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Virginia Meyer
University of Denver

Michael A. Swanson
University of Denver

Laura J. Clouston
University of Nebraska-Lincoln

Przemysław J. Boratyński
University of Nebraska-Lincoln

Richard A. Stein
Vanderbilt University, richard.a.stein@vanderbilt.edu

See next page for additional authors

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Authors

Virginia Meyer, Michael A. Swanson, Laura J. Clouston, Przemysław J. Boratyński, Richard A. Stein, Hassane S. Mchaourab, Andrzej Rajca, Sandra S. Eaton, and Gareth R. Eaton

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Room-Temperature Distance Measurements of Immobilized Spin-Labeled Protein by DEER/PELDOR

Virginia Meyer¹, Michael A. Swanson¹, Laura J. Clouston³, Przemysław J. Boratyński³, Richard A. Stein², Hassane S. Mchaourab², Andrzej Rajca³, Sandra S. Eaton¹, Gareth R. Eaton^{1*}

1. Department of Chemistry and Biochemistry, University of Denver, Denver, Colorado

2. Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee

3. Department of Chemistry, University of Nebraska, Lincoln, Nebraska

*. Corresponding author geaton@du.edu

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Abstract

Nitroxide spin labels are used for double electron-electron resonance (DEER) measurements of distances between sites in biomolecules. Rotation of *gem*-dimethyls in commonly used nitroxides causes spin echo dephasing times (T_m) to be too short to perform DEER measurements at temperatures between ~80 and 295 K, even in immobilized samples. A spirocyclohexyl spin label has been prepared that has longer T_m between 80 and 295 K in immobilized samples than conventional labels. Two of the spirocyclohexyl labels were attached to sites on T4

lysozyme introduced by site-directed spin labeling. Interspin distances up to ~ 4 nm were measured by DEER at temperatures up to 160 K in water/glycerol glasses. In a glassy trehalose matrix the T_m for the doubly labeled T4 lysozyme was long enough to measure an interspin distance of 3.2 nm at 295 K, which could not be measured for the same protein labeled with the conventional 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-(methyl)methanethio-sulfonate label.

Introduction

Pulsed electron paramagnetic resonance (EPR) dipolar spectroscopy (PDS) is among the best techniques for accurate measurement of the conformations of flexible regions of biomolecules ([1-4](#)). Nitroxide attachment to sites introduced by site-directed mutagenesis benefits from the relatively small size of spin labels that cause minimal structural perturbation. If the spin echo dephasing time, T_m , of the spin label is too short, it is difficult to perform PDS. The experiments are commonly done in a glassy matrix at ≤ 80 K because T_m for most nitroxides decreases rapidly at higher temperatures due to rotation of the gem-dimethyl groups at rates comparable to the anisotropy in the electron-proton hyperfine coupling. At temperatures above ~ 140 K, T_m in glassy matrices decreases due to softening of the matrix, which increases motions that modulate g and A anisotropy. For many purposes it would be desirable to study distances near ambient temperature, which will require probes without methyl groups and improved methods to decrease mobility of the probes and proteins to which they are attached.

PDS measures the magnetic dipole-dipole interaction between spin labels, detected by either double electron-electron resonance (DEER) or double

quantum coherence (DQC). DEER, also known as pulsed electron double resonance (PELDOR), was first performed using three microwave pulses (1). A fourth pulse was later added to compensate for instrument dead time (2), and this is currently the most widely employed pulse sequence. In the 4-pulse DEER experiment (Fig. 1) three microwave pulses at the probe frequency (ν_1) generate an electron spin echo. The times τ_1 and τ_2 between probe pulses are held constant. Pulses at the pump frequency (ν_2) flip the spins of neighboring unpaired electrons, which alters the magnetic field experienced by the spins that were excited at the probe frequency (3). Varying the time, T , between the second probe pulse and the pump pulse modulates the amplitude of the spin echo (4). The modulation frequency is proportional to the magnetic dipole-dipole interaction between the two spin labels. This dipolar interaction has a $1/R^3$ dependence, where R is the interspin distance.

For typical $R \approx 2$ to 4 nm measured in biomolecules by DEER, the dipolar interaction frequency is ~ 6.5 to 0.8 MHz, so the amplitude of the spin echo oscillates with a period ranging from $1/6.5$ to $1/0.8$, which is ≈ 0.15 to 1.2 μs . To define the oscillations accurately, the data acquisition period should encompass several oscillation periods. A sufficiently long data acquisition window is especially important when the dipolar oscillations are broadened because of the wide distributions of distances that are typical in doubly labeled biomolecules. The length of the data acquisition window is limited to the time between the second and third probe pulses. As the time between these pulses is increased, the amplitude of the echo decreases exponentially with time constant T_m and the signal/noise ratio (SNR) in the DEER data decreases. Thus, as T_m increases, so does the measurable interspin distance, along with the accuracy with which the distribution of distances can be

defined.

The dipolar interaction is averaged to zero by isotropic tumbling, so motional averaging has to be minimized, and doubly labeled biomolecules, including the spin-labeled side chains, must be immobilized. As R increases, a greater degree of immobilization is necessary, e.g., the relevant correlation times should be much longer than $1/(2\pi \times 6.5 \text{ MHz}) \approx 0.02 \mu\text{s}$ and $1/(2\pi \times 0.8 \text{ MHz}) \approx 0.2 \mu\text{s}$ for $R = 2$ and 4 nm , respectively.

Two works reporting pulsed dipolar spectroscopy measurements near physiological temperatures were published during the course of this work. Both used triarylmethyl (trityl) radicals as spin labels and immobilized the protein or nucleic acid on a solid support. Double mutants of T4 lysozyme (T4L) were attached to a solid sepharose bead and then labeled with a substituted trityl. T_m was 700 ns at 4°C . The DQC method (5) at 17.2 GHz and 4°C was employed to measure distances of 1.8 and 2.1 nm (6). Nucleic acid spin labeled with trityl radical was immobilized on an ion exchange sorbent, NucleosilDMA. D_2O was used to maximize T_m , which was $1.4 \mu\text{s}$ at 310 K . DQC evolution was acquired out to $\sim 2 \mu\text{s}$ and the distance measured was $\sim 4.6 \text{ nm}$ (7). The anisotropy in the EPR spectra of trityls is too small to permit DEER measurement of interspin distances at the fields and frequencies that are currently used.

Nitroxides are smaller, less hydrophobic, and less likely to perturb protein structures than trityl. The common nitroxide spin labels, such as 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-(methyl)methanethio-sulfonate (1), abbreviated MTSL (Fig. 2), have gem-dimethyl groups, which provide steric shielding of the N-O moiety for adequate persistence of the nitroxide radical.

The temperature dependence of $1/T_m$ for spin labels 1–3 at X-band is shown in [Fig. 3](#). Below ~ 80 K, spin echo dephasing for nitroxide radicals is dominated by nuclear spin diffusion involving predominantly solvent nuclei ([8](#)). As the temperature is increased above ~ 80 K, rotation of the methyl groups in 1 and in other methyl-containing nitroxides at rates comparable to the anisotropy in the hyperfine couplings to the methyl protons, becomes a highly effective dephasing mechanism ([9,10](#)). The resulting increase in $1/T_m$ above ~ 80 K ([Fig. 3](#)) ([8](#)) puts a practical limit of $T \leq 80$ K for PDS measurements of interspin distances for methyl-containing labels including 1. The maximum effect of this dephasing process is at ~ 150 K ([Fig. 3](#)). Replacing the gem-dimethyl groups with spirocyclohexyl groups eliminates the methyl group effect and decreases $1/T_m$ dramatically at temperatures $T > 80$ K, as previously illustrated for the spirocyclohexyl analog of TOAC, 2 ([11](#)), and other structurally related spirocyclohexyl nitroxides ([12](#)). The spirocyclohexyl nitroxides also are more resistant to bioreduction than MTSL-like nitroxides ([11,13,14](#)). In glassy or frozen solvents softening of the matrix with increasing temperature permits increasing motional modulation and averaging of g and A anisotropy, which also increases $1/T_m$. In water/glycerol, these motions dominate the increase in $1/T_m$ for 1 above ~ 180 K and for 2 above ~ 150 K ([Fig. 3](#)). In matrices with higher melting points, such as sucrose/trehalose or sorbitol, the matrix remains relatively rigid up to substantially higher temperature, and the increase in $1/T_m$ with increasing temperature is more gradual than in water/glycerol ([Fig. 3](#)).

Our strategy for obtaining DEER measurements at a temperature above 80 K relies on increasing T_m (decreasing $1/T_m$) by replacing methyl groups by spirocyclohexyl groups, and decreasing the motional averaging of g and A anisotropy by immobilization of the doubly labeled protein in glassy

water/glycerol (measurements up to 160 K) or in a disaccharide (trehalose) glassy matrix (measurements at room temperature). Trehalose matrices are commonly used to preserve proteins ([15–17](#)) and have been used to immobilize proteins for flash photolysis studies of reaction kinetics ([18](#)). We now report the synthesis of spirocyclohexyl iodoacetamide 3 ([Fig. 2](#)) and DEER distance measurements from 80 to 160 K and at room temperature using T4L spin labeled with 3.

Materials and Methods

Synthesis of iodoacetamide spirocyclohexyl spin label 3

Spirocyclohexyl piperidone ([11,19](#)) was subjected to reductive amination ([20](#)) to convert carbonyl to primary amine, which was then converted to an amide using chloroacetyl chloride ([21](#)). With the primary amine group protected as an amide, the secondary amine within the piperidine ring was oxidized to nitroxide using meta-chloroperoxybenzoic acid. Finally, replacement of chloride with iodide via nucleophilic substitution gave spin label 3 with chemical yield of 31% after 4 steps at optimum reaction conditions ([Scheme S1](#) in the [Supporting Material](#)). Using solutions of tempone in dichloromethane as a reference, the EPR spin count was calculated to be $96 \pm 5\%$. DPPH powder ($g = 2.0037$) was used as a standard for determination of g -values. Further details concerning synthesis and characterization of label 3 (and its synthetic intermediates) are described in the [Supporting Material](#).

EPR sample preparation

T4L mutants were expressed as previously described ([22](#)). Following cation exchange, the protein was incubated at room temperature for 2 h with a

fivefold molar excess of spin label 3 that was dissolved in dimethylformamide, and then stored on ice overnight. The samples were then gel filtered using a Superdex 75 column with 9/6 buffer (9 mM 3-(N-morpholino)propanesulfonic acid, 6 mM Tris, 50 mM NaCl, 0.02% sodium azide, and 0.1 mM EDTA at pH 7.2), at a flow rate of 0.7 mL/min. The sample was collected and concentrated with a 10 kDa molecular mass cutoff concentrator. Spin concentration was determined using a Bruker EMX spectrometer (Billerica, MA). The same procedure was used to prepare a sample of T4L labeled with MTSL at positions 65/80. The designation 65/80 indicates that spin-labeled cysteines are at positions 65 and 80.

DEER measurements were performed on T4L spin labeled at positions 61/135 (72 μ M), 65/135 (85 μ M), and 65/80 (130 μ M) in the amino acid sequence (22). Samples for DEER were contained in 1.6 mm outer diameter quartz EPR tubes. For measurements at liquid nitrogen temperatures, glycerol was added to the buffer to achieve a final concentration of 24%. To perform DEER at room temperature, the spin-labeled protein was immobilized in a thoroughly dried trehalose matrix that was prepared as follows. A 10-fold excess by mass of lysozyme was added to the 65/80 T4L in buffer to decrease local concentrations of spins in the glass and decrease intermolecular dipolar contacts (23–25). The mixture of lysozyme with spin-labeled T4L was combined with 0.2 M trehalose and allowed to dry on a watch glass in air. As the sample dried, trehalose replaced the hydrogen bonds that the protein lost with water (18). The resulting glassy residue was inserted into an EPR tube and was further dried by evacuation for \sim 48 h. The mole ratio of components in the resulting sample is \sim 2000:1 trehalose/T4L to minimize intermolecular spin-spin interactions in the glassy sample after the water evaporates.

EPR spectroscopy

Relaxation and DEER data were collected at Q-band (34 GHz) on a Bruker E580 system with a 1 W amplifier, Super Q-FT bridge, Bruker ER 5107D2 pulse ENDOR resonator, and an Oxford CF935 cryostat. T_m and T_1 were determined by spin echo decay and inversion recovery, respectively. The shot repetition time (SRT) used for DEER measurements was calculated as 1.2 times T_1 . The long component of T_1 fit to a double exponential function was used for this calculation. Before each DEER measurement, a field-swept echo-detected spectrum was recorded to determine the field position of the maximum echo amplitude ([Fig. S1](#)). The magnetic field and probe frequency were selected for resonance at this position in the spectrum. The pump frequency was set 37 MHz below that of the probe frequency, allowing adequate separation between pulses (see the [Supporting Material](#)). The probe pulse length was selected to maximize echo intensity and was typically set to $\pi/2 = 40$ ns for experiments from 80 to 160 K. The pump pulse length was held constant at $\pi = 40$ ns. The echo was digitized starting at the full-width at half-maximum amplitude using a gate equal to the echo width at this amplitude. Additional unwanted echoes were removed using 8-step phase cycling ([26](#)).

At temperatures between 80 and 160 K the spin echo decays for samples containing spin label 3 fit best to a stretched exponential, which is consistent with domination of the dephasing by nuclear spin diffusion ([8](#)). By contrast, the echo decays for methyl-containing 1 at temperatures above 80 K fit best with a simple exponential that occurs when a dynamic process dominates the decay ([11](#)). The difference in the shapes of the echo decays for labels 1 and 3 supports the assertion that the longer values of T_m for 3 than for 1 are due to replacement of the methyl groups by spirocyclohexyls

and elimination of the contribution of methyl rotation to the echo dephasing. The shapes of the echo decays at room temperature for T4L labeled with 3 or with MTSL are different than that measured for T4L labeled with 3 between 80 and 160 K. A simple exponential function fits well at 295 K, which is consistent with domination of echo dephasing by motional averaging of anisotropy (8). With these fitting parameters, the T_m for the dried trehalose sample of T4L labeled with 3 decreased from 3.6 μ s at 80 K to 920 ns at 295 K. As a consequence, the DEER acquisition window was decreased from 4 μ s at 80 and 160 K to 1.5 μ s at 295 K. T_1 was also dramatically shorter at room temperature, allowing the SRT for DEER to be reduced from 1500 μ s at 80 K to 50 μ s at 295 K. A Bruker SpecJet II was used to digitize the signal at this short SRT. At 295 K the maximum DEER echo was achieved with a 52 ns pump pulse. The data acquisition at room temperature was performed with 2900 scans, 8 phase cycles (26), 50 μ s SRT, 500 shots per point, and 390 points for a total time of 226,200 s, or 2 days and 15 h.

