Determination of an 8-Å Interatomic Distance in a Helical Peptide by Solid-State NMR Spectroscopy

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Abstract: The combination of transferred-echo double resonance (TEDOR) with rotational-echo double resonance (REDOR) has been used to measure an 8-Å fluorine–carbon internuclear distance in a nine-residue fragment of the peptide antibiotic emerimicin. The fragment is $^{19}FCH_2CO$-Phe-MeA-MeA-[1-^{13}C]MeA-[^{15}N]Val-Gly-Leu-MeA-MeA-OBzl (MeA = α-methylalanine or aminoisobutyric acid). The TEDOR part of this magic-angle-spinning, solid-state NMR experiment selects the ^{13}C label by its dipolar coupling to ^{15}N and suppresses the natural-abundance carbon background. The REDOR part of the experiment measures dipolar coupling of the selected carbon to ^{19}F. The TEDOR-REDOR combined experiment works with a variety of spin $^1/_2$ nuclei and can be used to characterize internuclear distances and geometry in macromolecular aggregates that do not crystallize.

Recent advances in X-ray crystallography and solution-state NMR spectroscopy have yielded three-dimensional structures of many soluble proteins and peptides.[1-3] Unfortunately, the same sort of information, which is essential in elucidating mechanisms of action, is largely unavailable for molecules which function within biological membranes. The problem lies primarily in the inability to prepare samples in a crystalline form suitable for X-ray analysis or to interpret NMR spectra displaying the broad lines of membrane-bound species with solid-like properties.

We have previously demonstrated[4] the utility of solid-state, rotational-echo double-resonance (REDOR) NMR spectroscopy[5,6] in providing accurate structural information for biological solids. REDOR involves the dephasing of transverse, S-spin magnetization by rotor-synchronized I-spin π pulses; I and S refer to different types of dipolar coupled rare spins. When the interactions with all other spins can be ignored or suppressed, the comparison of S-spin echo intensities with (S) and without (S_0) I-spin dephasing pulses leads directly to the strength of the I–S dipolar coupling and hence I–S internuclear distances.[4]

The natural-abundance S-spin background complicates the interpretation of REDOR experiments by a contribution to S_0 that is unrelated to I–S dipolar coupling. Sometimes this contribution can be measured in separate experiments on unlabeled samples.[7] This approach is generally successful if the concentration of label is high or the I–S dipolar coupling is strong. When these conditions are not met, we propose selecting the dipolar coupled spins from among the background of uncoupled spins by a coherence transfer from I to S. Dephasing of the selected S-signal by rotor-synchronized π pulses applied to a *third* rare spin (X) can now be used to measure S–X dipolar coupling and internuclear distances with no background interferences.

In this paper, we report the results of background-free distance measurements on a triple-labeled emerimicin 1–9. This peptide sequence is a fluorinated analogue of the N-terminal portion of the emerimicins III and IV, which are members of the peptaibol family of antibiotics known to function as ion channels in membranes.[8] The crystal structure of the nonfluorinated analogue has been previously determined to be α-helical.[9] The ^{19}F, which is part of a terminal CH_2F group, is one of the labels, while the other two labels tag the [1-^{13}C]MeA^4–[^{15}N]Val^5 peptide bond in the middle of the structure. Results of NMR dephasing experiments performed on this peptide show that when a high-γ nucleus like ^{19}F is used as a rare-spin label in background-free REDOR with ^{13}C observation, distances on the order of 10 Å can be measured with errors that are less than ±0.5 Å.

