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Through-bond correlation of imino and aromatic resonances in ^{13}C-,^{15}N-labeled RNA via heteronuclear TOCSY

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Summary

Novel HCCNH TOCSY NMR experiments are presented that provide unambiguous assignment of the exchangeable imino proton resonances by intranucleotide through-bond connectivities to the (assigned) nonexchangeable purine H8 and pyrimidine H6 protons in uniformly ^{15}N-,^{13}C-labeled RNA oligonucleotides. The HCCNH TOCSY experiments can be arranged as a two-dimensional experiment, correlating solely GH8/UH6 and GH1/UH3 proton resonances (**HCCNH**), or as three-dimensional experiments, in which additional chemical shift labeling either by GN1/UN3 (**HCCNH**) or by GC8/UC6 (**HCCNH**) chemical shifts is introduced. The utility of these experiments for the assignment of relatively large RNA oligonucleotides is demonstrated for two different RNA molecules.

The secondary and tertiary structures of nucleic acids are largely determined by hydrogen bonds within base pairs and, in the case of nonhelical interactions, nucleotide triples. For standard helical structures, assignment of the hydrogen-bonded imino protons is straightforward and relies on sequential imino–imino NOEs between successive base pairs (Feigon et al., 1992). Ambiguities due to overlap of the proton resonances can be resolved in the nitrogen dimension with three-dimensional HSQC-NOESY or related experiments using uniformly ^{15}N-labeled samples (Nikonowicz and Pardi, 1992,1993). However, many biologically interesting RNA molecules do not adopt a simple helical structure; instead, their folded structure contains a variety of nonstandard secondary and tertiary nucleotide–nucleotide interactions. In these cases, assignments cannot be obtained by sequential NOEs, since the pattern of NOE cross peaks cannot be predicted a priori. Assignment of the slowly exchanging imino proton resonances is crucial to determining the three-dimensional structure of folded nucleic acids, since even with complete nonexchangeable assignments the correct structure cannot be obtained without the additional constraints provided by the exchangeable hydrogen interactions. Here we present novel 2D and 3D HCCNH TOCSY NMR experiments, which provide unambiguous assignment of the exchangeable imino proton resonances by intranucleotide through-bond connectivities to the (assigned) nonexchangeable purine H8 and pyrimidine H6 protons in uniformly ^{15}N-,^{13}C-labeled RNA oligonucleotides. The triple-resonance HCCNH TOCSY experiments utilize a combination of INEPT (Morris and Freeman, 1979) and homo- and heteronuclear TOCSY (Bertrand et al., 1978; Mueller and Ernst, 1979; Braunschweiler and Ernst, 1983) coherence transfers. A simultaneous $^{13}C/^{15}N$ TOCSY allows an efficient transfer of polarization across up to five C-C and C-N bonds in guanine and uracil residues via two-bond ^{13}C-^{13}C and single-bond ^{13}C-^{15}N spin interactions. The HCCNH TOCSY experiment can be arranged as a 2D experiment, correlating solely GH8/UH6 and GH1/UH3 proton resonances (**HCCNH**), or as 3D experiments, in which additional chemical shift labeling either by GN1/UN3 (**HCCNH**) or by GC8/UC6 (**HCCNH**) chemical shifts is introduced. The utility of these experiments for the assignment of even relatively

