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Two- and three-dimensional HCN experiments for correlating base and sugar resonances in ^{15}N,^{13}C-labeled RNA oligonucleotides

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SUMMARY

New 2D and 3D ^{1}H-^{13}C-^{15}N triple resonance experiments are presented which allow unambiguous assignments of intranucleotide H1'-H8(H6) connectivities in ^{13}C- and ^{15}N-labeled RNA oligonucleotides. Two slightly different experiments employing double INEPT forward and back coherence transfers are optimized to obtain the H1'-C1'-N9/N1 and H8/H6-C8/C6-N9/N1 connectivities, respectively. The correlation of H1' protons to glycosidic nitrogens N9/N1 is obtained in a nonselective fashion. To correlate H8/H6 with their respective glycosidic nitrogens, selective ^{13}C-refocusing and ^{15}N-inversion pulses are applied to optimize the magnetization transfers along the desired pathway. The approach employs the heteronuclear one-bond spin–spin interactions and allows the 2D ^{1}H-^{15}N and 3D ^{1}H-^{13}C-^{15}N chemical shift correlation of nuclei along and adjacent to the glycosidic bond. Since the intranucleotide correlations obtained are based exclusively on through-bond scalar interactions, these experiments resolve the ambiguity of intra- and internucleotide H1'-H8(H6) assignments obtained from the 2D NOESY spectra. These experiments are applied to a 30-base RNA oligonucleotide which contains the binding site for Rev protein from HIV.

Until recently, NMR studies of nucleic acids have been largely limited to ^{1}H and some ^{31}P experiments. With the recent advent of methods for preparing uniformly labeled RNA oligonucleotides by enzymatic synthesis (Milligan et al., 1987) from ^{15}N- and/or ^{13}C-labeled NTPs isolated from *E. coli* (Nikonowicz et al., 1992; Michnicka et al., 1993) or methanolotrophic bacteria (Batey et al., 1992) grown on labeled media, it is now possible to adapt the heteronuclear multidimensional NMR methods developed for the study of protein structures (Kay et al., 1990; Clore and Gronenborn, 1991; Bax and Grzesiek, 1993) to the special problems of RNA oligonucleotides. Several such double resonance experiments have been reported, taking advantage of the

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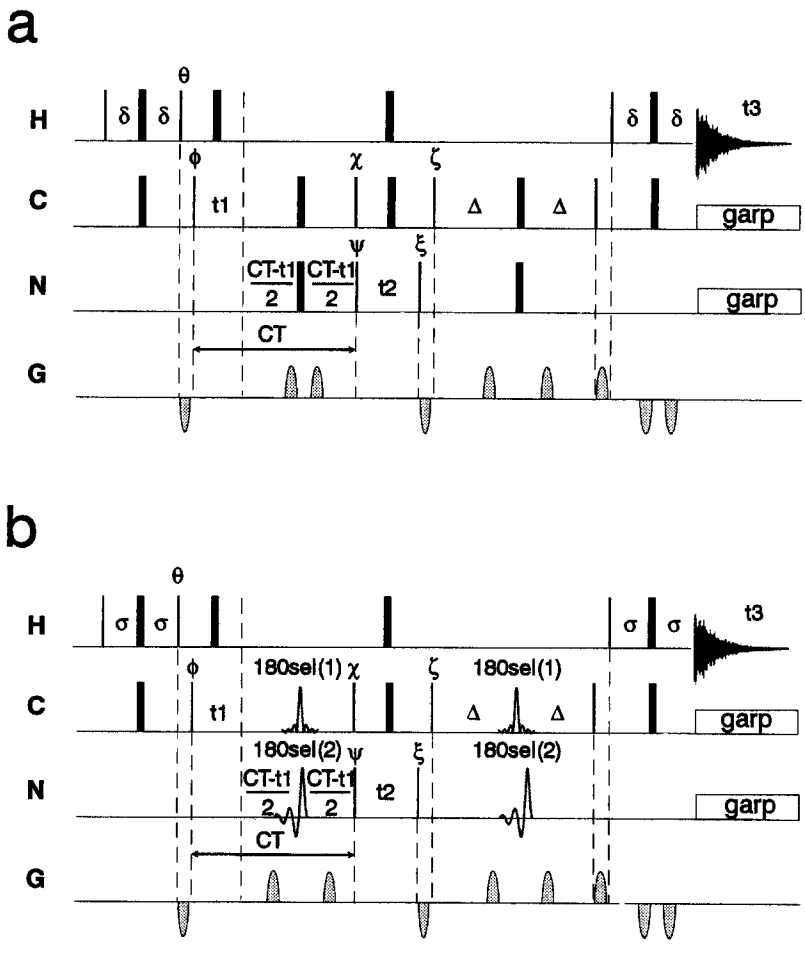


