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# A TROSY relayed HCCH-COSY experiment for correlating adenine H2/H8 resonances in uniformly $^{13}$C-labeled RNA molecules

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**Abstract**

A new TROSY relayed HCCH-COSY pulse sequence is introduced for correlating adenine H2 and H8 resonances in $^{13}$C-labeled RNA molecules. The pulse scheme provides substantial improvements in signal-to-noise compared to previously suggested experiments, and therefore will be suitable for NMR studies of larger RNA molecules. The experiment provides $^{13}$C chemical shifts for all carbon nuclei in the adenine base. This is advantageous for resolving spectral overlap in larger RNA molecules and provides a starting point for measuring additional parameters for these carbons in the adenine spin system.

*Abbreviations:* COSY, correlated spectroscopy; BSP, Bloch–Siegert phase; E-BURP, excitation band-selective uniform response pure-phase; NOE, nuclear Overhauser effect; TOCSY, total correlation spectroscopy; TPPI, time-proportional phase incrementation; TROSY, transverse relaxation-optimized spectroscopy.

The application of heteronuclear multidimensional NMR experiments in combination with isotope labelling (Batey et al., 1992; Nikonowicz et al., 1992) greatly facilitates NMR-based structure determination of RNA molecules in solution (Varani et al., 1996; Wijmenga and van Buuren, 1998). A number of pulse sequences have been developed for the assignment of chemical shifts in nucleic acids which exploit large one- or two-bond heteronuclear coupling constants (Sklenar et al., 1992; Farmer II et al., 1993; Heus et al., 1994; Marino et al., 1994b; Tate et al., 1995; Fiala et al., 1996; Ramachandran et al., 1996; Simorre et al., 1996; Sklenar et al., 1998). These experiments provide through-bond correlations, and thus overcome ambiguities associated with NOE-based assignment strategies (Wüthrich, 1986; Varani et al., 1996; Wijmenga and van Buuren, 1998). Recently, transverse relaxation-optimized spectroscopy (TROSY) has been introduced (Pervushin et al., 1997, 1998), and shown to dramatically improve the sensitivity of NMR experiments applied to larger proteins (Salzmann et al., 1998, 2000) and RNA molecules (Brutscher et al., 1998; Fiala et al., 2000; Riek et al., 2001). Thus, in combination with $^{2}$H-labeling (Scott et al., 2000), NMR-based structural studies of larger RNA molecules with MW $>$ 15 kDa become feasible.

The correlation of H2 and H8 protons in adenine spin systems allows to confirm sequential assignments in canonical A-helical RNA structures and is especially useful for the chemical shift assignment of H2 protons in non-canonical RNA structural regions (Legault et al., 1994; Marino et al., 1994a). Assignment of the nonexchangeable adenine base protons in $^{13}$C-labeled RNA molecules is currently based on HCCH-TOCSY experiments which employ isotropic mixing between the C8 and C2 nuclei (Legault et al., 1994; Marino et al., 1994a). However, the transfer efficiency is hampered by the small coupling constants and the large chemical shift dispersion of the $^{13}$C spins involved. This leads to poor sensitivity, especially when applied to larger RNA molecules.

The 3D ($^{13}$C,$^{13}$C,$^{1}$H) relayed HCCH-COSY experiment shown in Figure 1 intends to overcome the limitations of the TOCSY-based experiments. Magne-

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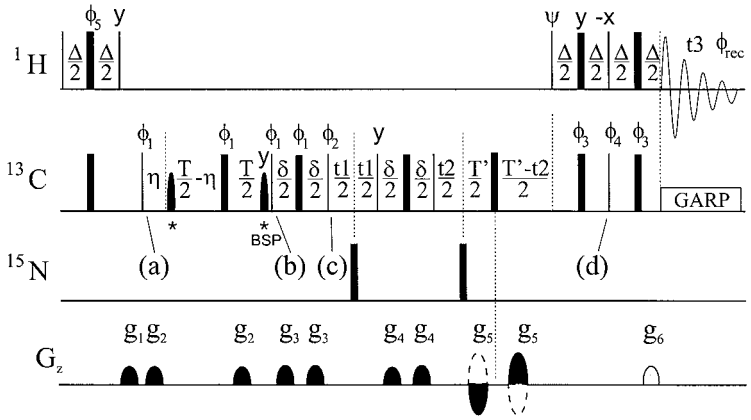


