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Accurate Measurement of Heteronuclear Long-Range Coupling Constants from 1D Subspectra in Crowded Spectral Regions

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The two-dimensional heteronucleus-coupled hetero half-filtered proton–proton correlation (HETLOC) experiment allows the elegant determination of heteronuclear long-range couplings at natural abundance (*1, 2*). The application of these experiments, particularly for the measurement of long-range carbon–proton coupling constants, is becoming quite common in conformational analysis. The relatively small splittings are conveniently measured in the directly detected F_2 dimension, whereas the signal displacement is obtained via the large direct $^1J(\mathrm{CH})$ coupling interaction in the less-resolved F_1 dimension. The great advantage of this technique, originally introduced for enriched compounds (*3*), is the possibility of measuring coupling constants smaller than the linewidth, since one observes the relative frequency shift of two signals residing on two separate F_1 slices of the 2D spectrum. Each slice corresponds to one of the two satellite peaks. However, as recently pointed out (*4*), improved experimental efficiency and greater accuracy can be achieved by a technique that separately excites either of the two given satellite lines in a 1D fashion. Such technique, named PASS-TOCSY (*4*), allows the recording of two 1D subspectra, corresponding to the two F_1 slices of the standard 2D HETLOC experiment. In this paper, we propose modified versions of PASS-TOCSY, which greatly extend the range of applicability of such a technique and we compare the relative performances of different pulse sequences using a test case.

The rationale of the PASS-TOCSY experiment (Fig. 1a) can be summarized as follows. Initially, a BIRD period (*5*) is used to suppress the signal due to protons not coupled to ^{13}C nuclei. The BD delay should bring such undesired magnetization component via relaxation from the $-z$ direction through the null point, exactly at the time of the execution of the ϕ_4 excitation pulse. In practice, due to the natural spread in T_1 relaxation times, the BD delay can only be the result of a compromise and some undesired magnetization results. The undesired signal can be minimized using a ^{13}C filter. A novel feature of the PASS-TOCSY sequence is the use of a selective 90° ^{13}C pulse (ϕ_6), which is designed to extract specifically the satellite lines corresponding to the carbon atom of interest. All other ^{13}C-coupled proton resonances are converted into nonobservable MQ coherences by a 90° pulse (ϕ_8). Finally, the τ delay [$\tau = 1/(4\ ^1J(\mathrm{XH}))$] allows the two satellite components to mutually dephase by 90°. At this point, a trim pulse can alternatively select either of the two satellite components to be used as the source of magnetization transfer for the MLEV-17 sequence. In practice, in separate experiments, the proton carrier is put on resonance with the individual satellite line to be selected. For the sake of clarity, it should be stated that the phase cycle of the PASS-TOCSY pulse sequence illustrated in Fig. 1a differs from the original phase cycle (*4*). The latter does not turn out to be correct. Moreover, the first 90° carbon pulse (ϕ_5) and the second 180° proton pulse (ϕ_7) are not necessary.

The limitations of this pulse sequence are mainly due to the limited selectivity of the ϕ_6 pulse which, being constrained within the Δ delay (for ^{13}C, $\Delta = 3.5$ ms), cannot be better than several hundred hertz. This is not sufficient in most cases. Let us consider the performance of the PASS-TOCSY pulse sequence using a test case. We were interested in the observation of some long-range carbon–proton coupling constants for the sugar ring of 3′-azido-3′-deoxythymidine (AZT) whose molecular structure is reported at the top of Fig. 2. For our purpose, it is useful to consider the results of the execution of the pulse sequence using zero mixing time, by recording the signal immediately after the first TP pulse. Ideally, we should observe only one of the two satellite lines corresponding to the selected ^{13}C atom. This observation corresponds to the starting point of the experiment, the accuracy of which will affect the quality of the final result. All subspectra in Fig. 2 were obtained by using zero mixing time. Figure 2a illustrates the result of the PASS-TOCSY experiment when C-3′ is irradiated. One of the two satellite lines of H-3′ is selected, but several undesired proton signals are clearly present, particularly those of H-5′,5″. This rather poor result is the consequence of the poor selectivity of the ^{13}C ϕ_6 pulse, as expected if we note that the chemical-shift difference between C-3′ and C-5′ is only 142 Hz.

