## Through-Bond Correlation of Adenine Protons in a $^{13}$C-Labeled Ribozyme

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Even though RNAs have been shown to be catalytically active in a variety of biological processes,[1] there is presently little X-ray or NMR structural data on ribozymes. The major reason for the lack of NMR structures has been difficulties in making proton resonance assignments in RNAs. Through-bond heteronuclear NMR experiments are now the methods of choice for resonance assignment of isotopically labeled proteins,[2–4] but assignments in nucleic acids still rely heavily upon observation of through-space nuclear Overhauser effects (NOEs).[5] These NOE-based assignment procedures have been successful for DNA and RNA helices. However, they require knowledge of the conformation of the molecule and therefore become problematic for many biologically active RNAs which contain residues in nonhelical structures such as hairpin loops, internal loops, or single strands.[6] Thus NMR structure determinations of RNA will clearly benefit from additional through-bond assignment techniques. Here we present a through-bond heteronuclear technique for unambiguous correlation of the A H2s and H8s in a 99% $^{13}$C/$^{15}$N labeled lead-dependent ribozyme, termed the leadzyme.[7]

All the A H8s in the leadzyme have been previously assigned by the conventional sugar proton to base proton NOE assignment procedure.[5,6,8] The A H2s of A·U base pairs in duplex regions are also normally assigned by NOE-based procedures.[5,6] However, only two of seven adenines in the leadzyme are predicted to be in A·U base pairs as illustrated in the secondary structural model shown in Figure 2.[7] Therefore the other five A H2s, including the two A's in the internal loop at the active site of the leadzyme, could not be specifically assigned by standard methods. To assign these protons, a 3D ($^1$H, $^{13}$C, $^1$H) HCCH-TOCSY experiment[9,10] was acquired on a 99% $^{13}$C/$^{15}$N labeled sample of the leadzyme. The goal of this experiment is to transfer magnetization between the A H2s and H8s through their intervening carbons.

Although adequate results were obtained with a standard 3D HCCH-TOCSY experiment,[9,10] improved signal to noise was obtained using the pulse sequence in Figure 1 which employs the method of sensitivity enhancement developed by Rance and coworkers.[11] Adenine has a complicated two-bond and three-bond $^{13}$C–$^{13}$C scalar coupling network (B. T. Farmer II, unpublished results) and optimum C2 to C8 magnetization transfer was achieved using a FLOPSY-8 spin lock sequence[12] and the parameters given in Figure 1. The $^{15}$N labeling, which is required for recently developed triple-resonance experiments on nucleic acids,[13–16] should not affect the sensitivity of this 3D HCCH-TOCSY spectrum since the $^{15}$N is effectively decoupled in the experiment. The pulsed $B_z$ gradients in Figure 1 serve as homospoil pulses in the context of $zz$ ($G_1$) and $z$ filters ($G_2$ and $G_3$).[17]

Figure 2 illustrates how 2D ($^1$H, $^{13}$C) planes of the 3D experiment were used to assign adenine H2 resonances in the leadzyme. For example, Figure 2A shows the correlation between the H8 and H2 resonances for A16. The upper and lower plots are centered on the C8 (142.6 ppm) and the C2 (154.5 ppm) resonances, respectively, and represent 2D planes taken at the H8 and H2 frequencies, respectively. Using this 3D HCCH-TOCSY spectrum, H2 to H8 correlations were identified for six of the seven adenines in the leadzyme, with the last H2 being assigned by default. No H2 to H8 connectivities were observed for A25 because its C2 resonance is severely broadened due to chemical exchange of this nucleotide between its protonated and unprotonated forms.[8]

The procedure described here allowed unambiguous assignment of the A H2 resonances in the leadzyme including the H2s in the internal loop and the hairpin loop, which cannot be assigned using standard methods. These assignments led to identification of additional NOEs involving the H2 on A25 at the active site of this ribozyme.[8] The spectral overlap in the 30 nucleotide leadzyme