Experimental Section

Synthetic Methods. The labeled peptide was prepared by fluoroacetylation of Phe-MeA-MeA-[1-^{13}C]MeA-[^{15}N]Val-Gly-Leu-MeA-MeA-OBzl, which was synthesized by solution-phase fragment condensation as shown in Figure 1. The [^{15}N]valine was purchased from MSD Isotopes, and [1-^{13}C]α-methylalanine was prepared by the general Bücherer–Bergs hydantoin method from K^{13}CN (98.5 atom % ^{13}C; MSD Isotopes), acetone, ammonium chloride, and ammonium carbonate.[10] H-[1-^{13}C]MeA-OMe was prepared with 2,2-dimethoxypropane and concentrated HCl. An efficient and relatively safe procedure for the preparation of the highly toxic and moisture-susceptible FCH_2COCl was used, involving the chlorination of dried sodium fluoroacetate by means of phthaloyl dichloride.[11] To introduce the fluoroacetyl group, a FCH_2COCl fraction boiling at 69.5–71.5 °C was used. Equimolar amounts of FCH_2COCl and N-deprotected nonapeptide in the form of its hydrochloride salt were stirred in CH_2Cl_2 solution at room temperature for 2 h in the presence of 2.5 equiv of triethylamine. The final fluoroacetyl nonapeptide was isolated and then crystallized from a mixture of EtOAc/MeOH/hexane: mp 210–212 °C; $[\alpha]^{23}_D$ +11.2° (c 0.5, MeOH); FABMS consistent with the structure. The preparation of the fluorononapeptide is similar to that used to prepare α-helical nonapeptide samples for X-ray analysis.[9] Conformational analyses have revealed that MeA residues prefer helical torsion angles,[12] and surveys of peptides rich in MeA show that they are universally helical.[9,13]

For NMR studies in which the effects of *inter*molecular interactions between labels were minimized, 20 mg of labeled peptide and 180 mg of the analogous nonfluorinated, nonlabeled peptide were dissolved in EtOAc/MeOH and lyophilized. Repeated recrystallizations of this mixture together with prolonged annealing resulted in some microscopic mixing but still left inhomogeneities, as indicated by the presence of separate exotherms from fluorinated and nonfluorinated components in differential scanning calorimetry experiments.

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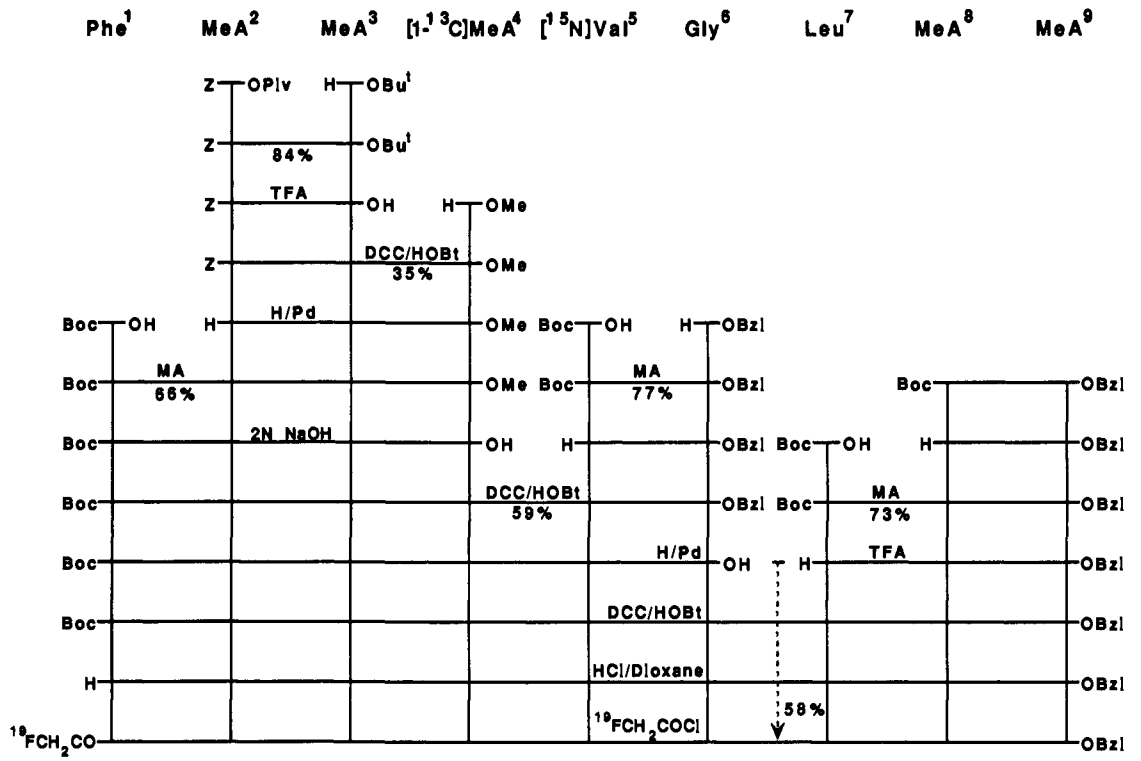