Data analysis

DEER data were analyzed using DEERAnalysis2011 (27). The inherent background arising from spin labels on neighboring proteins was subtracted using homogenous background subtraction (Fig. S2). The background for samples prepared in water/glycerol (80 and 160 K) fit best using a dimensionality of 3.3, although the trehalose sample at 80 and 295 K were fit using dimensionality between 3 and 3.3. These differences in dimensionality may arise from the dissimilar physical properties of the matrices. The starting point in the data array for background subtraction was chosen based on the shape of the Pake pattern (see Figs. S3, 5 B, and S5 B). Dipolar evolution curves were fit using Tikhonov regularization with $\alpha =$

100 for 61/135 and 65/135 and $\alpha = 10$ for 65/80 for the water/glycerol samples. A regularization parameter of $\alpha = 100$ was used for the trehalose sample at 80 and 295 K temperatures. The selection of different α parameters was guided by the corner of an L-curve formed by varying the fitting parameters with respect to sharpness of peaks in the distance distribution and smoothness of the fit function ([Fig. S4](#) for the water/glycerol samples, data not shown for the trehalose sample) ([28](#)). To select between neighboring points on the L-curve, the fit to the time domain background subtracted data was examined.

Results

Electron spin relaxation times of the spirocyclohexyl spin label 3

Values of $1/T_m$ for the iodoacetamide form of the spirocyclohexyl spin label moiety, 3, and the amino acid analog 2 in ~50% water/glycerol from 100 to 190 K at X-band are shown in [Fig. 3](#). Throughout this temperature range $1/T_m$ for 2 and 3 is substantially smaller (T_m is substantially longer) than for the widely used spin label MTSL (1), because these new labels do not contain the gem-dimethyls that enhance spin echo dephasing for MTSL in this temperature range. For all of the spin labels in water/glycerol, $1/T_m$ increases (T_m decreases) rapidly with increasing temperature above ~180 K due to softening of the glass and the onset of motions that average g and A anisotropy ([29](#)). When 2 or 3 is immobilized in matrices with higher glass transition temperatures (sorbitol or sucrose/trehalose) the temperature dependence of $1/T_m$ above 180 K is much more gradual than in water/glycerol ([Fig. 3](#)).

Spin-labeled T4L relaxation and DEER, 80–160 K

Relaxation time measurements at Q-band for spin label 3 attached to T4L in water/glycerol were conducted between 80 and 160 K ([Table 1](#)). For each of the spin labeled sites, the values of T_m at Q-band ([Table 1](#)) are similar to that for 2, and 3 in water/glycerol at X-band ([Fig. 3](#) and [Table S1](#)).

Frequency independence of echo dephasing is consistent with a dominant contribution from nuclear spin diffusion ([8](#)). Attachment of the label to the protein has little impact on T_m because the label is on the exterior of the protein in a largely aqueous environment. The weak temperature dependence between 80 and 160 K is attributed to the onset of low amplitude local motion of the probe, which is largely independent of its attachment to the protein. For example at 160 K T_m for 3 attached to the proteins in water/glycerol is 3.1 to 3.5 μs ([Table 1](#)) and for 3 in water/glycerol is 2.7 μs ([Fig. 3](#)). The strongly temperature-dependent values of T_1 for spin-labeled T4L in this temperature interval at Q-band ([Table 1](#)) are similar to those reported for 2 in 1:1 water/glycerol at X-band ([Table S1](#)) ([30](#)). The $\sim T^{-2.7}$ temperature dependence of T_1 is consistent with domination of the relaxation by a local mode, which is frequency independent ([29](#)). In this temperature range the Boltzmann population difference between the two electron spin states decreases approximately linearly with temperature. The decrease in T_1 permits shorter SRT, and averaging of more scans per unit time, at higher temperature. SNR improves approximately as the square root of the number of scans, so a $T^{-2.7}$ dependence of T_1 should permit sufficient decrease in SRT to compensate for the decrease in Boltzmann population, at constant T_m .

The SNR for the DEER experiments performed at 80 and 160 K are similar ([Fig. 4](#)). For each mutant total acquisition time was kept constant: 70 h for T4L 61/135, 40 h for 65/135, and 44 h for 65/80. The slight decrease in the

SNR of the raw data when temperature is increased from 80 to 160 K is attributed to the nonnegligible decrease in T_m . If T_m were constant, the use of shorter SRT that is permitted by the decrease in T_1 would compensate for the decrease in Boltzmann population at higher temperature (4). Even with slightly decreased SNR at 160 K, the same time window could be used at 80 and 160 K. The distance distributions are very similar at 80 and 160 K for all three samples (Fig. 4). Table 2 provides a comparison of distances obtained for T4L labeled with 3 measured at 80 and 160 K, in addition to distances obtained at 77 K by DQC using spin label 1. These data demonstrate that spin label 3 permits DEER measurements in water/glycerol up to substantially higher temperatures than are accessible when MTSL (1) is the spin label. At 140 to 150 K the T_m for MTSL either as a small molecule (Fig. 3) or attached to a protein is ~ 200 to 300 ns, which is too short to be useful for DEER.

Room temperature DEER

A central limitation to performing room-temperature DEER is the very short T_m relaxation time, which decreases the available acquisition window. T_m at Q-band was 550 ns at 295 K for a dried trehalose sample of T4L 65/80 labeled with methyl-containing MTSL. Spin label 3, without methyl groups, removes a significant contribution to T_m relaxation. Using spin label 3, T_m of a dried trehalose glass of T4L at Q-band increased to 920 ns, which was sufficient for DEER. The data were collected in 3 days at 295 K. The fast repetition rate allowed acquisition of 2900 scans during this time period. For the T4L samples immobilized in trehalose glass at 295 K, the longer T_m for spin label 3 than for MTSL is consistent with prior observations that T_m is significantly longer for a spirocyclohexyl nitroxide (1.8 μ s) than for MTSL (1.2 μ s) in a polyvinyl alcohol borate glass (11).

A Tikhonov parameter of $\alpha = 100$ was used to fit the room-temperature dipolar evolution curve. The background subtraction and Pake pattern (Fig. 5, A and B) are comparable to DEER collected at a lower temperature (Fig. S5). The dominant distance obtained (3.2 nm) is in good agreement with that obtained in 24% glycerol glass (3.0 nm) at 80 and 160 K (Table 2). Several factors contribute to broadening of the distribution observed for the room temperature of DEER. The shoulder in Fig. 5 D suggests the presence of additional rotamers at room temperature, and there likely is residual motion of the protein and spin label at this temperature (31). The data in trehalose were fit with $\alpha = 100$, whereas the data at 80 to 160 K in 24% glycerol were fit with $\alpha = 10$, which corresponds to a sharper distance distribution.

Discussion

For each of the three doubly labeled proteins the distances obtained by DEER are temperature independent between 80 and 160 K (Table 2). Distances for T4L labeled with 3 are in good agreement with distances obtained by DQC at 77 K for T4L labeled with MTSL at positions 61/135 and 65/80 (31), which indicates that for these samples the spin-labeled side chains of 3 adopt conformations similar to those for the widely studied MTSL. The distances measured for T4L spin labeled at positions 65/135 differed between the DEER measured here and the prior DQC measurement. The 4.0 nm distance measured by 4-pulse DEER (Table 2) correlates more closely to the distance measured for the same double mutant using 5-pulse DEER, which was 4.3 nm (32). The shoulder present in the distance distributions at both temperatures for this sample (Fig. 4 C) suggests that two rotamers may be present in this sample, one of which is a closer match to the DQC data. It is probable, therefore, that the samples studied here

adopt protein or label conformations upon freezing that differ from the samples studied by DQC (33). Similar bimodal distributions have been reported in prior studies of doubly spin-labeled T4L (31). Flash freezing of the 24% glycerol glass may select a smaller range of rotamers for 65/80 than for other labels, which may explain the much narrower distance distribution for 65/80 than for other pairs of labels (Fig. 4).

Shoulders present in the distance distributions for T4L 65/80 dried in trehalose measured at both 80 and 295 K also suggest additional rotamer conformations. The dominant conformation for the room temperature measurement (Fig. 5 D) appears to be present in the distance distribution from the 80 K measurement, and vice versa. Again, flash freezing of the sample for 80 K measurement may select a different set of label conformations than the sample that was equilibrated slowly at room temperature (33).

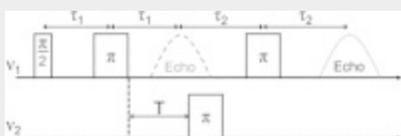
The weak temperature dependence of T_m for 3 between 80 and 160 K demonstrates that replacement of the methyl groups of MTSL with spirocyclohexyl groups dramatically lengthens T_m for fully immobilized nitroxides. Even with 3, local motion decreases T_m at room temperature sufficiently to limit the available time window for DEER data acquisition. Provided that the matrix is sufficiently rigid to limit global motions of the protein, local motions of the label are likely to dominate dephasing. The next challenge for development of nitroxides for room temperature DEER experiments is to decrease the mobility of the side chain that attaches the label to the protein.

Conclusions

PDS is a powerful tool for structural studies of biological macromolecules that has been greatly enhanced by site-directed spin labeling. Gem-dimethyl groups on the commonly used nitroxide spin label, MTSL, contribute to shortening of T_m , which in turn limits the achievable acquisition window. In this work, we show that removal of the methyl groups significantly increases T_m and allows for measurements at higher temperature. This enhancement is highlighted through DEER measurement at room temperature, where T_m for a typical nitroxide is too short to obtain a signal. The leap from 80 K DEER measurements to room temperature is an important step toward DEER measurement in physiological environments. The current fundamental requirement for DEER of protein immobilization provides additional avenues toward improvement of the technique.

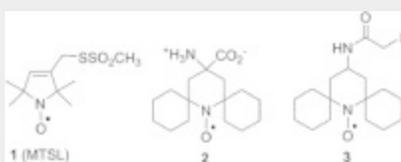
Author Contributions

V.M. designed, performed, and analyzed DEER experiments and contributed to the writing of this article; M.A.S. performed spin echo and saturation recovery measurements; L.J.C. synthesized spin label 3; P.J.B. synthesized spin label 3; H.S.M. designed experiments and contributed to the writing of this article; R.A.S. prepared spin-labeled T4L; A.R. designed experiments and contributed to the writing of this article; S.S.E. designed experiments and contributed to the writing of this article; G.R.E. designed experiments, supervised the DEER experiments, and contributed to the writing of this article.



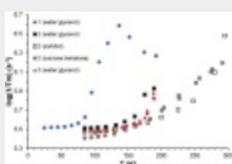
[View larger version](#)

Figure 1. Pulse sequence for 4-pulse DEER. The spin echo is formed with pulses at the probe frequency, ν_1 , with constant τ_1 and τ_2 , whereas the pump pulse at ν_2 is stepped by varying T . The resulting signal is a measure of the modulation of the amplitude of the spin echo from spins excited at ν_1 by the pump pulse at ν_2 .



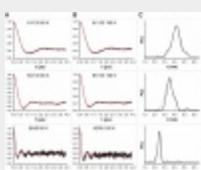
[View larger version](#)

Figure 2. Nitroxide radicals.



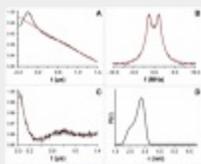
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Figure 3. Temperature dependence of $\log(1/T_m)$ at X-band for **1**, **2**, and **3** in glassy mixtures. Data for **1** and **2** in water/glycerol are reproduced from (11). To see this figure in color, go online.



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Figure 4. Background-subtracted DEER data at 80 K (A, black) and 160 K (B, black) for T4L labeled with **3** in 24% glycerol. Tikhonov fitting is shown in red. The distance distributions (C) were similar at 80 K (black) and 160 K (gray) for each double mutant. To see this figure in color, go online.



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Figure 5. Room temperature DEER of T4L 65/80 labeled with 3 in a glassy trehalose matrix: raw data (A, black) with background subtraction (A, red); Pake pattern (B, black) with Tikhonov regularization fitting, $\alpha = 100$ (B, red); background subtracted raw data (C, black) with Tikhonov fitting (C, red); distance distribution (D). To see this figure in color, go online.

Table 1
Temperature dependence of T_m and T_1 (μs) at Q-band for T4L samples labeled with 3 in

Temp (K)	65/135		65/135		65/80	
	T_m	T_1	T_m	T_1	T_m	T_1
80	4.0	1700	4.1	1600	4.1	1600
100	4.0	900	4.0	850	4.0	1000
120	3.7	620	3.8	520	3.9	370
140	3.4	400	3.7	300	3.7	400
160	3.1	260	3.3	190	3.3	280

[See full table](#)

Table 1. Temperature dependence of T_m and T_1 (μs) at Q-band for T4L samples labeled with 3 in 24% glycerol

Table 2
Distances in nm for samples studied by DEER and analyzed using Tikhonov regularization

Matrix	80 K	100 K	295 K	DQC distance ^a
65/135 24% glycerol	4.7	4.7	NA	4.7
65/135 24% glycerol	4.0	4.0	NA	4.6
65/80 24% glycerol	3.6	3.6	NA	3.6
65/80 Trehalose	2.9	NA	3.2	NA

Distances measured by DQC for T4L labeled with MTSL at 77 K (□) are included for α
^aDistances measured for MTSL spin label.

[See full table](#)

Table 2. Distances in nm for samples studied by DEER and analyzed using Tikhonov regularization in DEERAnalysis2011

Supporting Material

Supporting Citations

References ([34,35](#)) appear in the [Supporting Material](#).

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Footnotes

Przemysław J. Boratyński's present address is Department of Organic Chemistry, Wrocław University of Technology, 50-370 Wrocław, Poland.

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[\[Back\]](#)

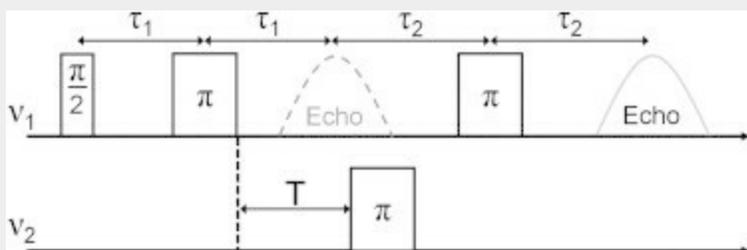


Figure 1.

Pulse sequence for 4-pulse DEER. The spin echo is formed with pulses at the probe frequency, ν_1 , with constant τ_1 and τ_2 , whereas the pump pulse at ν_2 is stepped by varying T . The resulting signal is a measure of the modulation of the amplitude of the spin echo from spins excited at ν_1 by the pump pulse at ν_2 .

[\[Back\]](#)

[\[Back\]](#)

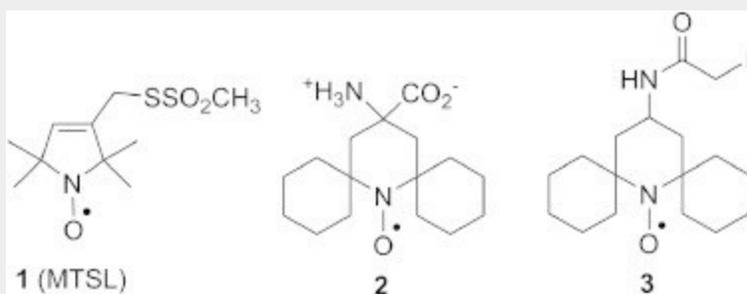


Figure 2.

