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Sensitivity-Enhanced Two-Dimensional Heteronuclear Shift Correlation NMR Spectroscopy

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Several years ago, it was demonstrated convincingly (*1–3*) that ^{1}H-detected chemical-shift correlation via heteronuclear multiple-quantum coherence (HMQC) (*3–5*) offers a substantial sensitivity advantage over the conventional shift-correlation experiment (*6–9*), in which the low-γ nucleus is detected directly during the data acquisition period. The ^{1}H-detected HMQC experiment, however, has not yet gained the popularity one might expect on the basis of its intrinsic advantages. Major problems with the HMQC experiment are the dynamic range problem that is introduced by the presence of large signals from protons that are not coupled to the low-γ nucleus and the required suppression of these intense signals in a difference experiment. In principle, both problems can be solved by presaturating the proton signals and transferring the NOE-enhanced low-γ signal to the protons (*10*), but the sensitivity advantage of the ^{1}H-detected experiments is partially lost in this process.

Here, we describe a simple method that alleviates the dynamic range problem and that facilitates the suppression of signals from protons that are not coupled to the low-γ nucleus. The idea is to saturate all protons not directly attached to the low-γ nucleus, leaving the protons coupled to the low-γ nucleus unaffected or slightly intensified by the homonuclear NOE effect (for molecules in the fast motion limit). The 2D pulse sequence is sketched in Fig. 1. In the following discussion, the low-γ nucleus is assumed to be ^{13}C. All protons not coupled to ^{13}C are inverted by the bilinear (BIRD) pulse (*11, 12*): $90^\circ_x(^1\text{H})$–$1/(2J)$–$180^\circ_x(^1\text{H}, ^{13}\text{C})$–$1/(2J)$–$90^\circ_{\pm x}(^1\text{H})$, where J is the one-bond ^{13}C–^{1}H scalar coupling constant. Protons coupled to ^{13}C are not affected by this bilinear pulse, whereas the magnetization of all other protons is inverted. At the time, τ, when the inverted magnetization changes from negative to positive (i.e., when protons not coupled to ^{13}C are nearly saturated), the first 90° pulse of the HMQC experiment is applied. In practice, the T_1's of the various protons in the molecule will vary, which at first sight makes it impossible to select a single τ value for which all protons not coupled to ^{13}C are near saturation. However, by keeping the delay time, T, between experiments short, one can largely circumvent this problem. This will be briefly explained below.

We define the delay period, T, as the time between the start of data acquisition in one scan and the end of the preparation period of the next scan. The bilinear pulse is

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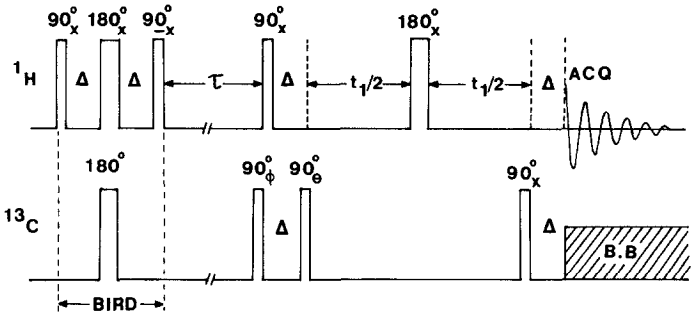

FIG. 1. Pulse scheme of the heteronuclear multiple-quantum experiment with presaturation of protons not coupled to ^{13}C. The phase cycling is given in Table 1. The BIRD pulse unit inverts protons that are not coupled to ^{13}C. The delay time, Δ, is set to $1/(2J)$. The use of an offset compensated 180° (^{13}C) pulse, $90^\circ_x 180^\circ_y 90^\circ_x$ (*20*) or $90^\circ_x 180^\circ_{-x} 270^\circ_x$ (*21*), is recommended to avoid partial saturation of protons that are coupled to ^{13}C nuclei that have a large offset from the ^{13}C carrier. The first 90°_ϕ (^{13}C) pulse serves to eliminate small artifacts from the 2D spectrum that otherwise arise from longitudinal ^{13}C magnetization present at the end of the preparation period. Broadband ^{13}C decoupling is used during ^1H data acquisition.

applied at a time, $\tau = T/2.7$, before the end of this delay period. The time T is chosen to be about 1.3 times the T_1 value of the fastest relaxing proton in the molecule. At the start of the data acquisition for the first scan, longitudinal magnetization may be assumed to be close to zero. The buildup of longitudinal magnetization during the delay period is depicted in Fig. 2. The bilinear pulse inverts the magnetization, and just after this inversion, the magnetization of the fastest relaxing proton is the most negative. At a time $T/2.7$ later, the longitudinal magnetizations of all protons not coupled to ^{13}C are close to zero. At this time the HMQC experiment is begun. In practice, we "fine tune" the duration of the delay time, τ, by choosing the value that minimizes the signal that is recorded in a single scan preceded by two dummy scans. It may appear that for the slowly relaxing protons in the molecule such a relatively short delay period between experiments is far from the optimum delay of $1.3T_1$ (*13*).

