification of $({}^{15}\mathrm{N}, {}^{13}\mathrm{C})$ resonances from residues adjacent to glycine in the peptide sequence [32]. Glycine ${}^{13}\mathrm{C}\alpha$ chemical shifts are typically offset by 5–10 ppm from the remainder of the protein residues making *soft*-${}^{13}\mathrm{C}\alpha$ filtration readily feasible [14].

Heteronuclear correlation spectra acquired with conventional (top) [7,8] and *soft*-triple resonance experiments (bottom) are shown in Fig. 2. The experiments were acquired on a sample of U-[^{13}C, ^{15}N]-*N*-formyl-Met-Leu-Phe-OH (MLF). The NCA correlation experiment appears in Fig. 2 (right). The three peaks correspond to the $({}^{15}\mathrm{N}^i, {}^{13}\mathrm{C}\alpha^i)$ correlations for Met [$(\Omega^{15}\mathrm{N}) = 125.2$ ppm, $(\Omega^{13}\mathrm{C}) = 52.0$ ppm)], Leu [$(\Omega^{15}\mathrm{N}) = 116.01$ ppm, $(\Omega^{13}\mathrm{C}) = 56.8$ ppm], and Phe [$(\Omega^{15}\mathrm{N}) = 107.8$ ppm, $(\Omega^{13}\mathrm{C}) = 54.4$ ppm]. Also shown are the ${}^{13}\mathrm{C}\alpha$ (center) and ${}^{13}\mathrm{CO}$ (right) regions of the NCOCA experiment in which the cross-peaks corresponding to ${}^{15}\mathrm{N}^i \rightarrow {}^{13}\mathrm{C}\alpha^{i-1}$ and ${}^{15}\mathrm{N}^i \rightarrow {}^{13}\mathrm{CO}^{i-1}$ appear. Note the gray box that highlights the bandwidth of the soft 180° inversion pulse utilized in the *soft*-triple resonance experiments below.

The spectra of MLF acquired with *soft*-triple resonance appear in the bottom panels of Fig. 2. The Gaussian profile only efficiently and selectively inverts the Leu ${}^{13}\mathrm{C}\alpha$ resonance. As expected by the coherence transfer pathway [1], magnetization which originates on the Leu residue is detected as a single resonance in the *soft*-(CACO)NCA experiment at the ^{15}N, ${}^{13}\mathrm{C}\alpha$ chemical shifts of the Phe residue (right). The two peaks that appear in the (CA)NCOCA experiment correspond to the coherence which has followed pathway [6] with peaks at the ^{15}N of Leu, ${}^{13}\mathrm{C}\alpha$ (center) and ${}^{13}\mathrm{CO}$ (left) of Met. Heteronuclear peaks associated with the two additional residues are effectively suppressed by the phase cycle.

The *soft*-triple resonance experiments of Fig. 1 may also be utilized to obtain *inter-residue* ^{13}C correlation spectra. These experiments are especially useful since two adjacent residues in the peptide sequence are correlated *within* a single experiment. Homonuclear spectra are obtained by setting the ^{15}N evolution time $t_1^{\mathrm{a}} = 0$ (omitting the 180° pulse in the center of the evolution period) and incrementing the ^{13}C evolution

