

## Robust refocusing of $^{13}\text{C}$ magnetization in multidimensional NMR experiments by adiabatic fast passage pulses

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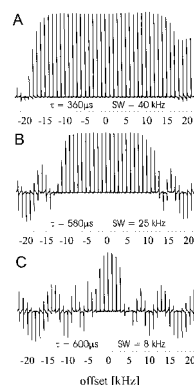
**Key words:** adiabatic fast passage pulses,  $^{13}\text{C}$ - $^1\text{H}$  HSQC, CBCA(CO)NH, refocusing

### Abstract

We show that adiabatic fast passage (AFP) pulses are robust refocusing elements of transverse  $^{13}\text{C}$  magnetization in multidimensional NMR experiments. A pair of identical AFP pulses can refocus selected parts or a complete  $^{13}\text{C}$  chemical shift range in  $^{13}\text{C}$  spectra. In the constant time  $^{13}\text{C}$ - $^1\text{H}$  HSQC, replacement of attenuated rectangular pulses by selective AFP pulses results in a sensitivity enhancement of up to a factor of 1.8. In the 3D CBCA(CO)NH the signal-to-noise ratio is increased by a factor of up to 1.6.

Rotations by  $180^\circ$  belong to one of the most basic manipulations of spin systems in NMR experiments (Abragam, 1961). Adiabatic fast passage (AFP) pulses (Abragam, 1961; Baum et al., 1983) are especially suited for these rotations because they provide basically unlimited bandwidth and accurate flip angles with high tolerance to spatial variations in RF field intensity (Baum et al., 1983; Hioe, 1984). A drawback of a single AFP pulse is that a spectral phase roll is observed as a function of frequency offset after an AFP pulse is used for refocusing (Uğurbil et al., 1988). High quality elimination of this phase roll can be achieved, however, with the even-numbered spin-echoes using a pair of identical AFP pulses (Levitt and Freeman, 1981; Conolly et al., 1991; Zweckstetter and Holak, 1998). Especially useful are AFP pulses when used for selective refocusing or in NMR experiments involving manipulations of  $^{13}\text{C}$  spins with their broad chemical shift range. Here, we describe multidimensional NMR experiments, including a fully  $^{13}\text{C}$ -refocused 2D  $^{13}\text{C}$ - $^1\text{H}$  HSQC, 2D  $^{13}\text{C}$ - $^1\text{H}$  CT-(constant time) HSQC, 3D HCCH-TOCSY and 3D CBCA(CO)NH, in which both broadband and selective refocusing of transverse  $^{13}\text{C}$  magnetization is achieved by a pair of identical AFP pulses.

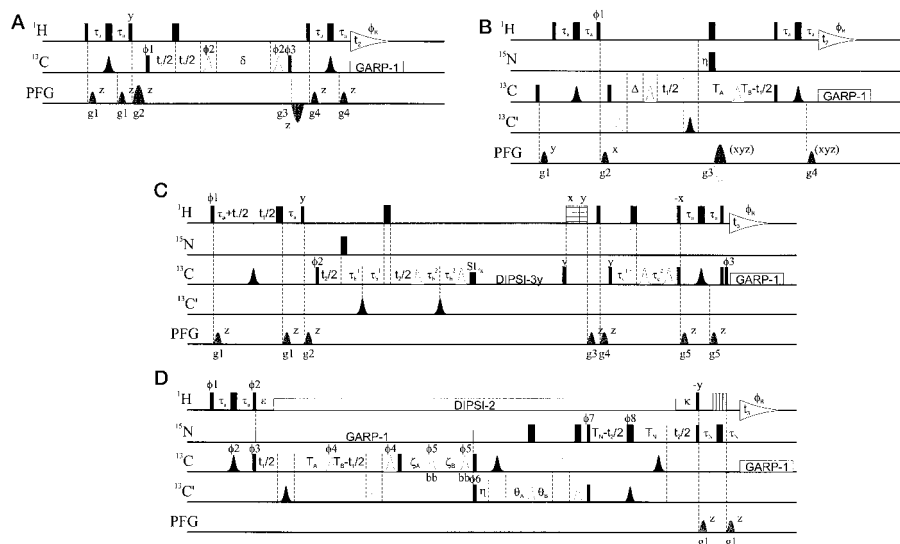
NMR experiments were performed on a Bruker DRX-600 spectrometer equipped with a triple-resonance ( $^1\text{H}/^{15}\text{N}/^{13}\text{C}$ ) three-axes gradient probe



