

## Accurate $^{13}\text{C}$ - $^{15}\text{N}$ Distance Measurements in Uniformly $^{13}\text{C}$ , $^{15}\text{N}$ -Labeled Peptides

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### Introduction

The ability to accurately measure  $^{13}\text{C}$ - $^{15}\text{N}$  dipolar couplings corresponding to internuclear distances in the 3-6 Å regime is important for constraining the three-dimensional structure of biological solids. Solid-state NMR (SSNMR) methods for heteronuclear distance measurements in isolated spin pairs are now well-established and will continue to provide valuable structural information [1,2]. However, these methods require synthesis of molecules isotopically labeled in a pairwise fashion, which can be both laborious and expensive. Thus, there is a clear motivation for the development of analogous SSNMR methods for larger spin systems, where multiple internuclear distances can be determined [3-5]. However, in multispin systems  $^{13}\text{C}$ - $^{15}\text{N}$  distance measurements have the potential to be complicated by the presence of multiple homonuclear and heteronuclear spin-spin couplings.

In the following we describe a magic angle spinning (MAS) NMR experiment for selective recoupling of  $^{13}\text{C}$ - $^{15}\text{N}$  dipolar interactions in uniformly  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled solids [6] and use it to measure multiple  $^{13}\text{C}$ - $^{15}\text{N}$  distances in the 3-6 Å range in a U- $^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled tripeptide, N-formyl-L-Met-L-Leu-L-Phe.

### Methods

The selective recoupling of  $^{13}\text{C}$ - $^{15}\text{N}$  interactions in a U- $^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled system is accomplished by the pulse sequence shown in Fig. 1. The experiment employs simultaneous frequency selective  $\pi$  pulses applied to the  $^{13}\text{C}$ - $^{15}\text{N}$  spin pair of interest,

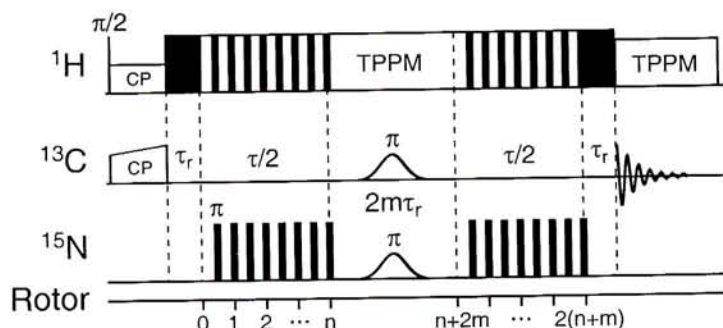


Fig. 1. Frequency selective REDOR pulse sequence. Ramped CP [7] creates the initial  $^{13}\text{C}$  magnetization and  $^{13}\text{C}$ - $^{15}\text{N}$  couplings are reintroduced in a nonselective fashion using REDOR [8]. Selective recoupling of  $^{13}\text{C}$ - $^{15}\text{N}$  spin pairs is achieved by the generation of a  $^{13}\text{C}$  spin echo in a frequency selective fashion using a pair of rotor-synchronized Gaussian  $\pi$  pulses. Couplings to  $^1\text{H}$  during the  $^{13}\text{C}$ - $^{15}\text{N}$  recoupling period are attenuated using a combination of CW and TPPM [9] decoupling (represented by filled and hollow rectangles, respectively). Reference ( $S_0$ ) experiments are obtained by acquiring spectra in the absence of the  $^{15}\text{N}$  selective pulse.

bracketed by two identical periods of nonselective dipolar recoupling. We assume: (i) heteronuclear recoupling sequences with an effective dipolar Hamiltonian of the form  $H_D = \sum_{i,j} \omega_D^{ij} 2C_i N_j$  (where the  $C$  and  $N$  operators refer to  $^{13}\text{C}$  and  $^{15}\text{N}$  spins, respectively, and  $\omega_D^{ij}$  is the orientation dependent  $C_i$ - $N_j$  dipolar coupling), and (ii) the weak coupling limit for  $^{13}\text{C}$ - $^{13}\text{C}$  J couplings ( $H_J = 2\pi J_{CC} C_{1z} C_{2z}$ ).

