Conformation of [1-^{13}C,^{15}N]Acetyl-L-carnitine. Rotational-Echo, Double-Resonance Nuclear Magnetic Resonance Spectroscopy

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Abstract: The conformation of [1-^{13}C,^{15}N]acetyl-L-carnitine is studied by rotational-echo, double-resonance (REDOR) NMR experiments. The REDOR results show that acetyl-L-carnitine adopts an extended molecular conformation in the solid state for both crystalline and lyophilized samples. These findings are in contrast to various X-ray-determined structures of racemic acetylcarnitine showing folded conformations.

Introduction

Acetyl-L-carnitine is a source of readily available activated acetyl groups,[1] which can be used as a substrate for oxidation mitochondria from several tissues such as brain, heart, kidney, testis, liver, and muscle,[2,3] or for biosynthetic purposes.[4] Through the activity of carnitine acetyltransferase (CAT) (EC 2.3.1.7), acetyl-L-carnitine is involved in the reversible transfer of acetyl groups between carnitine and coenzyme A,[5] thereby maintaining the proper CoASH/acetyl-SCoA ratio within the mitochondrial matrix.[6,7] The effect of acetyl-L-carnitine on aging has gained recent interest. In studies with rat heart mitochondria,[8] it has been proposed that acetyl-L-carnitine may restore the correct phospholipid composition (cardiolipin level) of the mitochondrial membrane, altered by aging, thereby restoring the original activity of the phosphate carrier. There is also recent literature on the possible utility of acetyl-L-carnitine in the treatment of age-related cholinergic deficits[9,10] and in the treatment of autonomic neuropathies,[11] a common complication in diabetes.

It has been suggested that the conformations of carnitine and acetylcarnitine play important roles in the activity of CAT where the enzyme requires the extended conformation of carnitine for the forward reaction and, once acetylated, the acetylcarnitine adopts a folded conformation. These conformational assumptions are supported by X-ray crystallographic data of carnitine and acetylcarnitine obtained by Gandour *et al.*[5] Their crystal structures show C(1) to N distances of 4.24 Å for acetyl-DL-carnitine, corresponding to a folded conformation, and 5.06 Å for carnitine, corresponding to an extended conformation. These X-ray results have also led to a structural hypothesis for the mode of action of CAT.[5] However, recent solution-state NMR investigations of L-carnitine and acetyl-L-carnitine indicate that the conformational difference between L-carnitine and acetyl-L-carnitine is minor, suggesting that both compounds have an extended conformation in solution.[12] Because the solid-state conformation of acetyl-DL-carnitine differs substantially from the solution-state conformation of acetyl-L-carnitine, we investigate the solid-state structure of acetyl-L-carnitine. Specifically, we will determine whether the solid-state conformation is folded or extended by examining the structure of a specifically labeled [1-^{13}C,^{15}N]acetyl-L-carnitine hydrochloride sample with rotational-echo, double-resonance (REDOR) NMR spectroscopy.[13]

Results and Discussion

[1-^{13}C,^{15}N]Acetyl-L-carnitine was prepared, as shown in Figure 1, via a modification of a procedure[14] for the synthesis of L-carnitine hydrochloride followed by acetylation.[2] Selective tosylation of (*R*)-(−)-α-monochlorohydrin[15] followed by nucleophilic displacement of the tosylate by potassium cyanide (^{13}C, 99%)[16] provided 4-chloro-3(*R*)-hydroxy[1-^{13}C]butyronitrile. Nucleophilic displacement of the chloride with a slight excess (1.5 equiv) of trimethylamine−HCl (^{15}N, 95%),[16] which had been initially converted to the free base (Amberlite IRA-400 (OH) resin in H_2O), provided 4-([^{15}N]trimethylammonio)-3(*R*)-hydroxy[1-^{13}C]butyronitrile (chloride salt) as a white crystalline solid after recrystallization from 95% EtOH. Hy-