Figure 1. Synthetic scheme for the preparation of ^{19}F,^{13}C,^{15}N-labeled emerimicin I–9 N-terminal segment. Abbreviations: MeA = α-methylalanine (or Aib), OPiv = pivalic acid mixed anhydride, MA = mixed anhydride with isobutyl chloroformate, DCC = dicyclohexylcarbodiimide, OHBt = 1-hydroxybenzotriazole, TFA = trifluoroacetic acid.

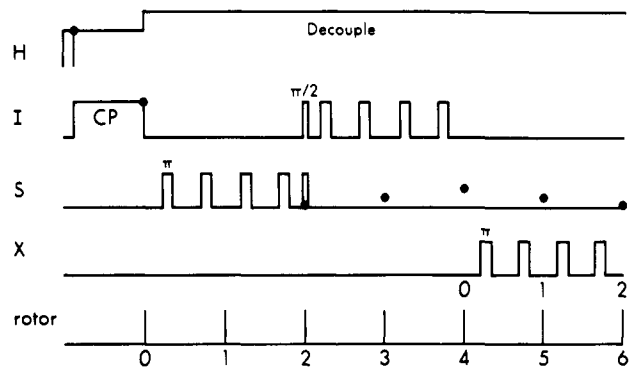

Figure 2. Pulse sequence for transferred-echo double resonance combined with rotational-echo double resonance (TEDOR REDOR). Following a cross-polarization transfer from abundant protons to generate initial magnetization, the coupling to protons is removed by resonant decoupling. The TEDOR part of the experiment (which for this illustration extends over the first four rotor periods following the cross-polarization transfer) selects S-spin magnetization by a coherence transfer from I spins. The REDOR part of the experiment immediately follows the TEDOR preparation and measures dipolar coupling of the selected S spin to a third rare spin X. The solid circles represent observable magnetization; π pulses are shown separated by half rotor periods.

Nuclear Magnetic Resonance Techniques. The pulse sequence for background-free distance measurements is shown in Figure 2. Transverse magnetization is first established in the I-spin system by a cross-polarization transfer from abundant protons. The dephasing of I-spin magnetization by dipolar coupled S spins through rotor-synchronized S-spin π pulses is then encoded by an I,S pair of $\pi/2$ pulses. These pulses occur together at the completion of a rotor cycle. Next, the coherence transferred to the S spins is translated into observable S-spin transverse magnetization by rotor-synchronized I-spin π pulses. Finally, the resulting S-spin signal is dephased by rotor-synchronized X-spin REDOR π pulses. The S-spin signal can be either observed synchronously (one sampling per rotor cycle) or observed as part of a two-dimensional experiment in which a full S-spin spectrum is obtained by normal acquisition starting at the beginning of a rotor cycle. The part of the experiment preceding the X-spin dephasing is called transferred-echo double resonance (TEDOR). The full procedure is called TEDOR REDOR. The TEDOR prepn. is itself rotational-echo double-resonance dephasing followed by a rotor-synchronized coherence transfer, the latter based on the same principles as an INEPT solution-state coherence transfer.[14]

TEDOR REDOR requires four radio-frequency channels in the same experiment. This has been accomplished for a spectrometer operating with a 4.7-T wide-bore Oxford magnet by the use of a coaxial transmission line connecting tuning components to a single, seven-turn, 9-mm-diameter solenoidal coil.[15] The spectrometer uses a commercial spinning system (Chemagnetics, Fort Collins, CO) and radio-frequency transmitters (ENI, Rochester, NY, for frequencies below 100 MHz, and Kalmus, Woodinville, WA, for frequencies above 100 MHz).