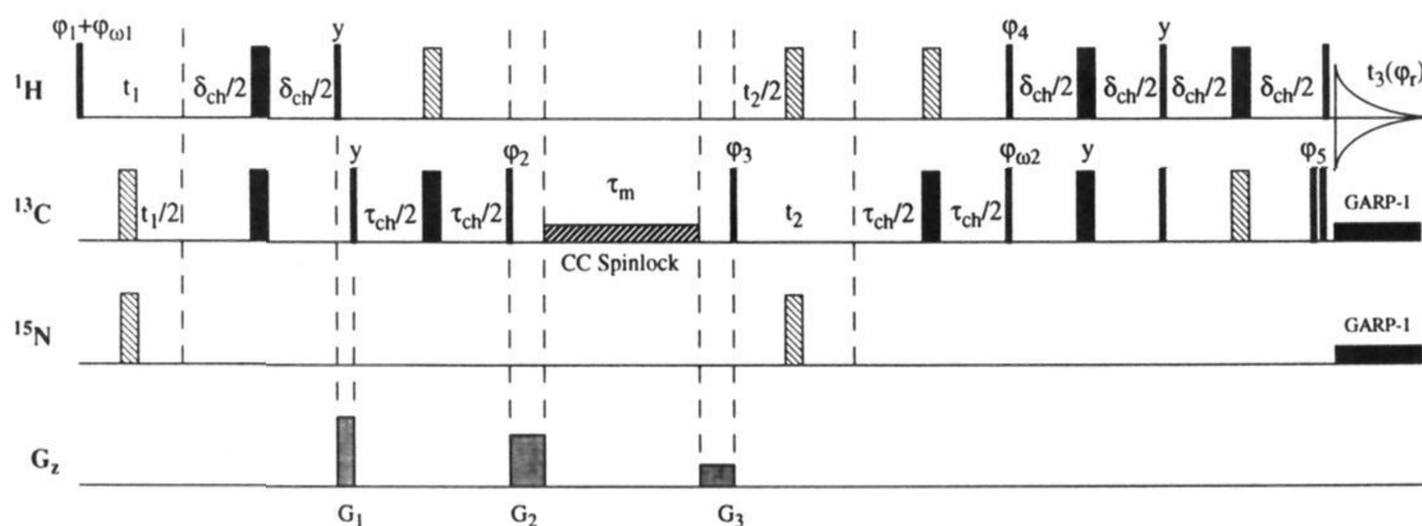


**Figure 1.** The 3D ($^1$H, $^{13}$C, $^1$H) HCCH-TOCSY experiment used to determine adenine H2 to H8 correlations in isotopically labeled RNAs. The narrow lines, black rectangles, and diagonally striped rectangles represent 90°, simple 180°, and composite $90_x240_y90_x$ pulses,[20] respectively. All pulses have phase $x$ unless otherwise indicated. The phase cycle is $\varphi_1 = x, -x$; $\varphi_2 = 2(x), 2(-x)$; $\varphi_3 = 4(y), 4(-y)$; $\varphi_4 = 8(x), 8(-x)$; $\varphi_5 = 16(x), 16(-x)$; and $\varphi_r = \varphi_1 + \varphi_2 + \varphi_3 + \varphi_4 - \pi/2$. Complex data were collected in $t_1$ by States-TPPI[21] with FIDs for $\varphi_{\omega 1} = (x, y)$ being stored separately and in $t_2$ with FIDs for $\varphi_{\omega 2} = (x, -x)$[11] being stored separately. The 3D hypercomplex data set was processed to achieve a quadrature-detected, phase-sensitive display along the $^{13}$C $t_2$ dimension with sensitivity enhancement.[11] A FLOPSY-8 $^{13}$C–$^{13}$C spin lock[12] was applied for 65.4 ms ($\tau_m$) using a 2.53-kHz RF field strength. For optimum efficiency in $^{13}$C isotropic mixing, the $^{13}$C carrier was placed midway between the A C2 and C8 resonances. All gradients were applied along the $z$ axis: $G_1$ = 8.0 G/cm for 0.5 ms, $G_2$ = 6.5 G/cm for 1.0 ms, and $G_3$ = 4.7 G/cm for 0.7 ms. $G_1$, $G_2$, and $G_3$ were followed by 1.5-, 2.0-, and 0.7-ms recovery times, respectively. Other relevant acquisition parameters are: $sw_{\omega 1}(^1H)$ = 1 kHz, $t_1^{max}$ = 27.00 ms, $sw_{\omega 2}(^{13}C)$ = 4.2 kHz, $t_2^{max}$ = 7.38 ms, $sw_{\omega 3}(^1H)$ = 4.45 kHz, $t_3$ = 57.53 ms, $\delta_{ch}$ = 2.0 ms, $\tau_{ch}$ = 2.4 ms, $\gamma B_2$($^{13}$C decouple) = 1.79 kHz, and $\gamma B_3$($^{15}$N decouple) = 1.23 kHz with GARP-1.[22] The total time of the experiment was 62 h. The NMR data were processed using an extensively modified version of the FELIX 1.0 program (Hare Research, Inc.).

