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In-Phase Selective Excitation of Overlapping Multiplets

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An editing experiment is presented that selects for a peak on the basis of its chemical shift and that of one of its scalar coupling partners. The selected multiplet is pure in-phase. The editing procedure can be used in conjunction with 1D TOCSY/HOHAHA and NOE measurements. The pulse sequence described is particularly suitable for small molecules; data is presented for Gramicidin S and dehydrotestosterone. © 2000 Academic Press

Key Words: **excitation sculpting; selective excitation; in-phase editing; spectral analysis.**

The use of selective excitation to reduce the dimensionality of NMR experiments is well established (*1–10*). When a limited amount of information is required the use of selective excitation can result in a considerable saving in time. For example, the COSY, TOCSY/HOHAHA, or NOE spectra of a pair of overlapping peaks may be resolved either by resort to an experiment of higher dimensionality or by editing the spectrum for one of the two peaks. The latter approach often implies the use of selective excitation (*11, 12*). In addition to the perennial problem of sensitivity, existing spectral editing techniques tend to suffer from two major drawbacks that can limit the extent of their application: the cleanness of the selection procedure and the phase with which the selected multiplet is prepared. Editing procedures that utilize selective pulses make the assumption that they excite a particular range of frequencies. However, even modern pulse shapes tend to perturb some signal outside their nominal excitation bands. While the spurious signals that this can result in may be relatively small compared to that of the chosen multiplet, they can be significant when small effects such as long range NOEs are being measured. Most editing procedures incorporate scalar coupling evolution times (*13*). A consequence of this is that the selected multiplet will typically consist of a mixture of in-phase and antiphase components. If TOCSY/HOHAHA (*14–16*) or NOE (*5, 7*) measurements are to be made it is desirable that the selected multiplet be pure in-phase. In the case of NOE measurements, antiphase magnetization may be converted into zero-quantum coherence that gives rise to signals in the final spectrum that can be difficult to separate from the NOEs. In this communication we present a pulse sequence that enables overlapping multiplets to be edited for cleanly and that produces a pure in-phase signal. This sequence is applicable to molecules of molecular weight up to about 1000 daltons. The technique is applied to 1D TOCSY/HOHAHA and NOE measurements. It is also suitable for quantitative NOE measurements.

The problem of selecting a band of frequencies cleanly can be overcome by using excitation sculpting (*17, 18*). The basic excitation sequence, which is applied to transverse magnetization, consists of: $-g1-S-g1-g2-S-g2-$, where $g1$ and $g2$ are magnetic field gradient pulses with unrelated amplitudes and S is a selective 180° pulse, typically modulated with a Gaussian waveform. Any magnetization that is not refocused by the two selective pulses is dephased by the magnetic field gradient pulses. The use of a train of two soft spin echos has two important beneficial effects: any phase errors that might result from the use of a single selective pulse are removed, and if a given fraction of the magnetization is refocused by the soft pulse at a given frequency, the use of two soft pulses results in the square of that fraction being refocussed in the sequence as a whole. The latter results in much cleaner excitation overall. Providing that the scalar coupling partners of the spin of interest are not perturbed by the soft pulse, any scalar coupling evolution during the pulse sequence will be reversed.

An editing sequence that utilizes excitation sculpting to select a spin l coupled to a spin k on the basis of their chemical shifts is given in Fig. 1A, where the selected coherence transfer pathway is

$$I_{kz} \xrightarrow{90^\circ_x} -I_{ky} \xrightarrow{\delta+\tau} 2I_{kx}I_{lz} \xrightarrow{90^\circ_y} -2I_{kz}I_{lx} \xrightarrow{\delta+\tau} I_{ly} \qquad [1]$$

Each half of the experiment incorporates an excitation sculpting sequence. To ensure that only the desired coherence transfer pathway survives, one of the soft pulses in the first excitation sculpting sequence selects only ω_k and one of the soft pulses in the second excitation sculpting sequence selects only ω_l. However, since coherence is transferred between the two spins via antiphase magnetization scalar coupling evolution