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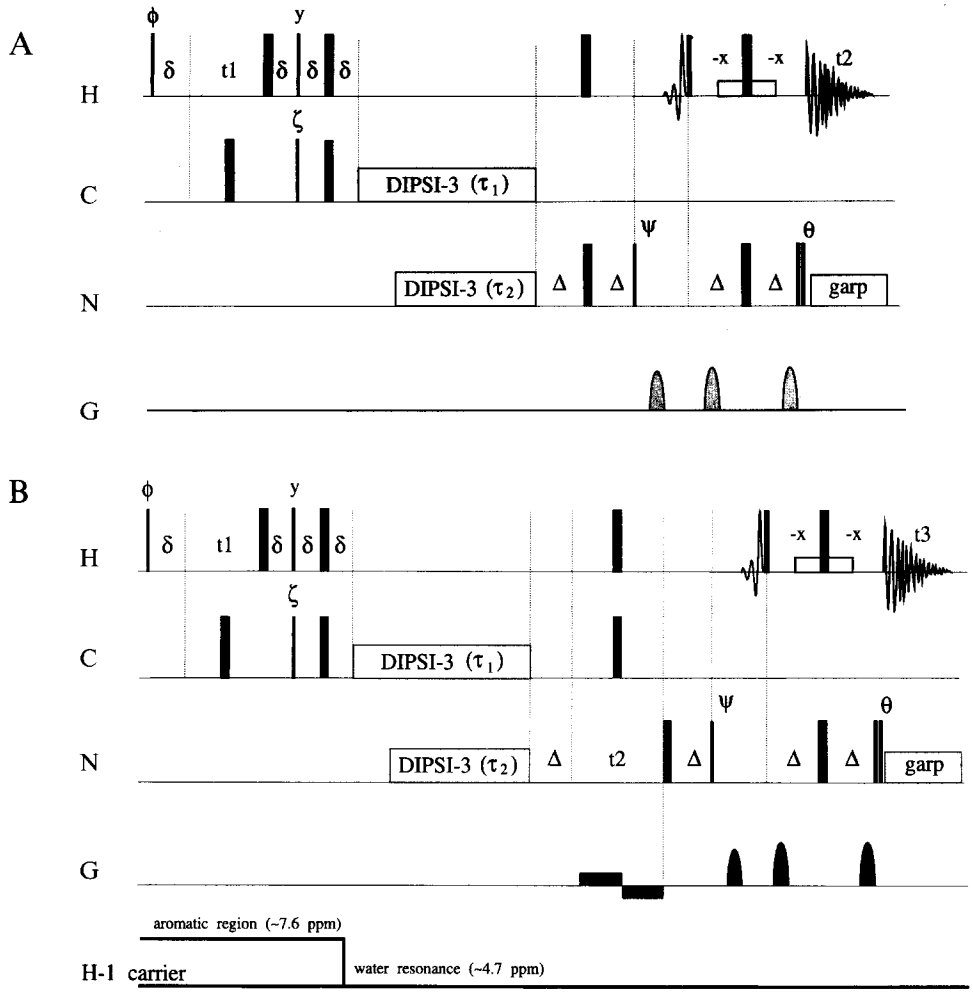

Fig. 1. Pulse schemes of (A) 2D **HCCNH** TOCSY; and (B) 3D **HCCNH** TOCSY experiments. The nonselective 90° and 180° pulses are indicated by thin and thick bars, respectively. The INEPT evolution delays are set to match 1/4J, both for H → C (J ~ 185–220 Hz) and N → H (J ~ 90 Hz) transfers (δ = 1.25 ms and Δ = 2.5 ms). In the 2D experiment, the proton carrier is centered at the water frequency. In the 3D experiment, the proton carrier is placed at the center of an aromatic region at ~ 7.6 ppm for the t_1-evolution period and switched to the H_2O position for the detection during t_3 in order to optimize the resolution and the size of the data matrix. DIPSI-3 mixing (Shaka et al., 1988) along the y-axis is used for the C-C and C-N TOCSY with an rf field of 2.9 kHz. The ^{13}C and ^{15}N carriers are set to 150 and 153 ppm, respectively. Water-flip back WATERGATE is applied during the ^{15}N-^{1}H INEPT step, using typically a 4 ms EBURP2 90° pulse and 1.3 ms selective 90° pulses flanking the nonselective 180° pulse. One scrambling gradient is applied during the ^{15}N-^{1}H INEPT, when the magnetization of interest is converted into H_zN_z order. Two identical gradients are used in the WATERGATE sequence. In the 3D experiment, the bipolar gradient echo (Sklenář, 1995) with an amplitude of 0.5 G/cm is used to suppress radiation damping during t_2. Unless otherwise specified, the pulses have phase = x. The phase cycle is: ϕ incremented in order to obtain States–TPPI (Marion et al., 1989) quadrature detection in t1; ζ = y,–y; ψ = y,y,–y,–y; θ = x,x,x,x,–x,–x,–x,–x; receiver = +,–,–,+. The phase of the 90° ^{1}H mixing pulse in the ^{1}H-^{13}C INEPT and the receiver phase are inverted after four scans. In addition, all phases of the WATERGATE and of the receiver are changed by 180° after eight scans for the 16-step cycle. The phase ψ is incremented according to the States–TPPI protocol to obtain quadrature detection in t_2 of the 3D experiment. Asynchronous GARP decoupling (Shaka et al., 1985) is used to suppress the heteronuclear spin–spin interactions during the acquisition.