**Figure 1.** Symmetric refocusing profiles of pairs of identical HS pulses (duration  $\tau$ , sweep width SW,  $\gamma H_1^{\text{max}} = 30$  kHz, 1% cut-off level). (A)  $^{13}\text{C}$  broadband. (B) non- $^{13}\text{C}$  selective. (C)  $^{13}\text{C}$  selective. A double spin-echo sequence with two surrounding gradients of opposite sign was employed.

head (TXI X/Y/Z-grad) operated at 300 K. For measurement of refocusing profiles a sample of 600  $\mu\text{l}$  of paramagnetically doped 90%  $\text{CH}_3\text{OH}/10\%$   $\text{D}_2\text{O}$  was utilized. The pulse schemes were applied to a sample containing 1.7 mM of uniformly  $^{13}\text{C}/^{15}\text{N}$ -labelled segment 4 of the rod domain of the gelation factor from *D. discoideum* dissolved in 90%  $\text{H}_2\text{O}/10\%$   $\text{D}_2\text{O}$  at pH 7 (Fucini et al., 1997).

The quality of refocusing achievable with pairs of identical hyperbolic-secant (HS) pulses is demon-

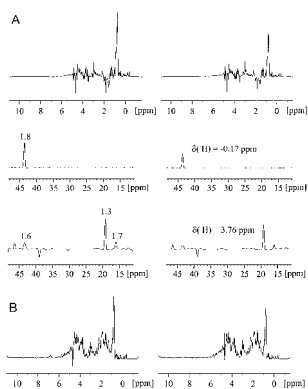


**Figure 2.** Pulse schemes of AFP-enhanced fully  $^{13}\text{C}$ -refocused  $^{13}\text{C}$ - $^1\text{H}$  HSQC (A),  $^{13}\text{C}$ - $^1\text{H}$  CT-HSQC employing coherence transfer pathway selection (B), 3D HCCH-TOCSY (C) and 3D CBCA(CO)NH (D). Narrow and wide rectangles indicate hard  $90^\circ$  and  $180^\circ$  pulses, respectively, with phase x, unless otherwise indicated.  $^{13}\text{C}$ -broadband, non- $^{13}\text{C}'$ -selective and  $^{13}\text{C}'$ -selective refocusing/inversion is achieved with the HS pulses (bell-shaped symbols) of Figures 1A, 1B and 1C (carriers at 106 ppm, 70 ppm and 176 ppm, respectively). Pairs of AFP pulses used for refocusing are shown in light grey. Quadrature detection in all indirect dimensions (except for the pulse scheme B) is obtained by STATES-TPPI (Marion et al., 1989) of  $\phi_1$  (A), of  $\phi_1$  and  $\phi_2$  (C) and of  $\phi_3$  and  $\phi_7$  (D). Delay durations are  $\tau_a = 1.6$  ms and (A)  $\delta = \text{pw}(\text{H} - 180) + t_1(\text{initial}) + 4/\pi\text{pw}(\text{C} - 90)$ ; (B)  $T_A = T - \text{pw}(\text{C}' - 180) - \text{pw}(\text{C} - 180) - t_1/2(\text{initial})$  and  $T_B = T - \text{pw}(\text{C}' - 180) - \text{pw}(\text{C} - 180) - 4/\pi\text{pw}(\text{C} - 90) - \Delta$ , where  $T = 29$  ms,  $\Delta = t_1/2(\text{initial})$  and  $\eta = 2/\pi\text{pw}(\text{C} - 90)$ ; (C)  $\tau_b^1 = \tau_b - 1/\pi\text{pw}(\text{C} - 90) - 0.5\text{pw}(\text{H} - 90)$ ,  $\tau_b^2 = \tau_b + 0.5\text{pw}(\text{H} - 90)$ ,  $\tau_c^1 = \tau_c - \text{pw}(\text{H} - 90) - 2/\pi\text{pw}(\text{C} - 90)$ ,  $\tau_c^2 = \tau_c + \text{pw}(\text{H} - 