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Sequential assignments in uniformly ^{13}C- and ^{15}N-labelled RNAs: The HC(N,P) and HC(N,P)-CCH-TOCSY experiments

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Summary

An approach for the simultaneous acquisition of HCN and HCP as well as HCN-CCH-TOCSY and HCP-CCH-TOCSY triple resonance data sets for ^{13}C-/^{15}N-labelled RNAs is presented. The new HCN-CCH-TOCSY scheme unambiguously links all sugar resonances to the base nitrogen. In addition, simultaneous acquisition of HCN-CCH-TOCSY and HCP-CCH-TOCSY data sets provides sequential and base-type information in a single experiment, thereby saving data acquisition time as well as providing complementary data sets that are useful in clarifying ambiguous assignments. Virtually complete sequence-specific phosphate–ribose ^{1}H, ^{31}P, and base $^{15}N1,9$ assignments as well as partial ^{13}C assignments could be obtained in a single experiment for a 0.5-mM sample of a 19-mer ribonucleotide.

RNAs play a critical role in a multitude of biological processes. However, RNA structure determination via ^{1}H NMR has been difficult due to poor ^{1}H chemical shift dispersion. Recently, techniques for the preparation of ^{15}N- and ^{13}C-labelled RNAs have been introduced (Wyatt et al., 1991; Batey et al., 1992; Nikonowicz et al., 1992) and several double and triple resonance schemes have been reported for facilitating sequence-specific resonance assignments and for the extraction of structurally relevant homo- and heteronuclear scalar couplings in labelled RNAs (Dieckmann and Feigon, 1994; Aboul-ela and Varani, 1995). Thus, the grouping of the ^{1}H and ^{13}C spins belonging to a particular ribose ring can be conveniently effected via the HCCH-COSY, HCCH-relay and HCCH-TOCSY experiments (Pardi and Nikonowicz, 1992). The linking of the ribose protons to a particular base can be performed by HCN-type experiments (Sklenář et al., 1993a,b,1994; Farmer II et al., 1993,1994; Tate et al., 1994) and sequential assignment of all ribose protons, ribose carbons and intervening phosphorus resonances in the RNA backbone is achieved via the HCP, HCP-CCH-TOCSY and P(CC)H-TOCSY experiments (Heus et al., 1994; Marino et al., 1994,1995; Tate et al., 1995; Wijmenga et al., 1995).

In the present paper, three aspects of obtaining assignments for ^{13}C,^{15}N-isotope-labelled RNA are addressed. Firstly, the extensive overlap of ribose C,H resonances encountered in RNA spectra can lead to ambiguity in identifying all nuclei within a given ribose spin system, even when the spectra are resolved by inclusion of a ^{31}P dimension like in the HCP-CCH-TOCSY experiment (Marino et al., 1995). Here the HCN-CCH-TOCSY experiment can help by taking advantage of the greater dispersion of the purine N9 and pyrimidine N1 resonances as compared to the ^{31}P resonances of the phosphate backbone. Secondly, in analogy to sequential assignment procedures in proteins, to resolve ambiguous sequential assignments in cases of spectral overlap it is advantageous to identify the type of the base associated with a particular ribose spin system. Here again we have found the HCN-CCH-TOCSY experiment to be very helpful, since unambiguous linking of the ribose spin system to the base can be achieved even in situations where the HCN experiment fails due to overlap of C1',H1' resonances. Finally, several different data sets are usually needed to obtain unambiguous assignments for isotope-labelled RNA, requiring considerable amounts of spectrometer time. In the present communication we introduce the HC(N,P)