large RNA oligonucleotides is demonstrated for two different RNA molecules, a 36-nucleotide RNA aptamer that binds ATP (Sassanfar and Szostak, 1993) and a 21-base oligonucleotide that contains the anticodon stem–loop of fMet-tRNA (Gale and Schimmel, 1995).

The pulse scheme of the 2D **HCCNH** TOCSY experiment is shown in Fig. 1A. During the initial t_1 period, the magnetizations of GH8 and UH6 protons are labeled by their respective chemical shifts and transferred via {^{1}H-^{13}C} INEPT to GC8 and UC6 carbons. After the carbon antiphase magnetizations $2H_zC_x$ have been refocused, a C-C TOCSY (Clore et al., 1990; Fesik et al., 1990) mixing sequence is applied to relay them via two-bond interactions (J ~ 8 Hz) between the guanine C8-C4 and C4-C6 (Fig. 2). Efficient single-bond transfers C4 → C5 → C6 in guanines and C6 → C5 → C4 in uracils are difficult to achieve using TOCSY mixing, despite the relatively large single-bond C-C coupling (60–90 Hz), due to the chemical shift dispersion of the carbons involved. Since the resonances of C5 both in guanines and uracils are shifted upfield (105–120 ppm) from other aromatic carbons (140–170 ppm), an excessively large rf field strength would be required to accomplish efficient transfer using the current state-of-the-art mixing sequences, such as DIPSI-3 (Shaka

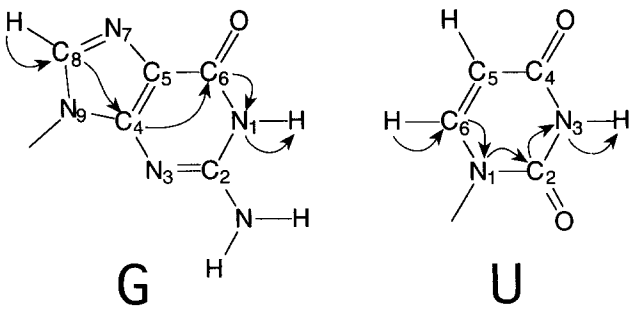

Fig. 2. Schematics of the nucleotide bases guanine and uracil, with their numbering system. Arrows indicate the coherence transfer pathways utilized in the HCCNH TOCSY experiments.

et al., 1988). Therefore, the experiment was optimized for the two-bond (GC4-GC6) transfers in guanines by setting the ^{13}C carrier to 150 ppm and the relative rf field strength to 23 ppm, corresponding to 2.9 kHz at 500 MHz. This eliminates the single-bond transfers C4-C5 and C5-C6 for guanines (and C6-C5 and C5-C4 for uracils). After the period of homonuclear C-C TOCSY mixing, a heteronuclear C-N TOCSY (Brown and Sanctuary, 1991; Ernst et al., 1991) is used to transfer the polarization from GC6 to GN1 and from UC6 to UN3 via the CN interactions UC6-UN1-UC2-UN3. The CN couplings involved are relatively large (~ 6–15 Hz). The nitrogen carrier is centered in between the GN1 and UN3 resonances (153 ppm). In the last transfer step, the in-phase nitrogen coherence is relayed to the directly bonded imino protons GH1 and UH3 via a ^{15}N-^{1}H INEPT coherence transfer. Since the HCCNH TOCSY experiments are performed in H_2O, a WATERGATE (Piotto et al., 1992; Sklenář et al., 1993b) read sequence was used to obtain efficient water suppression. In order to avoid partial saturation of the water resonance, the pulse scheme is designed as a water-flip back experiment (Grzesiek and Bax, 1993). During the heteronuclear C-C and C-N TOCSY, the water magnetization is driven back to the z-axis by radiation damping. The 180° pulse of the ^{15}N-^{1}H INEPT brings the H_2O magnetization to the –z-axis, and the following pair of selective EBURP2 (Geen and Freeman, 1991) and nonselective 90° pulses is used to return water magnetization to its equilibrium position.

