# Correlation of Adenine H2/H8 Resonances in Uniformly $^{13}C$ Labeled RNAs by 2D HCCH-TOCSY: A New Tool for $^{1}H$ Assignment

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The first step in NMR structural studies involves the unambiguous assignment of the $^{1}H$ resonances.[1] In RNA molecules, this initial procedure is quite difficult owing to both severe $^{1}H$ chemical shift degeneracy of the ribose protons and a lack of adequate unique starting points in the sequential assignment pathways.[2] Recently the development of efficient methods for the synthesis of isotopically labeled RNA[3-5] has allowed some of these problems to be addressed by the use of multidimensional heteronuclear NMR methods.[6-8] In this study, a 2D HCCH-TOCSY experiment[9-11] has been employed to obtain a through-bond correlation between the H2 and H8 protons within the same adenine base of a $^{13}C$-labeled RNA oligonucleotide. This correlation provides new unique starting points for sequential assignment of the $^{1}H$ spectra of RNA oligonucleotides by unambiguously assigning the adenine spin system. It also provides a connection between the imino and anomeric-aromatic assignment pathways which are often individually interrupted by termination of regular RNA structural elements. It can therefore be used to confirm assignments which are made on the basis of known NOE correlations in A-form helical regions,[1,12] as well as providing assignments of adenine protons in noncanonical RNA structural elements.

The 2D HCCH-TOCSY pulse scheme and parameters used in this study are given in Figure 1.[10,11] The experiment, which previously has been employed to correlate $^{13}C$ spin systems in amino acids of $^{13}C$ labeled proteins[13] and ribose sugar rings of $^{13}C$-labeled RNA,[14] is employed in this case to transfer $^{13}C$ magnetization over the three-carbon four-bond fragment of the adenine base via two-bond $^{13}C$–$^{13}C$ couplings (~8–10 Hz). The primary magnetization transfer pathway is shown in Figure 2A. After a $t_1$ $^{1}H$ evolution period, the H2 and H8 proton magnetization is transferred to their one-bond-coupled C2 and C8 carbons; the $^{13}C$ magnetization is then transferred by isotropic mixing (either directly via the C4, as indicated in Figure 2, or indirectly via the C6, C4 pathway) with a DIPSI-2 spin lock;[15] finally, the $^{13}C$ magnetization is transferred back to H2 and H8 protons for detection.

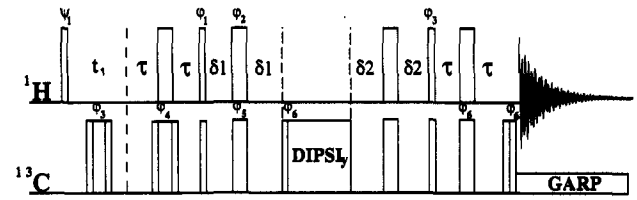


**Figure 1.** Pulse sequence diagram for 2D HCCH-TOCSY.[10,11] Narrow bars represent $\pi/2$ pulses, and wide bars represent $\pi$ pulses. The 16-step phase cycle is as follows: $\phi_1 = y, -y$; $\phi_2 = 4(x), 4(y), 4(-x), 4(-y)$; $\phi_3 = 8(x), 8(-x)$; $\phi_4 = 2(x), 2(-x)$; $\phi_5 = 2(x), 2(y), 2(-x), 2(-y)$; $\phi_6 = 4(x), 4(-x)$; Acq = $2(x, -x, -x, x), 2(-x, x, x, -x)$. The $180°\phi_3$ and $180°\phi_4$ are of the composite type $(90_x180_y90_x)$ and a 1.7-ms spin-lock trim pulse is applied with phase $\phi_6$. All other pulses are applied along the $x$ axis. The $^{13}C$ carrier is positioned in the center of the aromatic region (150 ppm), and a 100-ms spin-lock period using DIPSI-2 with an RF field of 3.5 kHz (covering the range of C2, C4, C6, C8) is applied along the $y$ axis (C5, which is 30 ppm upfield of the carrier, is not excited). The $\tau$, $\delta_1$, and $\delta_2$ delays were set to $1/(4J_{CH})$, 1.25 ms where $J_{CH} \sim 200$ Hz for aromatic resonances. Quadrature in the F1 dimension is obtained with the TPPI-states method[20] using $\Psi_1 = 16(x), 16(y)$. The receiver reference phase and $\Psi_1$ are incremented by 180° for each $t_1$ increment. Garp decoupling[21] of $^{13}C$ and $^{15}N$ was applied during the acquisition, as well as during $t_1$ for $^{15}N$.

The utility of the experiment is demonstrated on a uniformly $^{13}C$,$^{15}N$ labeled 19-mer stem-loop RNA, schematically represented in Figure 2B. The stem-loop is derived from RNA 1, a repressor molecule in the Col E1 replication control system.[16,17] The $^{1}H$ aromatic region of the 2D HCCH-TOCSY of the stem-loop is shown in Figure 2D. Two unique H2/H8 cross peaks are observed, as would be expected from an RNA oligonucleotide that contained two adenine bases. Assignment of the two cross peaks can be made easily in this case by utilizing imino and H2 sequential assignments made from a 2D NOESY experiment in $H_2O$ (data not shown). In the $H_2O$ NOESY, a strong NOE correlation between the A3 H2 and the U17 imino proton is observed for the A3–U17 base pair. In addition to the exchangeable proton assignments, the assignment of aromatic proton type has been made from $^{15}N$ and $^{13}C$ chemical shifts observed in HMQC and HSQC experiments.[18] The unique chemical shifts of the one-bond-coupled base carbon C2, C6, and C8 resonances[19] and two-bond-coupled base nitrogen N1, N3, N7, N9 (purines), and N1 (primidines) resonances[4] have been used to distinguish among the H2, H6, and H8 aromatic protons.