It can be shown [6] that the observable  $^{13}\text{C}$  signal for the complete pulse sequence with the selective pulses applied to spins  $C_k$  and  $N_l$  is given by [8]:

$$S(\tau) \propto \int_0^\pi d\beta \sin(\beta) \int_0^{2\pi} d\gamma \cos(\omega_D^{kl} \tau), \quad (1)$$

where  $\tau$  is the total dipolar evolution time (see Fig. 1),  $\omega_D^{kl}$  is the effective  $C_k$ - $N_l$  dipolar coupling (*vide infra*), which depends on the Euler angles,  $\beta$  and  $\gamma$ , defining the orientation of the dipolar tensor in the rotor-fixed reference frame (the integrals indicate the average over all orientations of the dipolar tensor in the powder sample). In other words, the  $^{13}\text{C}$ - $^{15}\text{N}$  dipolar coupling for the spin pair irradiated by both selective pulses is retained, while all remaining  $^{13}\text{C}$ - $^{15}\text{N}$  dipolar couplings and  $^{13}\text{C}$ - $^{13}\text{C}$  J couplings to the  $^{13}\text{C}$  spin of interest are refocused [6,10]. In the present implementation of the experiment REDOR [8] was employed for nonselective heteronuclear recoupling since it is well-compensated for pulse imperfections. Hence, we refer to the new experiment as frequency selective REDOR (FSR).

Since high-field studies of U- $^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled systems normally employ high MAS frequencies ( $\omega_r/2\pi \approx 10$ -30 kHz), we briefly consider the performance of REDOR

(a)

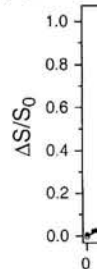


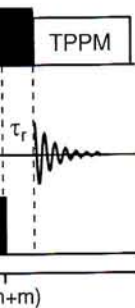
Fig. 2. Finite  $^{13}\text{C}$ ,  $^{15}\text{N}$  glyci... ( $\omega_r/2\pi = 15.1$  kHz) effective dipolar coupling of the effective dipolar coupling of the theoretical experiments. The experiments were performed at 15.152 kHz (15.152 kHz CW duration was 10  $\mu\text{s}$ .) from aqueous...

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[7] creates the initial  $^{13}\text{C}$  spin echo in a selective fashion using REDOR generation of a  $^{13}\text{C}$  spin echo using a combination of rectangular pulses, respectively). In the absence of the  $^{15}\text{N}$  selective

dipolar coupling. We assume: (i) the effective Hamiltonian of the form  $H_{\text{eff}}^{(1)}$  to  $^{13}\text{C}$  and  $^{15}\text{N}$  spins, (ii) the effective dipolar coupling, and (iii) the effective Hamiltonian. For the complete pulse sequence given by [8]:

(1)

the effective  $\text{C}_k\text{-N}_l$  dipolar coupling constant,  $b_{CN}$ , is proportional to the inverse cube of the internuclear distance,  $r_{CN}$ , and  $\varphi$  is the fraction of the rotor period,  $\tau_r$ , occupied by  $^{15}\text{N}$   $\pi$  pulses of length  $\tau_p$ :

Experiments normally employ high magnetic field to improve the performance of REDOR