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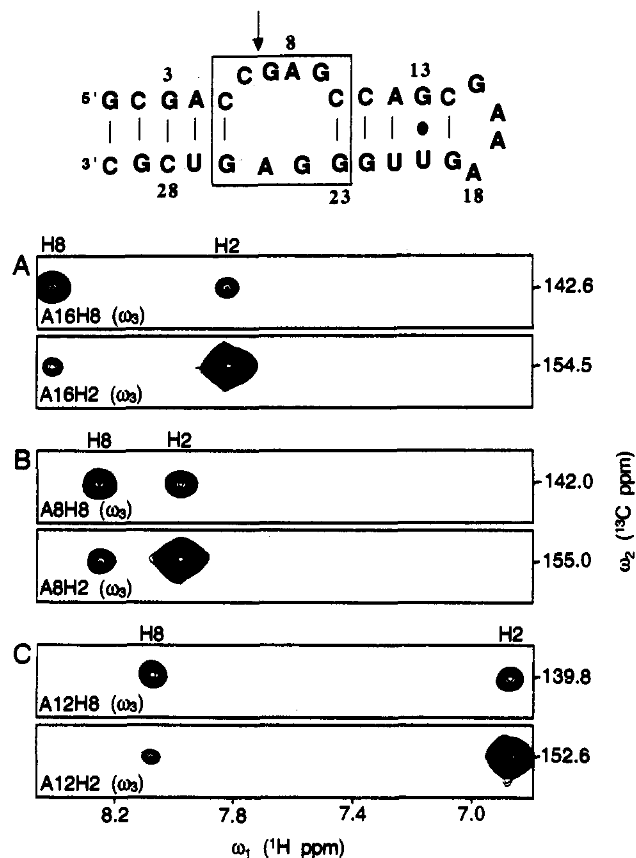


**Figure 2.** Contour plots of 2D ($^1$H, $^{13}$C) planes of the 3D HCCH-TOCSY spectrum of the 1.2 mM $^{13}$C/$^{15}$N-labeled leadzyme in 0.1 M NaCl, 10 mM sodium phosphate, 0.2 mM EDTA, pH = 5.5. The secondary structure model of the leadzyme[7] is shown above the plots. The box encloses nucleotides required for catalytic activity, and the arrow indicates the site of cleavage. (A) H2 to H8 correlations for A16, where the lower and upper plots are the aromatic proton regions at the C2 and C8 frequencies, respectively. The assignment of the $\omega_3$ proton plane is given in the left-hand corner. Similar plots of H2 to H8 correlations are shown for (B) A8 and (C) A12. The spectrum was acquired on a 600-MHz Varian Unityplus spectrometer at 25 °C. The 99% $^{13}$C/$^{15}$N labeled leadzyme was prepared by *in vitro* transcription as previously described.[23]

required acquisition of a 3D HCCH-TOCSY experiment, but this assignment procedure also can be employed as a 2D experiment in simpler systems. The through-bond linkage of the H2 and H8 resonances provides a direct connection between the sequential resonance assignments of the nonexchangeable base (H8/H6) and sugar (H1′, H2′, etc.) protons and the sequential assignments of the exchangeable imino protons. The combination of the procedure described here with other recently reported multidimensional heteronuclear NMR techniques[13-16,18,19] enormously simplifies resonance assignment and improves the structure determinations of biologically active RNAs.


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