Fig. 1. Pulse schemes of the 3D HCN experiments. (a) The nonselective version to detect the H1'-C1'-N9/N1 connectivities, and (b) the semiselective version to detect the H8-C8-N9 and H6-C6-N1 connectivities. The narrow and broad filled bars represent 90° and 180° pulses, respectively. For (a), $\delta = 1/4\ J_{H1',C1'} = 1.6$ ms, CT = 42 ms, $\Delta = 21$ ms. The ^{13}C and ^{15}N carriers and sweep widths are set to cover the range of C1' (88–96 ppm) and N9/N1 (142–176 ppm) chemical shifts. For (b), $\sigma \sim 1/4\ J_{C8,H8} \sim 1/4\ J_{C6,H6} \sim 1.25$ ms, CT = 38–40 ms, $\Delta = 19$–20 ms, 180sel(1) = 4 ms, REBURP refocusing pulse (Geen and Freeman, 1991) centered at 140.5 ppm, 180sel(2) = 2 ms, IBURP2 inversion pulse (Geen and Freeman, 1991) centered at 160 ppm. For both experiments, phase cycling is: θ = y,−y; Ψ = x,x,−x,−x; ϕ = 4x,4(−x); χ = 8y,8(−y); ξ = 32x,32(−x); ζ = 16(−y),16y; receiver = a, −a, −a, a where a = [x,−x,−x, x, 2(−x, x, x,−x), x,−x,−x, x]. All other pulses are applied along the x axis. In addition, ϕ and Ψ phases are cycled to obtain the States-TPPI t_1- and t_2-quadrature detection (Marion et al., 1989). The 1-ms gradient pulses (800-μs gradient pulse shaped to a 1% truncated sine envelope and 200 μs for the magnetic field recovery) are used with amplitudes −6/ 3 / 3/ −6/ 3/ 3/ 12/ −3/ −3 G/cm. The asynchronous GARP decoupling (Shaka et al., 1985) is used to suppress the heteronuclear spin–spin interactions during t_3 acquisition.

^{13}C label, for assignment of the ribose spin systems (Nikonowicz and Pardi, 1992a, 1992b, 1993). The ^{15}N label has been used in HMQC experiments for assignment of imino and amino resonances (Nikonowicz and Pardi, 1992b, 1993) and correlation of N7,N9 to purine H8 and N1,N3 to AH2 resonances (^1H-^{15}N HMQC) (Michnicka et al., 1993). The standard strategy for sequence-specific assignment of nucleic acids relies on sequential intra- and internucleotide base H8,H6-

sugar H1' connectivities observed in NOESY spectra (Wüthrich, 1986; Feigon et al., 1992). Such assignments are necessarily conformation dependent, and ambiguities can arise especially for the nonstandard base pairs and tertiary interactions found in many RNA oligonucleotides. In order to distinguish unambiguously between intra- and internucleotide base–ribose connectivities, experiments which correlate the ribose H1' and base H8/6 resonances via scalar rather than dipolar connectivities are needed.