Nitroxide radicals.

[\[Back\]](#)

[\[Back\]](#)

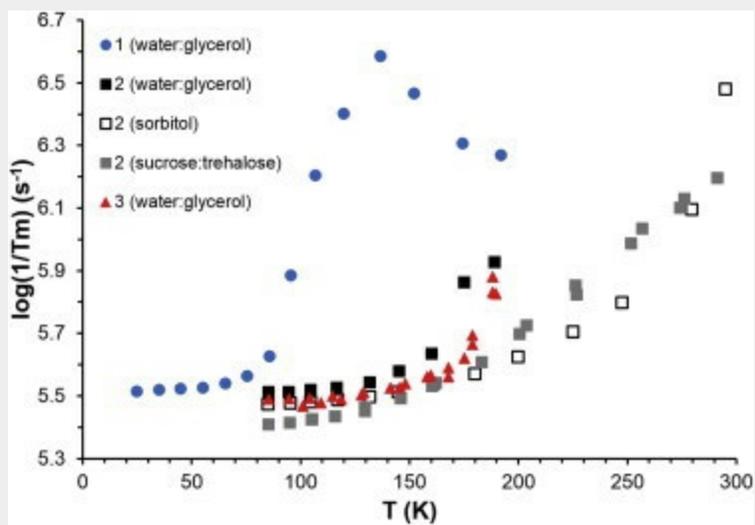


Figure 3.

Temperature dependence of $\log(1/T_m)$ at X-band for **1**, **2**, and **3** in glassy mixtures. Data for **1** and **2** in water/glycerol are reproduced from (11). To see this figure in color, go online.

[\[Back\]](#)

[\[Back\]](#)

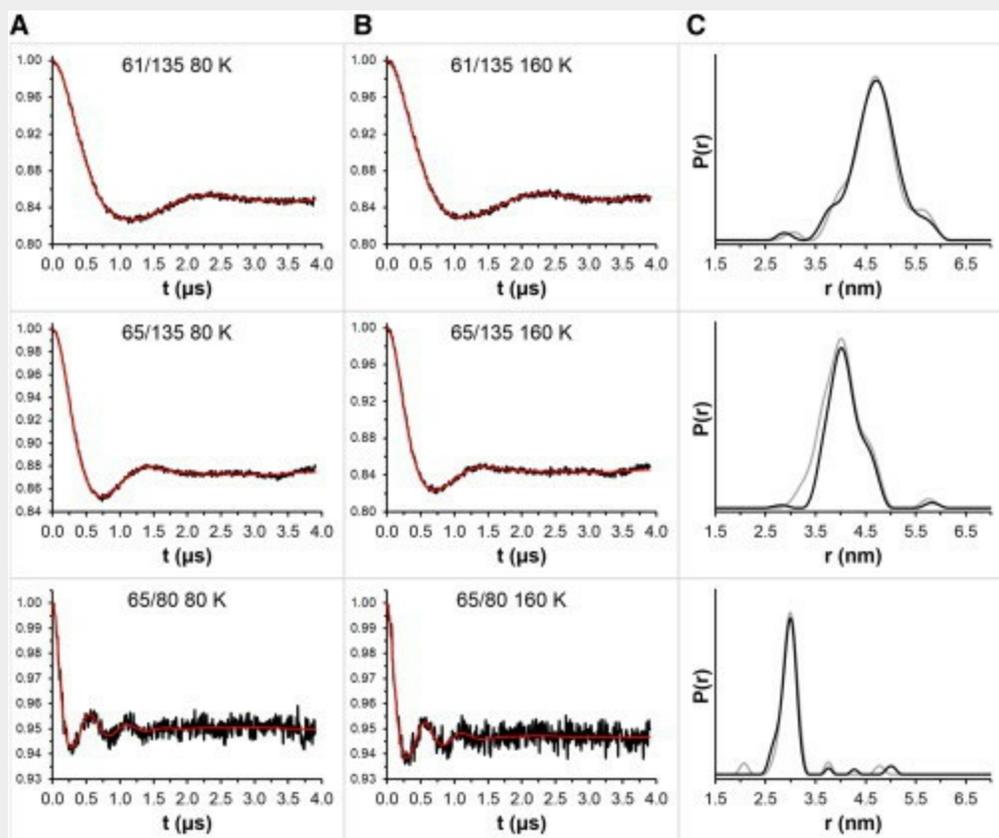


Figure 4.

Background-subtracted DEER data at 80 K (A, *black*) and 160 K (B, *black*) for T4L labeled with **3** in 24% glycerol. Tikhonov fitting is shown in red. The distance distributions (C) were similar at 80 K (*black*) and 160 K (*gray*) for each double mutant. To see this figure in color, go online.

[\[Back\]](#)

[\[Back\]](#)

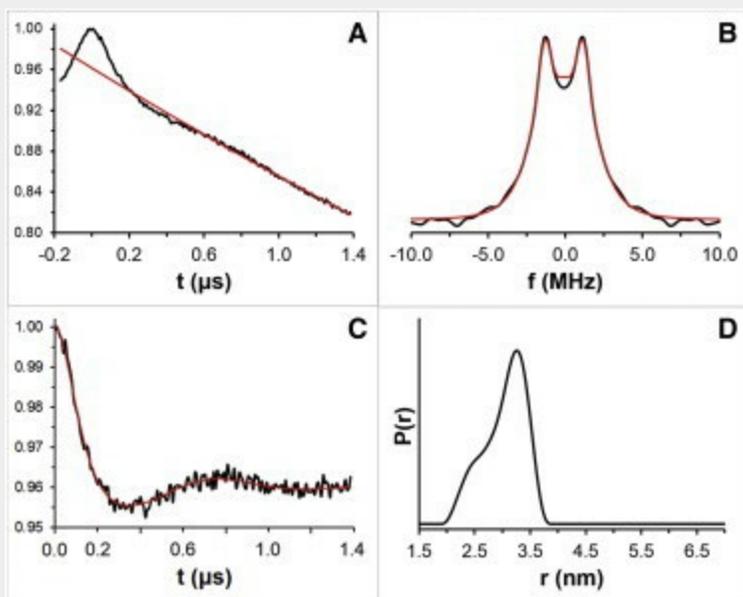


Figure 5.

Room temperature DEER of T4L 65/80 labeled with **3** in a glassy trehalose matrix: raw data (A, *black*) with background subtraction (A, *red*); Pake pattern (B, *black*) with Tikhonov regularization fitting, $\alpha = 100$ (B, *red*); background subtracted raw data (C, *black*) with Tikhonov fitting (C, *red*); distance distribution (D). To see this figure in color, go online.

[\[Back\]](#)

[\[Back\]](#)

Table 1.

Temperature dependence of T_m and T_1 (μs) at Q-band for T4L samples labeled with 3 in 24% glycerol

Temp (K)	61/135		65/135		65/80	
	T_m	T_1	T_m	T_1	T_m	T_1
80	4.0	1700	4.1	1600	4.1	1600
100	4.0	900	4.0	950	4.0	1000
120	3.7	620	3.8	520	3.9	570
140	3.4	400	3.7	330	3.7	400
160	3.1	260	3.5	190	3.3	280

[\[Back\]](#)

[\[Back\]](#)

Table 2.

Distances in nm for samples studied by DEER and analyzed using Tikhonov regularization in DEERAnalysis2011

	Matrix	80 K	160 K	295 K	DQC distance ^a
61/135	24% glycerol	4.7	4.7	N/A	4.7
65/135	24% glycerol	4.0	4.0	N/A	4.6
65/80	24% glycerol	3.0	3.0	N/A	3.0
65/80	Trehalose	2.9	N/A	3.2	N/A

Distances measured by DQC for T4L labeled with MTSL at 77 K ([31](#)) are included for comparison.

a. Distances measured for MTSL spin label.

[\[Back\]](#)

Table of Contents

Room-Temperature Distance Measurements of Immobilized Spin-Labeled Protein by DEER/PELDOR	1
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Supporting Information to Accompany:

Room-Temperature Distance Measurements of Immobilized Spin-Labeled Protein by DEER/PELDOR

Virginia Meyer,[†] Michael A. Swanson,[†] Laura J. Clouston,[§] Przemysław J. Boratyński,^{§,&}
Richard A. Stein,[‡] Hassane S. Mchaourab,^{*,‡} Andrzej Rajca,^{*,§} Sandra S. Eaton,[†] Gareth R.
Eaton^{*,†}

[†]Department of Chemistry and Biochemistry, University of Denver, Denver, Colorado 80208,
United States

[‡]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville,
Tennessee 37232, United States

[§]Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588, United States

[&]current address, Department of Organic Chemistry, Wrocław University of Technology, 50-370
Wrocław, Poland

Additional Figures

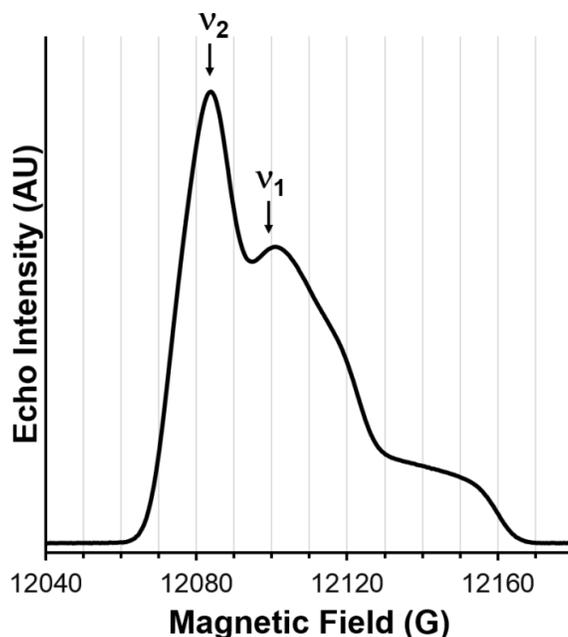


Figure S1. Field-swept echo-detected spectrum at Q-band and 80 K for T4L 61/135 doubly spin labeled with **3**. The pulses at ν_1 (probe) and ν_2 (pump) (Fig. 1) were positioned as shown, which corresponds to a separation of ~ 13.2 G. The excitation bandwidth calculated from the $\pi/2$ and π pulse lengths and the equation $\theta = \gamma_e \tau_p B_1$ was ~ 6.7 G.

Selection of Pump and Probe Pulse Positions to Prevent Pulse Overlap.

The frequencies of the pump and probe pulses for the DEER experiments were selected to be 37 MHz apart. This difference corresponds to 13.2 G (Fig. S1), calculated based on $h\nu = g\mu_B B$ where h is Planck's constant, ν is microwave frequency, g is the g -value of the radical (2.0060, see characterization of **3** below), μ_B is the Bohr magneton, and B is the magnetic field. A $\pi/2$ pulse length of 40 ns excites only 2.2 G of the full spectral width, as calculated by $\theta = \gamma_e \tau_p B_1$ (Fig. S1), where θ is the turning angle, γ_e the free electron gyromagnetic ratio, τ_p is pulse length, and B_1 is the microwave magnetic field. By the same equation, the 40 ns π pulse excites twice the spectral width of the $\pi/2$ pulse. The difference of 37 MHz between pulses is therefore sufficient to avoid pulse overlap ($13.2 \text{ G} > 2.2 + 4.5 \text{ G}$). The two pulses were positioned in regions of the spectrum where there are relatively large numbers of spins (Fig. S1).

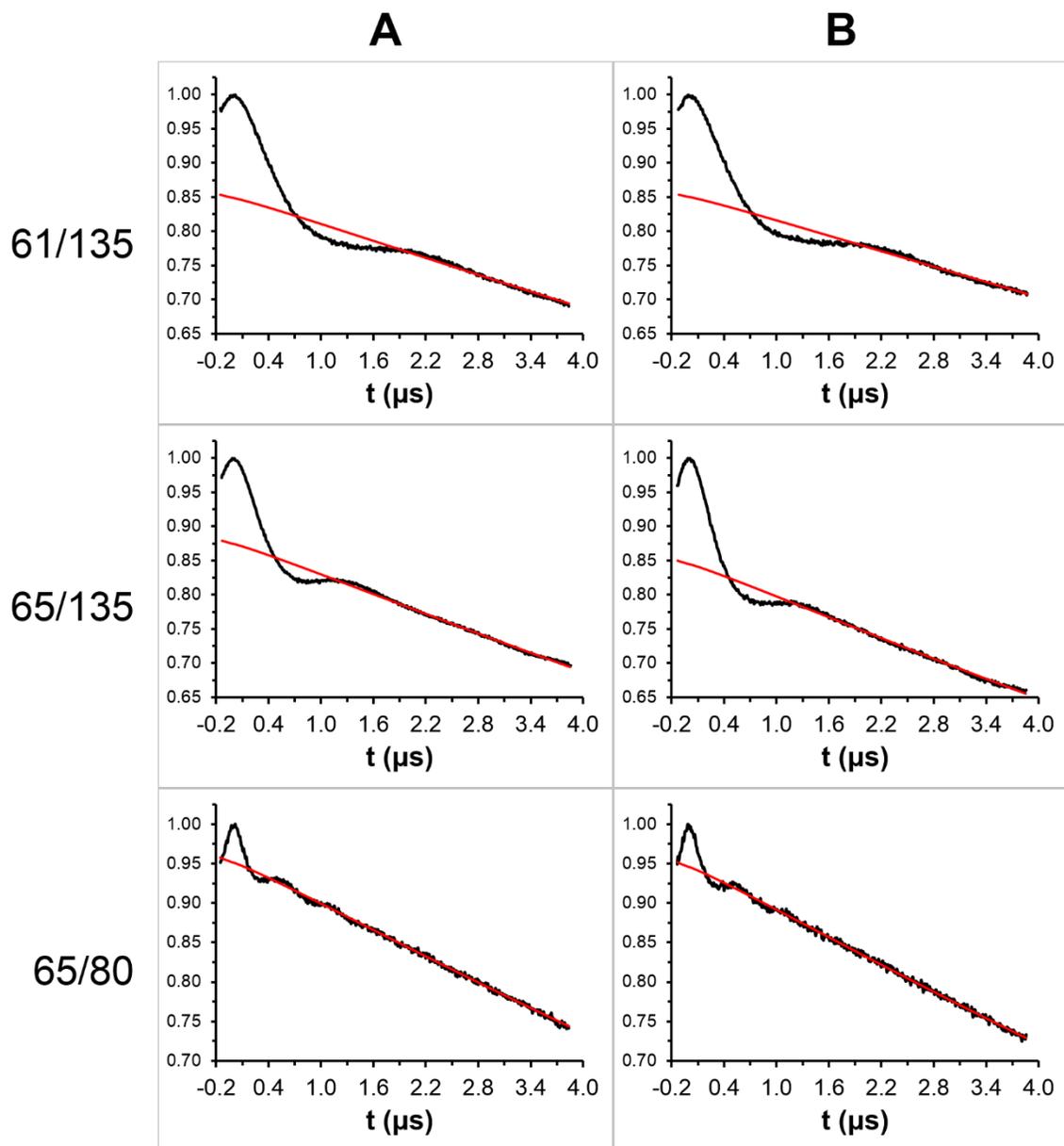


Figure S2. Raw DEER data (black) with homogeneous background subtraction (red) for T4L labeled with **3** in 24% glycerol measured at 80 K (A) and 160 K (B).