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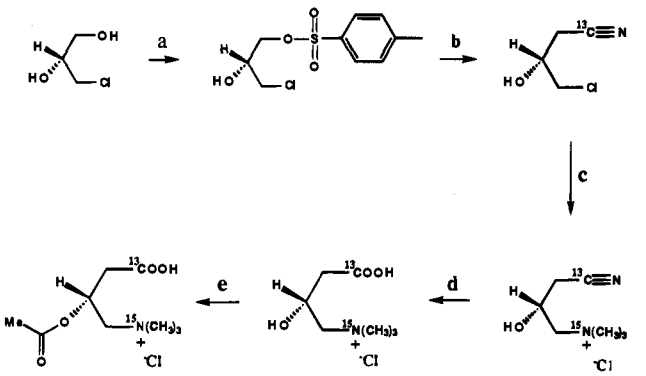

Figure 1. Synthesis of [1-^{13}C,^{15}N]acetyl-L-carnitine. Reagents and conditions: (a) TsCl, pyridine, 0 °C, 10 min, and then room temperature (RT), 1 h; (b) K^{13}CN, MeOH, RT, 3.5 h; (c) $(Me)_3{}^{15}N$, H_2O, 90 °C (bomb), 0.75 h; (d) HCl (37% aqueous), 100 °C, 4.5 h; (e) acetyl chloride, acetic acid, 52 °C, 4 h, and then RT, 18 h.

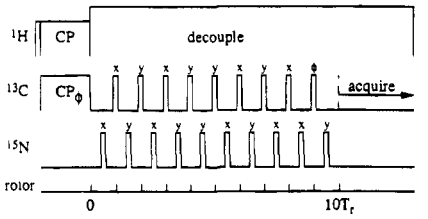

Figure 2. ^{13}C-observe REDOR NMR pulse sequence for a 10 rotor cycle evolution period. The carbon and nitrogen π pulses are phase cycled according to the *xy*-8 phase scheme to minimize off-resonance effects.[20,21]

drolysis of the nitrile provided L-carnitine hydrochloride (1-^{13}C, ^{15}N) and an equivalent amount of ammonium chloride. To remove the ammonium impurity, the material was made basic (Amberlite IRA-400 (OH) resin in H_2O), filtered, and lyophilized to provide the inner carnitine salt. Conversion to the HCl salt with 2 N HCl, followed by lyophilization, provided pure L-carnitine hydrochloride (1-^{13}C, ^{15}N). Acetylation provided the pure [1-^{13}C,^{15}N]acetyl-L-carnitine hydrochloride as a white crystalline solid. More details regarding the synthesis can be found in the Experimental Section.

The internuclear separation, r_{CN}, between a specifically labeled ^{13}C,^{15}N spin pair in a solid can be obtained by measuring the magnetic dipole coupling, D_{CN}, between the spin pair. The distance is related to the dipolar coupling through[17]

$$D_{CN} = \gamma_C\gamma_N h/2\pi r_{CN}^3 \qquad (1)$$

For complex solids, high-resolution ^{13}C NMR spectra are typically obtained by combining cross-polarization and high-power proton decoupling with magic-angle spinning (MAS).[18] Unfortunately, MAS averages the dipolar interaction to zero, and for very fast spinning conditions, no evidence of the interaction is present in the spectrum. Rotational-echo, double-resonance (REDOR) is a MAS NMR experiment designed specifically to recover the dipolar coupling between a heteronuclear spin pair while maintaining the requisite high resolution needed for complex problems.[13] The ^{13}C-observe REDOR pulse sequence used in this work differs from the original REDOR experiment[13] and is shown in Figure 2. This version of the REDOR is less sensitive to the effects of finite pulse widths and resonance offsets than its predecessor and does not scale the dipolar interaction.[19]