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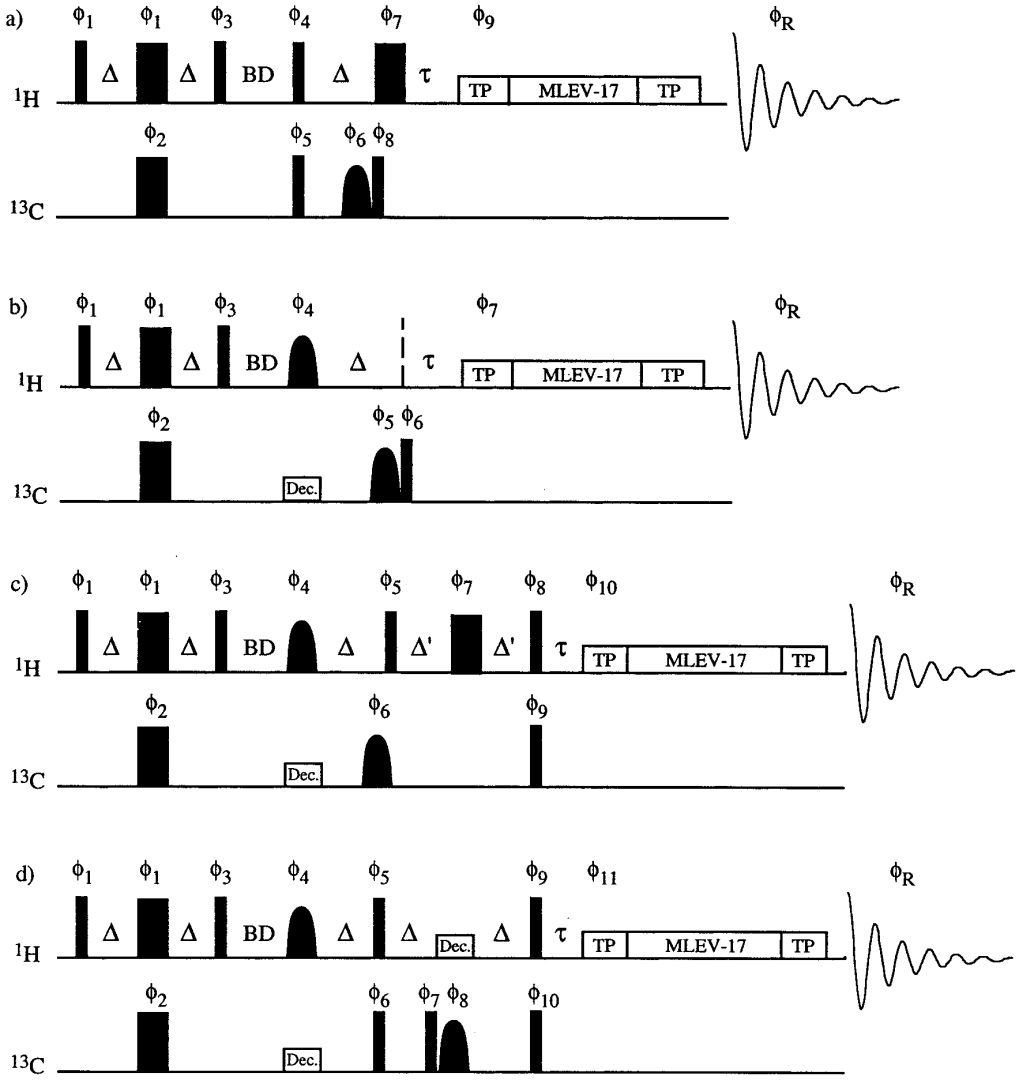