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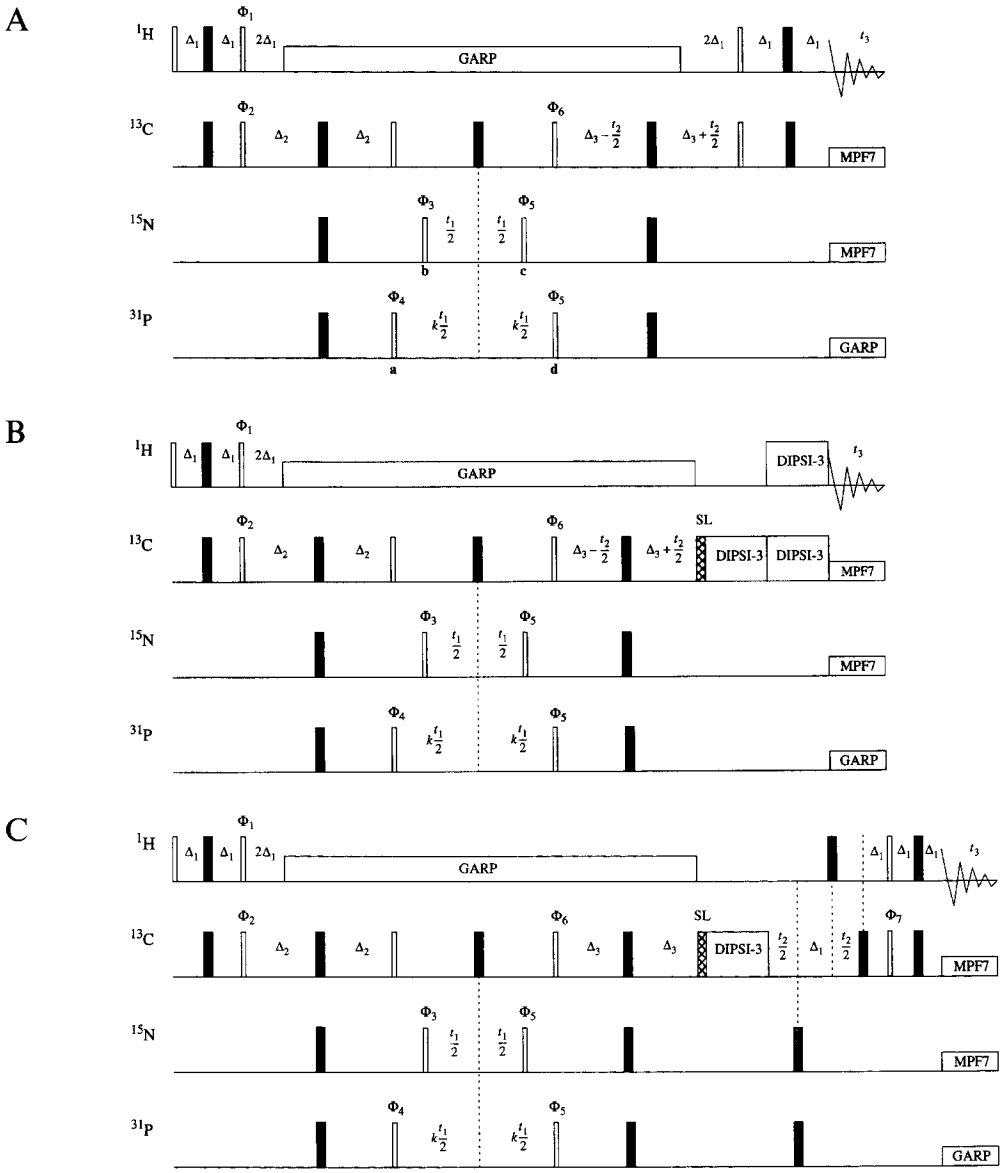