Figure 2C shows the H5/H1′ to aromatic region of a 2D NOESY of the stem-loop in $D_2O$. The arrows between panels D and C of Figure 2 show how the known assignments from the H2/H8 correlated spectra can be used to make assignments of the H8–H1′ and the H2–H1′ correlations observed in the H1′ to H6/H8 assignment pathway of an A-form RNA helix. In this RNA, the $D_2O$ NOESY identifies two NOEs for each adenine H8 proton, which indicates that both H8 protons are stacked in helical regions of the molecule. In addition, the assignment of two adenine H2 to H1′ cross peaks for A3 and one H2 to H1′ cross peak for A12 is as expected from known NOE correlations of AH2 protons to the 3′ H1′ proton on the same strand and the

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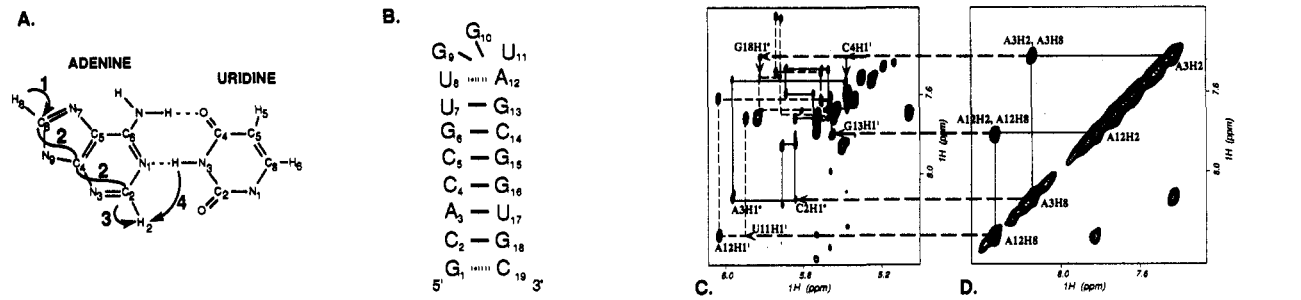


**Figure 2.** (A) Schematic of an AU Watson–Crick base pair with solid arrows indicating the primary magnetization pathway of the HCCH-TOCSY experiment: (1) the refocused inept sequence transfers $^1H$ magnetization into in-phase $^{13}C$ magnetization; (2) $^{13}C$ magnetization is transferred by DIPSI-2 isotropic mixing to its two-bond neighbors; and (3) a reverse refocused inept sequence transfers $^{13}C$ magnetization back to $^1H$ magnetization. The strong imino–H2 NOE correlation (4) that is normally seen in an AU Watson–Crick base pair in RNA is also indicated. (B) Sequence and secondary structure model of the RNA stem-loop used in this study. The RNA oligonucleotide was enzymatically synthesized using T7 polymerase runoff transcription.[22] The $^{13}C$,$^{15}N$-labeled NTPs were prepared from RNA isolated from *Methylophilus methylotrophus* grown in minimal media with $^{13}C$ methanol and $^{15}N$ $NH_4Cl$ as the sole carbon and nitrogen sources.[5] Solid lines indicate bases for which stable imino protons have been observed. Dashed lines between bases indicate base pairs for which stacking has been established but for which no stable imino proton is observed. (C) An expanded plot of the aromatic to H1′/H5 region of a 2D NOESY spectrum of the stem-loop illustrated in part B. The spectrum was recorded using a ~1.5 mM unlabeled RNA sample in $D_2O$ at 25 °C on a Bruker AM-500 spectrometer. The spectrum was collected with a standard NOESY pulse sequence with a 5000-Hz spectral width in both dimensions and a mixing time of 300 ms. Sixteen scans of 2048 complex data points in the $t_2$ dimension and 256 complex FIDs in the $t_2$ dimension were collected. The sequential assignments for the 5′ half of the helix (solid lines) and the 3′ half of the helix (dashed lines) are indicated. Cross peaks between H8, H2 aromatic, and H1′ anomeric resonances have been labeled with the assigned H1 proton of the pair. Correlations between AH2, H1′ cross peaks and the anomeric–aromatic sequential assignments are also indicated by arrows. (D) An expanded plot of the aromatic to aromatic region of a 2D HCCH-TOCSY spectrum of the stem-loop illustrated in part B. The spectrum was recorded using a uniformly $^{13}C$,$^{15}N$ labeled RNA sample (~1.5 mM) in $D_2O$ at 25 °C on a GE Omega500 spectrometer. The spectrum was collected with the pulse sequence indicated in Figure 1 with a 5000- Hz spectral width in both dimensions. Thirty-two scans of 512 complex data points in the $t_2$ dimension and 128 complex FIDs in the $t_1$ dimension were collected. Experiment time = 4 h. The chemical shifts of the A3 H2 and H8 and the A12 H8 and H2 protons are labeled, as well as the H2/H8 cross peaks. The dashed arrows between parts C and D connect assigned cross peaks in D with cross peaks in C.

3′H1′ on the opposite strand in A-form helices.1 The assignments indicate that the stacked helical region in this stem-loop extends through to an A12–U8 base pair and that the loop is bridged by only three nucleotides, of which U11 also appears to be stacked on the 3′ side.

In summary, the method described here allows unambiguous correlation of adenine H8 and H2 protons and thereby provides useful starting points for assignment of $^{13}C$-labeled RNA oligonucleotides. Taken together with other heteronuclear multidimensional NMR methods, this application of the HCCH-TOCSY experiment should provide a new tool for the assignment of both helical and nonhelical secondary and tertiary RNA structural motifs.

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