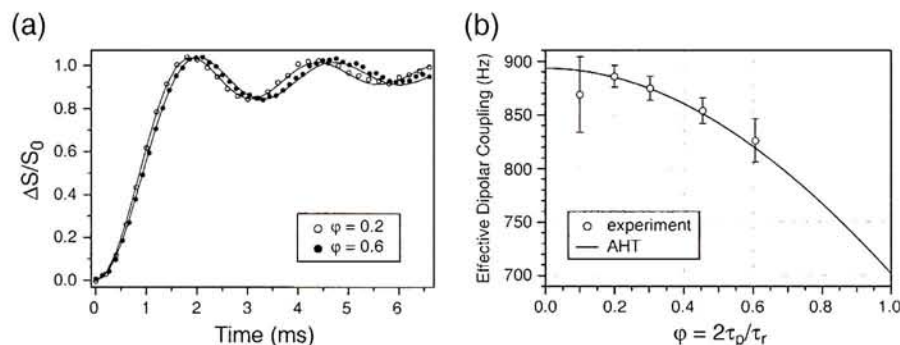


Fig. 2. Finite pulse effects during REDOR. (a) Experimental REDOR  $\Delta S/S_0$  curves for  $[2\text{-}^{13}\text{C}, ^{15}\text{N}]$ glycine recorded with: ( $\circ$ )  $\varphi=0.2$  ( $\omega_r/2\pi=10$  kHz,  $\omega_{rf}(^{15}\text{N})/2\pi=50$  kHz) and ( $\bullet$ )  $\varphi=0.6$  ( $\omega_r/2\pi=15.152$  kHz,  $\omega_{rf}(^{15}\text{N})/2\pi=25$  kHz). Simulations are shown as solid lines with best-fit effective dipolar couplings of  $886\pm 10$  Hz for  $\varphi=0.2$  and  $826\pm 20$  Hz for  $\varphi=0.6$ . (b) Comparison of the effective dipolar couplings determined for  $[2\text{-}^{13}\text{C}, ^{15}\text{N}]$ glycine as a function of  $\varphi$  ( $\circ$ ) and the theoretical curve (solid line) obtained using average Hamiltonian theory (cf. Eq. 3). All experiments were performed at 11.7 T. The spinning frequency was varied between 5.0 and 15.152 kHz (controlled to  $\pm 5$  Hz) and  $^{15}\text{N}$  rf fields were 25–50 kHz. The  $^1\text{H}$  decoupling was 83 kHz CW during REDOR and 83 kHz TPPM [9] during acquisition. The  $^{13}\text{C}$  refocusing pulse was 10  $\mu\text{s}$ .  $[2\text{-}^{13}\text{C}, ^{15}\text{N}]$ glycine (not diluted in natural abundance material) was recrystallized from aqueous solution, and experiments were performed at room temperature.

under conditions where the rf irradiation occupies a significant fraction of the rotor period. For the REDOR sequence shown in Fig. 1, where all  $\pi$  pulses are applied to the non-observed ( $^{15}\text{N}$ ) spins and phase-alternated according to  $xy\text{-}4$  or extensions thereof [11], the first-order effective Hamiltonian for the C-N dipolar coupling is given by [12]

$$H_D^{(1)} = \omega_D 2C_z N_z \quad (2)$$

with

$$\omega_D = -\frac{\sqrt{2}}{\pi} b_{CN} \frac{\cos(\frac{\pi}{2}\varphi)}{1-\varphi^2} \sin(2\beta)\sin(\gamma). \quad (3)$$

The C-N dipolar coupling constant,  $b_{CN}$  is proportional to the inverse cube of the internuclear distance,  $r_{CN}$ , and  $\varphi$  is the fraction of the rotor period,  $\tau_r$ , occupied by  $^{15}\text{N}$   $\pi$  pulses of length  $\tau_p$ :

$$\varphi = \frac{2\tau_p}{\tau_r}. \quad (4)$$

We note that Eq. 3 differs from the expression for ideal  $\delta$ -pulse REDOR [8] only by the scaling factor  $\cos(\varphi\pi/2)/(1-\varphi^2)$ . This means that  $b_{CN}$  can be scaled down by as much as  $\pi/4$  due to finite  $^{15}\text{N}$   $\pi$  pulses. However, for most experimental conditions of practical interest the scaling factor remains close to unity.