Simulations. The theoretical results were obtained by numerical calculations using a computer program[16] constructed to simulate NMR signals for a variety of magic-angle-spinning experiments on a spin system consisting of three coupled spins. All initial spatial orientations are specified with respect to the rotor axis. The program then computes the time-dependent elements of the evolution operator of this spin system. All possible combinations of chemical-shift and dipolar-interaction parameters can be considered during the calculation, assuming that no more than two of the three spins are involved in homonuclear coupling. Fourier transforms of summations of the resultant time-dependent NMR signals result in powder spectra. A sketch of the theoretical framework of the TEDOR-REDOR experiment is presented below.

The magic-angle-spinning Hamiltonian describing the dephasing of transverse I-spin magnetization (Figure 2) for an I–S dipolar coupled spin pair is

$$H(t) = \omega(t)\, I_Z S_Z \tag{1}$$

$$H(t) = \omega_D[G_1 \cos(\omega_R t + \phi) + G_2 \cos(2\omega_R t + 2\phi)] I_Z S_Z \tag{2}$$

where ω_D is the I–S dipolar interaction strength, ω_R is the spinning frequency, and G_1 and G_2 are geometric factors defined by the initial orientation of the distance vector between the spins and given by

$$G_1 = \tfrac{3}{4} \sin 2\theta_m \sin 2\theta \tag{3}$$

$$G_2 = \tfrac{3}{4} \sin^2 \theta_m \sin^2 \theta \tag{4}$$

In eqs 2–4, θ_m is the angle between the rotor spinning axis and the applied static magnetic field and θ and ϕ are the polar angles of the distance vector between the spins in the rotor-axis coordinate frame (see Figure

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1 of ref 6). When no dephasing pulses are applied on the I-spin system following the initial excitation, the I-spin chemical-shift anisotropy refocuses at the completion of each rotor period and can therefore be ignored. In the absence of pulses on the S-spin system, the coherent terms of the reduced I–S density matrix evolve as $I_X \cos \Omega_0(t) + 2I_YS_Z \sin \Omega_0(t)$, where $\Omega_0(t)$ is the time-dependent angle of dephasing:

$$\Omega_0(t) = \int_0^t \omega(t')\, dt' \quad (5)$$

which is zero at times equal to multiples of the rotor period, T_R.

In the presence of REDOR S-spin pulses, evolution of the I–S spin system depends on $\Omega(t)$ rather than $\Omega_0(t)$ where

$$\Omega(T_R) = \int_0^{t_1} \omega(t)\, dt - \int_{t_1}^{t_2} \omega(t)\, dt + \int_{t_2}^{T_R} \omega(t)\, dt \quad (6)$$

$$\Omega(T_R) = (4\omega_D/\omega_R)[G_1 \cos \phi \sin (\omega_R t_1) + \tfrac{1}{2}G_2 \cos \phi \sin (2\omega_R t_1)] \quad (7)$$

using the expression for $\omega(t)$ in eq 2 with $t_2 = T_R - t_1$. The times t_1 and t_2 are measured from the beginning of the rotor period until the first and second S-spin π pulses, respectively. We can define an effective (average) dephasing frequency

$$\omega_{eff} = \Omega(T_R)/T_R \quad (8)$$

such that the evolution of the spin system can be written as $I_X \cos (n\omega_{eff}T_R) + 2I_YS_Z \sin (n\omega_{eff}T_R)$. When the dephasing π pulses are separated by half-rotor periods ($t_1 = T_R/4$), the dephasing frequency from eqs 7 and 8 is $\omega_{eff} = 4\omega_D G_1 \cos \phi$. The I-spin signal for a powder is given by

$$S_I(t) = \int_0^{2\pi} d\phi \int_0^{\pi} \cos \theta\, d\theta \cos (n\omega_{eff}T_R) \quad (9)$$

where the integration takes into account the explicit dependence of ω_{eff} on θ and ϕ. This expression for the powder is the rotational-echo double-resonance dephased signal reported earlier.[6]