Fig. 1. Pulse sequences for acquisition of: (A) HC(N,P); (B) HC(N,P)-CCH-TOCSY with ^{13}C evolution before the homonuclear CC-TOCSY step; and (C) HC(N,P)-CCH-TOCSY with ^{13}C evolution after the homonuclear CC-TOCSY step. The narrow and wide bars represent 90° and 180° pulses, respectively. If not otherwise specified, the pulses are applied along the x-axis. The following phase cycle was used: $\Phi_1 = (y,-y)$, $\Phi_2 = (x,x,-x,-x)$, $\Phi_3 = \Phi_4 = 4(x),4(-x)$, $\Phi_5 = 16(x),16(-x)$, $\Phi_6 = 8(x),8(-x)$, $\Phi_{receiver} = (a,-a,-a,a,-a,a,a,-a)$ with $a = (x,-x,-x,x)$. For the pulse sequence of Fig. 1C, $\Phi_7 = 32(x),32(-x)$ is cycled additionally with $\Phi_{receiver} = (a,-a,-a,a,(-a,a,a,-a)_2,a,-a,-a,a)$. The spectral width in the ^{31}P dimension as compared to the ^{15}N dimension is controlled by the scaling factor k. For quadrature detection in t_2 according to the States procedure, the phase Φ_6 is incremented in the sequences A and B and Φ_6, together with the phases of the subsequent 180° and spin-lock pulse (SL) as well as of the DIPSI3 mixing step, are incremented in sequence C. The delays are set to $\Delta_1 = 1.6$ ms and $\Delta_2 = \Delta_3 = 11.5$ ms. This choice of Δ_2 and Δ_3 was found experimentally to give efficient polarization transfer and to minimize signal losses due to evolution of $^1J_{CC}$ scalar couplings. Quadrature detection in t_1 and extraction of nitrogen and phosphorus spectra are achieved as shown in Table 1. The phase cycle could be shortened by the use of gradients if an appropriate probehead were available, but in practice the HC(N,P)-CCH-TOCSY experiments require substantial signal averaging and the phase cycle is not a limiting factor in the time needed to record the data.

experiment for simultaneous collection of HCN and HCP spectra as well as the HC(N,P)-CCH-TOCSY experiment for simultaneous collection of HCN-CCH-TOCSY and HCP-CCH-TOCSY spectra. The efficiency of the pulse sequences is demonstrated with a 0.5 mM sample of a uniformly ^{13}C,^{15}N-labelled RNA hairpin containing a 5'-CG(UUU)CG-3' triloop, which is a common motif in eukaryotic 18S rRNA (Wolters, 1992).

Figure 1A shows the sequence that has been employed in this work for the simultaneous acquisition of HCN/HCP data sets. The pulse sequence utilizes nonselective rf pulses and sequential $^1H \rightarrow {}^{13}C$ and $^{13}C \rightarrow {}^{15}N/{}^{31}P$ polarization transfer steps to transfer magnetization via the $H1' \rightarrow C1' \rightarrow N1,9$ and $H_i^1 \rightarrow C_i^1 \rightarrow P$ ($i = 2, 3, 4$) pathways. In principle $H5' \rightarrow C5' \rightarrow P$ magnetization transfer is also possible, but signals from the C5' methylene groups have

not been observed in the present experiments due to the choice of $\Delta_1 = 1/(4\,{}^1J_{CH})$ to completely refocus antiphase methine coherences. Since the ${}^1J_{CN}$ and J_{CP} coupling constants are approximately of the same order of magnitude, the time taken for the second polarization transfer step has been kept identical for both the C → N and C → P transfer processes. However, due to the smaller ^{31}P spectral width, the chemical shift evolution of ^{31}P is started earlier and ended later than that of ^{15}N. In the sequence given in Fig. 1A, the ^{15}N magnetization is stored between points **a**,**b** and **c**,**d** in the $I_C^z I_N^z$ state while the ^{31}P magnetization is evolving. After evolution, the single-quantum ^{15}N/^{31}P magnetization is transferred back to the ^{13}C1'/^{13}C$_i'$ (i = 2, 3, 4) spins, followed by a transfer to the attached proton by a reverse INEPT step. Suitable phase cycling and data processing procedures (see Table 1 and the legend to Fig. 1) are employed to achieve quadrature detection in the t_1 and t_2 dimensions and to separate the HCN and HCP signals (Sørensen, 1990; Farmer II, 1991). Two-dimensional (^{15}N,^{1}H) and (^{31}P,^{1}H) spectra of a simultaneously acquired HCP/HCN experiment are presented in Fig. 2. In the nitrogen subspectrum, H1'-N1/9 connectivities are observed. Of the 19 expected peaks, 16 can be seen. Four of these show partial overlap. The three missing resonances belong to nucleotides that also appear as broad resonances for ^{13}C-^{1}H one-bond correlation, presumably due to an intermediate conformational exchange on the chemical shift time scale. The phosphorus subspectrum, showing H2',H3',H4'-P connectivities, does not provide many sequential assignments due to severe overlap. In a 3D HC(N,P) experiment, a variety of correlations involving $^{2,3}J_{CP}$ couplings were observed (Heus et al., 1994; Marino et al., 1995; Wijmenga et al., 1995). However, we were unable to obtain sequential assignments from this experiment due to the poor chemical shift dispersion of the nonanomeric CH resonances.