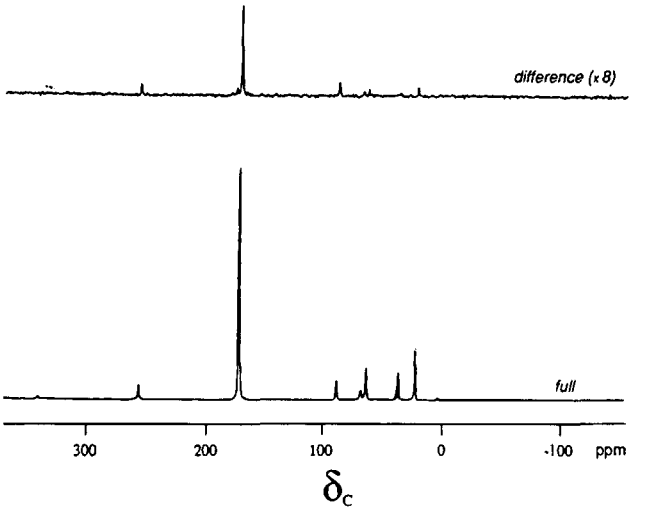

Figure 3. ^{13}C-observe REDOR NMR results for the crystalline powder sample of labeled [1-^{13}C,^{15}N]acetyl-L-carnitine hydrochloride diluted 1 part to 20 parts of natural abundance material: (bottom) *full* spectrum; (top) *difference* spectrum. These spectra were obtained with $N_c = 34$ and a spinning speed of 3205 Hz. Carbon and nitrogen radio frequency (rf) field strengths were 38 kHz. The proton rf field strengths were 38 kHz during cross-polarization (CP) and 110 kHz during the evolution and data acquisition periods.

The experiment consisted of ^{13}C signal preparation, by proton−carbon cross-polarization, followed by a dipolar evolution period lasting for N_c rotor cycles. Data acquisition immediately followed the dipolar evolution period. Strong proton decoupling was applied during the dipolar evolution and data acquisition periods. During the dipolar evolution period, a train of ^{13}C π pulses is always applied and a train of ^{15}N π pulses may be applied. The spacing between π pulses on each channel is one rotor period, T_r.

The REDOR experiment is performed as a difference experiment. In the absence of the ^{15}N π-pulse train, the ^{13}C−^{15}N dipolar interaction is spatially averaged to zero. The π-pulse train on the ^{13}C channel generates a spin-echo at the start of data acquisition. The ^{13}C signal obtained without application of the ^{15}N π-pulse train is the *full* signal, S_0, and serves as a control experiment to account for signal loss due to relaxation. When the ^{15}N π-pulse train is applied as illustrated in Figure 2, the ^{13}C−^{15}N dipolar interaction no longer averages to zero.[13] Consequently, the ^{13}C magnetization dephases during the evolution period, causing a net loss of ^{13}C signal intensity. This signal is the *reduced* signal, S_r. A particularly useful ratio in analyzing REDOR data is the *difference* signal, $\Delta S = S_0 - S_r$, divided by the *full* signal, S_0. An analytical expression for the dependence of $\Delta S/S_0$ on the dimensionless product $N_cT_rD_{CN}$ for a single spin pair with an arbitrary orientation has been derived.[13] For powder samples, the dependence of signal attenuation on the dipolar coupling, D_{CN}, and the dipolar evolution time, N_cT_r, can be calculated by computer.

Figure 3 shows ^{13}C-observe REDOR spectra of specifically labeled [1-^{13}C,^{15}N]acetyl-L-carnitine hydrochloride (crystallized, see Experimental Section) diluted in natural abundance acetyl-L-carnitine hydrochloride, 1:20. The dilution is necessary to minimize the *intermolecular* ^{13}C−^{15}N dipolar interaction.

The C(1) carbon resonance occurs at 171.9 ppm. Unfortunately, the ^{13}C resonance associated with the C(8) site occurs

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Table 1. REDOR NMR Results on [1-^{13}C,^{15}N]Acetyl-L-carnitine

sample form	N_c	$\Delta S^{(m)}/S^{(m)}$ [a]	$\Delta S^{(8)}/S_0^{(8)}$ [b]	$\Delta S^{(1)}/S_0^{(1)}$ [c]	$D_{C(1)-N}$ (Hz)	$r_{C(1)-N}$ [d] (Å)
crystalline	34	0.0498	0.290	0.0729	25.1	4.95
crystalline	42	0.0729	0.416	0.1068	24.8	4.97
lyophilized	42	0.0659	0.416	0.0961	23.5	5.06

[a] Computed by integration of center bands and spinning side bands. [b] Computed using a C(8)−N distance of 3.87 Å. [c] Computed using $\alpha = 0.652$ and $\beta = 0.007\,85$. [d] For the C(1)−N spin pair.

at the same spectral position. This spectral overlap complicates the analysis of the REDOR results, but this difficulty can be overcome.