FIG. 1. PASS-TOCSY pulse sequences. (a) Original PASS-TOCSY sequence. Narrow and wide bars represent hard 90° and 180° pulses, respectively. Gaussian shapes represent selective 90° pulses. An open rectangle indicates decoupling, and TP stands for trim pulse. The delay $\Delta = 1/[2\ ^1J(\mathrm{CH})]$ and $\tau = 1/[4\ ^1J(\mathrm{CH})]$. BD represents BIRD delay. Phase cycling is as follows: $\phi_1 = x$; $\phi_2 = x$; $\phi_3 = -x$; $\phi_4 = y$; $\phi_5 = x$; $\phi_6 = x, x, -x, -x$; $\phi_7 = x$; $\phi_8 = x, -x$; $\phi_9 = x$; $\phi_R = x, -x, -x, x$. (b) SEL1-PASS-TOCSY sequence; ϕ_4 is a selective pulse. See text for details. The carrier is sitting in the middle of the satellite lines. Phase cycling is as follows: $\phi_1 = x$; $\phi_2 = x$; $\phi_3 = -x$; $\phi_4 = 45°$ or 135°; $\phi_5 = x, x, -x, -x$; $\phi_6 = x, -x$; $\phi_7 = x$; $\phi_R = x, -x, -x, x$. (c) SEL2-PASS-TOCSY sequence. $\Delta' = 1/(4\ \Delta^{13}\mathrm{C})$ with $\Delta^{13}\mathrm{C}$ being the chemical shift between the ^{13}C under study and another ^{13}C resonance that happens to be also excited by the ϕ_6 pulse. Phase cycling is as follows: $\phi_1 = x$; $\phi_2 = x$; $\phi_3 = -x$; $\phi_4 = x$; $\phi_5 = y$; $\phi_6 = x, x, -x, -x$; $\phi_7 = x$; $\phi_8 = 45°$ or 135°; $\phi_9 = x, -x$; $\phi_R = x, -x, -x, x$. (d) SEL-PASS-TOCSY. ϕ_8 pulse is a truly selective ^{13}C-pulse. Phase cycling is as follows: $\phi_1 = x$; $\phi_2 = x$; $\phi_3 = -x$; $\phi_4 = x$; $\phi_5 = y$; $\phi_6 = x, x, -x, -x$; $\phi_7 = y$; $\phi_8 = x, -x$; $\phi_9 = 45°$ or 135°; $\phi_{10} = y$; $\phi_{11} = x$; $\phi_R = x, -x, -x, x$.

Some modifications to the pulse scheme can be implemented to improve the performances of the experiment. For example, a proton-selective excitation pulse (ϕ_4 in Fig. 1b) can be employed initially. The result of the selective excitation of H-3′ in our example is reported in Fig. 2b. The combination of two selective pulses ϕ_4 and ϕ_6 on both nuclei improves selectivity significantly. However, proton selectivity reaches a limit in undesired modulations due to homonuclear couplings that would be introduced by a long ϕ_4 selective pulse. In practice, pulse duration was 3.7 ms which can only guarantee zero excitation outside a region of several hundred hertz from the carrier. In order to further improve quality, a further change can be introduced (Fig. 1c). An additional delay is inserted after the selective ϕ_6 pulse, to allow undesired ^{13}C resonances to evolve in the transverse plane by 90°. These contributions are not converted into observable signals by the following ϕ_9 pulse and are filtered out. The improvement in the result is illustrated in Fig. 2c. The spectrum is now improved significantly. However, a further advance can be obtained by using the last scheme