TABLE 1
PHASE CYCLING FOR QUADRATURE DETECTION IN t_1 AND FOR DISTINGUISHING BETWEEN HCN AND HCP SPECTRAL TYPES

Data set	Φ_3	Φ_4	^{15}N signal	^{31}P signal
1	Φ_3	Φ_4	$(N+P)_{t_1}$	$(N+P)_{t_1}$
2	Φ_3	$\Phi_4+180°$	$(N+P)_{t_1}$	$-(N+P)_{t_1}$
3	$\Phi_3+90°$	$\Phi_4+90°$	$(N-P)_{t_1}$	$(N-P)_{t_1}$
4	$\Phi_3+90°$	$\Phi_4+270°$	$(N-P)_{t_1}$	$-(N-P)_{t_1}$

For the extraction of the nitrogen part of the interleaved experiment, the data set (1 + 2) gives the real part (N + P) and (3 + 4) yields the imaginary part (N − P) of the signal in t_1. For the equivalent phosphorus spectrum, (1 − 2) and (3 − 4) yield the real and imaginary parts in t_1, respectively. The phase cycling employed for Φ_3 and Φ_4 are indicated in Fig. 1. In the event that a four-channel probehead is not available, an HCN-CCH-TOCSY spectrum can be recorded by omitting all phosphorus pulses (Fig. 1) and recording data sets 1 and 3.

Figure 1B shows our approach to the simultaneous acquisition of the HCN-CCH-TOCSY and HCP-CCH-TOCSY data sets. The scheme is essentially identical to the HCN/HCP experiment, except that the single-quantum ^{13}C magnetization at the end of the t_2 period in Fig. 1A is subjected to a CC isotropic mixing period, which allows transfer of the ^{13}C magnetization to all the ribose carbons. While the in-phase ^{13}C magnetization at the end