## Results and Discussion

In Fig. 2 we investigate the finite pulse effects on REDOR experiments in a model two-spin system,  $[2-^{13}\text{C}, ^{15}\text{N}]$ glycine, where the fraction of the rotor period occupied by the rf pulses was varied between  $\sim 0.1$  and  $0.6$ . As predicted by average Hamiltonian theory (AHT) [13], a minor scaling ( $<10\%$ ) of the dipolar oscillation frequency is observed as  $\varphi$  increases from  $0.1$  to  $0.6$  and the overall agreement between the experiments and AHT is good.

Having considered the performance of REDOR at high MAS frequencies required for our experiments, we proceed with the discussion of the FSR experiment. In Fig. 3 we qualitatively investigate the selective recoupling of  $\text{C}^\beta\text{-N}'$  and  $\text{C}^\beta\text{-N}^{\delta 2}$  interactions in  $[U\text{-}^{13}\text{C}, ^{15}\text{N}]$ asparagine. (Asparagine represents a favorable model system for selective distance measurements in multispin clusters since the  $\text{C}^\alpha$  and  $\text{C}^\beta$  as well as  $\text{N}'$  and  $\text{N}^{\delta 2}$  resonances are well-resolved (spectra not shown).) A  $1.0$  ms Gaussian pulse is applied to the  $\text{C}^\beta$  resonance and  $^{15}\text{N}$  Gaussian pulses are applied at different

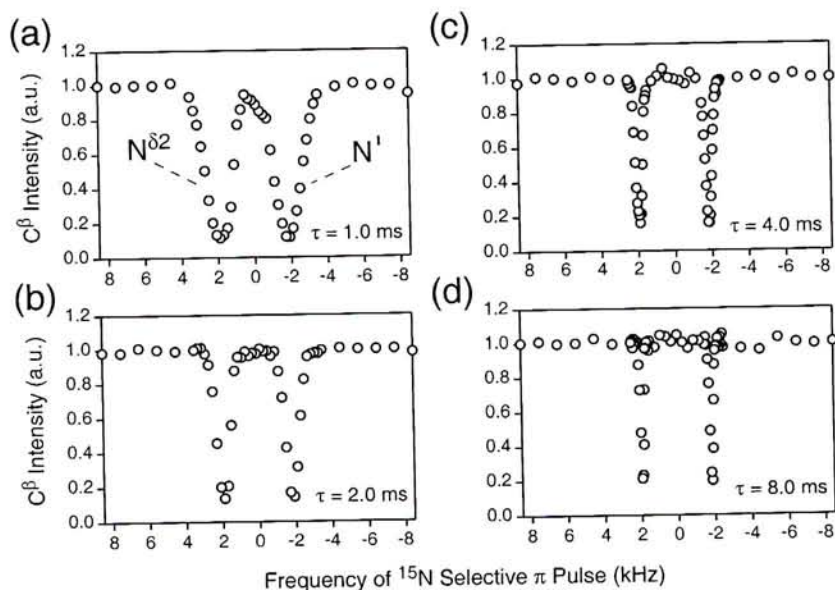


Fig. 3. Selective recoupling of  $\text{C}^\beta\text{-N}'$  and  $\text{C}^\beta\text{-N}^{\delta 2}$  dipolar interactions in  $[U\text{-}^{13}\text{C}, ^{15}\text{N}]$ asparagine. The FSR pulse sequence shown in Fig. 1 was used with the total dipolar evolution time  $\tau = 8.0$  ms. All experiments were performed at  $\omega_r/2\pi = 10$  kHz  $\pm 5$  Hz with a  $1.0$  ms Gaussian pulse applied to the  $\text{C}^\beta$  resonance. In (a)-(d) the results of 'frequency sweep' experiments are shown for  $^{15}\text{N}$  Gaussian pulses of: (a)  $1.0$ , (b)  $2.0$ , (c)  $4.0$  and (d)  $8.0$  ms. The bandwidths outside of which negligible recoupling occurs were found to be approximately: (a)  $\pm 2000$ , (b)  $1000$ , (c)  $500$  and (d)  $250$  Hz. Each experimental point ( $\circ$ ) corresponds to the ratio of the intensity of the  $\text{C}^\beta$  resonance in the presence and absence of the  $^{15}\text{N}$  Gaussian pulse.