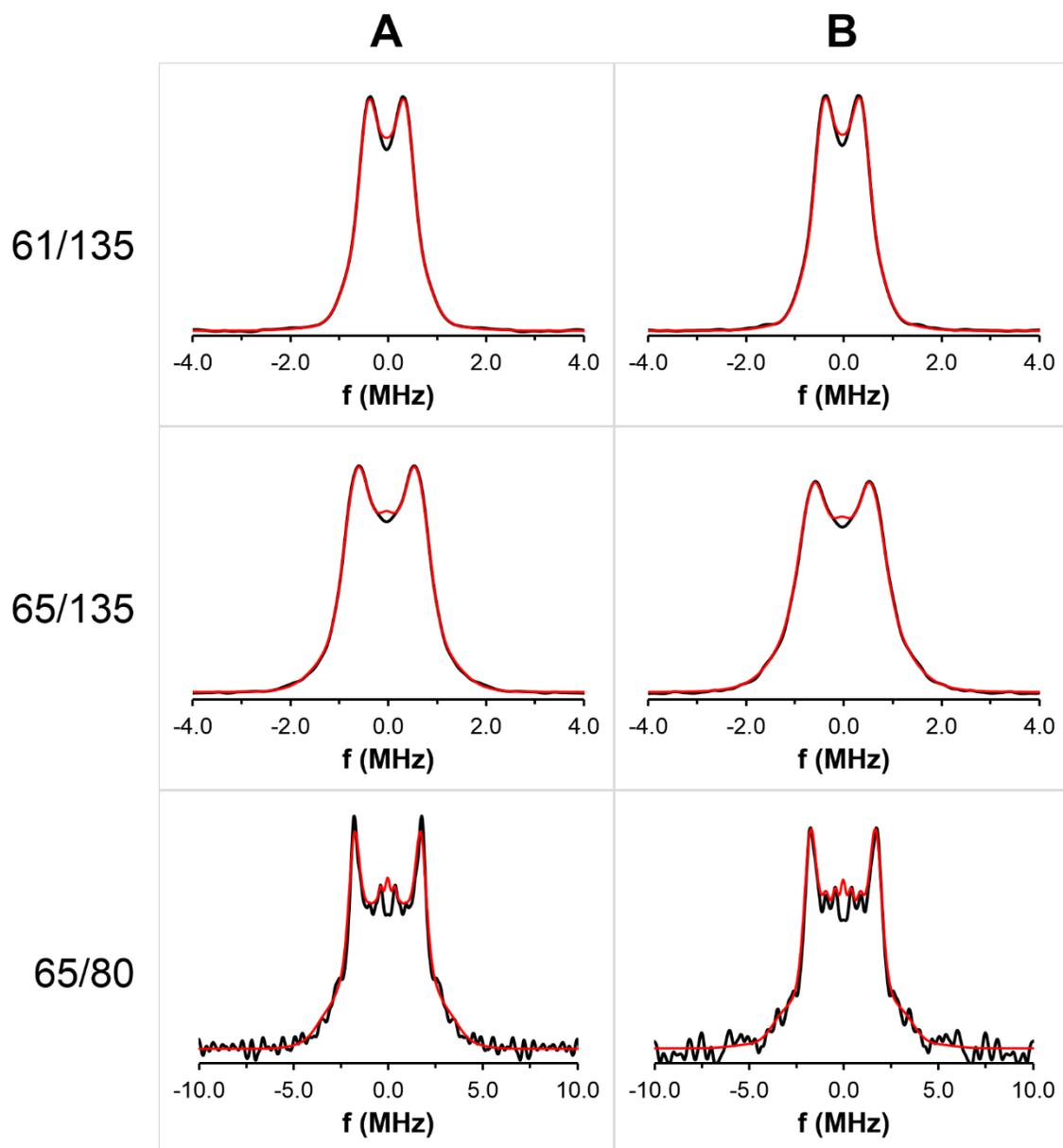


Figure S3. Pake patterns (black) with Tikhonov fitting (red) using regularization parameters of $\alpha = 100$ (61/135 and 65/135) and $\alpha = 10$ (65/80) for T4L labeled with **3** in 24% glycerol measured at 80 K (A) and 160 K (B).

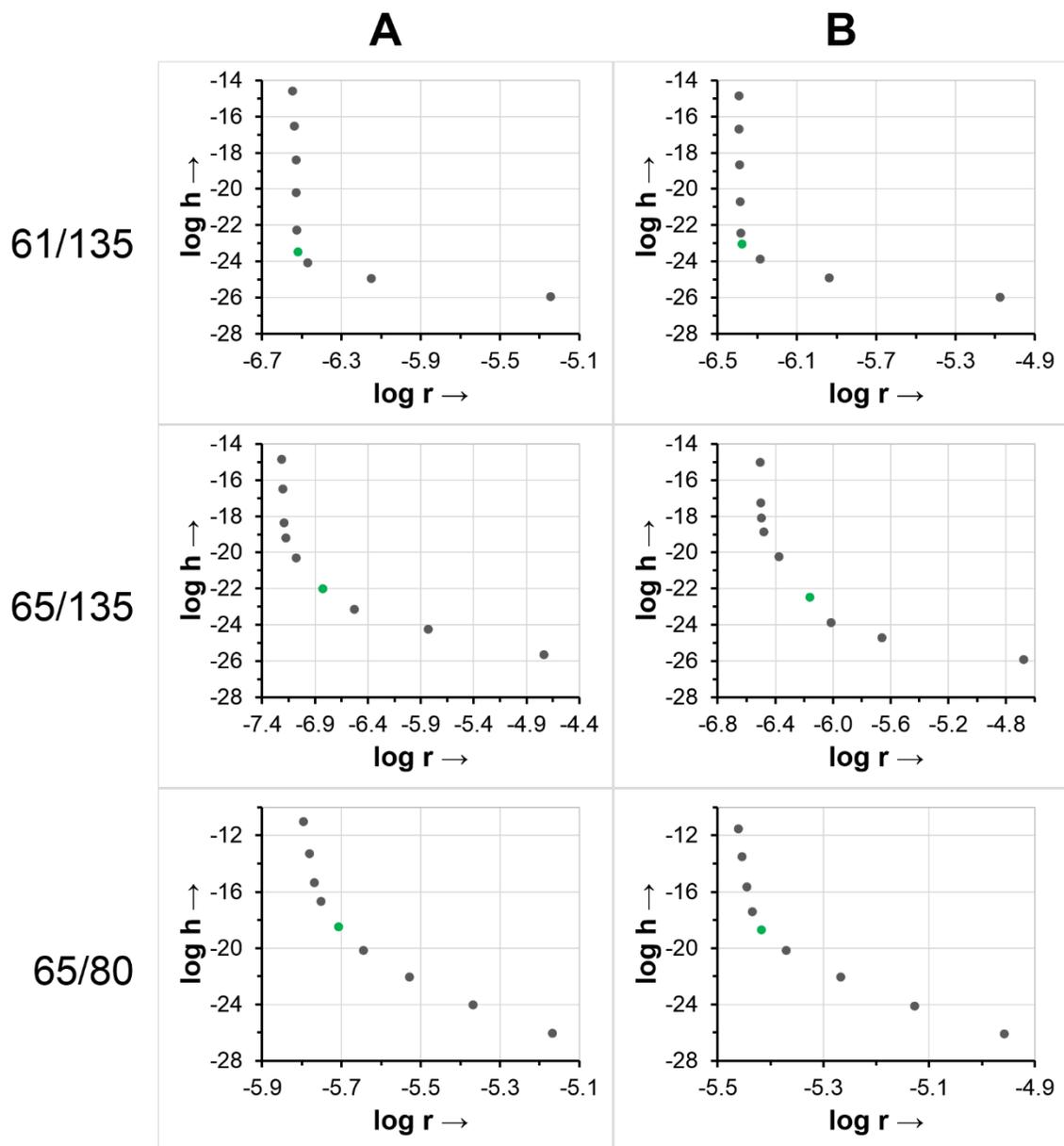


Figure S4. L-curves resulting from Tikhonov regularization for T₄L labeled with **3** in 24% glycerol measured at 80 K (A) and 160 K (B). The regularization parameter selected for analysis for each sample is highlighted in green ($\alpha = 100$ for 61/135 and 65/135 and $\alpha = 10$ for 65/80).

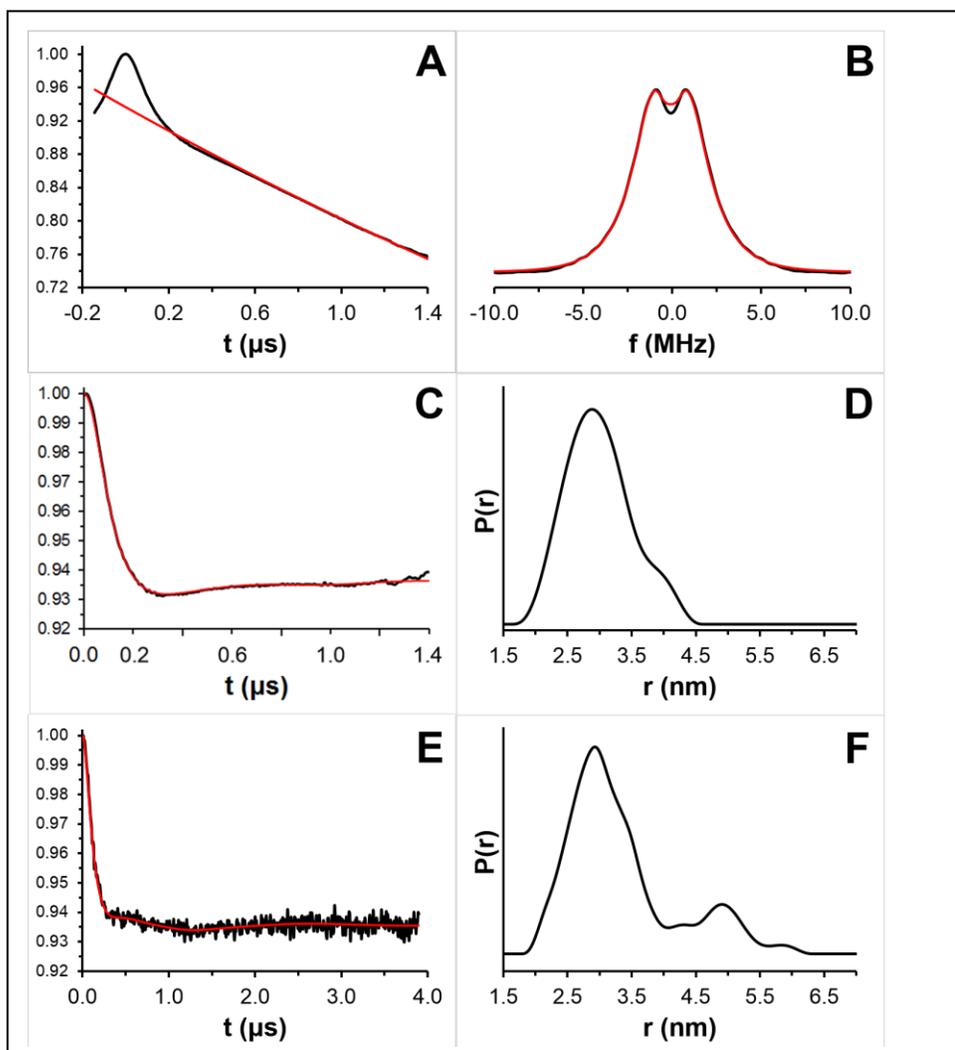


Figure S5. 80 K DEER of solid T4L 65/80 labeled with **3** in trehalose. Parts A-D are for a 1.5 μs acquisition window as shown for trehalose glass at room temperature in Fig. 5. Parts E and F are for a 4.0 μs acquisition window as shown for 24% glycerol at 80 K in Fig. 4.

Parts A – D: raw data (A, black) with background subtraction (A, red); Pake pattern (B, black) with Tikhonov regularization fitting using $\alpha = 100$ (B, red); background subtracted raw data (C, black) with Tikhonov fitting (C, red); distance distribution (D).

Parts E - F background subtracted data (E, black) and Tikhonov fitting with $\alpha = 100$ (E, red); distance distribution (F). The probability profile at distances longer than about 4.5 nm is strongly dependent on the dimensionality of the background between 3.1 and 3.3, and therefore attributed to artifacts. The profile between 1.5 and 3.5 nm is not strongly dependent on dimensionality, and the longer window provides a sharper distance profile.

Table S1. Relaxation times (μs) for Nitroxide **2** and MTSL at X-band in 1:1 water:glycerol

Temp (K)	Spirocyclohexyl nitroxide 2		MTSL	
	T_m^a	T_1^b	T_m^a	T_1^b
80	3.2	1400	2.5	500
100	3.2	540	1.3	450
120	3.2	340	0.31	230
140	2.9	240	0.25	190
160	2.7	170	0.50	110

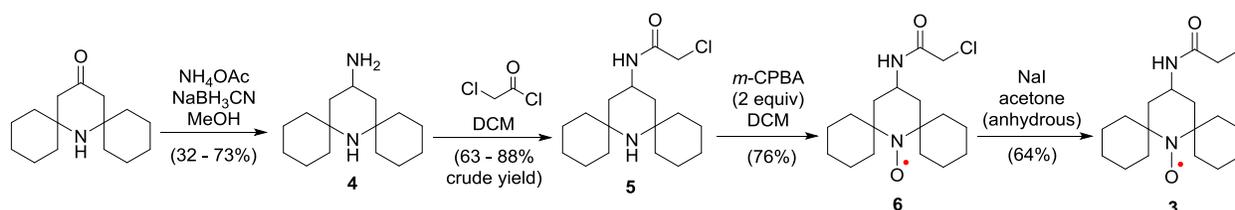
^a Values of T_m reported in Ref. S2.

^b Values of T_1 reported in Ref. S3 and calculated as a single exponential fit. These values are systematically shorter than the long component of a two component fit, which was used to select the SRT for the DEER experiments and reported in Table 1.

Additional Experimental Details

Synthesis of Spin Label **3**: Detailed Experimental Procedures and Spectral Characterization for Compounds **3** – **6**.

Throughout the following paragraphs labels “LC-1-67” and alike correspond to sample or experiment codes directly traceable to the laboratory notebooks or raw data. The synthesis of spin label **3** is outlined in Scheme S1.