After n rotor periods of S-spin dephasing pulses (Figure 2), a pair of I,S $\pi/2$ pulses transfers the coherent bilinear term of the spin-density operator to $2I_ZS_Y \sin (n\omega_{eff}T_R)$. This non-observable term evolves for m rotor cycles under the influence of the dipolar interaction, I_ZS_Z, and rotor-synchronized I-spin π pulses as $\sin (n\omega_{eff}T_R)[2I_ZS_Y \cos (m\omega_{eff}T_R) - S_X \sin (m\omega_{eff}T_R)]$. At the end of the m rotor cycles, the observable S-spin coherence can be monitored as a TEDOR S-spin transferred magnetization which is proportional to $\sin (n\omega_{eff}T_R) \sin (m\omega_{eff}T_R)$. Depending on ω_{eff}, convenient values of n and m can be chosen to maximize the amplitude of the transferred magnetization.

In the full experiment illustrated in Figure 2, the I–S TEDOR preparation is used to generate transverse magnetization from only those S spins that are dipolar coupled to I spins. This S-spin magnetization is dephased by l rotor periods of synchronized REDOR pulses applied to a third spin, X. The resulting REDOR S-spin signal is proportional to $\sin (n\omega_{eff}^{IS}T_R) \sin (m\omega_{eff}^{IS}T_R) \cos (l\omega_{eff}^{SX}T_R)$ where the superscripts on ω_{eff} identify the types of heteronuclear dipolar interactions. This signal measures the dephasing of coherent S-spin transverse magnetization by the dipolar interaction of S with X and so leads directly to a distance measurement between S and X spins. The relationship between ω_{eff}^{IS} and ω_{eff}^{SX} is evaluated using the dependences of θ^{IS} and ϕ^{IS} on θ^{SX} and ϕ^{SX}, which, in turn, describe the orientation of the I–S distance vector with respect to the S–X distance vector.

Results and Discussion

The selectivity of TEDOR is illustrated by the 50.3-MHz ^{13}C NMR spectra of $^{19}FCH_2CO$-Phe-MeA-MeA-[1-^{13}C]MeA-[^{15}N]Val-Gly-Leu-MeA-MeA-OBzl (Figure 3). The carbon TEDOR spectrum (Figure 3, bottom) was obtained by a cross-polarization transfer from protons to nitrogens. Two rotor cycles of ^{13}C dephasing pulses preceded the simultaneous ^{15}N,^{13}C $\pi/2$ pulses, and two rotor cycles of ^{15}N refocusing pulses followed the coherence transfer. These values are close to the optimum for a ^{15}N–^{13}C peptide bond with a dipolar coupling of 1.2 kHz.[17] The TEDOR spectrum has only one line arising from ^{13}C directly bonded to ^{15}N. The natural-abundance background has been eliminated. The carbonyl carbon signal intensity from the labeled methylalanine residue is about 33% of that observed in a standard ^1H–^{13}C cross-polarization, magic-angle-spinning experiment. Calculations suggest that under the conditions of these experiments, about 40% of the carbonyl carbon intensity could be expected.[17] We attribute the difference to pulse imperfections in the transfer and refocusing parts of the experiment.