*Figure 1.* Pulse sequence for the 3D TROSY relayed HCCH-COSY experiment. Narrow/wide bars represent 90°/180° pulses. Band-selective E-BURP-2 pulses (Geen and Freeman, 1991) (*) of 1.5 ms duration are applied at 100 ppm to suppress H6/C6 correlation peaks from uracil and cytidine bases. The second pulse (BSP) is applied to compensate for Bloch–Siegert phase shifts (Sattler et al., 1999). Sine-shaped pulsed field gradients were applied for 600 μs with relative peak amplitudes (in %): $g_1 = 70$, $g_2 = 13$, $g_3 = -7$, $g_4 = 9$, $g_5 = 45$, $g_6 = 22.6$, where 100% corresponds to 50 G/cm. Pulse phases are: $\phi_1 = x,-x$; $\phi_2 = y,-y$; $\phi_3 = x$; $\phi_4 = y$; $\phi_5 = x$; $\psi = -y$; $\phi_{rec} = x,-x$. $\phi_5$ is adjusted to add the $^{13}$C Zeeman magnetization ($\phi_5 = x$ on our Bruker DRX console). Quadrature detection in the $\omega_2$ dimension is achieved by recording a second FID for each $t_2$ point with $\psi = y$ and simultaneously inverting gradients $g_5$. Sign discrimination for $\omega_1$ is obtained by applying States-TPPI to $\phi_1$ and $\phi_2$. Delay durations are $\Delta = 2.5$ ms, $T = 28.4$ ms, $\delta = 2.0$ ms, $\eta = 7.12$ ms, and $T' = T - \Delta$. The $^1$H, $^{13}$C, and $^{15}$N carrier frequencies are placed at 4.8, 140, and 150 ppm, respectively.

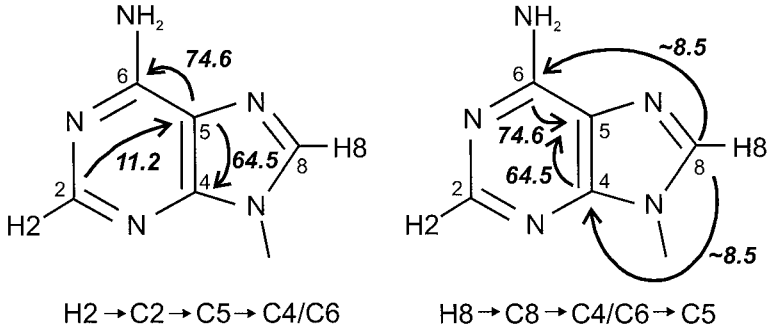


*Figure 2.* Schematic representation of the magnetization transfer in the TROSY relayed HCCH-COSY experiment originating from the H2 (left) and H8 (right) base protons. Bold italic numbers are coupling constants (Hz) between the carbon nuclei involved in the coherence transfer (Ippel et al., 1996).

tization is transferred simultaneously in an out-and-back manner from H2 and H8 to the three aromatic carbon spins, C4, C5 and C6 (Figure 2). At position (a) in the pulse sequence (Figure 1), proton carbon antiphase magnetization has been created for the H8/C8 and H2/C2 groups. The C2 and C8 carbons are then correlated with the aromatic C4, C5 and C6 nuclei via long-range interactions (Figure 2). C5-selective $^{13}$C 90° pulses are applied after a duration η in order to convert antiphase coherence between C5 and C6 carbons of pyrimidine bases into multiple-quantum coherence. Together with application of pulsed field gradients (g2 in Figure 1), this leads to suppression of undesired H6/C6 correlation peaks.