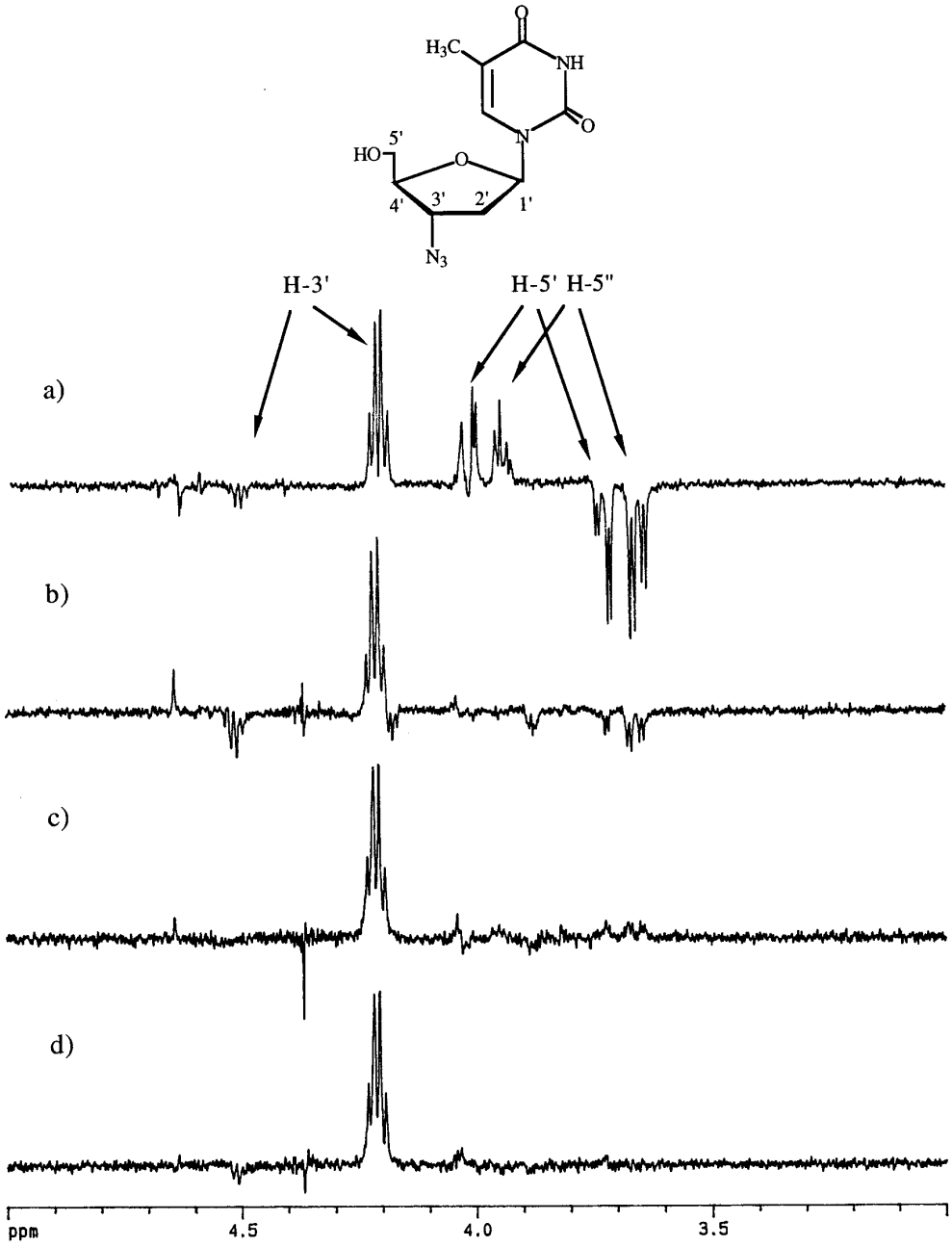

FIG. 2. Subspectra of AZT obtained by the application of pulse sequences reported in the legend to Fig. 1, in the same order (a–d). In all experiments, the first trim pulse is applied, and then the signal is detected (mixing time = 0). H-3′ (4.36 ppm) and corresponding C-3′ (70.72 ppm) are irradiated during selective pulses. ϕ_4 and ϕ_6 pulses can only be partially selective. ϕ_8 is a truly selective pulse. See text for details.

