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Triple resonance experiments for the simultaneous correlation of H6/H5 and exchangeable protons of pyrimidine nucleotides in $^{13}C,^{15}N$-labeled RNA applicable to larger RNA molecules

Jens Wöhnert[a,*], Matthias Görlach[b] & Harald Schwalbe[a]
[a]*Institut für Organische Chemie und Chemische Biologie, Center for Biomolecular Magnetic Resonance, Johann Wolfgang Goethe-Universität Frankfurt, Marie-Curie-Str. 11, 60439 Frankfurt/M, Germany;* [b]*Institut für Molekulare Biotechnologie, Beutenbergstr. 11, 07745 Jena, Germany*

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Abstract

Triple-resonance two-dimensional H6/H5(C4N)H and C6/C5(C4N)H experiments are described that provide through-bond H6/H5 or C6/C5 to imino/amino correlations in pyrimidine bases in $^{13}C,^{15}N$-labeled RNA. The experiments simultaneously transfer H6/H5 magnetization by an INEPT step to the C6/C5 nuclei and by homonuclear CC- and heteronuclear CN-TOCSY steps via the intervening C4 nucleus to the N3/N4 nuclei and then by a reverse INEPT step to the imino/amino hydrogens. The sensitivity of these experiments is high as demonstrated using a 30-nucleotide pyrimidine rich RNA at a concentration of 0.9 mM at temperatures of 10 °C and 25 °C. This indicates the general applicability of the experiments and the possibility to obtain correlations for imino resonances in non-canonical regions of the target RNA.

Due to the availability of isotopic labeling, virtually complete resonance assignments can be obtained for RNA molecules up to ∼ 45 nucleotides using triple-resonance heteronuclear NMR-experiments. A large number of pulse sequences have been proposed: (i) To identify individual sugar and base spin systems, (ii) to link base H6/H8 and ribose anomeric hydrogens and (iii) for the sequential assignment of non-exchangeable hydrogens (Wijmenga et al., 1998, Varani et al., 1996). Exchangeable protons of imino and amino groups in helical regions of RNA molecules can be assigned due to the presence of imino/imino- and imino/amino sequential or intra-basepair NOEs. However, in the structurally more interesting irregular regions such as loops and bulges this approach is not longer reliable since NOEs may arise from non-sequential spatial proximity. Triple resonance experiments that provide through bond connectivity between the nonexchangeable and exchangeable hydrogens of a base are then used to extend the sequential assignments of the nonexchangeable hydrogens to the imino/amino hydrogens (Simorre et al., 1995, 1996a, b; Sklenar et al., 1996; Fiala et al., 1996; Wöhnert et al., 1999).

For pyrimidine bases, NMR-experiments have been suggested that correlate the exchangeable hydrogens with either the H6 hydrogen (Simorre et al., 1995; Sklenar et al., 1996) or the H5 hydrogen (Wöhnert et al., 1999). However, in larger RNAs or in pyrimidine rich RNA sequences, such correlations might not be sufficient for the unambiguous assignment of the exchangeable hydrogens due to the limited chemical shift dispersion of either the H5 or the H6 hydrogens. In this case, additional information is needed to achieve unambiguous assignments of the exchangeable hydrogens.

Here, we present two novel 2D-triple resonance-experiments which simultaneously correlate the chemical shifts of the H6/H5 resonances or the C6/C5 carbon resonances with the imino proton and the amino

*To whom correspondence should be addressed. E-mail: jewoe@nmr.uni-frankfurt.de

proton chemical shifts in pyrimidine bases. In addition, these experiments allow the identification of the H6/H5 and C6/C5 resonances belonging to the spin system of the same base similar to 3D-HCCH-COSY or -NOESY-experiments. The proposed experiments have the advantage of increased resolution provided by the usually well resolved chemical shifts of the exchangeable protons. They are related to the HCCNH-TOCSY experiments developed by Sklenar et al. (1996) and Fiala et al. (1996) for the correlation of guanine H8 and imino proton resonances. In fact, Fiala et al. suggested the application of their experiment to pyrimidine bases but found it to be rather insensitive when applied to the uridines in a small 14-mer RNA at high concentrations.