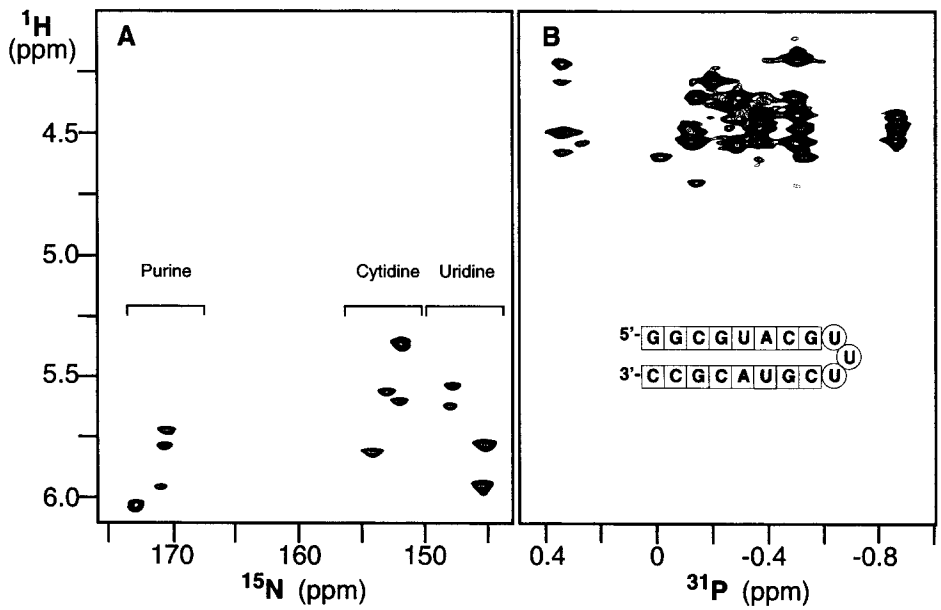

Fig. 2. Spectra obtained at 27 °C from a 2D HC(N,P) experiment on a 0.5 mM sample at pH 6.0 of the RNA hairpin shown in (B), using the pulse sequence shown in Fig. 1A. (^{15}N,^{1}H) and (^{31}P,^{1}H) subspectra recorded with $t_2 = 0$ are shown in (A) and (B), respectively. The spectral widths for ^{15}N and ^{31}P were chosen to be 2500 and 500 Hz, corresponding to a scaling factor of k = 5. In total, 48 t_1 increments were collected with 128 scans per data set and a recycle time of 1 s, resulting in a total experimental time of 8 h. A spectral width of 4500 Hz was chosen in the ^{1}H dimension and 384 complex points were collected. In (A) typical N1/9 shifts of purines, cytidines and uridines are given. All experiments were performed with a Varian Unity plus 600 spectrometer equipped with a modified Bruker QXI ^{1}H{^{13}C,^{15}N,^{31}P} four-channel probe.