At point (b), zero- and double-quantum coherences between C8/C4, C8/C6 and C2/C5 are created, followed by relayed COSY transfer during the delay δ via $^1J_{CC}$ couplings between the C4, C5 and C6 nuclei. During $t_1$ (point (c)), the $^{13}$C chemical shifts of all carbons in the adenine spin system are recorded, and the magnetization transfer is reversed back to the H8 and H2 protons. The $^{13}$C product operators present at points (a), (c) and (d) in Figure 1 are given below for the H8 (Equation 1) and H2 (Equation 2) transfer pathways. The pathways indicated in Equations 1/2 a, b and c give rise to diagonal, cross, and relayed cross peaks, respectively. Note that the cross peaks have opposite signs compared to diagonal and relayed cross peaks (see Equations 1 and 2). Therefore, the pulse

sequence should be run as a 3D experiment in order to avoid fortuitous cancellation of signals with opposite sign.

$$\mathrm{C8} \rightarrow \mathrm{C4/C6} \rightarrow \mathrm{C5} \rightarrow \mathrm{C4/C6} \rightarrow \mathrm{C8}$$

$$C_{8y} \rightarrow \quad C_{8x} \rightarrow +C_{8y} \tag{1a}$$

$$C_{8y} \rightarrow \quad -2C_{8z}C_{4/6y} \rightarrow -C_{8y} \tag{1b}$$

$$C_{8y} \rightarrow -4C_{8z}C_{4/6z}C_{5x} \rightarrow +C_{8y} \tag{1c}$$

$$\mathrm{C2} \rightarrow \mathrm{C5} \rightarrow \mathrm{C4/C6} \rightarrow \mathrm{C5} \rightarrow \mathrm{C2}$$

$$C_{2y} \rightarrow \quad C_{2x} \rightarrow +C_{2y} \tag{2a}$$

$$C_{2y} \rightarrow \quad -2C_{2z}C_{5y} \rightarrow -C_{2y} \tag{2b}$$

$$C_{2y} \rightarrow -4C_{2z}C_{5z}C_{4/6x} \rightarrow +C_{2y} \tag{2c}$$

Some additional multiple quantum terms are also observable during $t_1$. However, they only give rise to spurious cross peaks, and thus do not complicate the spectral analysis (see Supplementary material, available on request from the authors). The final C→H back transfer employs sensitivity-enhanced TROSY combined with gradient coherence selection (Meissner and Sørensen, 1999a,b). The TROSY principle is utilized by selecting the slowly relaxing doublet component during the long transfer delays with transverse $^{13}$C magnetization, which is particularly favourable for aromatic carbons (Pervushin et al., 1997, 1998; Brutscher et al., 1998; Meissner and Sørensen, 1999a, b; Fiala et al., 2000; Riek et al., 2001). In addition, a directed COSY transfer is employed, which avoids problems due to strong off-resonance effects during the TOCSY mixing (Wijmenga and van Buuren, 1998). Therefore, the sensitivity of the new pulse sequence is substantially improved compared to the TOCSY-based experiments (Legault et al., 1994; Marino et al., 1994a).

The correlation of C4, C5 and C6 chemical shifts with both the H2 and H8 protons establishes the connectivity within the adenine base spin system. The experiment in Figure 1 provides unambiguous assignments even in cases where H2 and H8 chemical shifts are degenerate, provided the combination of the three $^{13}$C chemical shifts is unique. With the TOCSY-based experiments, degenerate H2 and H8 chemical shifts within the same base can only be resolved by recording two $^{13}$C chemical shifts in a 3D experiment. The new pulse sequence was applied to