The pulse sequences for the correlation of the exchangeable proton resonances with either the H6/H5 or the C6/C5 chemical shifts are shown in Figures 1A and 1B, respectively. Due to different settings for delays and nitrogen carrier frequencies separate spectra are recorded for uridines and cytosines. Since the ^{1}H chemical shifts of the H5 resonances are close to the water resonance, it is optimal to record the chemical shifts of the H6/H5 resonances in the indirect dimension. The WATERGATE water (Piotto et al., 1992) or similar suppression schemes can then be incorporated for water suppression and minimal losses due to solvent exchange prior to detecting of the direct dimension. In addition, especially in the case of the uridine experiment it seems to be advantageous to combine the magnetization of both the H6 and H5 protons and transfer it to the imino proton rather than to split the steady state magnetization of the one imino proton and transfer it to the two aromatic protons.

Both experiments start with an INEPT step to transfer magnetization from both the H6 and H5 protons to the attached carbons. The antiphase carbon magnetization is refocused with respect to the CH scalar coupling. In the H6/H5(C4N)H experiment, the evolution period t_1 is concatenated with this first INEPT step, while in the C6C5(C4N)H experiment it is concatenated with the refocusing step. Coherence transfer between spins in the aromatic system is achieved by a combination of a short and broadband homonuclear TOCSY and a selective heteronuclear TOCSY step: Thereby, the C6/C5 magnetization is transferred to C4 by a 19 ms homonuclear TOCSY mixing step with a bandwidth of 80 ppm. Following this, the ^{13}C offset frequency is switched from 135 ppm to 175 ppm – the center of the C4 chemical shift region. The C4 magnetization is transferred to N3 (uridine) or N4 (cytosine) by a selective and heteronuclear CN-TOCSY mixing step for 50 ms. Switching of offsets and band width of the applied rf field has been optimized to avoid heating and unwanted coherence transfer. In the case of cytosine, leakage of C4 magnetization to the unwanted pathway involving the N3 nitrogen is avoided since the small chemical shift dispersion of both the C4 and the N4 region requires only a small excitation band width for the CN-heteronuclear TOCSY transfer and the N4/N3 nitrogen chemical shifts are separated by more than 50 ppm. A reverse INEPT step is then used to convert the N3/N4 magnetization into observable in-phase imino or amino hydrogen magnetization for uridine and cytosine, respectively. In order to reduce the spectral width in the indirect carbon dimension, an additional switch in the ^{13}C carrier frequency is employed in the C6/C5(C4N)H-experiment moving the ^{13}C carrier from 120 ppm during the first INEPT step and the carbon evolution period t_1 to 135 ppm – the center of the C4/C5/C6-chemical shift region at the beginning of the homonuclear TOCSY step.

The experiments described here were used to obtain assignments for the pyrimidine base spin systems of a 30mer RNA molecule containing 10 uridines and 8 cytidines (Figure 1D) at both 25 °C and 10 °C (Figures 2A and 2B) and at an RNA concentration of 0.9 mM. The spectrum at 25 °C obtained with the H6/H5(C4N)H experiment optimized for uridines is shown in Figure 2A, the one optimized for cytosine is shown in Figure 2C. At this temperature, H5/HN and H6/HN crosspeaks were observed for nine out of the ten uridine residues (Figure 2A). Only the correlations for U24 were missing but its imino resonance is only observable at lower temperatures as a broadened signal. However, the correlations for U24 could be observed when the H6/H5(C4N)H experiment was repeated at 10 °C (Figure 2B). In the cytosine experiment, correlations are observed for six of the eight cytosines (Figure 2C). The amino groups of C7 and C14 are broadened beyond detection in ^{15}N,^{1}H-HSQC-spectra at 25 °C presumably due to rotation about the C4-N4 bond and are only observed as broad peaks at 10 °C explaining the absence of observable cross peaks for these two bases in the present experiments. The cross peaks involving the H5 and the H6 resonances are of comparable intensity in both experiments when using the indicated CC-TOCSY mixing time of 19.5 ms. Upon changing the CC-TOCSY mixing time to 6.5 and 13 ms, spectra with dominating H5/HN or H6/HN cross peaks,