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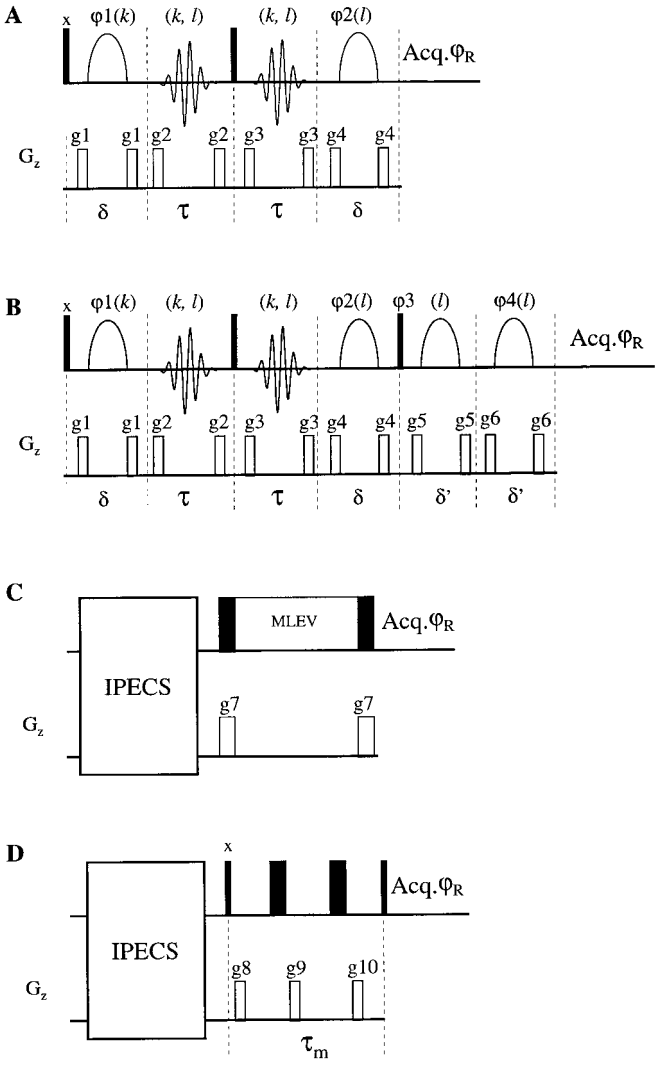

FIG. 1. Pulse sequences for resolving overlapping multiplets. (A) Basic experiment. (B) IPECS experiment incorporating an in-phase filter. (C) 1D TOCSY/HOHAHA experiment incorporating the IPECS editing sequence. (D) 1D NOE experiment incorporating the IPECS editing sequence. Narrow and wide rectangles denote nonselective 90° and 180° pulses, respectively; the default value for pulse phases is *y*. All other shapes denote selective pulses which perturb the spins indicated (*k* and *l*). τ is a scalar coupling evolution period with an optimal value of $\frac{1}{2}J_{kl}$ and δ and δ' are set to the minimum value consistent with the lengths of the events (soft pulses and gradient pulses) that they comprise. Phase cycling, where applicable: $\phi 1 = x, y, -x, -y$; $\phi 2 = 4(x), 4(y), 4(-x), 4(-y)$; $\phi 3 = 16(y), 16(-y)$; $\phi 4 = 32(x), 32(y), 32(-x), 32(-y)$. For (A) $\phi R = x, -x, x, -x, -x, x, -x, x$. For (B)–(D): $\phi R = 4(x, -x, x, -x, -x, x, -x, x,), 4(-x, x, -x, x, x, -x, x, -x,), 4(x, -x, x, -x, -x, x, -x, x,), 4(-x, x, -x, x, x, -x, x, -x,)$. In many instances using the first 4 or 16 steps of the phase cycle will produce acceptable results.