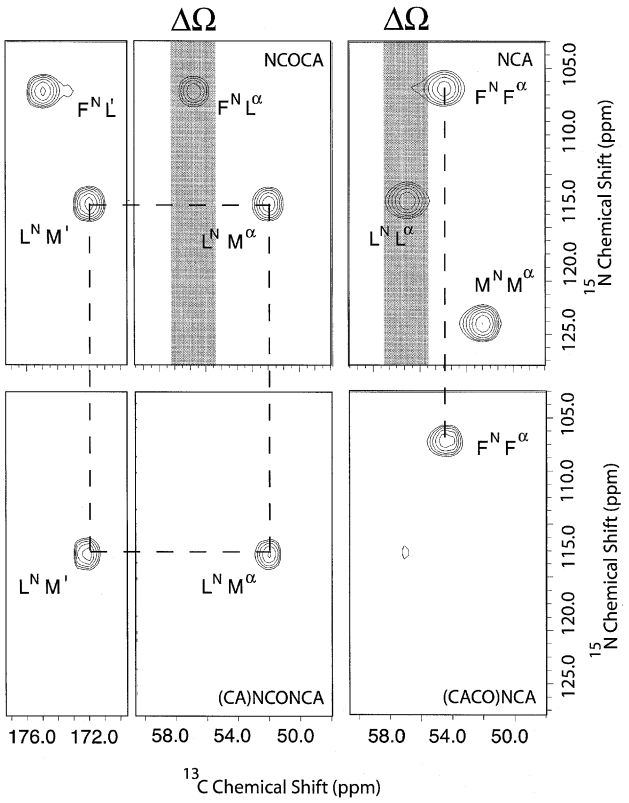

Fig. 2. Contour plots of heteronuclear correlation experiments. All spectra were demonstrated on a polycrystalline sample of U-[^{13}C, ^{15}N]-*N*-formyl-Met-Leu-Phe-OH (MLF) custom synthesized by SynPep (Dublin CA) with FMOC-protected U-[^{13}C, ^{15}N]-labeled amino acids purchased from Cambridge Isotope Labs (Andover, MA). The sample was re-crystallized from water and center packed in a 4-mm zirconia Chemagnetics (Fort Collins, CO) MAS PENCIL rotor NMR. Spectra were acquired at 8.4 T (360.336 MHz ^{1}H, 90.606 MHz ^{13}C, and 36.513 MHz ^{15}N) using a custom designed spectrometer (courtesy of David J. Rubin, FBML/MIT) and a Chemagnetics (Fort Collins, CO) triple resonance probe equipped with a 4-mm spinning module. The spinning frequency was set to 8.950 ± 2 Hz, regulated with a Doty-Scientific (Columbia, SC) spinning frequency controller. One hundred and twenty-eight scans were acquired per t_1 point. The ^{13}C and ^{15}N carrier were fixed at 62.5 and 110.3 ppm, respectively. The total acquistion time in ω_2 was 20 ms (1024 points, 50 kHz spectral width) and 22 ms in ω_1 (32 complex points, 1.48 kHz spectral width). The data were processed in the Molecular Simulations (Ventura, CA) software package FELIX. The first point of each data set was linear predicted in ω_2 followed by zero filling to 2 K points, Fourier transform and baseline correction. The data linear predicted to 64 complex points in ω_1, zero filled to 128 points and Fourier transformed to give the final spectrum (2048×128 points.) All spectra were indirectly referenced to a sample of solid U-[^{13}C, ^{15}N]-glycine assuming a $\Omega(^{13}\mathrm{CO})$ of 176.0 ppm. The NCA correlation spectrum appears in (a); a single $^{15}\mathrm{N} \rightarrow {}^{13}\mathrm{C}\alpha$ correlation appears for each residue (designated by three letter amino acid abbreviations.) The $^{13}\mathrm{C}\alpha$ and ^{13}CO regions of the NCOCA spectrum appear in (b) and (c), respectively. Highlighted in gray is the excitation profile of the selective pulse used in the *soft*-(CACO)NCA and *soft*-(CA)NCOCA experiments. The spectra were recorded using the pulse sequences in Fig. 1a and b, incrementing t_1^a with $t_1^b = 0$. Vertical dashed lines denote resonances that have evolved at the same frequency in ω_2 and horizontal lines note resonances that have evolved at identical frequency in ω_1.

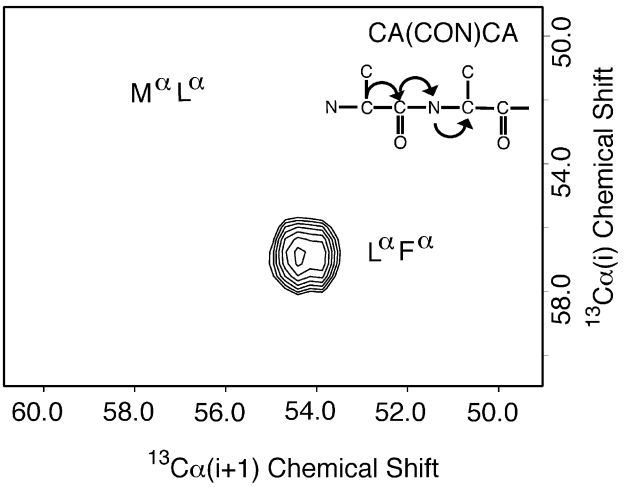

Fig. 3. Inter-residue ^{13}C homonuclear correlation spectrum recorded using the pulse sequence in Fig. 1a. The coherence transfer pathway is presented in the upper right. The spectrum was acquired setting $t_1^a = 0$ and incrementing the evolution period t_1^b. The sample and most experimental parameters are identical those presenting in Fig. 2. The total acquisition time in ω_1 is 7.2 ms (32 complex points, 4.45 kHz spectral width.) The data was processed and referenced as in Fig. 2. The single peak corresponding to the Leu-$^{13}\mathrm{C}\alpha \rightarrow$ Phe-$^{13}\mathrm{C}\alpha$ correlation is shown. The approximate position of the Met-$^{13}\mathrm{C}\alpha \rightarrow$ Leu-$^{13}\mathrm{C}\alpha$ is also noted.