90) + 2/\pi\text{pw}(\text{C} - 90)$ , where  $2\tau_b = 0.95$  ms and  $\tau_c = 1.1$  ms; (D)  $\epsilon = 2.3$  ms,  $T_A = T - \text{pw}(\text{C}' - 180) - \text{pw}(\text{C} - 180) - 4/\pi\text{pw}(\text{C} - 90) - t_1/2(\text{initial})$  and  $T_B = T - \text{pw}(\text{C}' - 180) - \text{pw}(\text{C} - 180)$ , where  $T = 3.3$  ms,  $\zeta_A = \zeta - \text{pw}(\text{C} - 180 - \text{bb}) - 4/\pi\text{pw}(\text{C} - 90)$  and  $\zeta_B = \zeta - \text{pw}(\text{C} - 180 - \text{bb})$ , where  $\zeta = 3.7$  ms,  $\eta = 4.7$  ms,  $\theta_A = \theta - \eta - \text{pw}(\text{C}' - 180) - \text{pw}(\text{C} - 180) - 4/\pi\text{pw}(\text{C}' - 90)$  and  $\theta_B = \theta - \text{pw}(\text{C}' - 180) - \text{pw}(\text{C} - 180)$ , where  $\theta = 11.4$  ms,  $T_N = 11.2$  ms,  $\kappa = 5.4$  ms and  $\tau_N = 2.4$  ms.  $\text{pw}(\text{H}/\text{C} - 90/180)$  and  $\text{pw}(\text{C}' - 180)$  are the durations of the  $90^\circ/180^\circ$   $^1\text{H}/^{13}\text{C}$  and the  $180^\circ$   $^{13}\text{C}'$  pulses, respectively ('bb' indicates a rotation of the complete  $^{13}\text{C}$  chemical shift range). Strength and duration of gradients (maximal strength of 50 G/cm) are (A)  $g_1 = (16\%, 0.5$  ms),  $g_2 = (50\%, 1$  ms),  $g_3 = (-60\%, 1$  ms),  $g_4 = (20\%, 0.5$  ms); (B)  $g_1 = (50\%, 4$  ms),  $g_2 = (50\%, 3.5$  ms),  $g_3 = (\pm 100.0\%, 2$  ms),  $g_4 = (50.4\%, 1$  ms); (C)  $g_1 = (16\%, 0.5$  ms),  $g_2 = (30\%, 2$  ms),  $g_3 = (75\%, 2$  ms),  $g_4 = (66\%, 2$  ms),  $g_5 = (50\%, 0.5$  ms); (D)  $g_1 = (50\%, 1$  ms). Phase cyclings are (A)  $\phi_1 = x, -x, \phi_2 = 2(x), 2(-x), \phi_3 = 4(x), 4(-x), \phi_R = x, 2(-x), x$ ; (B)  $\phi_1 = y, -y, \phi_R = x, -x$ ; (C)  $\phi_1 = x, -x, \phi_2 = 2(x), 2(-x), \phi_3 = 4(x), 4(-x), \phi_R = x, 2(-x), x$ ; (D)  $\phi_1 = y, \phi_2 = x, -x, \phi_3 = x, \phi_4 = 8(x), 8(y), 8(-x), 8(-y), \phi_5 = 4(x), 4(-x), \phi_6 = 2(x), 2(-x), \phi_7 = 4(x), 4(-x), \phi_8 = 8(x), 8(-x), \phi_R = x, 2(-x), x, -x, 2(x), -x$ . All  $^{13}\text{C}$  pulses are applied at high power. Exceptions are: (C) 2 ms  $\text{SL}_x$  pulse, DIPSI-3 ( $\pm y$ ) mixing scheme (Shaka et al., 1988) with the adjacent  $^{13}\text{C}$   $90^\circ$  pulse with RF field strengths of 8.2 kHz; (D)  $90^\circ$   $^{13}\text{C}$  and  $^{13}\text{C}'$  pulses are adjusted to yield zero excitation at the  $^{13}\text{C}'$  and  $^{13}\text{C}^\alpha$  position, respectively.  $^{13}\text{C}$  carrier positions are 43 ppm (C) and 46 ppm/177 ppm for  $90^\circ$   $^{13}\text{C}/^{13}\text{C}'$  pulses (D). The CBCA(CO)NH is a water flipback version.