must be allowed to occur between them and consequently the remaining two soft pulses are cosinusoidally modulated to select at both ω_k and ω_l simultaneously (*19*). This is achieved by cosinusoidally modulating the chosen waveform with a frequency of $(\omega_k - \omega_l)/2$ and applying the soft pulse midway between the two multiplets. Provided that the soft pulses in the first excitation sculpting sequence do not perturb other scalar coupling partners of *k* and that those in the second excitation sculpting sequence do not excite other scalar coupling partners of *l*, the amplitude of the observed signal will be a function only of J_{kl}. If $\tau = 1/2J_{kl}$, then pure in-phase magnetization will be generated. However, if J_{kl} is not known, a series of calibration experiments performed with different values of τ may be required. This is also the case for the pulse sequence proposed by Bourg and Nuzillard (*9*) for the simultaneous selection of the in-phase multiplets of a pair of coupled spins.

By appending a filter onto the end of the pulse sequence, Fig. 1B, it is possible to produce a spectrum in which *l* is in-phase regardless of the value of τ, thus removing the need for calibration experiments. For brevity we dub this sequence IPECS (*i*n-*p*hase *e*diting of *c*oupled *s*pins). The filter consists of a 90° pulse followed by the excitation sculpting sequence. To function properly the soft pulses in the excitation sequence must refocus *l*-spin magnetization, but not that of its scalar coupling partners. This sequence has no effect on in-phase magnetization of the chosen spin. However, the 90° pulse transfers antiphase magnetization either into antiphase magnetization of other spins or into multiple-quantum coherence. In either case the coherence is removed by the subsequent excitation sculpting sequence. Omitting relaxation effects and assuming scalar coupling evolution continues unaffected during the application of soft pulses, the observed signal intensity will be $\sin^2(\pi J_{kl}\tau)$. We note that Gradwell *et al.* (*6*) have resolved the in-phase spectra of overlapping multiplets by sandwiching a TOCSY/HOHAHA or ROESY period between two conventional excitation sculpting sequences.

1D NOE (*7*) and TOCSY/HOHAHA (*10*) sequences are appended onto the in-phase filtered editing experiment in Fig. 1D and C, respectively. Non-selective 180° pulses are incorporated into the mixing period of the NOE experiment to prevent the recovery of unselected magnetization (*7*).

The sequences are demonstrated on gramicidin S in Fig. 2. The amide proton of valine overlaps with the aromatic protons of phenylalanine in the conventional 1D spectrum, Fig. 2A. The editing sequences are demonstrated for this amide proton in Fig. 2B and C where *k* is the α-proton and *l* is the amide proton. Both sequences select the chosen peak cleanly. However, the spectrum given in Fig. 2B, acquired using the pulse sequence in Fig. 1A, clearly contains a mixture of in-phase and anti-phase components. The corresponding spectrum obtained with IPECS editing sequence given in Fig. 1B, which incorporates an in-phase filter, is pure in-phase. All parameters that are common to the two experiments have the same values in each case. 1D NOE spectra for the amide proton of valine obtained using both editing sequences are presented in Fig. 2D and E. The spectrum obtained using the basic editing sequence contains an antiphase signal at the valine α-proton frequency. This originated from antiphase magnetization generated by the editing sequence which was converted into zero-quantum coherence during the NOE mixing period and into antiphase

