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Facilitated Assignment of Adenine H2 Resonances in Oligonucleotides Using Homonuclear Long-Range Couplings

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NMR structural studies of nucleic acids require a nearly complete and unambiguous assignment of the proton resonances. This includes the adenine H2 protons, which, especially in B-type DNA, are generally far from other protons, rendering their assignment cumbersome. In both A- and B-type nucleic acid helices, the H2 protons are located in the minor grooves, where they serve as useful monitors for binding.[1] The conventional assignment method, which relies on nuclear Overhauser effects (NOEs) recorded in D_2O solution, generally does not allow complete assignment of the H2 resonances. In practice, these resonances are often assigned from their NOEs to the imino protons of A·U or A·T base pairs.[2,3] This requires performing the experiments in H_2O and the presence of detectable imino protons whose exchange with the solvent is sufficiently slow. For adenine residues in unpaired regions, other strategies are needed. Oligonucleotides that are ^{13}C-labeled allow magnetization transfer between the H2 and H8 protons via common couplings to intervening ^{13}C nuclei.[4,5] However, this approach is hampered by rapidly relaxing carbons. Subsequently, triple resonance experiments using [^{13}C,^{15}N]-labeled oligonucleotides were developed to correlate the H2 and H8 protons ($H \rightarrow {}^{15}N \rightarrow {}^{13}C \rightarrow H$).[6,7] Such schemes feature increased sensitivity but require labeled oligonucleotides and use of H_2O as the solvent. The development of higher-sensitivity NMR probes and the use of low-artifact gradient-enhanced experiments enabled the assignments of H2 and H8 protons at natural abundance using HMBC experiments in D_2O.[8] These experiments rely on a common heteronuclear long-range coupling of both H2 and H8 to C4. However, the inherently low sensitivity requires very long acquisition times and/or high sample concentrations. The adenine H2 resonances are readily identified in D_2O solution, since they appear as narrow singlets with long longitudinal (T_1) and transverse (T_2) relaxation times. The long T_1 is often a nuisance because it limits the repetition rate in many NMR experiments. On the other hand, the long T_2 permits the use of pulse sequences with longer durations, which aid in the detection of even small couplings. Long-range six-bond homonuclear couplings have been reported previously for polycyclic aromatic systems.[22] Therefore, we explored whether there is a homonuclear coupling between H2 and H8 of adenine that could be exploited.

Small coupling constants, particularly when they are on the order of the line widths, are difficult to measure.[18−20] Inspection of adenosine NMR spectra does not provide any obvious support for long-range H2−H8 couplings. Therefore, we used quantum-mechanical calculations to predict a long-range coupling of −0.18 Hz.[15−17] However, these calculations also forecast several other nonvanishing couplings of H2 (to H3′, H5′, and H5″) that were suspect (Figure S1A in the Supporting Information). In view of these results, experimental verification was clearly needed. The H2−H8 coupling was assessed using a long-range optimized COSY experiment on adenosine 5′-monophosphate.[14] The coupling became apparent for long-range evolution delays larger than 0.10 s. We also noted a long-range COSY correlation for the H8 and H1′ protons (Figure S1B). To estimate the H2−H8 coupling constant, the long-range evolution delay was varied (Figure 1).

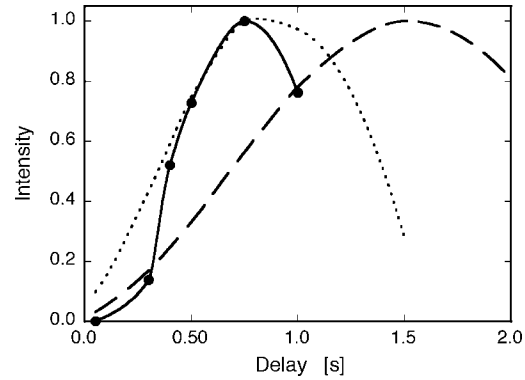

Figure 1. Dependence of the H2−H8 cross-peak height on the evolution delay in the long-range COSY experiment on 5′-AMP (50 mM in 99.9% D_2O, 10 mM sodium phosphate, 0.5 mM EDTA, pH* 6.6 at 298 K and 500 MHz): (●) Long-range COSY magnitude mode experiment. From the maximal response of the experimental curve, a coupling constant of ∼0.6 Hz was predicted using $J = 1/(2\tau)$. (•••••) NMR-SIM long-range COSY calculations for a coupling constant of 0.5 Hz. (- - -) Simulation for a coupling constant of 0.3 Hz. T_1 and T_2 relaxation times for 5′-AMP were determined from inversion recovery[11,12] and CPMG[13] experiments. Simulations were carried out with NMR-SIM 3.1 (Bruker) using $\delta = 8.17$ ppm, $T_1 = 6.0$ s, and $T_2 = 1.6$ s for H2 and $\delta = 8.54$ ppm, $T_1 = 2.1$ s, and $T_2 = 1.1$ s for H8.