TABLE 1

Phase Cycling Used in the Scheme of Fig. 1

Scan	ϕ	θ	Acq.[a]
1	x	x	x
2	x	y	x
3	x	$-x$	$-x$
4	x	$-y$	$-x$
5	$-x$	x	x
6	$-x$	y	x
7	$-x$	$-x$	$-x$
8	$-x$	$-y$	$-x$

[a] Data in odd- and even-numbered scans are stored in separate memory locations.

However, the presence of a ^{13}C nucleus adjacent to the protons of interest shortens the T_1 of these protons significantly. The dipolar interaction between the proton and its directly coupled ^{13}C nucleus is of the same order of magnitude as the dipolar coupling between geminal methylene protons. Therefore, all protons directly coupled to ^{13}C relax relatively efficiently, and a short delay period between experiments does not adversely affect the sensitivity.

As an example, we have applied the technique to a sample of 12 mg of the octapeptide angiotensin-II (MW 1044), dissolved in 0.35 ml D_2O, in a 5 mm sample tube, pH 3.5. The sample is not spun. Experiments are recorded on a Nicolet NT-500 spectrometer, using a Cryomagnet Systems probe (*14*), which has a broadband (^{15}N to ^{31}P) decoupling coil outside the ^1H observe coil. Eight scans preceded by two dummy scans are recorded for every t_1 value. A 1 s delay time between experiments (including the 128 ms t_2 acquisition period) and a 335 ms τ period are used. The experiment is repeated for 172 t_1 increments of 120 μs, resulting in a total measuring time of 29 min. Data for odd- and even-numbered scans are stored separately and the data are processed in the standard fashion (*15–17*) to yield a 2D phase-sensitive spectrum. Phase modulation effects due to homonuclear J coupling are very small for the short durations of the evolution period used in this experiment and consequently, the 2D spectrum can be phased, to a good approximation, to the purely absorptive mode. The spectrum obtained this way is shown in Fig. 3, along with the regular ^1H spectrum and the projection of the 2D spectrum onto the ^{13}C axis. The ^{13}C frequency scale is relative to hypothetical internal TSP. This referencing is obtained in a way similar to the method previously used by Live *et al.* (*18*) for ^{15}N. We measure the absolute frequency of the residual

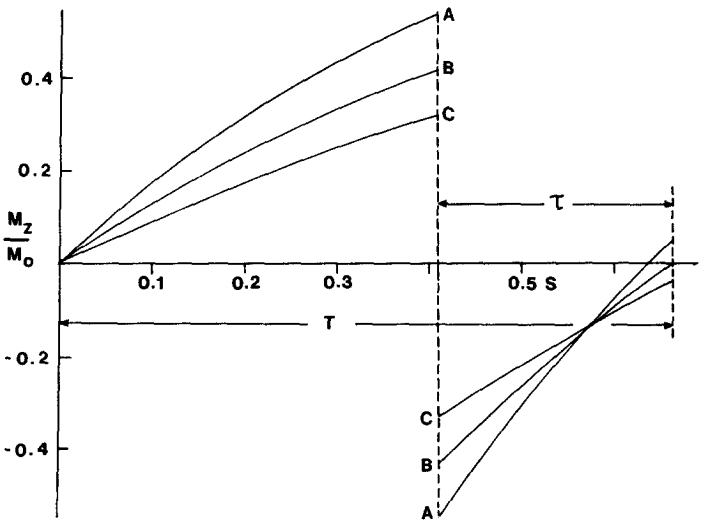

FIG. 2. Time evolution of longitudinal magnetization during the delay time between experiments for three spins, not coupled to ^{13}C with longitudinal relaxation times of (A) 0.5, (B) 0.7, and (C) 1.0 s. T is the time between the start of data acquisition of one scan and the end of the preparation period of the next scan. The BIRD pulse, which inverts magnetization of protons not coupled to ^{13}C is applied a time τ before the end of the preparation period, such that at the end of the preparation period the magnetizations of all three spins are close to zero. Recommended values are $T = 1.3T_1$ of the fastest relaxing proton and $\tau \approx 0.35T$.