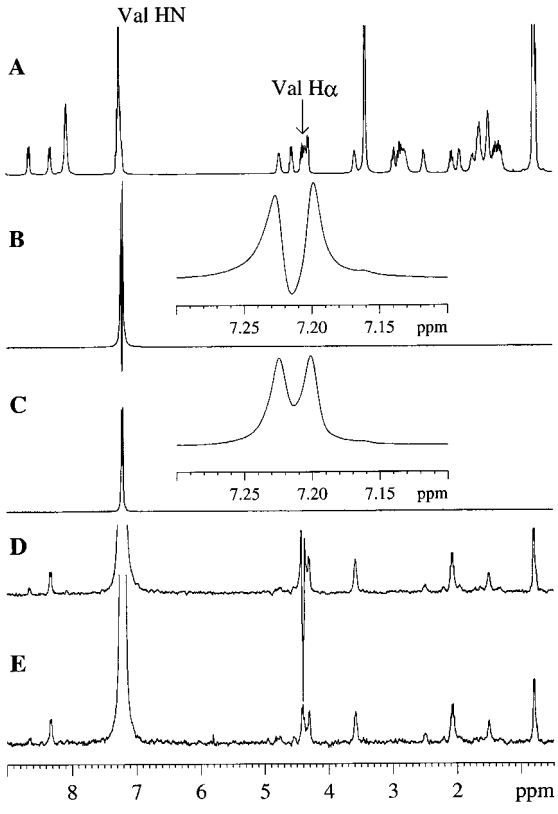

FIG. 2. 400 MHz ^{1}H spectra of 20 mM gramicidin S in DMSO-d_6. (A) Conventional spectrum. (B) Spectrum edited for valine H_N obtained using the pulse sequence given in Fig. 1A. (C) Spectrum edited for valine H_N obtained using the pulse sequence given in Fig. 1B. (D) 1D NOE spectrum of valine H_N obtained using the pulse sequence in Fig. 1A to select valine H_N. (E) 1D NOE spectrum of valine H_N obtained using the pulse sequence in Fig. 1D. In all cases k = valine Hα and l = valine H_N. In all cases all soft pulses were 20 ms gaussian waveforms, $\delta = \delta' = 22.4$ ms, $\tau = 32.4$ ms, $t_m = 97.2$ ms. The modulated soft pulses were cosinusoidally modulated at 562 Hz. Magnetic field gradient pulses were applied for 1 ms. Gradient amplitudes (100% = 50 G cm^{-1}) where applicable: g1 = 61%, g2 = 26%, g3 = 33%, g4 = 71%, g5 = 45%, g6 = 86%, g8 = 5%, g9 = 13%, g10 = 17%.

magnetization of both amide and α-protons at the end of the experiment. The corresponding spectrum obtained using the in-phase filtered editing sequence as a basis, Fig. 2E, does not suffer from this problem.

A further demonstration using dehydrotestosterone is given in Fig. 3 where k corresponds to 6α and 6β and l, the spin edited for, is 7α. Both 6 multiplets were selected because their close proximity in the spectrum made it impractical to select just one of them at the field strength used in this study. Omitting relaxation effects, this will modify the observed signal intensity to: $\{\sin^2(\pi J_{6\alpha 7\alpha}\tau) + \sin^2(\pi J_{6\beta 7\alpha}\tau)\}\cos(\pi J_{6\alpha 6\beta}\tau)$.

The multiplet of 7α while unresolved in the conventional proton spectrum, Fig. 3A, is clearly resolved in the edited spectra, Fig. 3B–3C. It is apparent from the appearance of the 7α multiplet in Fig. 3B obtained with the basic editing sequence, Fig. 1A, that the observed signal contains a mixture of in-phase and anti-phase components. However the spectrum obtained with the IPECS editing sequence, Fig. 3C, is clearly in-phase; all parameters common to the two experiments had the same value in each case. It should be noted that both edited spectra are free of any artefacts above noise level which is a prerequisite if an editing procedure is to be incorporated into experiments designed to observe relatively small perturbations of the magnetization such as long range NOEs.

Since two coupled spins, 6α and 6β, are selected by pulses denoted as perturbing k in Fig. 1, some components of multiplet-quantum coherence such as $I^+_{6\alpha}I^-_{6\beta}I^-_{7\alpha}$ may be present at the end of the basic editing sequence. While these cannot be directly observed, they may result in observable signals if the editing sequence is used in conjunction with other techniques such as 1D NOE measurements. The IPECS editing experiment removes these multiple-quantum coherences, so this problem does not arise. The 1D TOCSY/HOHAHA version of the experiment, Fig. 1C, is demonstrated for 7α in Fig. 3D and produces a largely in-phase spectrum, as expected.