Here we present an approach which utilizes HCN triple resonance experiments to correlate intranucleotide ribose H1' and base H8/6 indirectly via the shared N9/1 of the glycosidic bonds. Owing to variations in ^{1}H-^{13}C, ^{13}C-^{13}C and ^{13}C-^{15}N spin–spin scalar couplings, different coupling networks, and relatively large differences in the ^{13}C chemical shifts of the ribose C1' and the aromatic C8/6 carbons (~ 50 ppm), the correlations H1'-N9/1 and H8/6-N9/1 are optimally detected by using two slightly different experiments. Depending on the complexity and overlap in the measured spectra, the experiments can be implemented in a 2D or 3D fashion to obtain the connectivities of two (H,N) or three (H,C,N) nuclei along the given H1'-C1'-N9/N1 or H8/H6-C8/C6-N9/N1 linkage.

The pulse schemes for the 3D HCN experiments are shown in Fig. 1. Two pulse schemes (a) and (b) have been designed to optimize the forward and back coherence transfers along the pathways H1'-C1'-N9/N1 and H8/H6-C8/C6-N9/N1, respectively. The preferred coherence transfer pathways as well as the undesired coherence transfer pathways due to other scalar interactions in RNA nucleotides are illustrated in Fig. 2. The basic principles already established for triple resonance experiments in proteins have been applied in these experiments (Kay et al., 1990; Clore and Gronenborn, 1991; Bax and Grzesiek, 1993), and only the points important for the measurement of RNA oligonucleotides are discussed below. For both experiments, two consecutive $\{^{1}H$-$^{13}C\}$ and $\{^{13}C$-$^{15}N\}$ INEPT and reverse INEPT steps (Morris and Freeman, 1979) are used to transfer the magnetization from the protons H1', H8 or H6 to their respective glycosidic nitrogens N9 or N1 and back. During the $\{^{13}C$-$^{15}N\}$ J evolution, the C1' and C8 and C6 carbons are labeled by their ^{13}C chemical shifts. In order to optimize the transfer from carbons to nitrogens, the constant time (CT) version of ^{13}C chemical shift labeling (Santoro and King, 1992; van de Ven and Philippens, 1992; Vuister and Bax, 1992) during t_1 evolution is applied in both experiments.

Pulse scheme (a): H1'-C1'-N9/N1. The δ value in the $\{^{1}H$-$^{13}C\}$ INEPT steps is set for J(H1',C1') ~ 160 Hz (Nikonowicz and Pardi, 1992b). The only nonproductive scalar interaction in the H1'-C1'-N9/N1 pathway is the C1'-C2' coupling. This interaction can be easily suppressed

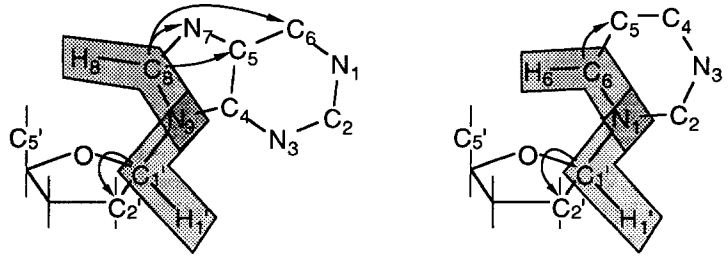


Fig. 2. Schematics of purine (left) and pyrimidine (right) ribonucleotides, showing the coherence transfer pathways for the two HCN experiments (shaded regions). Additional undesired coherence pathways are illustrated by the arrows.