The maximum cross-peak intensity was obtained for a delay of 0.75 s (Figure 1). NMR-SIM simulations of this experiment that accounted for relaxation were consistent with a coupling of ∼0.5 Hz. Sørensen and co-workers[9,10] introduced an innovative experiment named XLOC that allows a direct measurement of small homonuclear couplings. This approach disentangles small homonuclear J_{HH} couplings in the F2 dimension by the large one-bond $^1J_{CH}$ coupling in F1. Using XLOC on a 5′-AMP sample yielded a coupling constant of 0.5 ± 0.2 Hz, consistent with the previous result (Figure S2). The coupling between H2 and H8 protons also resulted in a cross-peak in the total correlation spectroscopy (TOCSY) experiment at long mixing times (0.20−1.25 s). Such durations are possible because of the slow relaxation behavior of the A H2 proton.

A selective TOCSY experiment using an E-Burp pulse for excitation of the base region was applied to several DNA samples in D_2O. The selective pulse limits the appearance of unwanted signals and permits the use of large receiver gains. For a 10-mer DNA duplex at mixing times larger than 0.25 s, a clear correlation of all of the H2 and H8 protons was observed (Figure 2). The cross-peak intensity remained nearly constant for mixing times between 0.40 and 1.0 s (Figure S3). An unambiguous assignment was also obtained for a 0.75 mM 18-mer DNA hairpin in just 45 min using a mixing time of 0.30 s (Figure 3).

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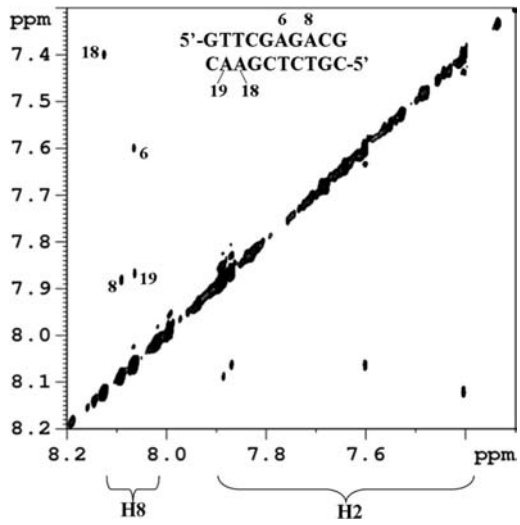

Figure 2. Selective 600 MHz TOCSY spectrum of a 10-mer DNA duplex (0.5 mM in 99.99% D_2O, 10 mM sodium phosphate, 0.1 mM EDTA, 100 mM NaCl, pH* 6.6) recorded at 298 K with a mixing time of 500 ms and a relaxation delay of 5.0 s. An E-Burp pulse of 4.5 ms was used for excitation at 7.7 ppm. A 1024 × 100 data-point matrix was acquired in a spectral window of 2.4 × 2.4 ppm, resulting in an acquisition time of 5 h.

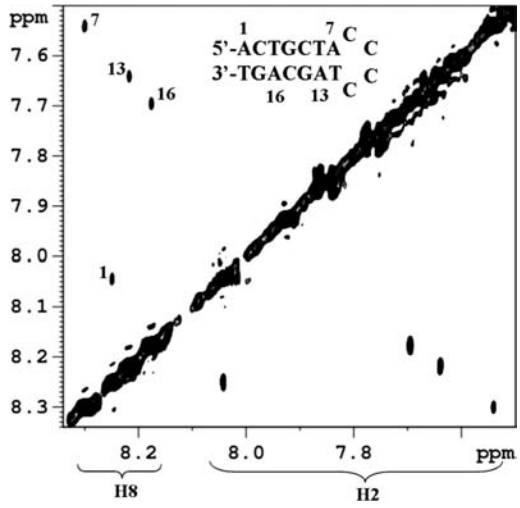

Figure 3. Selective 500 MHz TOCSY spectrum of an 18-mer DNA hairpin (0.75 mM in 99.99% D_2O, 10 mM sodium phosphate, 0.1 mM EDTA, 50 mM NaCl, pH* 6.84) recorded at 298 K with a relaxation time of 3.5 s and mixing time of 300 ms. A 1024 × 128 data-point matrix was acquired using four scans per increment, resulting in an experiment time of 45 min on a TXI cryogenic probe.

Depending on sequence and structural peculiarities, H2 protons can be assigned from regular NOESY experiments, particularly for RNA.[21] Often, however, the relevant cross-peaks are missing, weak, ambiguous, or in overlapped regions (Figure S4). Although not observed for the nucleic acids shown here (Figures 2 and 3), a rotational NOE (ROE) artifact could potentially also arise if H2 is close to another proton. However a direct ROE has the opposite sign of a TOCSY cross-peak, and a through-space correlation is also readily recognized from a companion NOESY experiment.

These examples demonstrate that a simple semiselective TOCSY experiment optimized for very small couplings allows straightforward and rapid assignment of A H2 protons in unlabeled oligonucleotides via a 6/7 bond homonuclear coupling. The through-bond correlations are unambiguous, while NOE-based assignments can be problematic, especially in nonhelical regions.

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Supporting Information Available: Complete ref 15, long-range optimized COSY and XLOC spectra of 5′-AMP, H2−H8 TOCSY cross-peak intensity of a DNA decamer, and 200 ms NOESY spectrum of the DNA hairpin. This material is available free of charge via the Internet at http://pubs.acs.org

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