Since the dispersion of proton chemical shifts in the regions of the imino and aromatic resonances may be limited, the 2D **HCCNH** TOCSY correlation can be extended into the 3D ^{1}H-^{15}N-^{1}H **HCCNH** TOCSY experiment by adding a t_2 period for GN1/UN3 chemical shift labeling (Fig. 1B). In order to minimize radiation damping during the incremented t_2 delay, a bipolar gradient is applied to keep the water magnetization along the –z-axis (Sklenář, 1995). The onset of radiation damping at later stages of t_2 incrementation would otherwise lead to partial saturation of the water resonance. To optimize the resolution of the 3D experiment, the proton carrier is set to the center of the aromatic region for the t_1 evolution and switched to the water frequency for detection of imino resonances during t_3. Alternatively, the proton carrier can be centered on the imino region if the 3-9-19 version (Sklenář et al., 1993a) of WATERGATE with phase order for off-resonance water suppression is applied during the ^{15}N-^{1}H INEPT step. A 3D ^{1}H-^{13}C-^{1}H **HCCNH** TOCSY version of the experiment, in which the GC8 and UC6 chemical shifts are labeled, can also be implemented as a straightforward variation of the experiment described above, with the t_2 interval incorporated before the TOCSY step.

Application of the 2D **HCCNH** TOCSY experiment to the 36-base ATP-binding RNA aptamer (Sassanfar and Szostak, 1993), uniformly ^{13}C,^{15}N labeled at all guanine nucleotides in complex with unlabeled AMP, is illustrated in Fig. 3. The lengths of the C-C and C-N mixing periods were optimized experimentally for the guanine imino protons (only) to $\tau_1 = 77.4$ ms and $\tau_2 = 58$ ms. The sample was prepared enzymatically by in vitro transcription (Milligan et al., 1987) with T7 RNA polymerase and a single-strand DNA template using uniformly ^{13}C-,^{15}N-labeled GTP and unlabeled ATP, CTP, and UTP, essentially as previously described for fully labeled samples

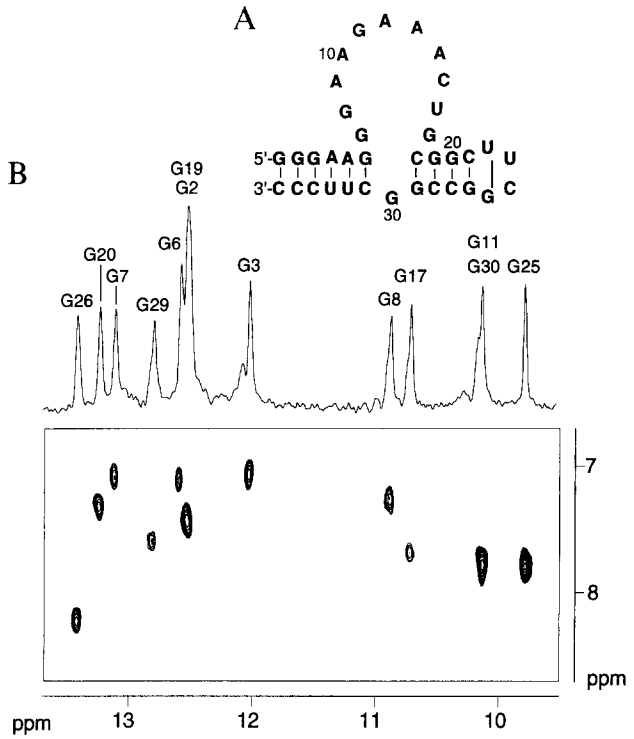

Fig. 3. (A) Sequence and secondary structure of the 36-nucleotide RNA ATP-binding aptamer. (B) 2D **HCCNH** TOCSY spectrum, showing the cross peaks between the guanine imino protons and GH8 resonances. Assignments of the imino resonances are indicated in the one-dimensional spectrum shown at the top. The spectrum was acquired with 48 × 2048 points in t_1 and t_2, respectively, $\tau_1 = 77.4$ ms, $\tau_2 = 58$ ms, spectral widths of 10 204 Hz in F1 and 1500 Hz in F2, 512 scans per t_1 increment, and a total measurement time of 36 h. One 800 μs (15 G/cm) and two 800 μs (20 G/cm) gradient pulses, shaped to a 5% truncated sine envelope, followed by a 200 μs recovery delay, were applied before and during the WATERGATE. The size of the processed data matrix was 2048 × 512 points.