The C(1)−N distance can be obtained once $\Delta S^{(1)}/S_0^{(1)}$ is determined. Here, $\Delta S^{(1)}$ and $S_0^{(1)}$ are the *difference* and *full* signal intensities from the specifically labeled spin 1-^{13}C, ^{15}N spin pair, respectively. Since the labeled sample is diluted with natural abundance material and since there is spectral overlap between the C(1) and C(8) carbon resonances, $\Delta S^{(1)}/S_0^{(1)}$ differs from the experimentally determined ratio $\Delta S^{(m)}/S^{(m)}$.[22] The desired ratio $\Delta S^{(1)}/S_0^{(1)}$ can be obtained by noting that the experimentally determined *difference* signal, $\Delta S^{(m)}$, is the sum

$$\Delta S^{(m)} = \Delta S^{(1)} + \Delta S^{(8)} \tag{2}$$

This result, divided by $\Delta S^{(m)}$, can be rearranged to obtain

$$\frac{\Delta S^{(1)}}{S_0^{(1)}} = \left(\frac{1}{\alpha}\right)\frac{\Delta S^{(m)}}{S^{(m)}} - \left(\frac{\beta}{\alpha}\right)\frac{\Delta S^{(8)}}{S_0^{(8)}} \tag{3}$$

The ratio of the number of 1-^{13}C, ^{15}N spin pairs, $S_0^{(1)}$, to the total number of ^{13}C spins, $S^{(m)}$, is α. Similarly the ratio of the number of 8-^{13}C, ^{15}N spin pairs, $S_0^{(8)}$, to $S^{(m)}$ β. This analysis assumes that the ^{13}C spin dynamics, such as cross-polarization and relaxation during the dipolar evolution period, are similar for the C(1) and C(8) positions. The $\Delta S^{(8)}/S_0^{(8)}$ term is calculated by assuming a "known" C(8)−N distance. Spectra such as those shown in Figure 3 are used to obtain the ratio $\Delta S^{(m)}/S^{(m)}$. For example, measurement of the center band and the spinning side band intensities associated with the carboxylic acid ^{13}C resonance in the *difference* spectrum (Figure 3, top) produces $\Delta S^{(m)}$; $S^{(m)}$ is obtained similarly from the *full* spectrum (Figure 3, bottom).

Two REDOR experiments, one with $N_c = 34$ and the other with $N_c = 42$, were performed on the labeled crystalline powder sample. The data are summarized in Table 1.

Initially, the $\Delta S^{(8)}/S_0^{(8)}$ was calculated using a C(8)−N distance of 4.17 Å reported for an acetyl-DL-carnitine monohydrate sample.[5] With that result, our REDOR data produced C(1)−N distances of 4.84 and 4.96 Å for the 34 and 42 rotor cycle experiments, respectively. Each of these distances is much greater than previously reported C(1)−N distances for samples of acetyl-DL-carnitine, including 4.35 Å for a monohydrate,[5] 4.24 Å for a hydrochloride,[23] and 4.33 Å for a hydrochloride·H_2O.[24]

Such a large difference between our calculated C(1)−N distance in acetyl-L-carnitine and the distances reported for acetyl-DL-carnitine is not due to any uncertainty in the C(8)−N distance and its corresponding correction in eq 2. For example, assuming relatively extreme C(8)−N distances of 3.0 and 4.8

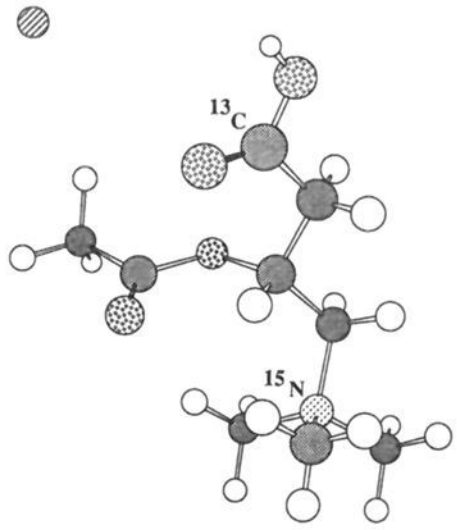

Figure 4. X-ray-determined structure of [1-^{13}C,^{15}N]acetyl-L-carnitine hydrochloride used in this work. The sites of isotopic labeling are shown.