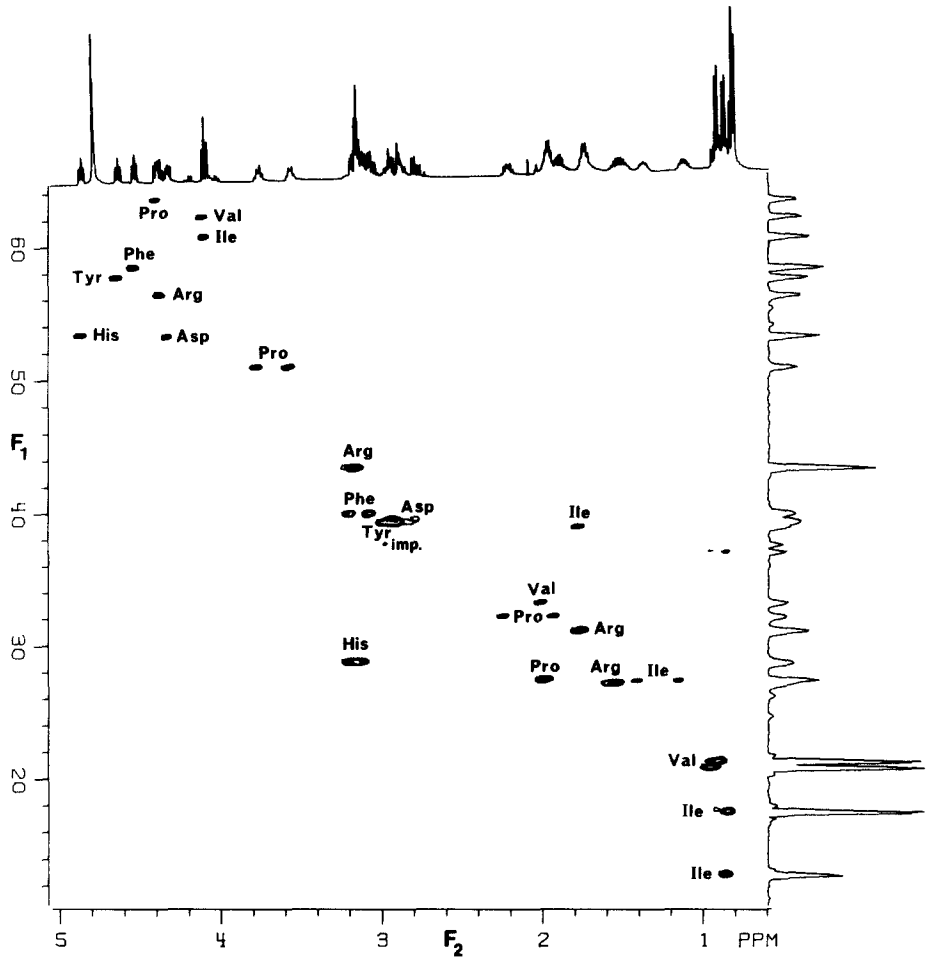

FIG. 3. Absorption-mode heteronuclear chemical-shift-correlation spectrum of 12 mg angiotensin-II, recorded in 29 min. The spectrum results from a 172 × 2048 data matrix. 30 and 8 Hz Gaussian line broadening have been used in the t_1 and t_2 dimensions, respectively. Some small artifacts at the ^{13}C carrier frequency (37.3 ppm), and an impurity at 38.0/2.96 ppm are also visible. The ^{13}C carrier artifacts disappear if four instead of two dummy scans are recorded for every t_1 value. The projection of the 2D spectrum onto the ^{13}C (F_1) axis and the regular high-resolution ^1H spectrum are shown along the sides of the 2D spectrum.

HDO present in the sample, which at 22°C resonates at 4.80 ppm relative to TSP. By multiplying the ^1H frequency of the HDO resonance by 0.25144954 (the $^{13}C/^1H$ frequency ratio in TSP) one obtains the carbon frequency that corresponds to 4.8 ppm downfield from internal TSP. In all cases used we find this method of referencing accurate to at least 0.1 ppm. For TMS, the $^{13}C/^1H$ frequency ratio is 0.25145002. All ^{13}C chemical shifts agree fairly well (±1.5 ppm) with typical values reported for linear peptides (*19*). This suggests that this type of heteronuclear chemical-shift correlation may be of use for assignment of ^1H spectra of linear peptides.

The signal-to-noise ratio on cross sections taken parallel to the F_1 axis through the spectrum of Fig. 3 ranges from 8:1 for the Ile Cγ methylene resonances that have a

broad multiplet structure in the F_2 dimension to 15:1 for the Cα resonances and 40:1 for the methyl groups. For relatively small molecules (<2000 daltons) we find in practice that N μg of sample of molecular weight, N, generally yields heteronuclear shift-correlation spectra with an acceptable sensitivity in less than 12 hours. The main advantage of the modification proposed here is that on larger quantities (but still small relative to conventional ^{13}C NMR quantities) it becomes possible to record 2D shift-correlation spectra that are essentially free of t_1 noise in a very short period of time. We find the indirect detected ^{13}C method attractive for routine use in our laboratory since no probe changes are necessary and the time needed for shimming on our 5 mm probe is much less than usually needed for our broadband 10 mm probe. The addition of a bilinear pulse as described in this communication is not recommended for the study of proteins and other macromolecules because during the delay time, τ, the negative NOE effect decreases the intensity of the protons coupled to ^{13}C.

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