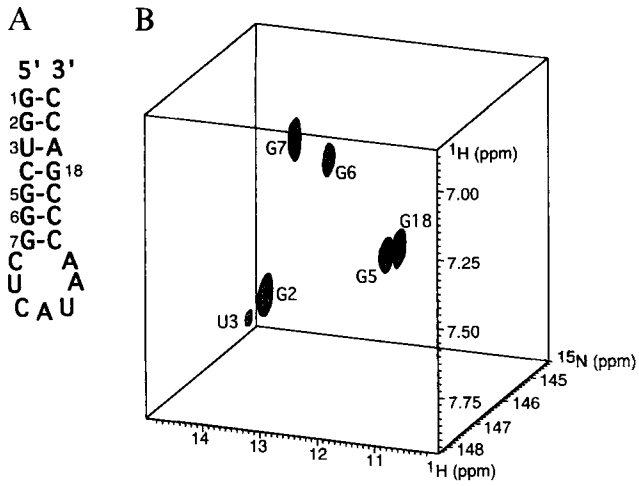

Fig. 4. (A) Sequence and secondary structure of the 21-nucleotide *E. coli* fMet-tRNA anticodon stem–loop. (B) 3D **HCCNH** TOCSY spectrum, showing the cross peaks correlating GH8-GN1-GH1 and UH6-UN3-UH3. The spectrum was acquired with $16 \times 20 \times 2048$ points in t_1, t_2, and t_3, respectively, $\tau_1 = 77.4$ ms, $\tau_2 = 58$ ms, spectral widths of 650.17, 204.74, and 10 204 Hz in F1, F2, and F3, respectively, 272 scans per t_1 increment, and a total experiment time of 36 h. Forward linear prediction to 28 and 24 data points in t_1 and t_2, respectively, was used before the Fourier transform. The size of the processed data matrix was $64 \times 64 \times 2048$ points.

(Batey et al., 1992; Nikonowicz et al., 1992; Sklenář et al., 1993a). The NMR sample was 1.3 mM in RNA oligonucleotide, 100 mM NaCl, pH 6.0 in a volume of 450 µl. Resonances of 13 out of the 14 guanine residues are observed in the 1D ^{15}N-decoupled ^{1}H spectrum. Nine of the guanines are found in the helical stems of the RNA, while five are present in the loop regions, where it was not possible to obtain imino assignments from either the proton NOESY or the ^{1}H-^{15}N HSQC-NOESY spectra. In the 2D **HCCNH** TOCSY spectrum, acquired over a period of 36 h, correlations are observed between all of the imino and H8 resonances. These H8 resonances had been previously assigned using HCN experiments (Sklenář et al., 1993a) and selectively adenine and guanine labeled samples, in combination with ^{13}C- and ^{15}N-filtered NOESY (Nikonowicz and Pardi, 1993) experiments. The assignments indicate that the missing guanine imino resonance is from the terminal G1•C36 base pair, which is exchanging too rapidly at 20 °C to be observed.