Å, C(1)−N distances of 5.0 and 4.9 Å are calculated for the 34 rotor cycle experiment, respectively. Additional experiments were performed on a similarly labeled sample that had been lyophilized and on a natural abundance sample. The signal for the resonance at 171.9 ppm in the lyophilized labeled sample is dominated by the C(1) position whereas in the natural abundance sample the C(1) and C(8) positions are expected to make comparable contributions to the resonance. The magnetization buildup during cross-polarization was similar, but not identical, for the two samples, and the signal decay following cross-polarization was nearly identical for the two samples. These results suggest that the assumptions in eqs 1 and 2 are reasonable. Our conclusion is that the C(1)−N distance in the acetyl-L-carnitine hydrochloride crystalline form is longer than that found for acetyl-DL-carnitine and results in full extension of the molecular backbone.

Since our REDOR results suggest that the C(1)−N distance in acetyl-L-carnitine hydrochloride is very different from that reported elsewhere,[5,23,24] a single crystal of this sample was grown for X-ray analysis. Results of that work will be presented later,[25] but this X-ray study finds a C(1)−N distance of 5.05 Å and a C(8)−N distance of 3.87 Å. The agreement between the REDOR-determined and the X-ray-determined C(1)−N distances is excellent. For completeness, the C(1)−N REDOR-determined distances in Table 1 use the C(8)−N distance found in this recent X-ray work for the correction term in eq 2. The X-ray-determined structure of our sample is shown in Figure 4 and shows nearly full extension of the molecule. The space group and hence the crystal packing of acetyl-L-carnitine is different from that obtained for acetyl-DL-carnitine, and we believe that this is the reason for the different solid-state structures.

The C(1)−N distances measured by REDOR and X-ray diffraction differ by approximately 0.1 Å, with the REDOR providing the smaller value. NMR measurements typically report distances that are longer than those reported by X-ray diffraction studies, with the longer NMR-determined distance attributed to motional averaging. The uncertainty measured in the quantities $\Delta S^{(m)}/S^{(m)}$ obtained from analyzing the REDOR spectra is approximately 0.0003, corresponding to uncertainties in the C(1)−N distance of about 0.01 Å. This small uncertainty does not account for this 0.1 Å difference. The source of the difference in distances between the REDOR and X-ray scattering measurements comes from two other sources. First, although the ratio of labeled to unlabeled material was 1:20, there are still *intermolecular* contributions to $\Delta S^{(m)}/S^{(m)}$ which are difficult

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to calculate. From the X-ray diffraction data, the closest C(1)−N and C(8)−N intermolecular distances are 5.23 and 4.83 Å, respectively. Such additional dipolar interactions cause the REDOR-determined distance to be short compared to the true distance value. For example, in recent intramolecular REDOR distance measurements of highly diluted samples of melanostatin (1 part labeled material to 49 parts unlabeled material), corrections of 0.05 Å were still necessary to account for the long-range intermolecular contributions to the dipolar interaction. Second, the cross-polarization spin dynamics of the labeled sample and the natural abundance material were similar but not exactly the same. We have assumed in eqs 1 and 2 that the cross-polarization of the C(1) and C(8) carbons is the same. However, if the cross-polarization enhancements of the two carbon positions are different, then the values of α and β will change. For example, if a C(8) carbon is enhanced relative to a C(1) carbon by 20%, then the calculated C(1)−N distance would be about 0.04 Å greater than the reported value and closer in agreement with the X-ray-diffraction-determined distances. In any event, our general conclusion that the acetyl-L-carnitine has an extended conformation is still correct.