(Fig. 1d) which we refer to as SEL-PASS-TOCSY. In this case, the magnetization is transferred to the carbon atom using hard pulses (ϕ_5, ϕ_6). Then the direct heteronuclear coupling is refocused and the magnetization is converted into longitudinal magnetization. At this point, an optimized ^{13}C selective pulse, whose duration can be matched to the desired selectivity, will excite only the ^{13}C resonance under examination. The magnetization is then retransferred back to its directly bonded proton and the satellite selection is performed in the usual way. The result is reported in Fig. 2d, for our test case. The H-3′ satellite line is now neatly selected as the starting point for the MLEV-17 magnetization transfer.

The relative sensitivity loss is evident by comparing the noise level in the different spectra (Figs. 2a–2d). The corresponding gain in selectivity and absence of artifacts is also

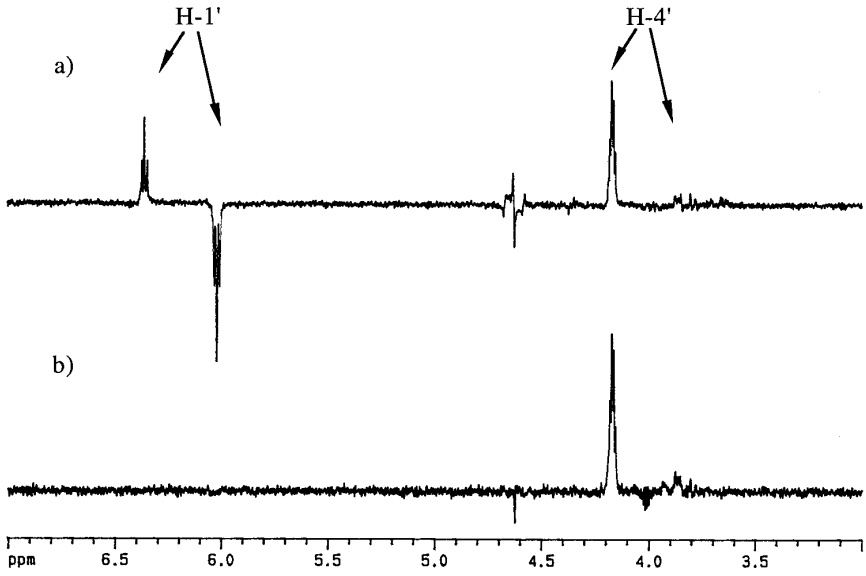

FIG. 3. (a) Subspectrum of AZT obtained by the application of PASS-TOCSY (Fig. 1a). (b) Subspectrum of AZT obtained by the application of SEL-PASS-TOCSY (Fig. 1d). H-4′ (4.02 ppm) and corresponding C-4′ (93.54 ppm) are irradiated during selective pulses. Note that frequency of 4′ satellite line is the same of 3′ satellite line reported in Fig. 2.

shown by the same comparison. We believe that the most convenient sequence must be chosen according to the user's specific need. However, the availability of the sequence reported in Fig. 1d makes the PASS-TOCSY experiment feasible in virtually all practical cases. A practical different feature of the new pulse schemes (Figs. 1b–1d) with respect to the original one (Fig. 1a) is the position of the proton carrier, which is positioned exactly in the middle of the two satellite lines in order to apply a correct selective proton pulse (ϕ_4). This aspect must be taken into account in the choice of the relative pulse phases for a correct selection of the desired satellite component (see legend to Fig. 1).

As an additional example, we report the results obtained by the application (with zero mixing time) of PASS-TOCSY