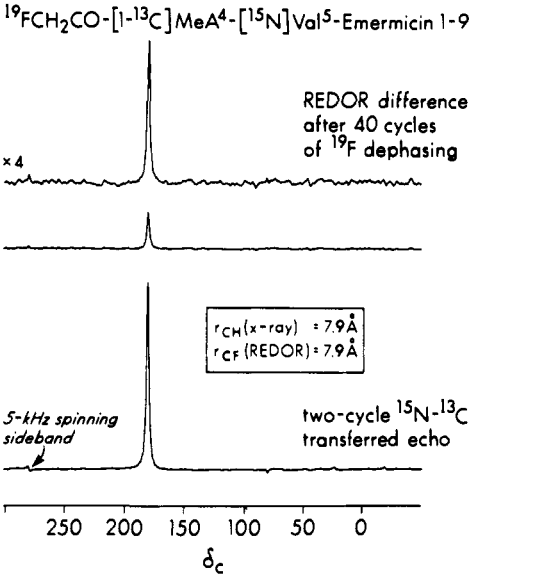

Figure 3. TEDOR-REDOR ^{13}C NMR spectra of a ^{19}F,^{15}N,^{13}C-triple-labeled peptide. The TEDOR spectrum (bottom) was obtained using a ^{15}N–^{13}C coherence transfer sandwiched between two rotor cycles of dephasing and refocusing pulses. This is close to the optimum for a coherence transfer between the ^{15}N and ^{13}C of a peptide bond. The TEDOR-REDOR difference spectrum (the difference between spectra with and without dephasing pulses) is shown at the top of the figure. This spectrum was obtained by dephasing the ^{15}N TEDOR-selected ^{13}C magnetization by 40 rotor cycles of ^{19}F π pulses. The ratio of the difference signal to the transferred-echo signal gives an r_{CF} of 7.9 ± 0.3 Å. Magic-angle spinning was at 5.000 kHz and decoupling at 76 kHz.

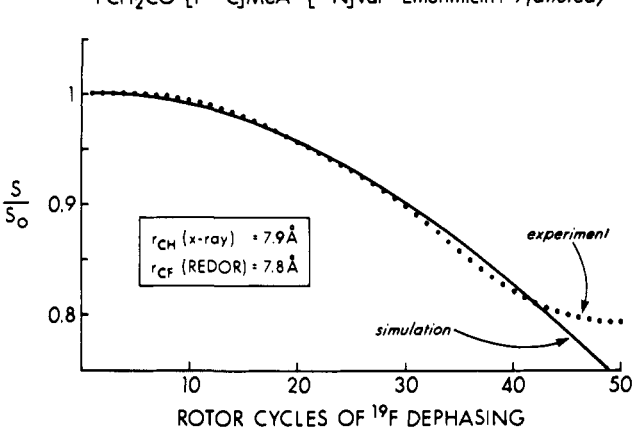

Figure 4. TEDOR-REDOR ^{13}C NMR data for a ^{19}F,^{15}N,^{13}C-triple-labeled peptide diluted 10-fold by natural-abundance peptide. The ^{15}N TEDOR-selected ^{13}C signal (which arises from 10 μmol of ^{13}C label and 40 000 accumulated scans) is sampled once each rotor period either with (S) or without (S_0) ^{19}F REDOR dephasing π pulses. The experimental ratios agree with the predictions of a three-spin simulation (with a best-fit r_{CF} of 7.8 Å) up to about 45 rotor cycles, at which point both S and S_0 are too small to yield an accurate ratio.

The second half of the TEDOR-REDOR experiment involves REDOR dephasing of the TEDOR-selected ^{13}C signal. The dephasing in the example of Figure 3 is by 40 rotor cycles of ^{19}F π pulses at half-rotor-period intervals. The difference between echo intensities with and without the dephasing ^{19}F pulses (ΔS) is 19% of the full-echo intensity (S_0). The difference spectrum is shown at the top of Figure 3. Three-spin simulations show that, for this experiment, the TEDOR preparation of S-spin magnetization does not seriously affect the REDOR powder averaging necessary to interpret $\Delta S/S_0$ in terms of D and hence the internuclear distance, r_{CF}.[15] A value of $\Delta S/S_0 = 0.19$ means r_{CF} is 7.9 Å,[6] consistent with the X-ray-determined value, assuming