(a)

(b)

1.0-  
0.8-  
 $\Delta S/S_0$   
0.6-  
0.4-  
0.2-  
0.0-

Fig. 4. (a)  $^{13}\text{C}$  Leu-Phe re-  
measurements  
and analytic  
Leu( $\text{C}^\beta$ ), (b)  
kHz  $\pm 5$  Hz  
2.0 and 10.0  
 $^{13}\text{C}, ^{15}\text{N}]$ Met-  
measurements

frequencies  
and  $\text{C}^\beta\text{-N}^{\delta 2}$   
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duration of  
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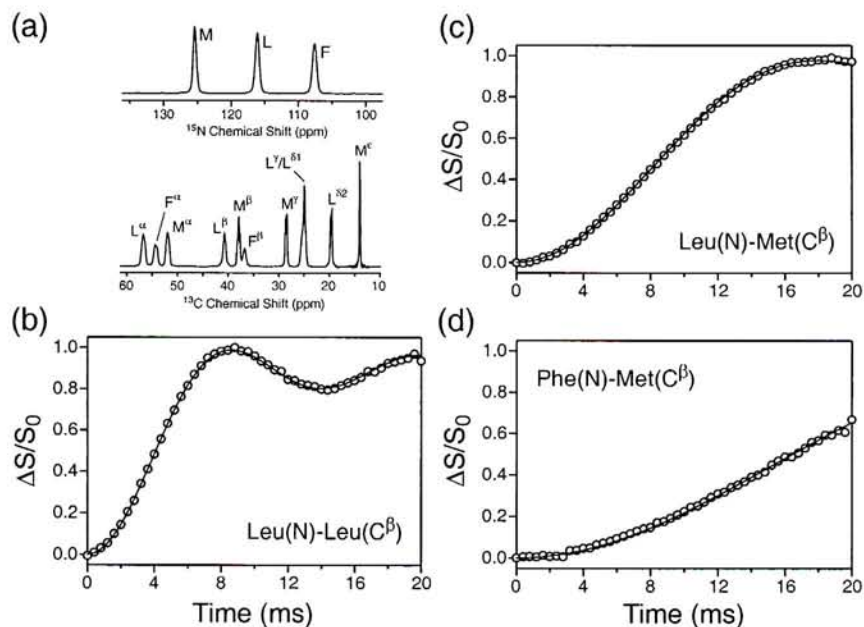


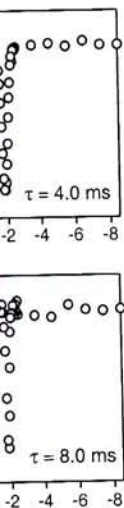
Fig. 4. (a)  $^{13}\text{C}$  (only aliphatic region shown) and  $^{15}\text{N}$  MAS spectra of N-formyl-[U- $^{13}\text{C}$ ,  $^{15}\text{N}$ ]Met-Leu-Phe recorded at  $\omega_r/2\pi=10$  kHz. In (b)-(d) representative internuclear distance measurements in N-formyl-[U- $^{13}\text{C}$ ,  $^{15}\text{N}$ ]Met-Leu-Phe are shown. Experimental  $\Delta S/S_0$  curves ( $\circ$ ) and analytical simulations (solid lines) are shown for selective recoupling of: (a) Leu(N)-Leu( $\text{C}^\beta$ ), (b) Leu(N)-Met( $\text{C}^\beta$ ) and (c) Phe(N)-Met( $\text{C}^\beta$ ). Experiments were performed at  $\omega_r/2\pi=10$  kHz  $\pm 5$  Hz and 32 scans were averaged for each time point.  $^{13}\text{C}$  and  $^{15}\text{N}$  Gaussian pulses were 2.0 and 10.0 ms, respectively. The experiments shown here were performed on N-formyl-[U- $^{13}\text{C}$ ,  $^{15}\text{N}$ ]Met-Leu-Phe undiluted in the natural abundance peptide. The summary of all distance measurements in a diluted N-formyl-[U- $^{13}\text{C}$ ,  $^{15}\text{N}$ ]Met-Leu-Phe sample is given in Fig. 5.