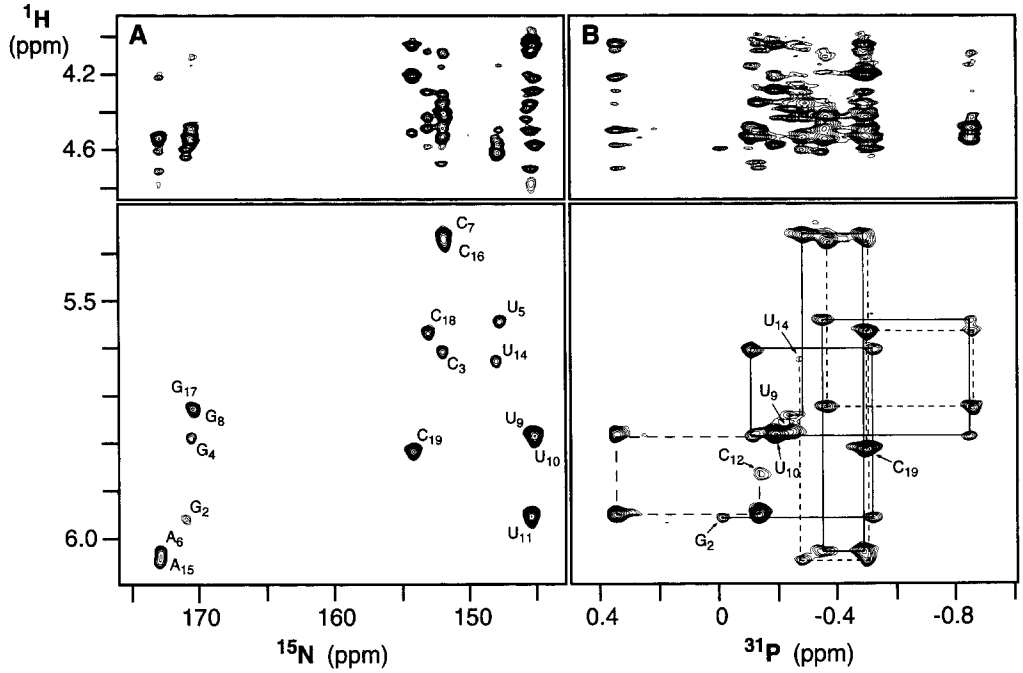

Fig. 3. Spectra from a 2D HC(N,P)-CCH-TOCSY experiment of the RNA hairpin using the pulse sequence shown in Fig. 1B. (^{15}N,^{1}H) and (^{31}P,^{1}H) subspectra are shown in (A) and (B), respectively. In spectrum (A) assignments of the nucleotides obtained from spectrum (B) are given. In (B) the sequential walks for three fragments are shown: G2 to U9, U10 to C12 and U14 to C19, providing almost complete sequential assignment except for the G1 and G13 nucleotides. These can be distinguished due to the characteristic chemical shifts of the terminal nucleotide (Varani and Tinoco Jr., 1991). For the CC-TOCSY step a mixing period of 15 ms was used with a ^{13}C rf field strength of 7.2 kHz and the ^{13}C carrier centered in the ribose region (72.1 ppm). The hetero-TOCSY mixing time was 5.6 ms. For each data set, 48 t_1 increments with 768 scans per increment were collected. Otherwise the acquisition parameters corresponded to the spectrum of Fig. 2.

of the TOCSY period is transferred back immediately to the ^{1}H spins in the scheme shown in Fig. 1B, in the pulse sequence of Fig. 1C the evolution frequencies of the ^{13}C magnetizations at the end of the TOCSY period are mapped out during the free evolution period t_2 and then transferred back to the ^{1}H spins via a reverse INEPT procedure. The latter pulse sequence is less sensitive due to the additional delays involved, but provides all ^{13}C and ^{1}H chemical shifts of the ribose ring.

The results of an interleaved 2D HC(N,P)-CCH-TOCSY experiment obtained by the sequence of Fig. 1B are depicted in Fig. 3. The nitrogen subspectrum shows the same signals as the HC(N,P) data set as well as the ribose ring proton chemical shifts. In the phosphorus subspectrum, sequential assignment could be achieved due to the better resolved H1' region and to the base-type information obtained from the nitrogen subspectrum of this experiment. Figure 4 shows typical ^{13}C planes of a simul-