Although the available sample used for the 2D **HCCNH** TOCSY experiment was labeled only on the guanines, the experiment also works for the imino resonances found on uracils. This is illustrated in the 3D ^{1}H-^{15}N-^{1}H **HCCNH** TOCSY spectrum of the 21-nucleotide RNA containing the anticodon stem–loop of *E. coli* fMet-tRNA (Gale and Schimmel, 1995). The sample was prepared as described above, but with all NTPs uniformly ^{13}C,^{15}N labeled. The RNA oligonucleotide concentration was only 1.0 mM in a total volume of 200 µl (5 mm Shigemi NMR tube), 100 mM NaCl, pH 5.8. All but the terminal base pair iminos in the stem region are observed in the 1D ^{1}H spectrum, and all show correlations in the 3D experiment acquired over a period of 36 h. Note that both the guanine and uracil correlations are obtained in a single experiment, although the correlations for uracil under the conditions used here are somewhat weaker. Optimal correlations for Gs are obtained using the homonuclear C-C TOCSY prior to the heteronuclear C-N TOCSY, which minimizes the competing GC8-GN9 transfer. However, for Us, optimal results are obtained by setting τ_1 and τ_2 to the same value (75–80 ms) for the two DIPSI-3 spin-locks (results not shown).

Application of double- and triple-resonance experiments to labeled RNA samples (Dieckmann and Feigon, 1994; Pardi, 1995) has been somewhat limited by the short T_2 values of many of the RNA oligonucleotides of interest. We have shown that the HCCNH TOCSY approach for assigning exchangeable imino resonances works even for RNAs at the larger end of the range of molecules currently being studied. Since assignment of the imino resonances helps in defining the global fold of unusual RNAs, these experiments should greatly facilitate the structure determination of biologically interesting RNA molecules.

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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.

Bertrand, R.D., Moniz, W.B., Garroway, A.N. and Chingas, G.C. (1978) *J. Am. Chem. Soc.*, **100**, 5227–5229.

Braunschweiler, L. and Ernst, R.R. (1983) *J. Magn. Reson.*, **53**, 521–528.

Brown, L.R. and Sanctuary, B.C. (1991) *J. Magn. Reson.*, **91**, 413–421.

Clore, G.M., Bax, A., Driscoll, P.C., Wingfield, P.T. and Gronenborn, A.M. (1990) *Biochemistry*, **29**, 8172–8184.

Dieckmann, T. and Feigon, J. (1994) *Curr. Opin. Struct. Biol.*, **4**, 745–749.

Ernst, M., Griesinger, C. and Ernst, R.R. (1991) *Mol. Phys.*, **74**, 219–252.

Feigon, J., Sklenář, V., Wang, E., Gilbert, D.E., Macaya, R.F. and Schultze, P. (1992) *Methods Enzymol.*, **261**, 235–253.

Fesik, S.W., Eaton, H.L., Olejniczak, E.T. and Zuiderweg, E.R.P. (1990) *J. Am. Chem. Soc.*, **112**, 886–888.

Gale, A.J. and Schimmel, P. (1995) *Biochemistry*, **34**, 8896–8903.

Geen, H. and Freeman, R. (1991) *J. Magn. Reson.*, **93**, 93–141.

Grzesiek, S. and Bax, A. (1993) *J. Am. Chem. Soc.*, **115**, 12593–12594.

Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.

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.

Mueller, L. and Ernst, R.R. (1979) *Mol. Phys.*, **38**, 963–992.

Nikonowicz, E.P. and Pardi, A. (1992) *Nature*, **355**, 184–186.

Nikonowicz, E.P., Sirr, A., Legault, P., Jucker, F.M., Baer, L.M. and Pardi, A. (1992) *Nucleic Acids Res.*, **20**, 4507–4513.

Nikonowicz, E.P. and Pardi, A. (1993) *J. Mol. Biol.*, **232**, 1141–1156.

Pardi, A. (1995) *Methods Enzymol.*, **261**, 350–380.

Piotto, M., Saudek, V. and Sklenář, V. (1992) *J. Biomol. NMR*, **2**, 661–665.

Sassanfar, M. and Szostak, J.W. (1993) *Nature*, **364**, 550–553.

Shaka, A.J., Barker, P. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.

Shaka, A.J., Lee, C.J. and Pines, A. (1988) *J. Magn. Reson.*, **77**, 274–293.

Sklenář, V., Peterson, R.D., Rejante, M.R. and Feigon, J. (1993a) *J. Biomol. NMR*, **3**, 721–727.

Sklenář, V., Piotto, M., Leppik, R. and Saudek, V. (1993b) *J. Magn. Reson. Ser. A*, **102**, 241–245.

Sklenář, V. (1995) *J. Magn. Reson. Ser. A*, **114**, 132–136.