A 42 rotor cycle REDOR experiment was also performed on a labeled acetyl-L-carnitine sample lyophilized from aqueous solution. The isotopic labeling and dilution conditions of the lyophilized sample were identical to those used for the labeled crystalline sample. The REDOR spectra of the lyophilized sample are not shown, but were similar to the spectra shown in Figure 2 for the crystalline powder sample. The experimental NMR conditions for the lyophilized sample were the same as those for the crystalline powder sample. Using a C(8)−N distance of 3.87 Å, we obtained a C(1)−N distance of 5.06 Å, similar to that for the crystalline sample. As suggested above, relatively large variations in the C(8)−N distance will have only a minor effect on the calculated C(1)−N distance. Thus, the lyophilized sample has an extended backbone conformation similar to that found for the crystalline sample. The REDOR data for the crystalline and lyophilized samples show that acetyl-L-carnitine is in an extended conformation in the solid state.

Earlier models for the conformation of acetylcarnitine bound to CAT were based on a folded conformation of acetylcarnitine. These new results, which are in agreement with the solution studies by Brewster[12] and our X-ray data,[25] should be considered regarding the enzyme-bound conformation of acetylcarnitine.

Experimental Section

General Methods. Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Solution-state proton and carbon NMR spectra were obtained on a Bruker AC300 or a Bruker AM500 instrument. The spectra were measured in deuteriochloroform solution, unless otherwise stated, relative to tetramethylsilane (δ 0.00). Each signal is described in terms of chemical shift in parts per million from tetramethylsilane. AB patterns are reported as observed line separations.

Solid-state NMR experiments were performed on a home-built triple-channel spectrometer with a Larmor frequency for protons of 151 MHz. All experiments were performed at a spinning speed of 3205 Hz with a Chemagnetics double-bearing spinning head. The spinning speed was stabilized to within 2 Hz of the set point by a spinning speed controller. The home-built triple-tuned probe uses the transmission line design.[26] Carbon and nitrogen rf field strengths were 38 kHz. Proton rf field strengths were 38 kHz during proton−carbon cross-polarization and 120 kHz during proton decoupling. The rf field strengths were monitored and controlled to within 0.25% by an electronic feedback circuit.

Fourier transform infrared spectra were recorded on a Nicolet 550 Magna-IR spectrometer using KBr pellets unless otherwise noted. Mass spectra were recorded on a Finnigan 4600 GC−MS or VG 7070E mass spectrometer. Optical rotations were performed on a Perkin-Elmer 241MC polarimeter. Microanalyses were performed by the Physical Chemistry Department, Sandoz Research Institute.

All nonaqueous reactions were run under N_2 and a calcium sulfate drying tube. All organic extracts were dried over anhydrous sodium sulfate. All solvents were reagent grade and used as received. Thin-layer chromatography (TLC) was performed on 0.25 mm Macherey-Nagel precoated silica gel plates with fluorescent indicator (Polygram Sil G/UV$_{254}$). Silica gel 60 (230−400 mesh), supplied by E. Merck, was used for flash chromatography.

3-Chloro-2(*R*)-hydroxy-1-tosylpropane. (*R*)-(−)-α-Monochlorohydrin[16] (10.00 mL, 120 mmol) was dissolved in 133 mL of anhydrous pyridine and cooled in ice water. *p*-Toluenesulfonyl chloride (25.2 g, 132 mmol) was then added in small portions over 0.25 h, and the resulting light yellow reaction mixture was allowed to warm to room temperature and was then stirred for 1 h. The reaction mixture was then washed with 1.2 L of ice cold 2 N HCl(aq) and the mixture extracted three times with 500 mL of CH_2Cl_2. The combined CH_2Cl_2 solutions were washed with H_2O (500 mL) and brine (250 mL) and dried, and the solvent was removed to give 31.2 g (98.6%) of the tosylate as a clear yellow oil: ^{1}H NMR δ 7.81 (d, J = 8 Hz, 2H), 7.37 (d, J = 8 Hz, 2H), 4.16−4.04 (m, 3H), 3.61−3.58 (m, 2H), 2.67 (br d, J = 5 Hz, 1H), 2.46 (s, 3H); MS, *m/e* 265 (MH^+).