period t_1^b. The signal for the CA(CON)CA experiment is:

$$\sigma(\mathbf{I}+\mathbf{II}) = \exp[\mathrm{i}\Omega(^{13}\mathrm{C}\alpha^{i+1})t_2]\cos[\Omega(^{13}\mathrm{C}\alpha^{i})t_1^b] \quad (8)$$

In large or heterogeneous systems, homonuclear *J*-decoupling should be applied during the indirect evolution period to further improve the resolution [33].

Presented in Fig. 3 is a CA(CON)CA spectrum of MLF. As expected, there is a single peak in the two-dimensional spectrum, located at the Leu $^{13}\mathrm{C}\alpha$ chemical shift in the indirect dimension and the Phe $\Omega^{13}\mathrm{C}\alpha$ chemical shift in the directly detected dimension. A peak from the correlation of Met and Leu, expected in a non-selective version of this experiment, has been suppressed; its approximate position is also noted in Fig. 3.

A CA(N)COCA experiment may also be obtained in a similar fashion. The detected signal for this experiment is

$$\sigma(\mathbf{I}+\mathbf{II}) = \{\exp[\mathrm{i}\Omega(^{13}\mathrm{CO}^{i-1})t_2] + \exp[\mathrm{i}\Omega(^{13}\mathrm{C}\alpha^{i-1})t_2]\}\cos[\Omega(^{13}\mathrm{C}\alpha^{i})t_1^b] \quad (9)$$

Additional resolution in ω_1 of both experiments can be obtained if the $(\Omega^{13}\mathrm{C}\alpha^i)$ and $(\Omega^{15}\mathrm{N}^i)$ are simultaneously encoded by setting $t_1^b = \kappa t_1^a$ with κ the chemical shift scaling factor [34–37].

Sequential resonance assignments for each member of the "couplet" may be obtained by recording a pair of inter-residue ^{13}C homonuclear correlation experiments. The $^{13}\mathrm{C}\alpha^i$ chemical shift of the central spin in the "couplet" is recorded in the indirect dimension in both experiments. The $^{13}\mathrm{C}\alpha^i$ is correlated to the $^{13}\mathrm{C}\alpha^{i-1}$ in the CA(N)COCA and to the $^{13}\mathrm{C}\alpha^{i+1}$ in the CA(CON)CA