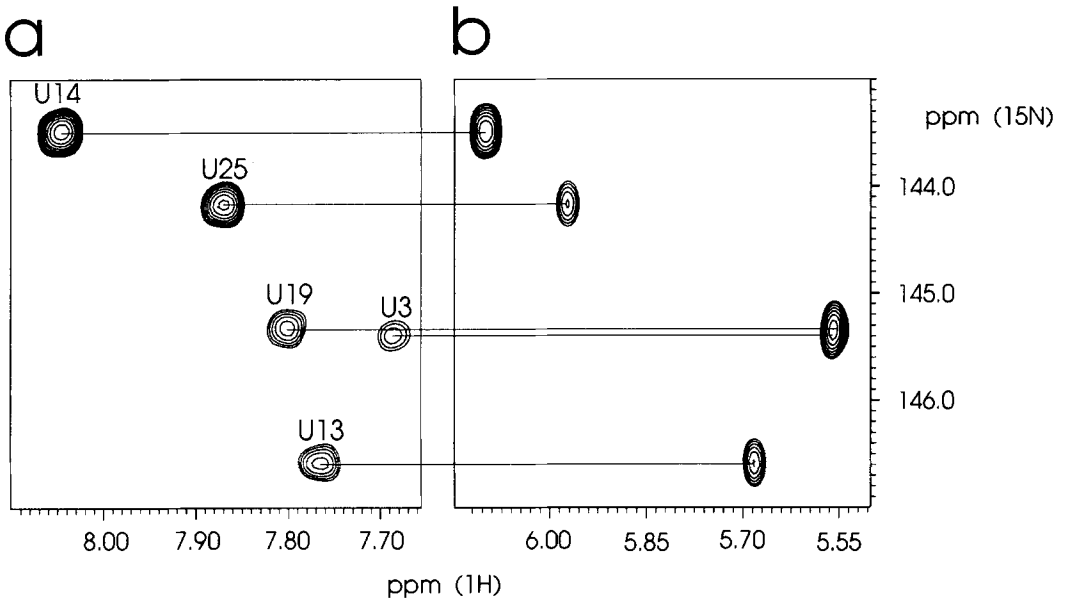


Fig. 3. 500 MHz (Bruker AMX 500) spectra of ^{13}C,^{15}N-labeled RBE3 at 40 °C. (a) Portion of a H8/H6-C8/C6-N9/N1 selective 2D HCN spectrum showing the UH6-N1 cross peaks. (b) Portion of a nonselective 2D HCN spectrum showing the UH1'-N1 cross peaks. The lines between the two spectra indicate the intranucleotide UH6-UH1' correlations obtained from analysis of these spectra. (c and d) Slices through ^{13}C planes of a nonselective (H1'-C1'-N9/N1) 3D HCN spectrum showing the G4H1'-N1 and G23H1'-N1 connectivities, respectively. These cross peaks overlap in both the ^{1}H and ^{15}N dimensions, but are resolved in the ^{13}C dimension. The selective and nonselective 2D HCN experiments were acquired with 512 and 256 points in the ^{1}H and ^{15}N dimensions, respectively. Fifty-two scans were acquired for each t_1 increment. Total measuring time for each experiment was 3.3 h. The processed data matrix was 1K × 1K points. The nonselective 3D HCN spectrum was acquired with 256 × 80 × 160 points in the ^{1}H, ^{13}C, and ^{15}N dimensions, respectively. Eight scans were acquired for each t_1 and t_2 increment. The processed data matrix was 256 × 128 × 512 points. Total measuring time was 33 h. External chemical shift references are DSS and liquid $^{15}NH_3$ for the ^{1}H and ^{15}N dimensions, respectively.

during t_1 by setting CT ~ $2/J_{C1',C2'}$. The uniformity of the one-bond ribose ^{13}C-^{13}C J couplings ($J_{C1',C2'}$ ~ 45 Hz) and the amplitude of the one-bond ^{13}C-^{15}N interactions ($J_{C1',N9/N1}$ ~ 11–13 Hz) make it possible to optimize both the decoupling of $^{13}C1'$-$^{13}C2'$ J interactions and the {^{13}C-^{15}N} INEPT transfer efficiency at the same time. By setting CT = 42 ms, the transfer coefficient for this step [$\alpha = \cos(\pi J_{C1',C2'} \cdot CT) \cdot \sin(\pi J_{C1',N9/1} \cdot CT)$] is greater than 0.9, neglecting transverse relaxation of C1'. After the transfer to ^{15}N and labeling by N9/N1 chemical shifts, the relevant coherences are relayed back to the originating H1' protons for detection via reverse {^{15}N-^{13}C} and {^{13}C-^{1}H} INEPT steps. With appropriate phase cycling, an absorption-mode spectrum with fully decoupled heteronuclear and single-bond ^{13}C-^{13}C interactions is obtained in all dimensions. To minimize the required phase cycling, gradient pulses are used to purge unwanted coherences in periods when the magnetizations of interest are converted into zz spin orders (Brühwiler and Wagner, 1986; Bax and Pochapsky, 1992; Sklenář et al., 1993b). In addition, two symmetrical gradients are applied along with each 180° refocusing pulse (Bax and Pochapsky, 1992; Sklenář et al., 1993b). These two pulses have the same effect as the four-step EXORCYCLE and generate a perfect echo by removing the imperfections of the 180° refocusing pulses.