The number of soft pulses and scalar coupling evolution periods incorporated into the two editing sequences dictate their minimum effective lengths. Typically this will be in the region of 150–200 ms. While for molecules in the fast tum-

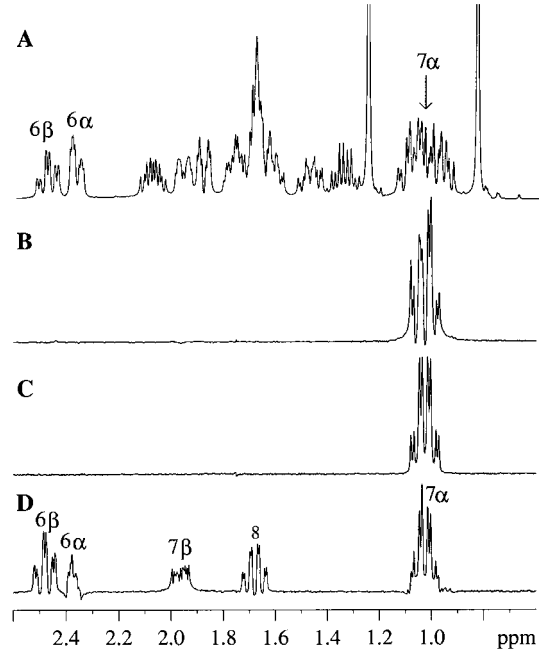

FIG. 3. 400 MHz ^{1}H spectra of 0.1 M dehydrotestosterone in $CDCl_3$. (A) Conventional spectrum. (B) Spectrum edited for 7α obtained using the pulse sequence given in Fig. 1A. (C) Spectrum edited for 7α obtained using the pulse sequence given in Fig. 1B. (D) 1D TOCSY spectrum obtained using the pulse sequence given in Fig. 1C. In all cases k = 6α and 6β, and l = 7α. In all cases all soft pulses were 20 ms gaussian waveforms, $\delta = \delta' = 22.4$ ms, $\tau = 28.4$ ms, the MLEV-17 mixing sequence was applied for 20 ms and was sandwiched between 2.5 ms trim pulses. The modulated soft pulses were cosinusoidally modulated at 292 Hz. Gradient values were the same as for Fig. 3 except g7 = 6%, which was applied for the length of the trim pulses (2.5 ms). All experiments were performed on a Bruker DRX-400 operating at 400 MHz for ^{1}H.

bling regime relaxation attenuation during this period may not be expected to be too severe, for molecules in the slow tumbling regime signal attenuation due to relaxation is likely to preclude its use. For 7α of dehydrotestosterone signal intensity of the edited spectrum is low at approximately 10% of the normal 1D spectrum. However, it should be taken into account that coherence was transferred to 7α from both 6α and 6β, resulting in a dependence of the observed signal intensity upon three scalar couplings, as noted above, instead of the more usual one. Consequently much of the signal lost will be due to the inability to simultaneously optimise all three terms in the intensity expression. A more normal case involving transfer from H17 to H16α (results not given), which is resolved in the conventional 1D spectrum, gave an efficiency of 49% for the optimum τ-value indicating that approximately half of the signal has decayed due to relaxation. Gramicidin S is in the slow tumbling regime and is probably towards the upper limit, in terms of correlation time, of molecules that can usefully be edited with this technique; after passing through the IPECS editing experiment signal intensity was reduced to approximately 10% of its initial value.

In conclusion, the pulse sequence presented in Fig. 1B enables the pure in-phase multiplets of overlapping peaks to be resolved. The technique has been successfully combined with 1D NOESY and TOCSY measurements. The length of the editing sequence is likely to restrict its use to molecules in the fast tumbling regime.

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