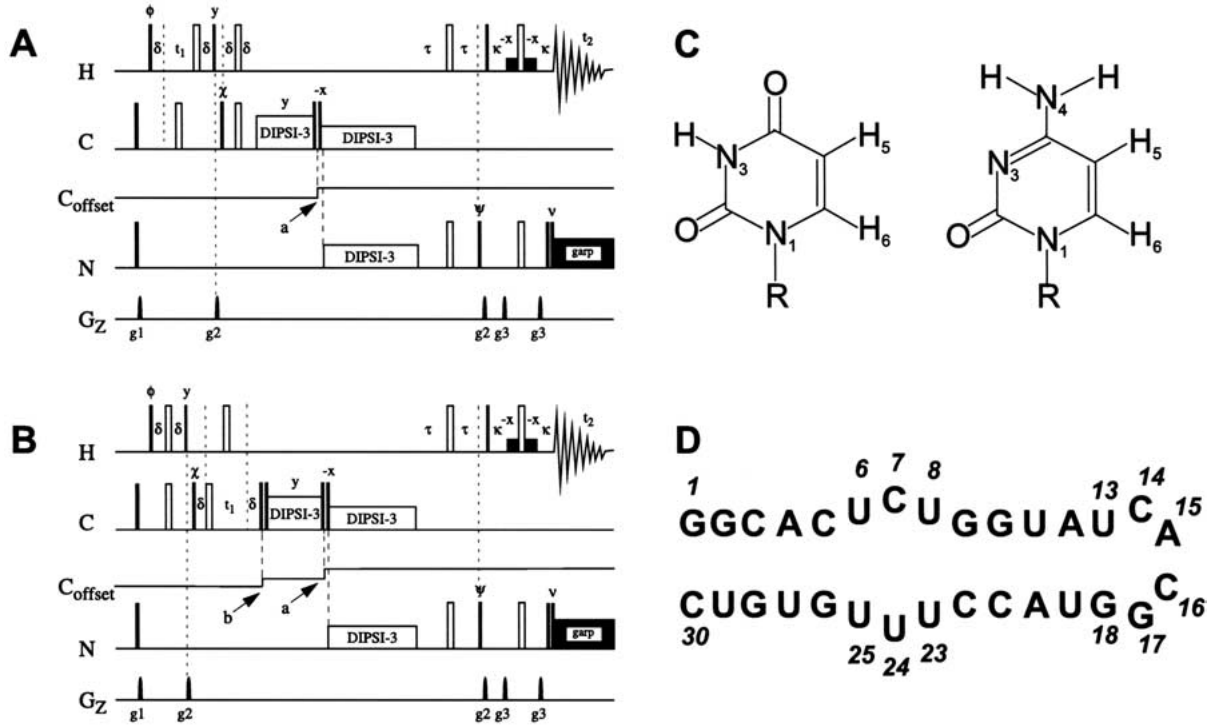

Figure 1. The 2D-H6/H5(C4N)H (A) and the 2D-C6/C5(C4N)H (B) pulse sequences used to establish simultaneous H6/H5-imino/amino and C6/C5-imino/amino correlations for pyrimidine bases in $^{13}C,^{15}N$-labeled RNA. Filled and open rectangles represent 90° and 180° pulses, respectively. The hydrogen carrier frequency is kept at the water frequency. The ^{13}C carrier frequency is shifted from 135 ppm to 175 ppm at point **a** in both sequences. In addition, in (B) the ^{13}C carrier frequency is shifted from 120 ppm to 135 ppm at point **b** in the sequence. The nitrogen carrier is set to 155 ppm in the experiments for uridines and to 100 ppm in the experiments for the cytosines, respectively. The DIPSI-3 (Shaka et al., 1988) spin-lock for the homonuclear ^{13}C-TOCSY mixing step was applied for 19.5 ms with a bandwith of 12 kHz and a y-phase. The DIPSI-3 spin-lock for the heteronuclear $^{13}C,^{15}N$-TOCSY mixing step was applied for 50 ms with a bandwidth of 1 kHz. The magnetization transfer delays employed were $\delta = 1.25$ ms, $\kappa = 2.5$ ms and $\tau = \kappa$ in the uridine experiments or $\tau = \kappa/2$ in the cytosine experiments, respectively. The WATERGATE-technique (Piotto *et al*., 1992) is applied during the$^{15}N,^{1}H$ reversed INEPT step using typically 880 μs selective 90° pulses flanking the nonselective 180° pulse. The GARP (Shaka et al., 1985) sequence was used for ^{15}N-decoupling during the acquisition time. G1 to G3 denote the z-axis pulsed-field gradients employed. Unless otherwise specified, the pulses are applied with phase = x. The phase cycle is $\phi = 8^*(x), 8^*(-x)$, $\chi = (y, -y)$, $\psi = (y, y, -y, -y)$, $\nu = 4^*(x), 4^*(-x)$ and receiver = $2^*(x, -x, -x, x), 2^*(-x, x, x, -x)$. In addition, to obtain quadrature detection in t_1 according to the hypercomplex method (States et al., 1982) the phase ϕ was incremented in (A) and the phase χ was incremented in (B), respectively. (C) Numbering scheme in pyridimine nucleobases. (D) Secondary structure of the 30-mer RNA used in this study. The RNA was synthesized in uniformly $^{13}C,^{15}N$-labeled form by *in vitro* transcription with T7-RNA polymerase as described previously (Stoldt et al., 1998).

respectively, were obtained. The sensitivity of both the uridine and the cytosine optimized experiments is rather high requiring 7.5 and 15 h of instrument time, respectively. The uridine experiment recorded at 10 °C required 15 h measurement time. Spectra of similar quality were obtained in the C6/C5(C4N)H-experiments (see supplementary material, Figure S1, obtainable from the author) with similar sensitivity. The sensitivity of the cytosine experiment might be further improved by the introduction of synchronous proton-nitrogen-Carr-Purcell-Meiboom-Gill (CPMG) pulse trains (Meiboom and Gill, 1958) during the nitrogen-proton reverse INEPT transfer to suppress the decay of amino proton magnetization caused by chemical exchange due to rotation of the amino group around the C4-N4 bond (Mueller et al., 1995).

In summary, the combination of the H6/H5(C4N)H- and the C6/C5(C4N)H experiments were used to derive complete and unambiguous assignments for 15 of the 18 pyrimidine base spin systems in a pyrimidine-rich 30mer RNA. Due to their satisfactory inherent sensitivity they should be also applicable for larger RNAs.