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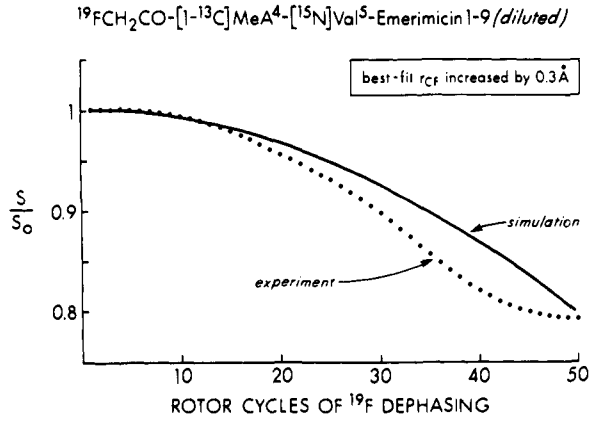

Figure 5. Comparison of the data of Figure 4 with a simulation assuming an r_{CF} of 8.1 Å.

the fluorinated analogue is isomorphous with the nonfluorinated peptide. This comparison also assumes rotameric averaging for the fluoromethyl group and ignores intermolecular F–C coupling. Small-amplitude main-chain motions that affect the dipolar coupling of the fluoromethyl group were taken into account in an approximate way as described in ref 4. This correction reduces the rigid-lattice dipolar coupling of a ^{19}F–^{13}C pair by about 5%.

Large-amplitude motions (other than C_3 rotation) which could affect the fluoromethyl group were judged to be not present. In particular, the fluoromethyl group is unlikely to be part of a floppy, dynamically active structural region because the amplitudes of spinning sidebands arising from aromatic side-chain carbons at the fluorinated end of the peptide were as great after 20 ms as those from main-chain carbonyl carbons in the middle of the peptide (data not shown). Furthermore, the middle of the peptide is known to be dynamically inactive.[4] In fact, the ^{15}N–^{13}C one-bond coupling for the MeA^4 peptide carbonyl carbon is close to the full rigid-lattice value.

Because the directly observed, TEDOR-selected ^{13}C signal is only a single line, it is practical to perform the ^{19}F REDOR dephasing with on-resonance synchronous ^{13}C detection (see Experimental Section). The amplitude of the ^{13}C signal is sampled once each rotor period. This version of the experiment is performed in two halves, each of which consists of a TEDOR preparation followed by synchronous detection. The first half has no dephasing ^{19}F pulses and yields S_0, while the second half has dephasing ^{19}F pulses and yields S. The experimental ratio, S/S_0, for a sample of $^{19}FCH_2CO$-[1-^{13}C]MeA^4-[^{15}N]Val^5-emerimicin 1–9 which had been diluted 10-fold by natural abundance material is plotted in Figure 4 and compared to the results of a three-spin simulation. The two are in agreement up to about $N_c = 45$, at which point the experimental S and S_0 are both small, as most transverse magnetization disappears. Changing r_{CF} by as little as 0.3 Å results in a misfit between experiment and simulation that is many times larger than the experimental error (Figure 5). However, uncertainties in the homogeneity of the labeled–unlabeled emerimicin mixture and the actual crystallographic r_{CF} make impossible establishing a rigorous error limit at this time. Additional experiments to determine the extent of heterogeneity are in progress.

In addition to ^{13}C, ^{15}N, and ^{19}F, we have performed TEDOR-REDOR experiments using ^{13}C, ^{15}N, and ^{31}P. Other combinations of spin-$^1/_2$ and spin-1 nuclei should also be possible. The three labels need not all be in the same molecule as was the case for the triple-labeled emerimicin described here. For example, it may be possible to measure the distance from ^{19}F or ^{31}P in a labeled inhibitor to an ^{15}N–^{13}C selected site of a labeled enzyme. The ability of TEDOR-REDOR NMR spectroscopy to measure selected, long-range distances suggests the technique may be applicable to structure determinations for macromolecular aggregates of a variety of types that are not amenable to analysis by conventional methods.

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