Scheme S1. Synthesis of Spin Label **3**.

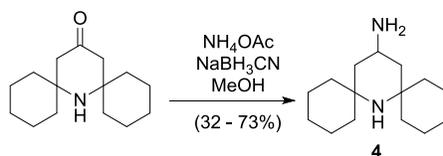
Chemicals and per-deuterated solvents for NMR spectroscopy were obtained from commercial sources and used as received unless otherwise indicated. Reagent grade acetone was allowed to dry over DrieriteTM for 4-6 days, then distilled under N_2 . Purification and titration of *m*-chloroperoxybenzoic acid (*m*-CPBA) were implemented as previously described (S4). Column chromatography was carried out on flash grade silica gel or activated aluminum oxide (neutral Al_2O_3 , 50–200 microns), using 0 – 20 psig pressure.

CW EPR spectra for **3** and its precursor nitroxides were obtained on Bruker EMX spectrometer equipped with frequency counter and nitrogen flow temperature control. Spectra of nitroxides were obtained in dichloromethane (DCM) at room temperature. DPPH powder ($g = 2.0037$) and solutions of tempone in DCM were used as g -value and spin counting references, respectively.

NMR spectra were obtained using Bruker Avance with QNP probe spectrometer (^1H , 300 MHz) using chloroform-*d* (CDCl_3) as solvent. The chemical shift references were as follows: (^1H) chloroform, 7.26 ppm. For diamagnetic compounds, typical 1D FID was subjected to exponential multiplication with an exponent (LB) of 0.3 Hz and 1.0 Hz for ^1H and ^{13}C NMR, respectively. IR spectra were obtained using a commercial instrument, equipped with an ATR sampling accessory. MS analyses were carried out at the local mass spectrometry facility; for

HR FABMS analyses, two matrices were typically used: 2-nitrophenyl octyl ether (ONPOE) and 3-nitrobenzyl alcohol (3-NBA).

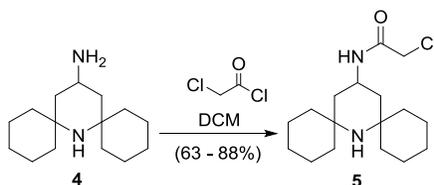
Procedure for Diamine 4.



Run	SM (mg/mmol)	SM label	NH ₄ OAc (g/mmol/equiv)	NaBH ₃ CN (mg/mmol/equiv)	TM (mg)	Yield (%)	TM label
LC-1-67	24.3 / 0.103	LC-1-67SM	0.169/2.172/21	16.2/0.258/2.5	12.1	50	LC-1-68-LC1
LC-1-69	510.3 / 2.17	LC-1-67SM	3.475/45.12/20.7	336/5.35/2.46	81.4 99.2	35	LC-1-69-LC1 LC-1-69b-LC1
LC-1-77	687.6 / 2.93	LC-1-67SM	4.580/59.48/20.3	453/7.21/2.46	503.6	73	LC-1-77-LC1

LC-1-77: The 7-azadispiro[5.1.5.3]hexadecan-15-one (687.6 mg, 2.93 mmol) was dissolved in methanol (59 mL) and AcONH₄ (4.580 g, 59.48 mmol, 20.3 equiv) was added and stirred vigorously until solid was dissolved. After 2 min, NaBH₃CN (453 mg, 7.21 mmol, 2.46 equiv) was added and the solution was stirred at room temperature for 112 h. The methanol was evaporated and the remaining oil was basified with 10% aqueous NaOH, extracted with CHCl₃, and washed with saturated aqueous NaCl. Combined organic extracts were dried over Na₂SO₄ and evaporated. Purification on neutral aluminum oxide column with EtOAc to EtOH gave 503.6 mg (73%) of diamine 4. M.p. 225 – 231 °C (dec. under air), 256 – 259 °C (under N₂). ¹H NMR (300 MHz, CDCl₃, LC-1-77-lc1d): δ 3.041 (tt, *J* = 11.8 Hz, 3.6 Hz, 1H), 2.708 (br s, 2H), 2.091 (dd, *J* = 9.5 Hz, 3.4 Hz, 2H), 1.317-1.724 (m, 20H), 0.893 (t, *J* = 12.3 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃, LC-1-77-lc1d): above 90 ppm region, expected 0 resonances, found 0 resonances, below 90 ppm region, expected 8 resonances, found 8 resonances at δ 55.0, 45.5, 42.8, 42.7, 36.7, 26.1, 22.9, 22.4. IR (ZnSe, cm⁻¹, LC-1-77-lc1): 3347 (N-H), 2919, 2849 (C-H), 1452, 1259, 1183, 925, 847, 677. HR-FABMS (LC-1-77-LC1, 3-NBA matrix): (%RA = percent relative abundance for *m/z* = 235-239, deviation from formula), 236.2243 [M]⁺ (8%, +3.9 ppm for ¹²C₁₅H₂₈N₂), 237.2332 [M+H]⁺ (100%, -0.6 ppm for ¹²C₁₅H₂₉N₂), 238.2368 [M+H+1]⁺ (18%, -1.7 ppm for ¹²C₁₄¹³C₁H₂₉N₂).

Procedure for chloro-acetamide 5.

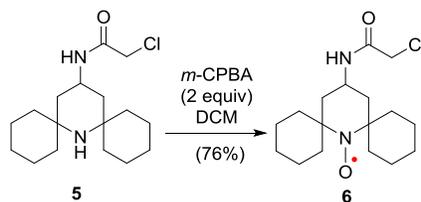


Run	SM (mg/mmol)	SM label	Chloroacetyl chloride (mg/mmol)	TM (mg)	Yield (%)	TM label
LC-1-72	9.4/0.040	LC-1-69b-lc1	4.5/0.040	11	88	LC-1-72-cr
LC-1-75	17.4/0.074	LC-1-69b-lc1	8.3/0.074	19.5	85	LC-1-75-cr
LC-1-81	68.5/0.290	LC-1-77-lc1	32.8/0.290	61.8	68	LC-1-81-cr
LC-1-84	334.7/1.42	LC-1-77-lc1	160/1.42	189 90	63	LC-1-84-cr1 LC-1-84-cr2 ^a
LC-1-90	220.4/0.934	LC-1-68-lc1 LC-1-69-lc2 LC-1-69b-lc1 LC-1-77-lc1	105/0.936	220	76	LC-1-90-cr

^aA second extraction into acid was done after low amount of the product was recovered in first extraction

LC-1-81: The diamine **4** (68.5 mg, 0.290 mmol) was weighed into a round bottom flask, dissolved in DCM (2.2 mL) and cooled in an ice bath while stirring. Chloroacetyl chloride (32.8 mg, 0.290 mmol, 1 equivalent) in DCM (0.78 mL) was added to the solution and stirred for 3 h in an ice bath. Solution was diluted with diethyl ether, and extracted with 1 M HCl. The aqueous fractions were combined, made alkaline with 10% aqueous NaOH, and extracted with DCM. DCM extractions were combined, dried over Na₂SO₄, and evaporated, resulting in crude chloro-acetamide (68%). M.p. 131 – 134 °C. ¹H NMR (300 MHz, CDCl₃, LC-1-84-cr2c): δ 6.302 (d, *J* = 7.1 Hz, 1H), 4.111-4.150 (m, 2H), 4.004 (s, 2H), 2.067 (dd, *J* = 9.3 Hz, 2.8 Hz, 2H), 1.221-1.722 (m, 20H), 0.841 (t, *J* = 12.3 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃, LC-1-84-cr2c): above 90 ppm region, expected 1 resonance, found 1 resonance at 165.0, below 90 ppm region, expected 9 resonances, found 9 resonances at 52.9, 43.8, 43.0, 42.9, 42.5, 37.4, 26.2, 22.9, 22.3. IR (ZnSe, cm⁻¹, LC-1-84-cr2): 3281, 3081, 2924, 2851, 2360, 2342, 1653, 1549, 1454, 1341, 1260, 712, 669. HR-FABMS (LC-1-84-cr2, 3-NBA matrix): (%RA = percent relative abundance for *m/z* = 310-315, deviation from formula), 312.1960 [M]⁺ (11, +2.5 ppm for C₁₇H₂₉N₂O₁³⁵Cl₁), 313.2035 [M+H]⁺ (72, +3.5 ppm for C₁₇H₃₀N₂O₁³⁵Cl₁), 315.2013 [M+H+2]⁺ (23, +1.1 ppm for C₁₇H₃₀N₂O₁³⁷Cl₁).

Procedure for chloro-nitroxide **6**.



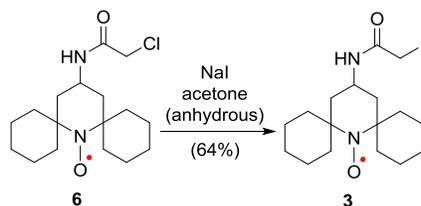
Run	SM (mg/mmol)	SM label	<i>m</i> -CPBA (mg/mmol/equiv)	TM (mg)	Yield (%)	TM label	Hydroxylamine ^b (mg)/(label)
LC-1-73	11/0.035	LC-1-72-cr	- ^a	-	-	-	-
LC-1-76	19.5/0.063	LC-1-75-cr	10.8/0.063/1	8	38	LC-1-76-p1	-
LC-1-82	61.8/0.198	LC-1-81-cr	51.4/0.298/1.5	19.2	30	LC-1-82-lc1	21.9/LC-1-82-lc2
LC-1-85	189/0.578	LC-1-84-cr1	149.6/0.870/1.5	27.8	15	LC-1-85-lc1	33.0/LC-1-85-lc2
LC-1-91	214.6/0.688	LC-1-90-cr	177.9/1.034/1.5	54.8	24	LC-1-91-lc1	65.6/LC-1-91-lc2
LC-2-01	72.3/0.232	LC-1-84-cr2	79.6/0.463/1.99	57.9	76	LC-2-01-lc1	0

^a Oxidation using hydrogen peroxide, tungstate, and EDTA resulting in no yield of the target product.

^b In reactions LC-1-82,85,91 using 1.5 equivalents of *m*-CPBA, hydroxylamine was isolated as a byproduct by flushing column with ethyl acetate. Combined fractions of hydroxylamine (106.5 mg, 0.325 mmol) were then further oxidized with *m*-CPBA to provide additional amount (36.2 mg) of the target chloro-nitroxide.

LC-2-01: Crude chloro-acetamide **5** (72.3 mg, 0.232 mmol) and a stir bar in a round bottom flask were flushed with nitrogen, dissolved in DCM (3 mL) and cooled in an ice bath. Purified *m*-CPBA (79.6 mg, 0.463 mmol, 1.99 equivalent) in DCM (5 mL) was added, and the solution was stirred in an ice bath for 2.25 h in the absence of light. The flask was removed from the ice bath, and stirring was continued for 5 h in the exclusion of light at room temperature. The resulting orange solution was diluted with ether, washed several times with pH 7.3 phosphate buffer, dried over Na₂SO₄, and evaporated. Purification on silica gel column with benzene:acetone 20:1 gave 57.9 mg (76%) of chloro-nitroxide **6**. M.p. 164 – 166 °C. HR-FABMS (LC-1-82-lc1, ONPOE matrix): (%RA = percent relative abundance for *m/z* = 325-330, deviation from formula), 327.1841 [M]⁺ (100, -0.5 ppm for ¹²C₁₇H₂₈N₂O₂³⁵Cl₁), 328.1915 [M+H]⁺ (85, +0.7 ppm for ¹²C₁₇H₂₉N₂O₂³⁵Cl₁), 330.1895 [M+H+2]⁺ (34, -2.3 ppm for ¹²C₁₇H₂₉N₂O₂³⁷Cl₁). ¹H NMR (500 MHz, CDCl₃, c = 0.41 M, LC-1-91-lc1, spectrum on p. S18). IR (ZnSe, cm⁻¹, LC-1-82-lc1): 3294, 2928, 2862, 2360, 1661, 1542, 1450, 1337, 1257, 1180, 792, 757, 719. EPR (DCM, LC-1-82-lc1): *g* = 2.0060, *a*_N = 1.542 mT.

Procedure for spin label **3**.

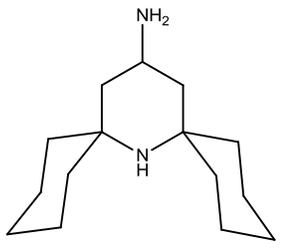


Run	SM (mg/mmol)	SM label	NaI (mg/mmol/equiv)	TM (mg)	Yield (%)	TM label	Characterization
LC-1-88	15.8/0.0483	LC-1-85-1c1	11.4/0.076/1.57	5.1	25.3 ^a	LC-1-88-2 ^b	¹ H NMR; EPR; ESI-MS
LC-1-93	7.8/0.0239	LC-1-85-1c1	3.9/0.0262/1.1	4.0	39.9 ^a	LC-1-93-P1 ^b	IR; m.p.
LC-1-95	46.2/0.141	LC-1-91-1c1	24.2/0.161/1.14	21.4	36.2 ^a	LC-1-95-1c1 ^b	¹ H NMR
LC-1-98	55.9/0.171	LC-1-96-1c1 LC-1-82-1c1 LC-1-95-1c2 LC-1-79-pc1 LC-1-89-1 LC-1-93-p2	28.5/0.189/1.11	13.8 31.8	63.6	LC-1-98-1c1 LC-1-98-1cb1 ^b	EPR, pulsed EPR

^a Purification by chromatography (silica, pentane:EtOAc, 3:1)

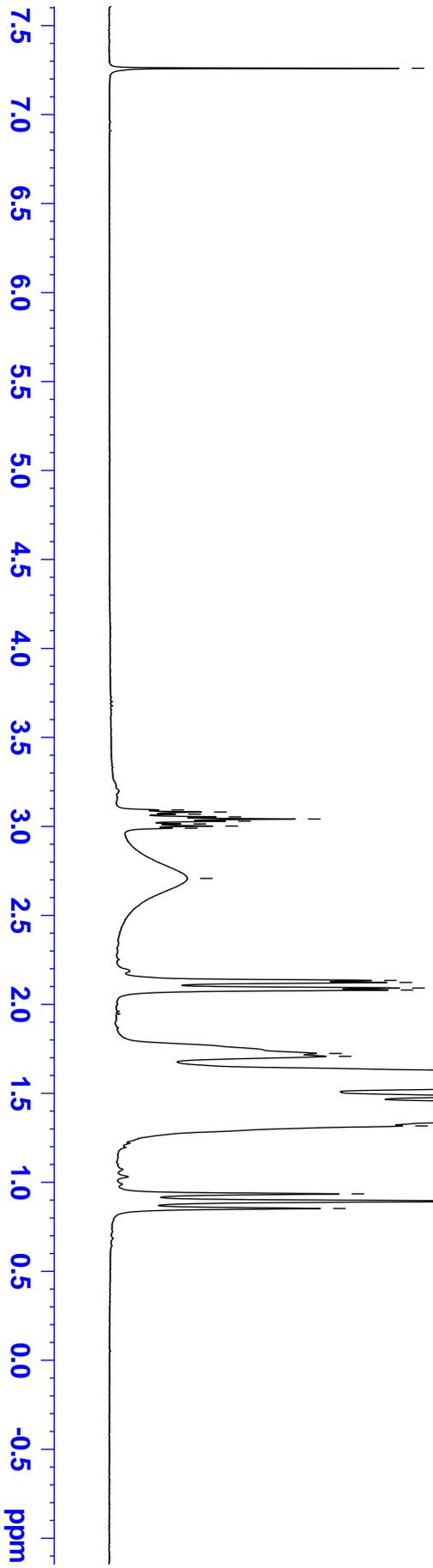
^b These samples were combined, and the spin concentration was found to be approx. 96% for LC-1-98-1c1 (EPR label: LC-2-06).