4-Chloro-3(*R*)-hydroxy[1-^{13}C]butyronitrile. 3-Chloro-2(*R*)-hydroxy-1-tosylpropane (31.1 g, 118 mmol) was dissolved in 194 mL of methanol, and to this was added potassium cyanide (^{13}C, 99%)[16] (9.59 g, 148 mmol). The initially clear reaction (no exotherm) became cloudy after stirring at room temperature for 0.10 h. After stirring for a total of 3.5 h at room temperature, the precipitate was removed by filtration, washed with methanol, and concentrated on a rotary evaporator (low vacuum due to the volatile nature of the product) to give a light yellow-orange, oily solid. Flash chromatography (hexane−ethyl acetate, 4:1) yielded 5.27 g (37.2%) of the nitrile product as a clear, light yellow liquid: ^{1}H NMR δ 4.21−4.14 (m, 1 H), 3.66 (d, J = 5 Hz, 2 H), 3.07 (br s, 1 H), 2.76−2.67 (m, 2 H); ^{13}C NMR (partial) δ 117.42 (^{13}CN); IR (partial) 2204 cm^{-1} (^{13}CN); MS, *m/e* 138 (MNH_4^+).

4-([^{15}N]Trimethylammonio)-3(*R*)-hydroxy[1-^{13}C]butyronitrile (Chloride Salt). To an ice−water-cooled mixture of Amberlite IRA-400 ($^-$OH) (Aldrich) (25 mL, 35.1 mmol) in H_2O (17.00 mL) was added $^{15}N(CH_3)_3$·HCl[16] (2.50 g, 26.3 mmol), and the mixture was allowed to stir at ice−water temperature in a sealed glass bomb for 2 h. The aqueous solution carrying the free base was then pipet-removed from the Amberlite IRA-400 (OH) and transferred to another sealed glass bomb containing 4-chloro-3(*R*)-hydroxy[1-^{13}C]butyronitrile (2.09 g, 17.5 mmol), and the initially heterogeneous solution was stirred at 90 °C for 0.75 h and then cooled. The water was removed via lyophilization to give a dark, orange, oily precipitate. Crystallization (95% EtOH) provided 0.693 g (22.0%) of the ammonium salt as a white crystalline solid: mp > 230 °C dec; ^{1}H NMR (CD_3OD) δ 4.60−4.50 (m, 1 H), 3.52 (d, J = 6 Hz, 2 H), 3.26 (s, 9 H), 2.88−2.66 (m, 2H); ^{13}C NMR (CD_3OD) (partial) δ 117.88 (^{13}CN); IR 3427, 3260, 3215, 2196, 1475, 1091, 964.7, 953.4, 927.3 cm^{-1}; OR $[\alpha]^{25}$ = −33.0° (c = 1.00, H_2O); FAB MS, *m/e* 145 (M^+). Anal. Calcd for $C_6{}^{13}C(1)H_{15}N_1{}^{15}N_1Cl_1O_1$: C, ^{13}C, 47.09; H, 8.37; N, ^{15}N, 16.06; Cl, 19.62. Found: C, ^{13}C, 46.91; H, 8.40; N, ^{15}N, 15.43; Cl, 19.87.

[1-^{13}C,^{15}N]-L-Carnitine Hydrochloride. 4-([^{15}N]Trimethylammonio)-3(*R*)-hydroxy[1-^{13}C]butyronitrile (chloride salt) (0.609 g, 3.38 mmol) was dissolved in 1.69 mL of 37% HCl (aqueous), and the resulting clear, colorless solution was stirred at 100 °C for 4.5 h. The resulting clear, light yellow solution, containing a white precipitate, was diluted with 75.0 mL of H_2O and lyophilized. Microanalysis indicated an equimolar amount of ammonium chloride was still present in the [1-^{13}C,^{15}N]-L-carnitine hydrochloride sample. To a solution of the product dissolved in 20 mL of H_2O was added 10 mL of Amberlite IRA-400 (OH), and the reaction mixture was allowed to stir at room temperature for 1 h. The mixture was filtered, and the Amberlist was washed with 10 mL of H_2O. The combined filtrates were lyophilized to provide 0.290 g of [1-^{13}C,^{15}N]-L-carnitine inner salt as an off-white solid. The inner salt was dissolved in 10 mL of 2 N HCl(aq) and stirred at room temperature for 1 h. The solution was then diluted with 40 mL of H_2O and lyophilized to provide 0.248 g (36.8%) of pure

(26) McKay, R. A. U.S. Patent 4446431, 1984.