strated in Figure 1. Within about 70% of the sweep width of the RF of the HS pulses, uniform refocusing is achieved with no phase errors remaining. No signal losses occur due to frequency offsets. Different bandwidths of refocusing or inversion were obtained by adjusting the duration and sweep width of the HS pulses. Broader chemical shift ranges or better selectivity can be achieved by variation of the sweep width of the RF of HS pulses or by optimizing the shapes of the AFP pulses (Rosenfeld et al., 1997; Hwang et al., 1998). At 600 MHz and for the current application this

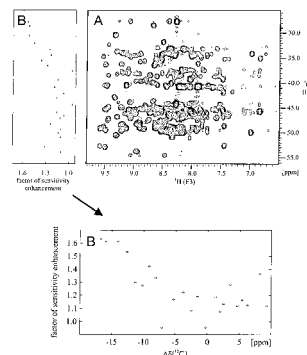
proved to be unnecessary making the implementation of AFP-based refocusing very easy.

Figure 2 shows pulse sequences of the fully  $^{13}\text{C}$ -refocused  $^{13}\text{C}$ - $^1\text{H}$  HSQC,  $^{13}\text{C}$ - $^1\text{H}$  CT-HSQC, 3D HCCH-TOCSY and 3D CBCA(CO)NH experiments in which refocusing of transverse  $^{13}\text{C}$  magnetization is achieved by HS pulses (Silver et al., 1984). The fully  $^{13}\text{C}$ -refocused  $^{13}\text{C}$ - $^1\text{H}$  HSQC experiment (Figure 2A) is similar to the one proposed by Ogura et al. (1996). Instead of a shaped, non-adiabatic V1 pulse (broadband refocusing pulse described in Table 2.C of Abramovich and Vega, 1993), however, a pair of



**Figure 3.** Comparison of  $^{13}\text{C}$ - $^1\text{H}$  CT-HSQC (A) and HCCH-TOCSY (B; mixing time of 19 ms) (pulse schemes of Figure 2) employing pairs of identical HS pulses (left column) or single rectangular pulses (right column; attenuated with the carrier at 37 ppm (A) or high power (B)) for refocusing.  $^{13}\text{C}$  carrier positions are 29.5 ppm (A) and 43 ppm (B). First transients and  $^1\text{H}$ - $\text{F}_1$  cross sections (A) are shown ( $^{13}\text{C}$ - $^1\text{H}$  CT-HSQC: acquired data size  $256^* \times 1024^*$ ; spectral widths of 5640.79 and 7507.5 Hz in  $\omega_1(^{13}\text{C})$  and  $\omega_2(^1\text{H})$ , respectively; zero-filled to final matrix size of  $512^* \times 1024^*$ ).