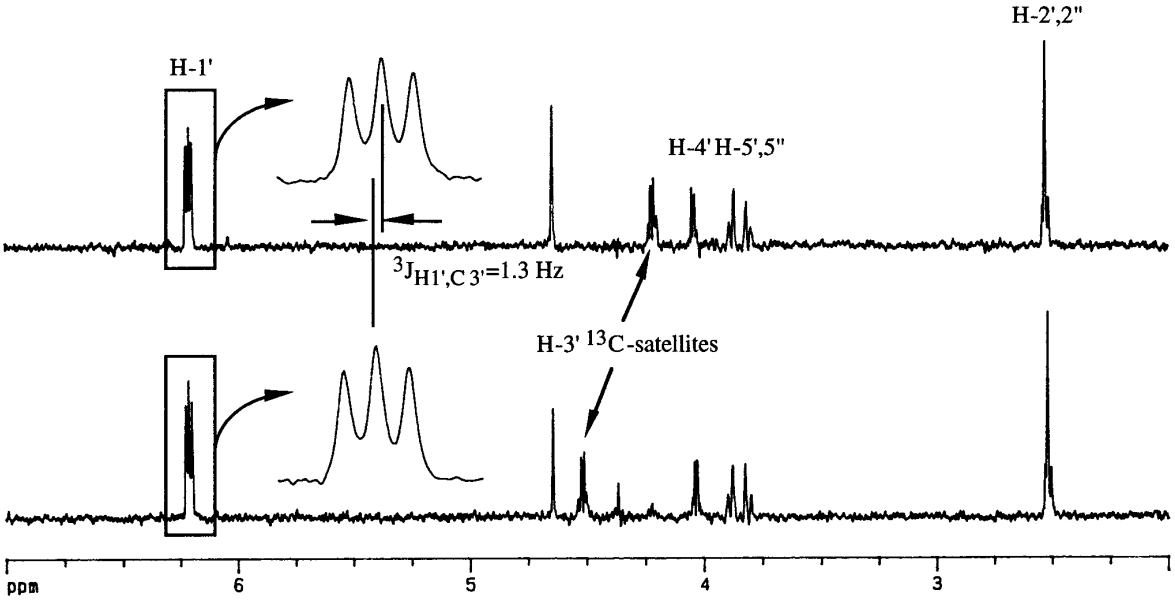

FIG. 4. An application of SEL-PASS-TOCSY (Fig. 1d) with irradiation of H-3′ (4.36 ppm) and corresponding C-3′ (70.72 ppm). MLEV-17 mixing time duration is 60 ms.

and SEL-PASS-TOCSY (Figs. 1a, 1d) for the selective excitation of H-4′ satellite resonance (Figs. 3a and 3b). The undesired excitation of H-1′, caused by the proximity of corresponding ^{13}C chemical shifts (112 Hz), is completely avoided with the new sequence. Incidentally, the two satellite lines appearing in Figs. 2d and 3b have virtually the same chemical shift. Therefore, in a standard 2D HETLOC experiment, they completely overlap, thereby impairing the possibility of measuring the corresponding coupling constants from the extraction of the two cross sections from the 2D experiment. In this particular case, only the 1D PASS-TOCSY experiment can solve the problem, in its best-resolved version (SEL-PASS-TOCSY).

Finally, we report in Fig. 4 the result of the complete PASS-TOCSY experiment (Fig. 1d), for the measurement of long-range heteronuclear coupling constants involving C-3′. All spectra were obtained on a Bruker AMX 500 MHz spectrometer at 37°C. AZT (25 mg) was dissolved in 0.5 ml DMSO.

Ultimately, there is a final observation that is relevant for the quality of the results. All these experiments rely on the total cancellation of non-^{13}C-coupled proton signals by the BIRD sequence. In practice, any residual signal must be canceled by the phase cycle on carbon pulses. Best suppression is obtained in our experience by avoiding any phase cycling on proton pulses, including trim pulses (TP) and the MLEV-17 mixing sequence. Any such phase change results in a disturbance for the stationary state of the undesired magnetization, which in turn generates artifacts in the final spectrum that spoil the accuracy of measurements.

In summary, the implementation of improved versions (especially SEL-PASS-TOCSY) of the PASS-TOCSY original scheme (Fig. 1a) greatly expands the range of applicability of this experiment to virtually all cases of interest. Accurate values of long-range heteronuclear couplings can be obtained at natural abundance. The use of these accurate measurements for conformational studies is already becoming widespread.

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