frequencies in the  $^{15}\text{N}$  spectrum. Fig. 3 demonstrates the selective recoupling of  $\text{C}^\beta\text{-N}'$  and  $\text{C}^\beta\text{-N}^{\delta 2}$  interactions using the frequency selective spin-echo generated by the two selective pulses. The  $\text{C}^\beta$  signal is dephased only by the  $^{15}\text{N}$  spins on-resonance with the Gaussian pulse, while couplings to off-resonance nuclei are refocused by the pulse sequence. The spectral selectivity of the FSR experiment can be tuned by adjusting the duration of the  $^{15}\text{N}$  Gaussian pulse (e.g., the recoupling bandwidth obtained with a 8 ms pulse is  $\sim \pm 250$  Hz).

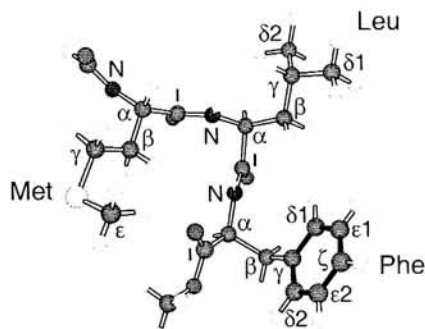
Representative FSR distance measurements in N-formyl-[U- $^{13}\text{C}$ ,  $^{15}\text{N}$ ]Met-Leu-Phe (MLF) (sample not diluted in natural abundance peptide) are shown in Fig. 4. The  $^{13}\text{C}$  and  $^{15}\text{N}$  MAS spectra of MLF (Fig. 4a) suggest that distance measurements between most sidechain  $^{13}\text{C}$  and backbone  $^{15}\text{N}$  should be feasible. Indeed, the use of  $^{15}\text{N}$  pulses in the 6-10 ms range enables the selective  $^{13}\text{C}$ - $^{15}\text{N}$  recoupling, and in a [U- $^{13}\text{C}$ ,  $^{15}\text{N}$ ]MLF sample diluted to  $\sim 10\%$  in natural abundance MLF we have measured 15 sidechain to

s in a model two-occupied by the hamiltonian theory cy is observed as experiments and

MAS frequencies SR experiment. In  $\beta\text{-N}'$  and  $\text{C}^\beta\text{-N}^{\delta 2}$  ble model system and  $\text{C}^\beta$  as well as ms Gaussian pulse ifferent



$^{13}\text{C}$ ,  $^{15}\text{N}$ ]asparagine. evolution time  $\tau = 8.0$  ms Gaussian pulse experiments are shown bandwidths outside of  $\pm 2000$ , (b) 1000, (c) of the intensity of the



atoms		NMR (Å)	X-ray (Å)
M(N)	M(C <sup>β</sup> )	2.52 ± 0.02	2.50
	M(C <sup>γ</sup> )	3.20 ± 0.03	3.04
	M(C <sup>ε</sup> )	5.4 ± 0.3	5.71
	L(C <sup>β</sup> )	5.7 ± 0.7	6.03
	L(C <sup>δ2</sup> )	5.5 ± 0.3	6.28
L(N)	M(C <sup>β</sup> )	3.12 ± 0.03	3.20
	M(C <sup>γ</sup> )	4.17 ± 0.10	4.56
	M(C <sup>ε</sup> )	5.5 ± 0.3	5.93
	L(C <sup>β</sup> )	2.46 ± 0.02	2.50
	L(C <sup>δ2</sup> )	3.64 ± 0.09	3.63
F(N)	M(C <sup>β</sup> )	4.12 ± 0.15	4.06
	M(C <sup>γ</sup> )	4.8 ± 0.2	5.43
	M(C <sup>ε</sup> )	5.2 ± 0.3	5.62
	L(C <sup>β</sup> )	3.24 ± 0.12	3.12
	L(C <sup>δ2</sup> )	5.4 ± 0.3	5.38