Pulse scheme (b): H8/H6-C8/C6-N9/N1. The situation for correlating aromatic purine H8

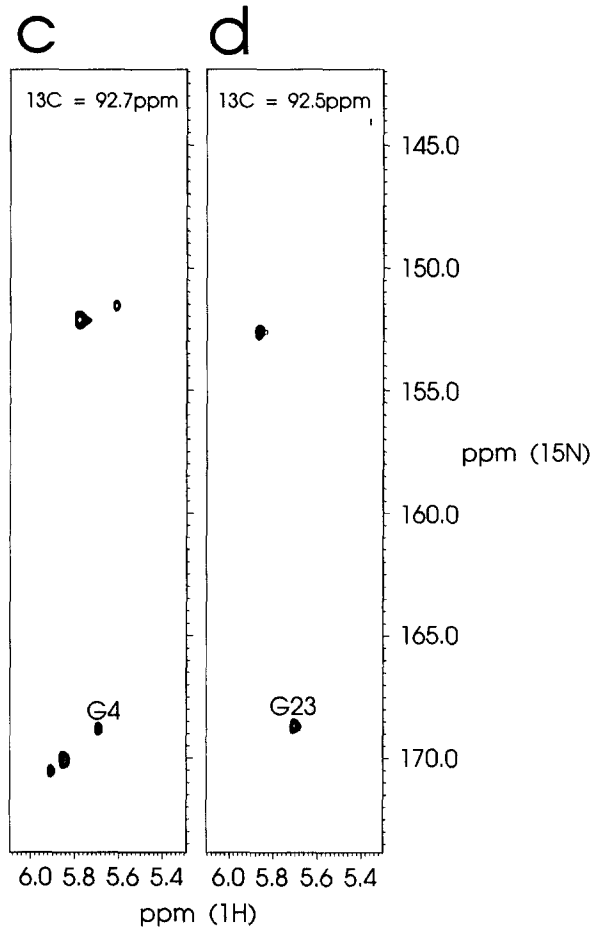


Fig. 3. (continued).

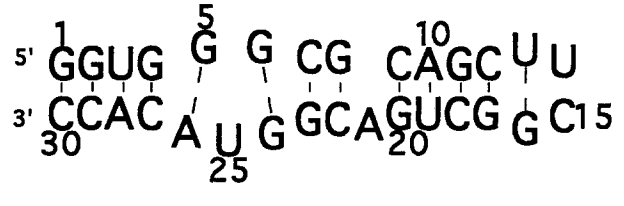
and pyrimidine H6 with their respective glycosidic N9 and N1 is complicated by alternate coherence transfer pathways. The nonselective version of the HCN experiment would result in substantial loss of sensitivity, especially for the purines, because of competing coherence transfers C8-C5, C8-C4 and C8-N7 which have J values similar to $J_{C8,N9}$. The basic strategy of the experimental design remains the same, but selective pulses are used to select for the desired coherences only. Only the deviations from the pulse scheme (a) will now be briefly discussed. The size of ^{1}H-^{13}C couplings in purine and pyrimidine residues is substantially larger than in the ribose residues (180–225 Hz) (Varani and Tinoco, 1991) and $\sigma \sim 1/4J_{C,H}$ is set to 1.25 ms accordingly. The adenine and guanine C8 have a relatively large two-bond ^{13}C-^{13}C coupling to C5 and C4 ($J_{C8,C5/4} \sim 8$–11 Hz (Rejante, M., unpublished results)). In addition to the ^{13}C-^{15}N interaction between C8 and N9 ($J_{C8,N9} \sim 4$–8 Hz), this carbon is also coupled to N7 ($J_{C8,N7} \sim 10$ Hz). The uracil and cytosine C6 have large one-bond ^{13}C-^{13}C couplings to carbons C5 ($J_{C6,C5} \sim 66$ Hz) and small two-bond couplings to C2 ($J_{C6,C2} \sim 2$–3 Hz). The one-bond ^{13}C-^{15}N coupling between C6 and N1 is ~ 13 Hz. In order to optimize the sensitivity for the H8-C8-N9 transfers, the ^{13}C-^{15}N