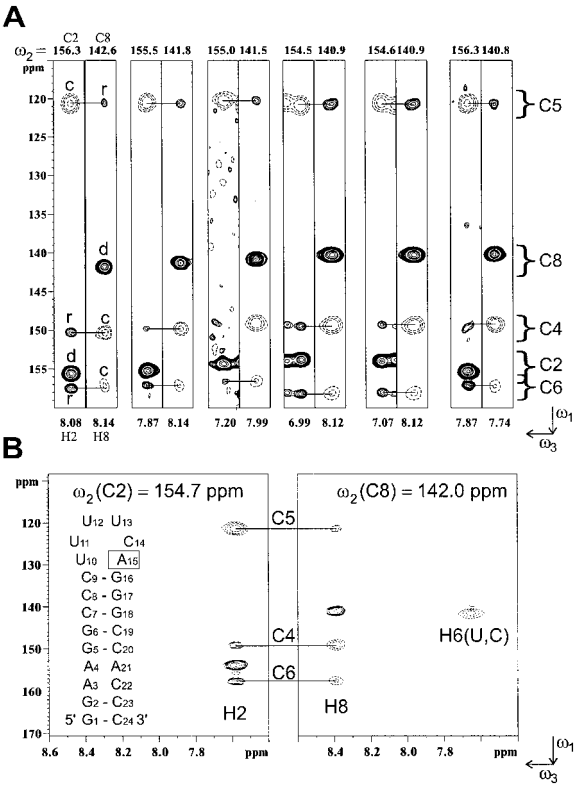


*Figure 3.* (A) Correlation of H2 and H8 in the six adenine base spin systems in a 0.8 mM sample of a single-stranded $^{13}$C/$^{15}$N-adenosine labeled RNA, 5′-GGUAUACUAACAA. Strips on the left and right are extracted at the C2 and C8 chemical shifts in $\omega_2$, respectively. Diagonal, cross and relayed cross peaks are labeled d, c and r, respectively; positive and negative contours are drawn with solid and dashed lines, respectively. (B) Assignment of A15 in a 24-mer RNA hairpin derived from the 3′ tail of histone pre-mRNA (Zanier and Sattler, in preparation). A 0.8 mM, uniformly $^{13}$C/$^{15}$N-labeled RNA sample in $D_2O$ was used. 3D experiments were recorded for 15 h. Evolution times of 5.5, 12.6, and 60.3 ms were acquired with 50, 115 and 1024 complex points for $t_1$, $t_2$ and $t_3$, respectively. A small residual signal originating from incompletely suppressed H6/C6 correlation peaks is annotated. The intensities of the H6/C6 cross peaks are reduced by 93% as compared to the experiment run without the selective E-BURP-2 pulses (data not shown).

a single-stranded $^{13}$C/$^{15}$N adenosine-labeled 13-mer RNA oligonucleotide, and to a uniformly $^{13}$C/$^{15}$N-labeled 24-mer RNA hairpin (Zanier and Sattler, in preparation). The spectra shown in Figures 3A and 3B are taken from the corresponding 3D experiments. Four peaks are expected for each strip, at the C2/C8, C4, C5, and C6 chemical shifts. As shown in Figure 3A, all six correlations are obtained for the 13-mer RNA. This is remarkable considering the low $^{13}$C chemical shift dispersion in this unstructured single-stranded RNA molecule. Figure 3B shows the assign-

ment for the A15 residue in the loop of the 24-mer hairpin. The observation of the C4, C5 and C6 chemical shifts allows unambiguous correlation of the H2 and H8 protons. The sensitivity of the new experiment (Figure 1) is significantly improved compared to the HCCH-TOCSY version (see Supplementary material).

In summary, we have introduced a novel pulse scheme for correlating adenine H2 and H8 resonances in $^{13}$C-labeled RNA molecules which provides considerable improvements with respect to sensitivity compared to available experiments. Since the TROSY principle is applied during the evolution of transverse $^{13}$C magnetization, the experiment will be especially useful for NMR studies of larger RNA molecules (MW $>$ 15 kDa). The experiment provides $^{13}$C chemical shifts for all carbon nuclei within the adenine base. This is advantageous for resolving spectral overlap in larger RNA molecules, and provides a starting point for measuring additional NMR parameters for these carbon nuclei.

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## Supplementary material

A more detailed product operator analysis, a figure showing the transfer efficiencies for the relayed COSY pulse sequence and an experimental comparison of the new pulse sequence, to an HCCH-TOCSY experiment are available via the Internet at http://www.embl-heidelberg.de/nmr/sattler or upon request from the authors.

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