Fig. 5. X-ray structure of N-formyl-Met-Leu-Phe-OMe [14] and the summary of internuclear distances in N-formyl-[U-<sup>13</sup>C,<sup>15</sup>N]Met-Leu-Phe determined using frequency selective REDOR. The distances were measured in a N-formyl-[U-<sup>13</sup>C,<sup>15</sup>N]Met-Leu-Phe sample diluted to ~10% in natural abundance peptide to minimize intermolecular <sup>13</sup>C-<sup>15</sup>N couplings (uncertainties are reported at the 95% level). For comparison with the NMR results, we include the corresponding X-ray distances in N-formyl-Met-Leu-Phe-OMe.

backbone <sup>13</sup>C-<sup>15</sup>N distances in the 3-6 Å range with the precision of ~0.1-0.3 Å [6]. The distance measurements are summarized in Fig. 5. Since no X-ray structure has been reported for N-formyl-L-Met-L-Leu-L-Phe, the structure of its methyl ester analogue, N-formyl-L-Met-L-Leu-L-Phe-OMe [14] is shown for comparison. We note here that a set of solid state NMR constraints for N-formyl-Met-Leu-Phe has been compiled by combining the <sup>13</sup>C-<sup>15</sup>N distances determined above with multiple φ, ψ and χ dihedral angle constraints [15]. The calculation of the three-dimensional structure of the peptide based on these constraints is currently in progress [16].

### Conclusions

To date most solid state NMR structural studies have employed methods applicable to molecules containing labeled spin pairs (<sup>13</sup>C-<sup>13</sup>C, <sup>13</sup>C-<sup>15</sup>N, etc.). Furthermore, it has been assumed that certain of these methods function best at slow spinning frequencies where resolution is compromised. Here we have shown that it is possible to perform heteronuclear recoupling experiments such as REDOR in the rapid MAS regime, and to accurately measure multiple <sup>13</sup>C-<sup>15</sup>N distances in uniformly <sup>13</sup>C,<sup>15</sup>N-labeled molecules. In the studies of the tripeptide N-formyl-Met-Leu-Phe we have performed accurate measurements of 15 distances that constrain the structure of the molecule. This is one of the initial investigations to achieve this goal, and we anticipate that the approaches described here will be applicable to larger systems, and will stimulate further developments along these lines.

### Acknowledgments

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(Å)	X-ray (Å)
0.02	2.50
0.03	3.04
0.3	5.71
0.7	6.03
0.3	6.28
0.03	3.20
0.10	4.56
0.3	5.93
0.02	2.50
0.09	3.63
0.15	4.06
0.2	5.43
0.3	5.62
0.12	3.12
0.3	5.38

Summary of internuclear  
 distance selective REDOR  
 experiments diluted to ~10% in  
 samples (uncertainties are  
 included the corresponding

of ~0.1-0.3 Å [6]. The  
 crystal structure has been  
 determined for the methyl ester analogue,  
 and we note here that a  
 list has been compiled by  
 us for the  $\phi$ ,  $\psi$  and  $\chi$  dihedral  
 angles of the peptide

methods applicable to  
 peptides. Furthermore, it has  
 been shown that by using  
 magic spinning frequencies  
 it is possible to perform  
 distance MAS regime, and to  
 measure distances in  
<sup>15</sup>N-labeled molecules.  
 We have performed accurate  
 distance measurements on a  
 peptide molecule. This is one of  
 the first examples to show  
 that the approaches  
 described here will stimulate further

## Acknowledgements

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