[1-^{13}C,^{15}N]-L-carnitine hydrochloride as a white solid: mp 142 °C dec;[28] ^{1}H NMR (D_2O) δ 4.83−4.69 (m, 1 H), 3.58−3.54 (m, 2 H), 3.30 (s, 9 H), 2.82−2.62 (m, 2 H); ^{13}C NMR (D_2O) δ 176.91, 72.46, 65.65, 57.02, 56.95, 43.14, 42.40; IR 3420, 2923, 2659, 1683, 1475, 1403, 1290, 1181, 1092, 956, 928, 876, 610 cm^{-1}; OR $[\alpha]^{25} = -21.0°$ (c = 1.00, H_2O);[28] FAB MS, *m/e* 164 (M^+). Anal. Calcd for $C_6{}^{13}C(1)H_{16}{}^{15}N_1Cl_1O_3$ with 0.30 mol of H_2O: C, ^{13}C, 41.49; H, 7.86; ^{15}N, 7.32; Cl, 17.31. Found: C, ^{13}C, 41.49; H, 8.06; ^{15}N, 6.89; Cl, 17.86.

[1-^{13}C,^{15}N]Acetyl-L-carnitine Hydrochloride. [1-^{13}C,^{15}N]-L-Carnitine hydrochloride (0.188 g, 0.944 mmol) was dissolved in 0.625 mL of warm acetic acid, and to this solution was added acetyl chloride (1.29 mL, 18.1 mmol). The reaction mixture was stirred at 52 °C for 4 h and then at room temperature for 18 h. The reaction was concentrated on a rotary evaporator, and the dull yellow residue was crystallized as follows:[2] ethanol (5 mL) and anhydrous diethyl ether (18 mL) were added to the oil, and the resulting solution was allowed to sit at ice−water temperature for 0.5 h. The solvent was removed via decanting, and a mixture of methanol (1 mL) and acetone (5 mL) was added to the clear yellow oil to give a clear solution. Diethyl ether (~4 mL) was added dropwise to the point of cloudiness and the first precipitate formation. Diethyl ether (10 mL) was then added to maximize precipitation. The solution was then filtered with diethyl ether washing to provide 0.155 g (68.1%) of the final product as a white solid: ^{1}H NMR (CD_3OD) δ 5.58 (br s, 1 H), 3.85 (dd, J = 12, 7 Hz, 1 H), 3.69 (dd, J = 12 Hz, 1 H), 3.20 (s, 9 H), 2.75 (m, 2 H), 2.10 (s, 3 H); ^{13}C NMR (CD_3OD) δ 170.06, 167.71, 69.47, 66.45, 54.60, 38.12, 21.03; IR 3432, 2977, 2917, 2854, 1743, 1681, 1382, 1236, 1170, 1049, 959, 617 cm^{-1}; FAB MS, *m/e* 206 (M^+). Anal. Calcd for $C(8){}^{13}C(1)H_{18}{}^{15}N_1Cl_1O_4$ with 0.20 mol of H_2O: C, ^{13}C, 44.48; H, 7.56; ^{15}N, 6.12; Cl, 14.45. Found: C, ^{13}C, 44.26; H, 7.60; ^{15}N, 5.75; Cl, 14.72. For the REDOR and X-ray analysis, a 1:20 mixture of [1-^{13}C,^{15}N]acetyl-L-carnitine hydrochloride−acetyl-L-carnitine hydrochloride (Aldrich), dissolved in methanol, was crystallized by slow vapor diffusion of acetone into this initially saturated salt solution: $[\alpha]^{25} = -27.4°$ (c = 1.00, H_2O); mp 193−195 °C dec (lit.[27] $[\alpha]^{25} = -28°$ (c = 1.00, H_2O); mp 194 °C dec).

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(27) Aldrich catalog (acetyl-L-carnitine hydrochoride (1-^{12}C,^{14}N)): $[\alpha]^{25} = -28°$ (c = 1.00, H_2O); mp 194 °C dec.

(28) Aldrich catalog (L-carnitine hydrochloride (1-^{12}C,^{14}N)): $[\alpha]^{22} = -22.0°$ (c = 1, H_2O); mp 142 °C dec.