INEPT steps have been designed as semiselective. Owing to the fact that C6 and C8 resonate between 136–145 ppm, the H8-C8-N9 and H6-C6-N1 transfers can be optimized simultaneously in the semiselective pulse scheme. The ^{13}C refocusing 180° pulse is chosen to invert only the region of the C6 and C8 resonances. The semiselective nitrogen 180° inversion pulse is set to cover the range of N9 and N1 chemical shifts (143–173 ppm) while leaving the N7 resonances (220–240 ppm) unaffected. For the relatively large RNA oligonucleotide used here, the best empirically determined compromise for optimal evolution of $J_{C6,N1}$ and $J_{C8,N9}$ and short T_2 relaxation is 38–40 ms for the constant-time t_1 evolution period. The semiselective 180° ^{13}C and ^{15}N pulses in the ^{13}C-^{15}N INEPT steps decouple the C8-C5, C8-C4, C8-N7, C6-C5 and C6-C2 interactions for $t_1 = 0$ and during the reverse ^{15}N-^{13}C INEPT step. However, these couplings remain active during the t_1 evolution, leading to a corresponding in-phase splitting of the ^{13}C resonances in the F_1 dimension. Gradients and appropriate phase cycling for pure absorption-mode spectra are applied as in the selective experiment.

These 3D HCN experiments can be easily converted into the 2D versions by leaving out the ^{13}C chemical shift labeling. The resulting 2D HCN pulse schemes correlate the corresponding protons with their glycosidic nitrogens.

The HCN experiments presented here have been applied to the 30-base RNA oligonucleotide RBE3 (see scheme below). This oligonucleotide contains the binding site for the Rev protein of HIV and forms a folded structure that comprises a stem-bulge-stem-loop. RBE3 was enzymatically synthesized on a DNA template by using T7 RNA polymerase to incorporate labeled NTPs following procedures described by Milligan et al. (1987) and scaled up to yield NMR quantities (Heus and Pardi, 1991; Wyatt et al., 1991). The ^{13}C,^{15}N-labeled NTPs were prepared from RNA isolated from *E. coli* grown in ^{13}C-glucose and ^{15}N-$(NH_4)_2SO_4$ containing media (Nikonowicz et al., 1992) following the procedures of Batey et al. (1992). The RNA was purified by polyacrylamide gel electrophoresis and electroelution, followed by ethanol precipitation and dialysis (Amicon Centricon-SR 3 filters). The NMR sample was 1.2 mM RBE3 in 450 µl D_2O containing 10 mM phosphate, pH 6.0, 100 mM NaCl. Portions of the 2D HCN spectra containing the cross peaks from the five U nucleotides are shown in Figs. 3a and b. By combining the H1'-N9/N1 and H8/H6-N9/N1 correlations obtained from the nonselective and selective experiments, respectively, each H1' can be correlated with the intranucleotide H8/H6. In the 2D HCN spectra of RBE3, all of the expected H8/H6-N9/N1 and H1'-N9/N1 are observed, although there is some overlap especially for the purine resonances. Some slices through ^{13}C planes of the 3D spectra are shown in Figs. 3c and d. In the example shown, the ^{13}C dimension was used to resolve ambiguities caused by overlap of G4H1' and G23H1' in both the ^{15}N and ^{1}H dimensions.