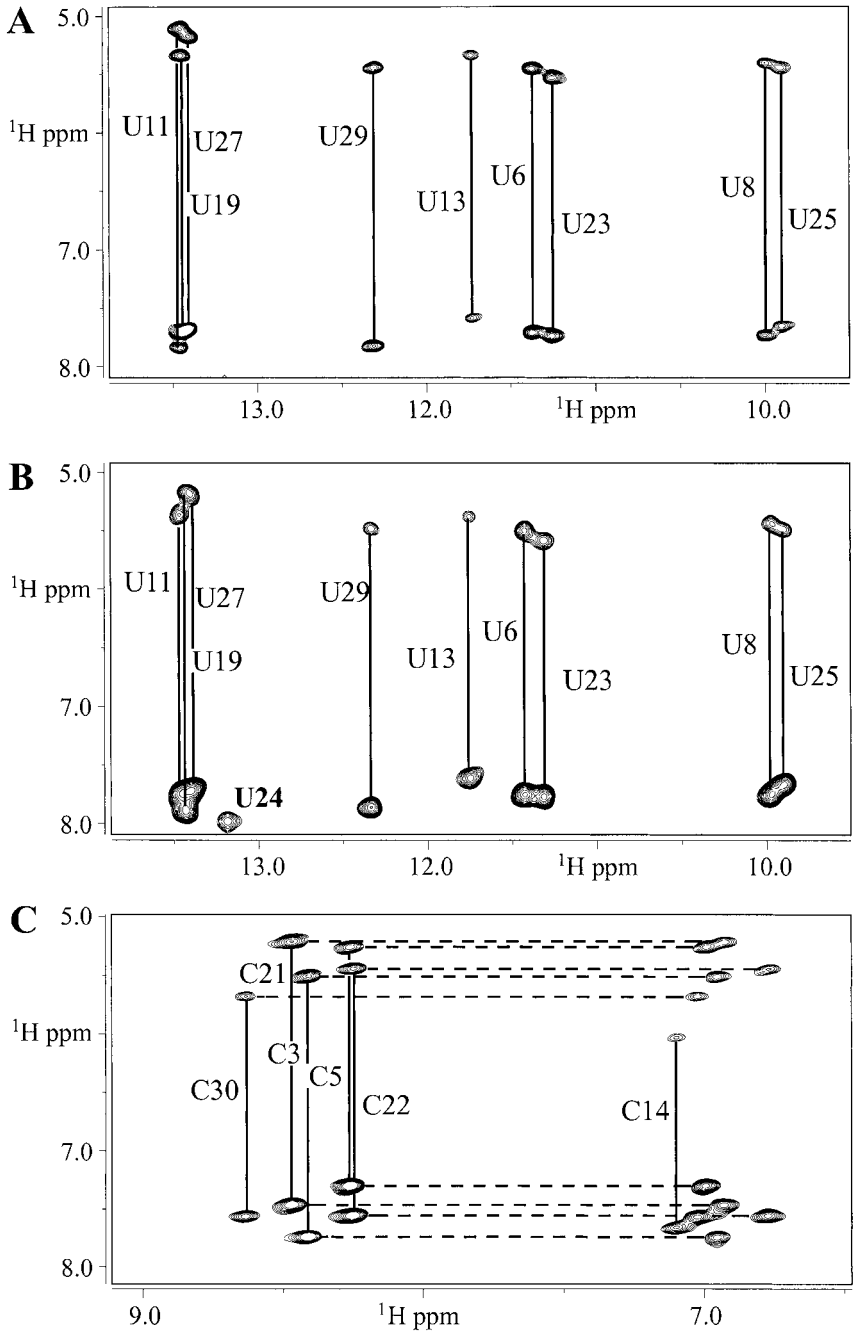

Figure 2. (A) Two-dimensional H6/H5(C4N)H spectrum showing the cross peaks between the uridine imino and H6/H5 resonances with the assignments indicated. The spectrum was acquired with 96*1024 complex points in t_1 and t_2, respectively, spectral widths of 5000 Hz in F1 and 13000 Hz in F2, 64 scans per t_1 increment, and a relaxation delay of 2s. H6 and H5 resonances correlated with the same imino proton resonance are connected by a solid line. (B) Uridine optimized H6/H5(C4N)H spectrum recorded at 10 °C using 128 scans per t_1 increment. The H6/NH cross peak for U24 not observable at higher temperatures is indicated in bold. The intensity of the H5/NH cross peak for this residue is below the threshold. (C) Two-dimensional H6/H5(C4N)H spectrum showing the cross-peaks between the cytosine amino and H6/H5 resonances with the assignments indicated. Resonances belonging to the same amino group are connected by a dotted line. H6 and H5 resonances correlated with the same amino proton resonance are connected by a solid line. The spectrum was acquired with 96*1024 complex points in t_1 and t_2, respectively, spectral widths of 5000 Hz in F1 and 13000 Hz in F2, 128 scans per t_1 increment, and a relaxation delay of 2s. All experiments were performed on a 0.9 mM uniformly ^{13}C,^{15}N-labeled RNA sample at 25 °C on a Varian ^{Unity}*INOVA* 600 MHz spectrometer.

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