LC-1-98: Chloro-nitroxide **6** (55.9 mg, 0.171 mmol) was evacuated in a vial, and suspended in freshly distilled acetone (0.6 mL). Then a freshly prepared solution of sodium iodide in acetone (0.34 mL, 0.558M, 0.189 mmol, 1.11 equiv) was added. The vial was sealed and sonicated until **6** dissolved completely, then stirred for 22 h in the absence of light. A white precipitate was observed, solution was filtered and acetone evaporated. A portion of the product that dissolved in pentane/EtOAc 3:1 was purified on a silica gel column using the same solvent giving 13.8 mg of **3**. The undissolved crude material was chromatographed on silica gel using benzene/acetone 20:1 to give an additional 31.8 mg of product. A total of 45.6mg (64%) of spin label **3** was obtained as orange crystals. M.p. 180 – 181 °C. ESI-MS (LC-1-88-2, +Na): 442.1097 [M+Na]⁺ (0.9 ppm). NMR (500 MHz, CDCl₃, *c* = 0.15 M, LC-1-95-1c1, spectrum on p. S19). IR (ZnSe, cm⁻¹, LC-1-93-p1): 3273, 2924, 2857, 2360, 1648, 1547, 1456, 1326, 1309, 1255, 1171, 908, 719, 669. EPR (DCM, LC-1-98-1c1r2): *g* = 2.0060, *a_N* = 1.548 mT; spin concentration (DCM, LC-1-98-1c1): 96%.



7.260

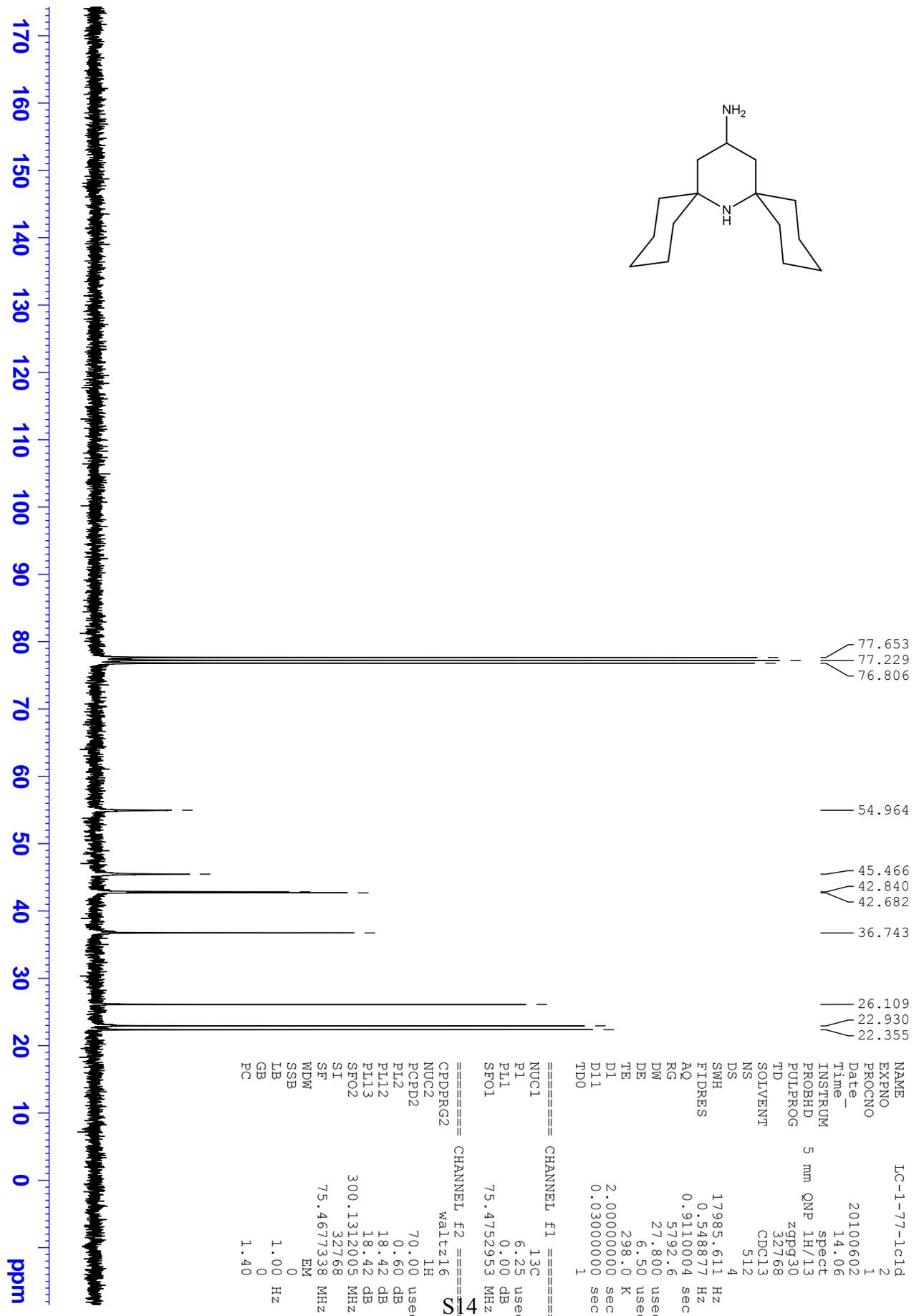
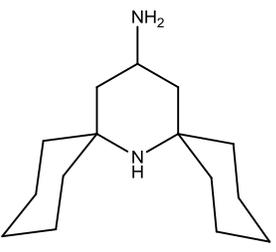
- 3.092
- 3.081
- 3.068
- 3.053
- 3.041
- 3.029
- 3.014
- 3.002
- 2.990
- 2.708
- 2.134
- 2.123
- 2.091
- 2.080
- 1.724
- 1.707
- 1.625
- 1.588
- 1.550
- 1.481
- 1.446
- 1.413
- 1.386
- 1.350
- 1.317
- 0.935
- 0.893
- 0.853



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===== CHANNEL f1 =====
NAME          LC-1-77-1c1d
EXPNO         1
PROCNO        1
Date_         20100602
Time_         13.50
INSTRUM       spect
PROBHD        5 mm QNP 1H/13
PULPROG       zg30
TD            65536
SOLVENT       CDCl3
NS            32
DS            2
SWH           6172.839 Hz
FIDRES       0.094190 Hz
AQ           5.3084660 sec
RG           181
DM           81.000 usec
DE           6.50 usec
TE           298.0 K
D1           1.00000000 sec
TD0          1

===== CHANNEL f2 =====
NUC1          13C
P1            9.00 usec
PL1          0.60 dB
SFO1         300.1318534 MHz
SI           32768
SF           300.1300064 MHz
WDW          EM
SSB          0
LB           0.30 Hz
GB           0
PC           1.00
  
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77.653
77.229
76.806
54.964
45.466
42.840
42.682
36.743
26.109
22.930
22.355

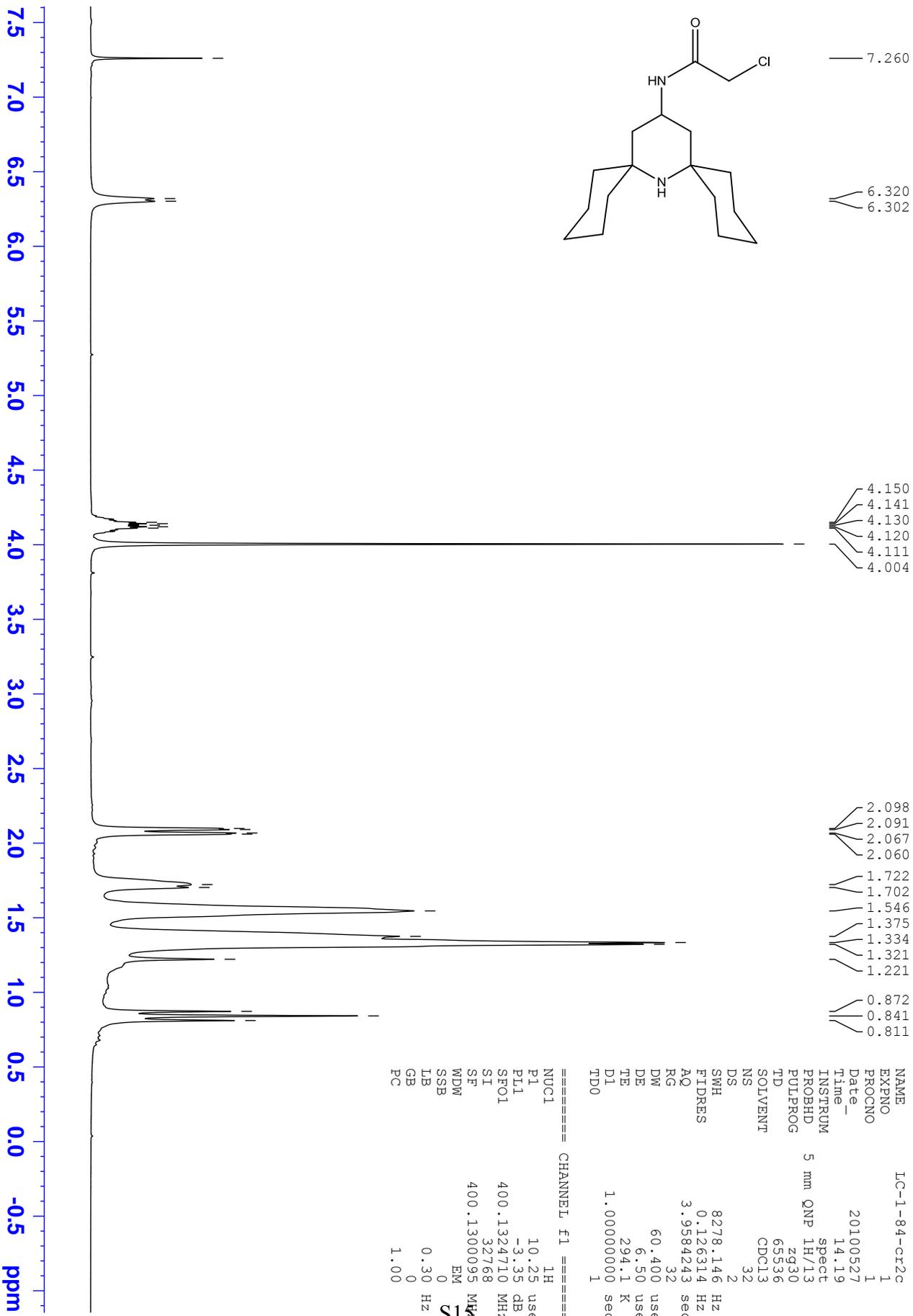
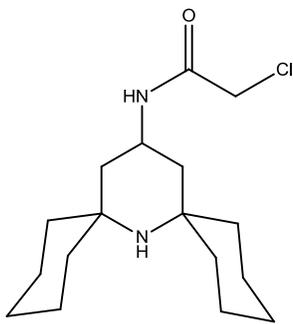
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PROCNO        1
Date_         20100602
Time_         14.06
INSTRUM       spect
PROBHD        5 mm QNP 1H/13
PULPROG       zgpg30
TD            32768
SOLVENT       CDCl3
NS            512
DS            4
SWH           17985.611 Hz
FIDRES        0.548877 Hz
AQ            0.9110004 sec
RG            5792.6
DM            27.800
DE            6.50 usec
TE            298.0 K
D1            2.00000000 sec
D11           0.03000000 sec
TD0           1

===== CHANNEL f1 =====
NUC1          13C
P1            6.25 usec
PL1           0.00 dB
SFO1         75.4752953 MHz

===== CHANNEL f2 =====
CPDPRG2      waltz16
NUC2          1H
PCPD2        70.00 usec
PL2           0.60 dB
PL12         18.42 dB
PL13         18.42 dB
SFO2         300.1312005 MHz
SI           32768
SF           75.4677338 MHz
WDW           EM
SSB           0
LB           1.00 Hz
GB            0
PC           1.40
  
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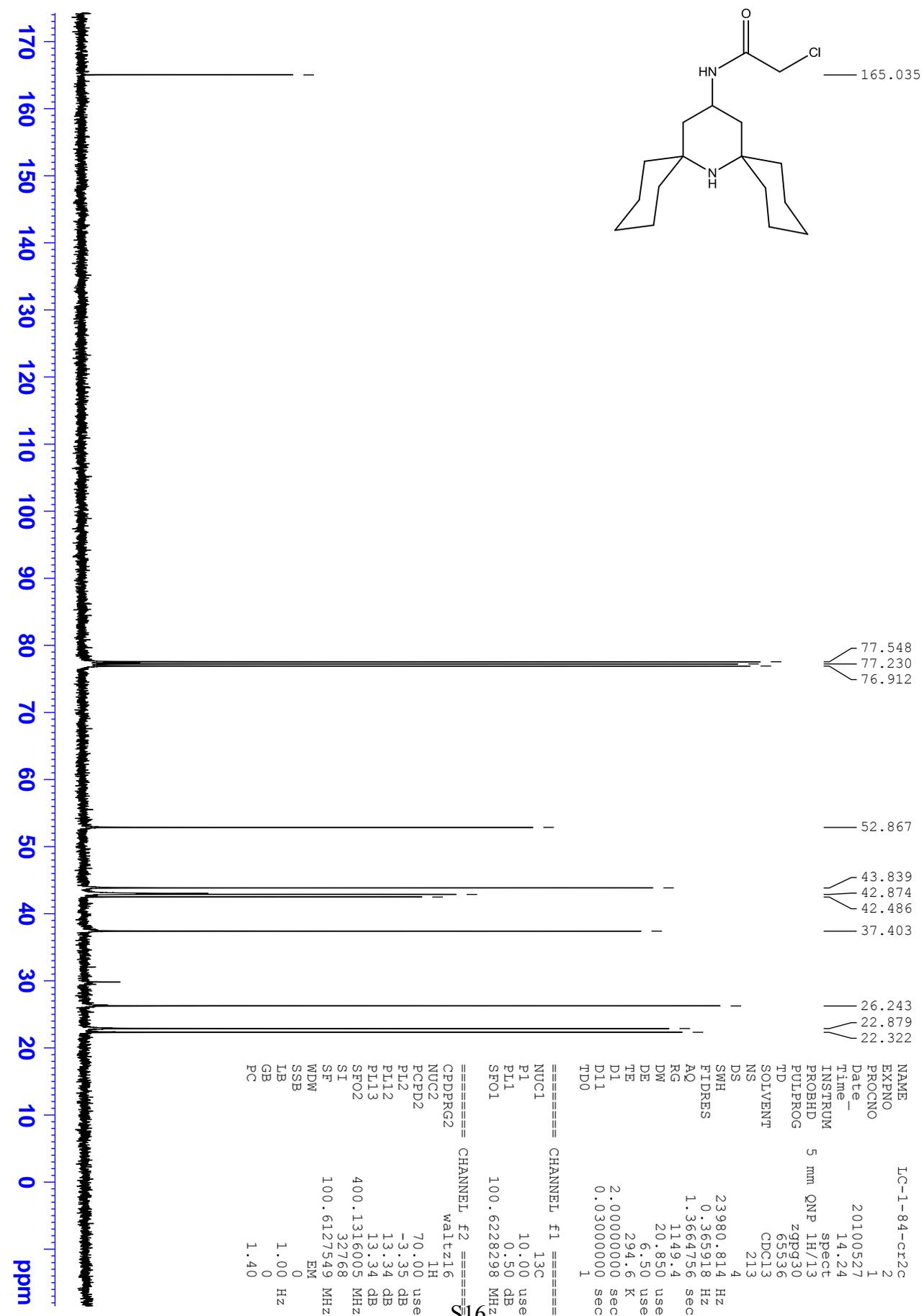
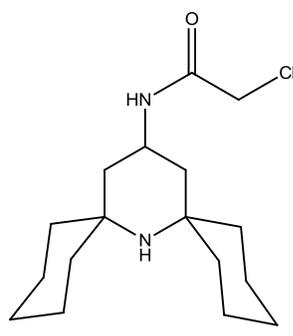
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm



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NAME          LC-1-84-cr2c
EXPNO         1
PROCNO        1
Date_         20100527
Time_         14.19
INSTRUM       spect
PROBHD        5 mm QNP 1H/13
PULPROG       zg30
TD            65536
SOLVENT       CDCl3
NS            32
DS            2
SWH           8278.146 Hz
FIDRES        0.126314 Hz
AQ            3.9584243 sec
RG            32
DM            60.400 usec
DE            6.50 usec
TE            294.1 K
D1            1.00000000 sec
TD0           1

===== CHANNEL f1 =====
NUC1          1H
P1            10.25 usec
PL1          -3.35 dB
SFO1         400.1324710 MHz
SI           32768
SF           400.1300095 MHz
WDW           EM
SSB           0
LB           0.30 Hz
GB           0
PC           1.00
  
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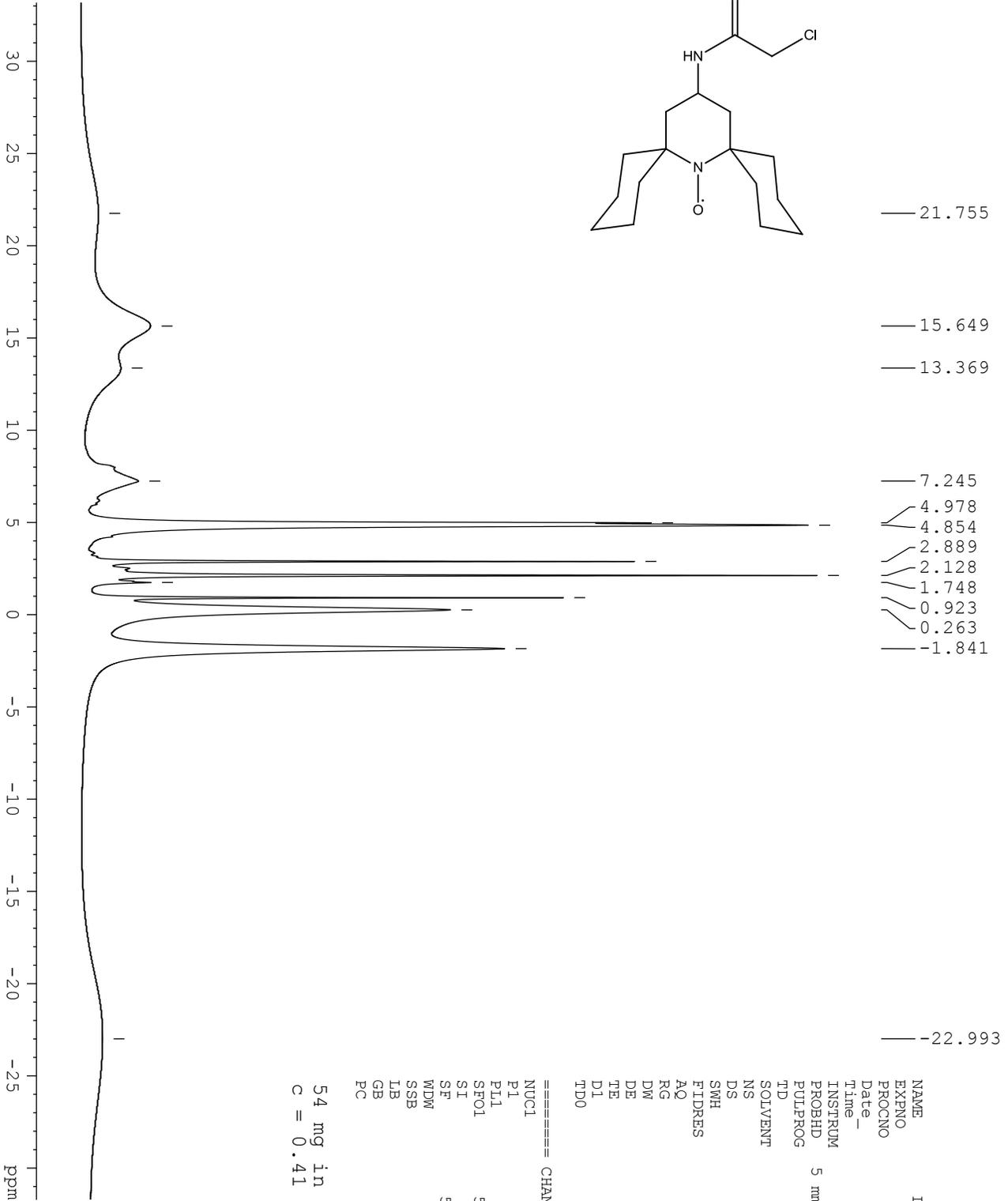
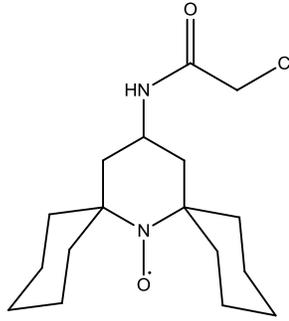
77.548
77.230
76.912
52.867
43.839
42.874
42.486
37.403
26.243
22.879
22.322

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NAME          LC-1-84-cr2c
EXPNO         2
PROCNO        1
Date_         20100527
Time          14.24
INSTRUM       spect
PROBHD        5 mm QNP 1H/13
PULPROG       zgpg30
TD            65536
SOLVENT       CDCl3
NS            213
DS            4
SWH           23980.814 Hz
FIDRES        0.365918 Hz
AQ            1.3664756 sec
RG            1149.4
DE            20.850 usec
TE            294.6 K
D1            2.0000000 sec
D11           0.0300000 sec
TD0           1

===== CHANNEL F1 =====
NUC1          13C
P1            10.00 usec
PL1           0.50 dB
SFO1         100.6228298 MHz

===== CHANNEL F2 =====
CPDPRG2      waltz16
NUC2          1H
PCPD2        70.00 usec
PL2          -3.35 dB
PL12         13.34 dB
PL13         13.34 dB
SFO2         400.1316005 MHz
SI           32768
SF           100.6127549 MHz
WDW          EM
SSB          0
LB           1.00 Hz
GB           0
PC           1.40
  
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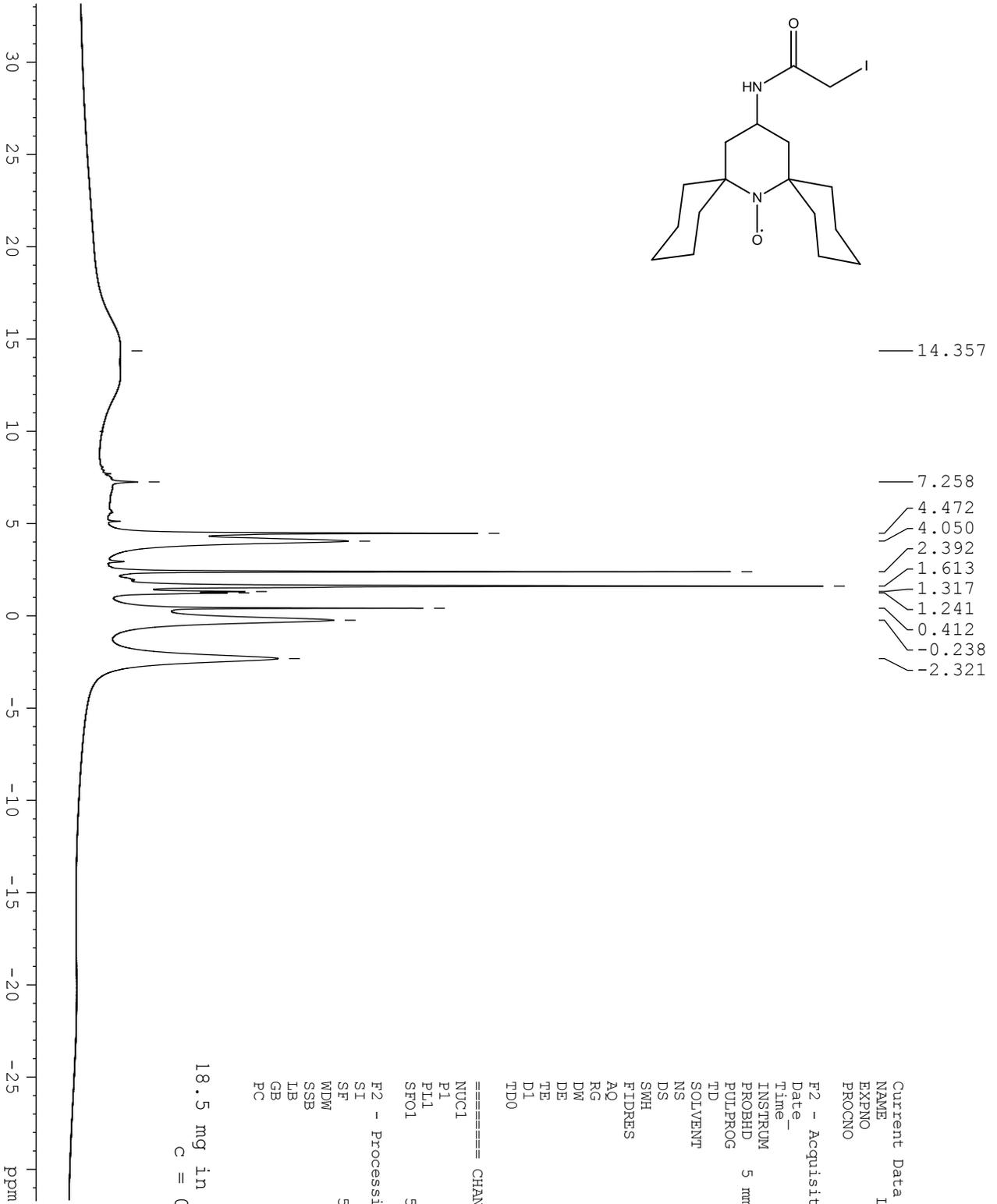
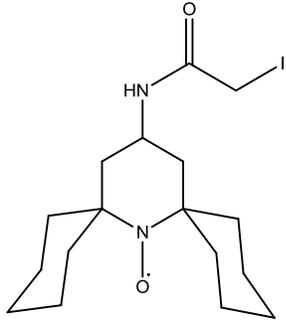
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NAME          IC-1-91-1c1
EXPNO         3
PROCNO        1
Date_         20100611
Time_         10.56
INSTRUM       spect
PROBHD        5 mm CPTXI 1H-
PULPROG       zg30
TD            131072
SOLVENT       CDCl3
NS            64
DS            2
SWH           50125.313 Hz
FIDRES       0.382426 Hz
AQ           1.3075032 sec
RG           35.9
DW           9.975 usec
DE           6.00 usec
TE           298.0 K
D1           1.00000000 sec
TD0          1

===== CHANNEL f1 =====
NUC1          1H
P1           8.30 usec
PL1          -4.30 dB
SFO1         500.1350013 MHz
SI           131072
SF           500.1300000 MHz
WDW          EM
SSB          0
LB           0.30 Hz
GB           0
PC           1.00

54 mg in 0.4 mL CDCl3
C = 0.41 M

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Current Data Parameters
 NAME LC-1-95-1c1
 EXPNO 1
 PROCNO 1

F2 - Acquisition Parameters

Date_ 20100624
 Time_ 12.51
 INSTRUM spect
 PROBHD 5 mm CPTXI 1H-
 PULPROG zg30
 TD 131072
 SOLVENT CDCl3
 NS 64
 DS 2
 SWH 50125.313 Hz
 FIDRES 0.382426 Hz
 AQ 1.3075032 sec
 RG 256
 DW 9.975 usec
 DE 6.00 usec
 TE 298.0 K
 D1 1.00000000 sec
 TD0 1