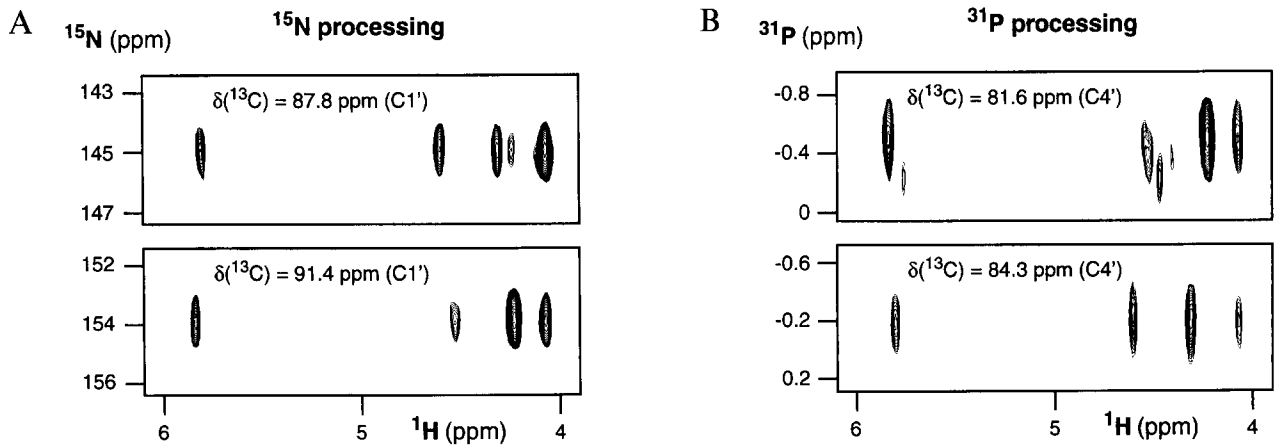

Fig. 4. Typical ^{13}C planes of a 3D HC(N,P)-CCH-TOCSY experiment using the pulse sequence of Fig. 1B. Subspectra for (^{15}N,^{13}C,^{1}H) (A) and (^{31}P,^{13}C,^{1}H) (B) recorded for the RNA hairpin are shown. The spectral widths were 2400, 5200 and 4500 Hz for ^{15}N/^{31}P, ^{13}C and ^{1}H, respectively; 14, 26 and 576 complex points were acquired in t_1, t_2 and t_3 with 64 scans per data set, leading to a total experiment time of 58 h. In t_1 and t_2, 11 and 12 additional complex points were linearly predicted. Homo- and hetero-TOCSY mixing times were 16.2 and 6.6 ms, respectively.

taneously acquired 3D HC(N,P)-CCH-TOCSY experiment, obtained by the pulse sequence of Fig. 1B with identical spectral widths for ^{15}N and ^{31}P. Apart from the three nucleotides with broad resonances due to chemical exchange, all ^{1}H resonances of the ribose rings could be identified, where the different overlap of resonances in the ^{15}N and ^{31}P subspectra was very helpful in resolving ambiguities. Even at the low resolution used in the present experiment, all of the C1' and many of the C2', C3', C4' resonance frequencies could be identified. Low-resolution experiments with the pulse sequence of Fig. 1C (data not shown) suggest that complete ^{13}C assignments could be obtained with this experiment. Alternatively, the partial ^{13}C assignments obtained with the sequence of Fig. 1B might be completed with the aid of an HCCH-TOCSY spectrum.

The pulse schemes given here allow for simultaneous acquisition of spectral data sets that are essential for the assignment of moderate-sized RNA molecules and therefore provide considerable savings in spectrometer time, without sacrificing much signal-to-noise ratio. The HCP part of the sequence is identical to the noninterleaved experiment, therefore no loss in sensitivity is expected. In the HCN part the signal-to-noise ratio is expected to be identical to that of a single HCN experiment, except for the losses due to relaxation of the J-ordered states during the time $(k-1)t_1$. In the system under investigation these losses have been found to be less than 20%. The simultaneous acquisition of the different data sets has the further advantage that all data sets are collected under exactly the same experimental conditions. This can be a very important aid in deciphering assignments for resonances with very small chemical shift differences. In the present case, the simultaneous HC(N,P)-CCH-TOCSY experiment has allowed us to obtain virtually complete ^{1}H, ^{15}N and ^{31}P assignments of the ribose–phosphate backbone in a single experiment. Because of the dynamic properties of the 19-mer, the information on base type embodied in the different ^{15}N chemical shifts of the uridine N1, cytidine N1 and purine N9 nuclei was an essential component in obtaining these assignments. In particular, the resonances of G1, C12 and G13 showed broad lines, apparently due to fraying at the end of the stem (G1) and to an as yet uncharacterized conformational equilibrium (C12, G13). Due to the broad lines, it was not possible to follow the entire connectivity for the ribose–phosphate backbone, but the correct sequential assignments could nonetheless be obtained by incorporating information on the RNA sequence and the base type as obtained from the simultaneous HC(N,P) CCH-TOCSY data set.

NMR spectroscopy of RNA molecules is characterized by limited chemical shift dispersion of resonances, even in ^{13}C,^{15}N-labelled molecules, and by magnetization transfers involving relatively small scalar couplings. At the present stage of development, a variety of NMR experiments provide effective ways to assign the spectra of molecules of up to about 30–35 nucleotides, but even in molecules of this size it is not uncommon to have strong overlap of critical resonances such as C1'/H1'. As NMR studies proceed to larger RNA molecules, problems of spectral overlap are likely to become increasingly acute. We anticipate that the ability to label entire ribose rings with ^{15}N frequencies characteristic of C, U and purines and to use this information in conjunction with simultaneously acquired data from the HCP-CCH-TOCSY experiment as well as the known sequence of the molecule will extend the size of RNA molecules for which unambiguous sequential assignments can be obtained.

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