AFP pulses is used for broadband refocusing of  $^{13}\text{C}$  magnetization. This has the advantage of high insensitivity against a wide range of experimental errors (Baum et al., 1983; Hioe, 1984). Additionally, since the V1 pulse is shaped but not adiabatic it requires high power which causes stability problems when the linear range of the amplifier is exceeded. Another alternative, composite pulses (Levitt and Freeman, 1981), are also inferior to AFP pulses for broadband refocusing or inversion. Even very elaborate composite pulses have limited bandwidths and are far more sensitive to RF field inhomogeneity (Uğurbil et al., 1988). The AFP-enhanced  $^{13}\text{C}$ - $^1\text{H}$  CT-HSQC of Figure 2B is similar to the one recently proposed by Ottinger et al. (1998) for measuring residual dipolar couplings. To achieve a more reliable sequence, however, the  $^{13}\text{C}'$  magnetization is inverted adiabatically and the inversion is compensated for Bloch–Siegert shifts (Freeman, 1987). In contrast to the  $^{13}\text{C}$ - $^1\text{H}$  CT-HSQC proposed by Hallenga and Lippens (1995), the AFP-enhanced  $^{13}\text{C}$ - $^1\text{H}$  HSQC experiments of Figure 2 do not require any higher-order phase correction along the  $^{13}\text{C}$  axis. This makes the incorporation of AFP-based refocusing elements into multidimensional experiments like, for example, 3D HCCH-TOCSY (Kay et al., 1993) or 3D CBCA(CO)NH (Grzesiek and Bax, 1992) feasible (Figures 2C and 2D). The introduction of AFP pulses for refocusing into CT-elements does not change the duration of these elements. Accordingly, longer, more



**Figure 4.** First  $^1\text{H}(\text{F}_3)$ - $^{13}\text{C}(\text{F}_1)$  plane (A) and sensitivity enhancement with respect to  $^{13}\text{C}$ -offset  $\Delta\delta(^{13}\text{C})$  (B) of the AFP-enhanced 3D CBCA(CO)NH spectrum (pulse scheme of Figure 2D). (A) No baseline correction and no phase correction along  $\text{F}_1$  was applied (acquired data size  $42^*(\text{t}_1) \times 1024^*(\text{t}_3)$ ; spectral widths of 5713.1 and 7507.5 Hz in  $\omega_1(^{13}\text{C})$  and  $\omega_3(^1\text{H})$ , respectively; zero-filled to final matrix size of  $256^*(\text{t}_1) \times 1024^*(\text{t}_3)$ ). (B) The gain in signal-to-noise ratio is given with respect to a 3D CBCA(CO)NH experiment in which all three AFP-based refocusing elements were replaced by attenuated square pulses ( $180^\circ$   $^{13}\text{C}^{\alpha/\beta}$ : carrier at 46 ppm,  $\gamma\text{H}_1 = 11.4$  kHz;  $180^\circ$   $^{13}\text{C}'$ : carrier at 177 ppm,  $\gamma\text{H}_1 = 10.5$  kHz; additional  $180^\circ$   $^{13}\text{C}'$  inversion pulse in the middle of  $\zeta$ : sinc shape, duration of 202  $\mu\text{s}$ ). The factor of sensitivity enhancement with respect to frequency offset from the carrier of the  $180^\circ$   $^{13}\text{C}^{\alpha/\beta}$  refocusing pulses was determined by summing the absolute value of signal intensity along  $\text{F}_2$  for both the AFP-enhanced CBCA(CO)NH and the non-AFP-enhanced spectrum. Data points shown are amplitude ratios for the most prominent peaks of these projections.

adiabatic AFP pulses may be used; however, at the expense of a reduced number of possible increments for frequency discrimination in the  $^{13}\text{C}$  dimension. In non-CT-elements, on the other hand, the total duration is slightly increased upon incorporation of AFP pulses due to the requirement for two AFP pulses and the longer duration of the AFP pulses. The second drawback, however, can be relaxed by employing AFP pulses optimized for fast inversion (Hwang et al., 1998).