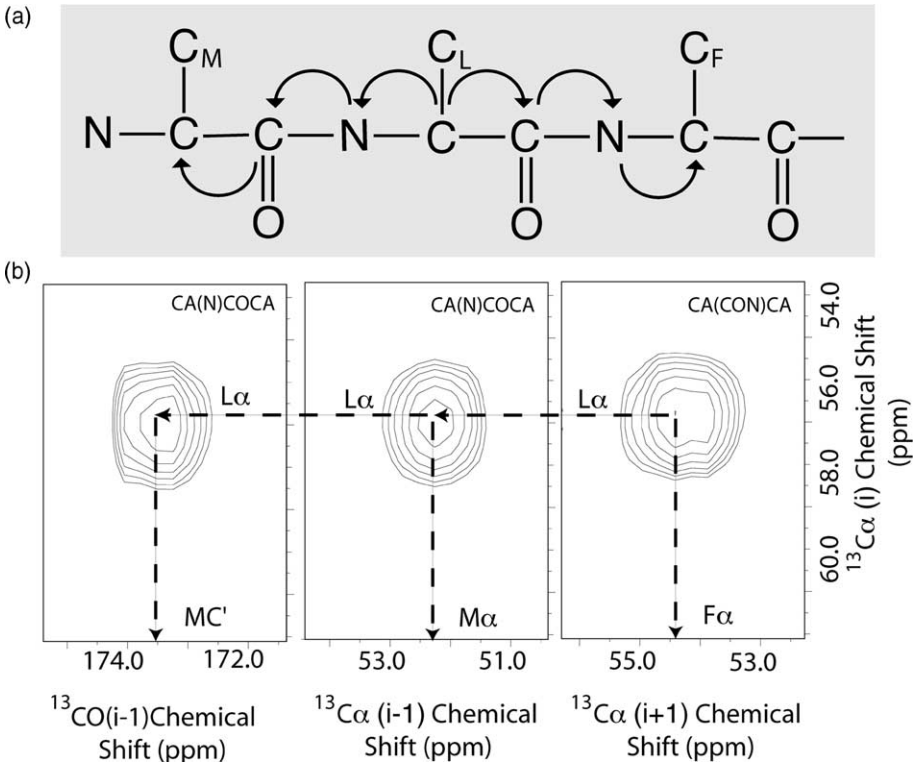

Fig. 4. Coherence transfer pathways (top) and magnified contour plots (bottom) of *soft*-homonuclear ^{13}C–^{13}C correlation spectra using the pulse sequences in Fig. 1a and b. Both spectra were acquired setting $t_1^a = 0$ and incrementing the evolution period t_1^b. The sample and most experimental parameters are identical those presenting in Figs. 2 and 3. Vertical and horizontal lines indicate resonances that have evolved at the same frequency in ω_1 and ω_2. The three panels correspond to the $^{13}C\alpha$ region of the CA(CON)CA experiment, the $^{13}C\alpha$ region of the CA(N)COCA experiment and the ^{13}CO region of the CA(N)COCA experiment. The arrows mark the 'sequential walk' from $\omega_2 = \text{Phe-}\Omega(^{13}C\alpha^{i+1}) \rightarrow \omega_1 = \text{Leu-}\Omega(^{13}C\alpha^{i})$ in the CA(CON)CA $\rightarrow \omega_1 = \text{Leu-}\Omega(^{13}C\alpha^{i}) \rightarrow \omega_2 = \text{Met-}\Omega(^{13}C\alpha^{i-1})$ and Met-$\Omega(^{13}CO^{i-1})$ in the CA(N)COCA experiment.

(Fig. 4a). By identifying resonances in the two experiments with identical shifts in the indirect dimension, the sequential walk through the "couplet" may be obtained.

The 'sequential walk' assignment strategy is demonstrated in Fig. 4b. The correlation of $\Omega(^{13}C\alpha^{i+1})$ of Phe in ω_2 and $\Omega(^{13}C\alpha^{i})$ of Leu in ω_1 of the CA(CON)CA experiment is indicated by the dashed line in Fig. 4b (right). The correlation of $\Omega(^{13}C\alpha^{i-1})$ and $\Omega(^{13}CO^{i+1})$ of Met in ω_2 and $\Omega(^{13}C\alpha^{i})$ of Leu in ω_1 of the CA(N-CO)CA experiment appears in Fig. 4 (center-left). The sequential walk through the 'couplet' from $\Omega(^{13}C\alpha^{i+1})$ to $\Omega(^{13}C\alpha^{i})$ in the CA(CON)CA experiment 'across' the common ω_1 in the CA(N)COCA experiment at $\Omega(^{13}C\alpha^{i})$ to $\Omega(^{13}C\alpha^{i-1})$ and $\Omega(^{13}CO^{i-1})$ completes the correlation.

Well resolved or clusters of side-chain $^{13}C\beta$ resonances may also be used to enhance the spectral resolution. A *soft*-(CBCA)NCA experiment is generated by modifying the pulse sequence of Fig. 1a (data not shown). Both SPECIFIC CP periods should be optimized for transfer between $^{13}C\alpha$ and ^{15}N. The coherence transfer pathway for this experiment is given by:

$$^{13}C\beta^{i} \rightarrow {}^{13}C\alpha^{i} \rightarrow {}^{15}N^{i} \rightarrow {}^{13}C\alpha^{i} \qquad (10)$$

$^{13}C\beta \rightarrow {}^{13}C\alpha$ transfer is optimized by positioning the carrier in the aliphatic region of the spectrum and reducing the power of the homonuclear correlation mixing sequence. Despite the relative sensitivity of ^{13}C vs. ^{15}N detection, in some cases it may be preferable to apply ^{13}C homonuclear decoupling in the indirect dimension and detect on the ^{15}N channel [7,33].

In conclusion, we have demonstrated a new family of triple resonance experiments that utilize pre-existing resolution of one or more spins to obtain additional resolution in the more crowded regions of homonuclear and heteronuclear spectra. One potential application is to exploit the unique ^{15}N (i.e., proline), $^{13}C\alpha$ (i.e., glycine, threonine), and $^{13}C\beta$ (threonine, alanine) chemical shifts and dipolar coupling topologies of several amino acids as the basis for amino acid selective triple resonance correlation experiments [32,38–42]. Other applications include crosschecking established resonance assignments and observation of selective regions of the spectrum for structural or ligand binding studies.

Acknowledgments

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