The intranucleotide base-H1' connectivities obtained using the HCN experiments provide a starting point for the sequential assignments obtained from NOESY spectra, since the intranucleotide NOEs can now be unambiguously distinguished from internucleotide NOEs. The different



chemical shift ranges for CN1, UN1 and purine N9 are also useful for identification of proton type (Sklenář et al., 1993a), which provides a useful check from the primary sequence of the RNA on the sequential connectivities. The 3D HCN experiments should be especially useful in extending the range of NMR studies to even larger ribonucleotides such as tRNA.

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REFERENCES

Batey, R.T., Inada, M., Kujawinski, E., Puglisi, J.D. and Williamson, J.R. (1992) *Nucleic Acids Res.*, **20**, 4515–4523.
Bax, A. and Grzesiek, S. (1993) *Acc. Chem. Res.*, **26**, 131–138.
Bax, A. and Pochapsky, S. (1992) *J. Magn. Reson.*, **99**, 638–643.
Brühwiler, D. and Wagner, G. (1986) *J. Magn. Reson.*, **69**, 546–551.
Clore, G.M. and Gronenborn, A.M. (1991) *Prog. Nucl. Magn. Reson. Spectrosc.*, **23**, 43–92.
Feigon, J., Sklenář, V., Wang, E., Gilbert, D.E., Macaya, R.F. and Schultze, P. (1992) *Methods Enzymol.*, **176**, 235–253.
Geen, H. and Freeman, R. (1991) *J. Magn. Reson.*, **93**, 93–141.
Heus, H.A. and Pardi, A. (1991) *J. Mol. Biol.*, **217**, 113–124.
Kay, L.E., Ikura, M., Tschudin, R. and Bax, A. (1990) *J. Magn. Reson.*, **89**, 496–514.
Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.
Michnicka, M.J., Harper, J.W. and King, G.C. (1993) *Biochemistry*, **32**, 395–400.
Milligan, J.F., Groebe, D.R., Witherell, G.W. and Uhlenbeck, O.C. (1987) *Nucleic Acids Res.*, **15**, 8783–8798.
Morris, G.A. and Freeman, R. (1979) *J. Am. Chem. Soc.*, **101**, 760–762.
Nikonowicz, E.P. and Pardi, A. (1992a) *J. Am. Chem. Soc.*, **114**, 1082–1083.
Nikonowicz, E.P. and Pardi, A. (1992b) *Nature*, **355**, 184–186.
Nikonowicz, E.P. and Pardi, A. (1993) *J. Mol. Biol.*, **232**, 1141–1156.
Nikonowicz, E.P., Sirr, A., Legault, P., Jucker, F.M., Baer, L.M. and Pardi, A. (1992) *Nucleic Acids Res.*, **20**, 4507–4513.
Santoro, J. and King, G. (1992) *J. Magn. Reson.*, **97**, 202–207.
Shaka, A.J., Barker, P. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.
Sklenář, V., Peterson, R.D., Rejante, M.R. and Feigon, J. (1993a) submitted.
Sklenář, V., Piotto, M., Leppik, R. and Saudek, V. (1993b) *J. Magn. Reson. Ser. A*, **102**, 241–245.
van de Ven, F.J.M. and Philippens, M.E.P. (1992) *J. Magn. Reson.*, **97**, 637–644.
Varani, G. and Tinoco Jr., I. (1991) *J. Am. Chem. Soc.*, **113**, 9349–9354.
Vuister, G.W. and Bax, A. (1992) *J. Magn. Reson.*, **98**, 428–435.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Wyatt, J.R., Chastain, M. and Puglisi, J.D. (1991) *BioTechniques*, **11**, 764–769.