The AFP-enhanced pulse sequences were compared to corresponding square-pulse refocused experiments (Figures 3 and 4). For the AFP-enhanced fully  $^{13}\text{C}$ -refocused  $^{13}\text{C}$ - $^1\text{H}$  HSQC the sensitivity is now similar to that of the non-refocused  $^{13}\text{C}$ - $^1\text{H}$  HSQC experiment with a signal enhancement of 15% and 20% for the methyl and aromatic resonances, respectively (data not shown). In contrast to the non-refocused  $^{13}\text{C}$ - $^1\text{H}$  HSQC experiment, however, all signals have the same phase irrespective of the degree of folding. Similar or even more pronounced improvements are obtained when the AFP-enhanced

fully  $^{13}\text{C}$ -refocused  $^{13}\text{C}$ - $^1\text{H}$  HSQC is incorporated into three- and four-dimensional  $^{13}\text{C}$  NOESY-HSQC experiments (Muhandiram et al., 1993; Vuister et al., 1993; Baur et al., 1998). Another potential application for broadband AFP-based refocusing are HCC-type experiments, for example the three-dimensional HCCH-TOCSY. In Figure 3B first transients of the AFP-enhanced and the high power square-pulse refocused HCCH-TOCSY are compared. Sensitivities of these two experiments are comparable for the entire spectral width with the AFP-enhanced version giving a significant improvement for the aromatic resonances and a 10% to 20% higher signal-to-noise ratio in the methyl region. More spectacular improvements were observed for CT-experiments with selective refocusing pulses. This is demonstrated in Figures 3A and 4. In the  $^{13}\text{C}$ - $^1\text{H}$  CT-HSQC experiment the signal-to-noise ratio of resonances in the aliphatic region is enhanced by up to a factor of 1.8, and in the aromatic region, only the AFP-enhanced version results in undisturbed correlation peaks. The signal-to-noise ratio of signals with different  $^{13}\text{C}$  chemical shifts does no longer depend on the specific value of the excitation frequency of the refocusing pulse. Some improvement with respect to sensitivity can also be achieved by employing high power, non-selective square pulses for refocusing of  $^{13}\text{C}$  spins. However, high power square pulses still have only a limited bandwidth, and due to their non-selectivity, artefacts are caused for nuclei that are coupled to  $^{13}\text{C}'$  spins (Vuister and Bax, 1992). A similar enhancement as in the  $^{13}\text{C}$ - $^1\text{H}$  CT-HSQC is expected for the  $^1\text{H}$ -detected long range  $^{13}\text{C}$ - $^{13}\text{C}$  correlation experiment (Bax et al., 1992).

The sensitivity gain which can be achieved by AFP-based refocusing in experiments with several selective  $^{13}\text{C}$  refocusing elements is highlighted in Figure 4 for 3D CBCA(CO)NH. A high quality spectrum with no phase roll and a very flat baseline in the  $^{13}\text{C}$  dimension is obtained (Figure 4A). As no attenuation of off-resonance signals is introduced, a factor of up to 1.6 in signal-to-noise ratio is gained for the  $\text{C}'$  resonances. The square-pulse refocused CBCA(CO)NH has the same sensitivity as the AFP-enhanced version only for signals that are on-resonance. The high number of pulses, especially of selective  $180^\circ$  pulses, does no longer limit the efficiency of the CBCA(CO)NH experiment.

In conclusion, efficient refocusing of transverse  $^{13}\text{C}$  magnetization in various multidimensional NMR experiments by AFP pulses was demonstrated. Performing all  $180^\circ$  rotations of  $^{13}\text{C}$  magnetization by

AFP pulses allows NMR experiments which achieve highest sensitivity, despite off-resonance effects, pulse miscalibration or inhomogeneity of the static magnetic field or the RF field intensity. Since the bandwidth of AFP pulses is basically unlimited, the increased chemical shift ranges at even very high magnetic field strengths do not result in an increased offset-dependent sensitivity degradation.

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