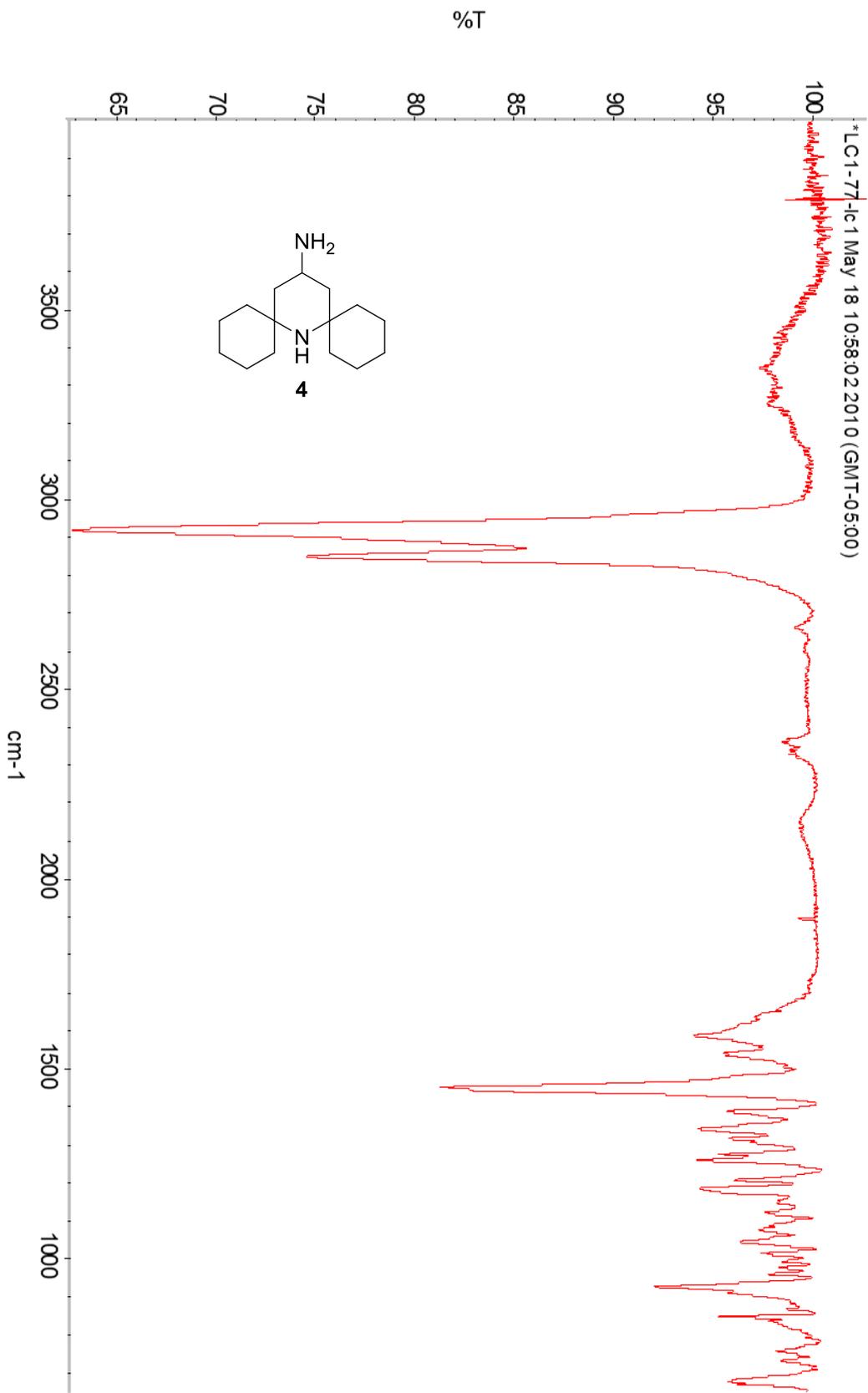
==== CHANNEL F1 =====

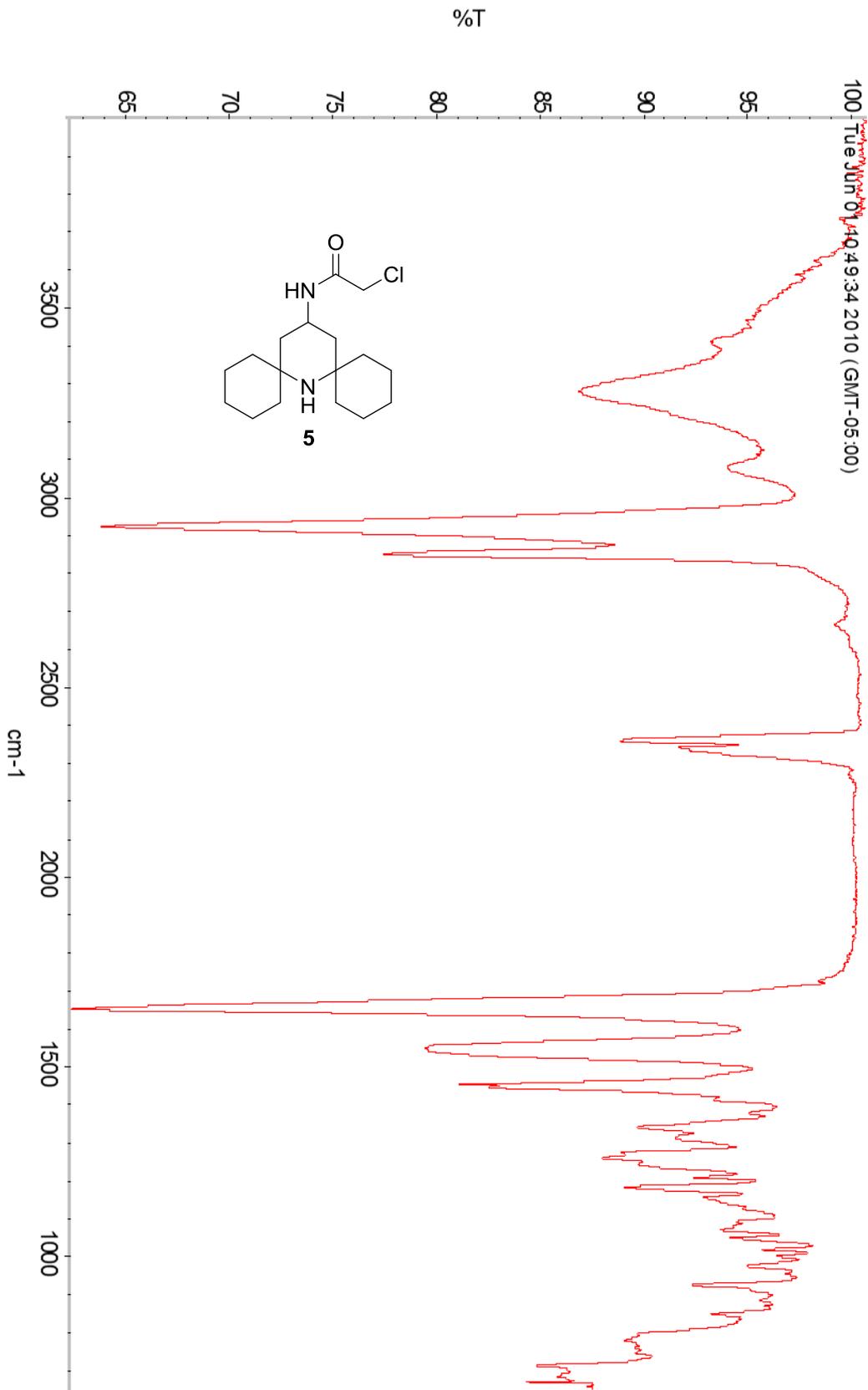
NUC1 1H
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 PL1 -4.30 dB
 SF01 500.1350013 MHz

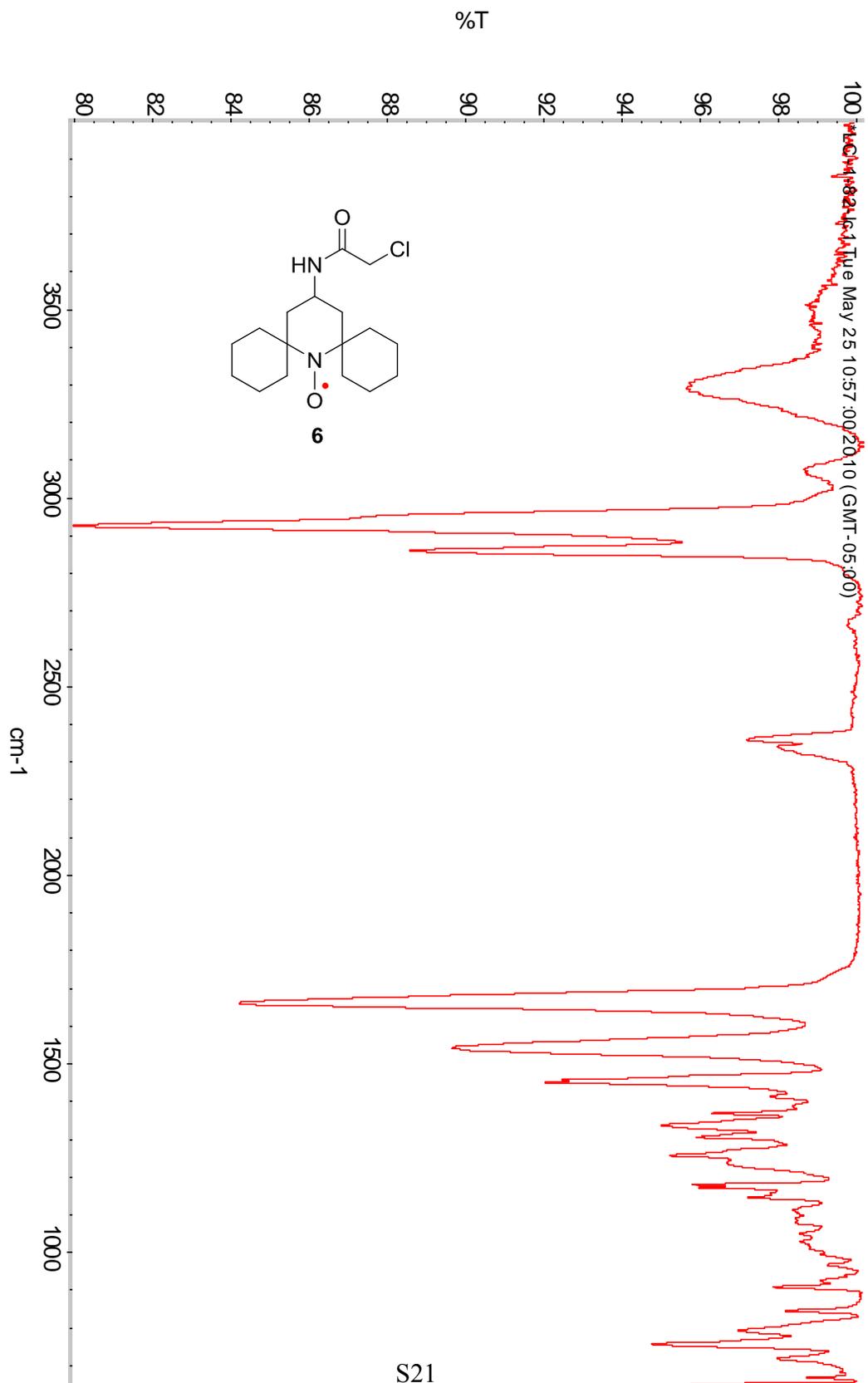
F2 - Processing parameters

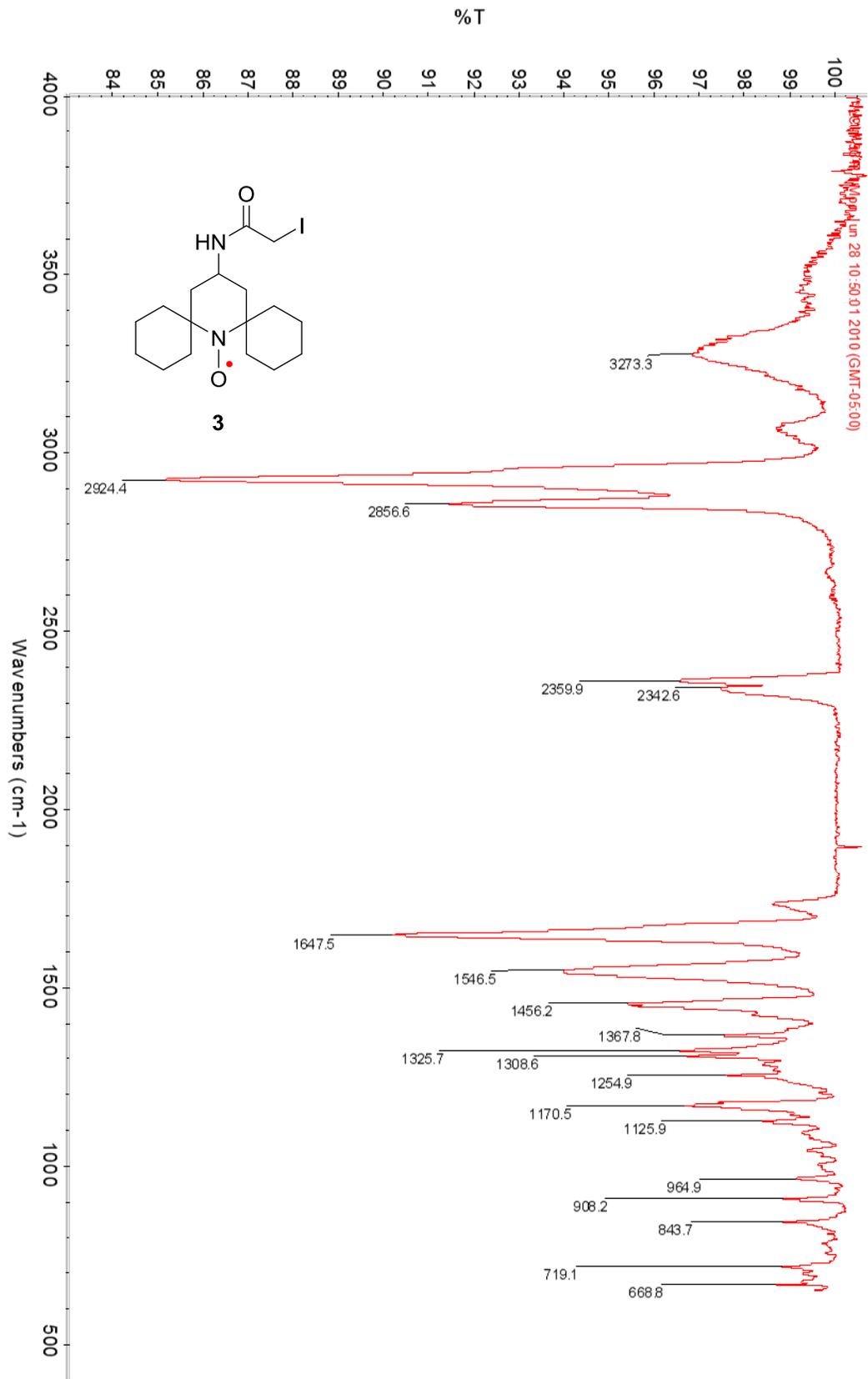
SI 131072
 SF 500.1300029 MHz
 WDM EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

18.5 mg in 0.3 mL CDCl3
 c = 0.15 M









Supporting References

- S1. Mims, W. B. 1972. Electron Spin Echoes. In *Electron Paramagnetic Resonance*. S. Geschwind, editor. Plenum Press. New York. 263-351.
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- S3. Yu, Z., R. W. Quine, G. A. Rinard, M. Tseitlin, H. Elajaili, V. Kathirvelu, L. J. Clouston, P. J. Boratyński, A. Rajca, R. Stein, H. Mchaourab, S. S. Eaton, and G. R. Eaton. 2014. Rapid-Scan EPR of Immobilized Nitroxides. *J. Magn. Reson.* 247: 67-71 (2014).
- S4. Rajca, A., K. Shiraishi, and S. Rajca. 2009. Stable Diarylnitroxide Diradical with Triplet Ground State. *Chem. Commun.*:4372 - 4374.