Ultrashort Broadband Cooperative Pulses for Multidimensional Biomolecular NMR Experiments

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Abstract: NMR spectroscopy at ultra-high magnetic fields requires improved radiofrequency (*rf*) pulses to cover the increased spectral bandwidth. We introduce optimized 90° pulse pairs as Ramsey-type cooperative (Ram-COOP) pulses for biomolecular NMR applications. The Ram-COOP element provides broadband excitation with enhanced sensitivity and reduced artifacts even at magnetic fields >1.0 GHz ^{1}H Larmor frequency (23 T). A pair of 30 μs Ram-COOP pulses achieves an excitation bandwidth of 100 kHz with a maximum *rf* field of 20 kHz, more than three-fold improved compared to achievable excitation by rectangular pulses. Ram-COOP pulses exhibit little offset-dependent phase errors and are robust to *rf* inhomogeneity. The performance of the Ram-COOP element is experimentally confirmed with heteronuclear multidimensional NMR experiments, applied to proteins and nucleic acids. Ram-COOP provides broadband excitation at low *rf* field strength suitable for application at current magnetic fields and beyond 23 T.

The continuous development of high-field NMR spectrometers greatly enhances the sensitivity and resolution of NMR experiments and enables application to ever more challenging and complex biomolecular systems.[1] Next to technological hurdles of designing ultrahigh-field NMR magnets, spectroscopic challenges inevitably emerge. One critical aspect is the requirement for *rf* pulses with large bandwidths for excitation, refocusing, inversion and decoupling, while being constrained by a technical upper limit of applicable *rf* power. We and others have recently introduced novel heteronuclear decoupling pulses, that were developed using optimal control theory, and enable very broadband decoupling, as required at ultrahigh magnetic fields, at low *rf* power.[2]

The rectangular pulse, which is the most frequently used pulse shape in NMR spectroscopy, has limited off-resonance performance with respect to amplitude and phase of the excited magnetization. In particular, currently available pulses cannot cover the excitation bandwidths required for large heteronuclear chemical shift ranges at large magnetic fields or in paramagnetic proteins.[3] Shaped and composite pulses offer significant improvements over rectangular pulses in terms of bandwidth and B_1 robustness, however, at the cost of considerably longer pulse durations.[3-4] Based on optimal control theory, the limits of individually optimized broadband pulses were explored.[5]

The concurrent optimization of pulses that act in a cooperative way makes it possible to further improve the overall performance of pulse sequences.[6] Here, we focus on so-called s^2-COOP pulses[6b] that compensate each other's imperfections in a single scan based on the concept of global pulse sequence compensation.[7] The additional degrees of freedom for optimization results in highly efficient, short pulses. In the following, we denote these pairs of excitation ($S^{(1)}$) and flip-back pulses ($S^{(2)}$) with mutually matched phase properties for Ramsey-type sequence elements as Ram-COOP pulses. The Ramsey-type sequence can be described schematically as $S^{(1)} - \tau - S^{(2)}$. Starting from longitudinal magnetization the Ram-COOP element creates an offset-dependent modulation of the longitudinal magnetization component of the form $S_z \cos 2\pi\nu\tau$, where ν is the offset of the spin and τ is the effective evolution time between the excitation and the flip-back pulse. The building block can also be applied to coupled spins and can be used as a frequency-labeling element for indirect evolution periods of many multi-dimensional experiments.

Here we focus on the following questions: (a) How can Ram-COOP pulses be best implemented in biomolecular NMR experiments? (b) Can the superior performance over conventional experiments be utilized at current and future available magnetic fields?

Following our recently reported protocol,[6b] Ram-COOP pulse pairs were optimized for 70 and 100 kHz excitation bandwidth and a maximum *rf* field of 10 and 20 kHz, respectively. The amplitude and phase as a function of pulse duration are shown in Figure S2A,B. These pulses are in the following referred to as Ram-COOP$_{10\ kHz}$ and Ram-COOP$_{20\ kHz}$. The pulse length for each of the Ram-COOP$_{10\ kHz}$ (Ram-COOP$_{20\ kHz}$) pulses was 75 μs (30 μs). The performance of rectangular versus Ram-COOP pulses was first compared by numerical simulations (Figure 1A,B) by analyzing the signal amplitudes $A(\nu)$ and phase errors $\Delta\phi(\nu)$ as a function of the resonance offset (SI Equation (1,2)). At an *rf* field of 10 kHz the Ram-COOP$_{10\ kHz}$ pulse yields a signal amplitude of about 100% over the full target bandwidth of 70 kHz, while the rectangular pulse drops to 2.3% towards the maximal offset (solid lines, Figure 1A). Increasing the *rf* field to 20 kHz improves the performance of the rectangular pulse to 36.3%, while the corresponding Ram-COOP$_{20\ kHz}$ pulse still yields 100%

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performance over the target bandwidth considered here of 100 kHz and beyond, up to ~120 kHz (Figure 1B).

Note, that covering large bandwidths is valuable for concurrent detection of all proton-attached aromatic and aliphatic resonances in proteins. Ideally, a ^{13}C bandwidth of ~132 ppm (~40 kHz at a ^{1}H Larmor frequency of 1.2 GHz) needs to be covered for diamagnetic proteins, when considering two standard deviations of the ^{13}C chemical shift (Figure S9-10). Considering also paramagnetic proteins, increases the required bandwidth to ~143 ppm (~43 kHz). However, for particular paramagnetic centers, this value can be significantly exceeded by several tens of ppm, owing to large contact and pseudocontact shifts (Figure S11).[8] This indicates, that excitation pulses that provide an excitation window far beyond the typical ~143 ppm will be useful for such extreme band widths. The required bandwidths for excitation of aromatic and aliphatic protein signals are depicted in Figure 1A,B by dashed vertical lines in orange (950 MHz, ±17 kHz) and cyan (1.2 GHz, ±21.6 kHz), respectively.

We also compared the expected phase error $\Delta\phi(\nu)$ (SI Equation (2)), plotted in the lower panel of Figure 1A,B (solid lines). Nonlinear contributions cannot be easily corrected by phase correction. To evaluate the phase performance we plotted $\Delta\phi(\nu)$ for both pulses employed in the $S^{(1)} - \tau - S^{(2)}$ element, which represents the sum of all nonlinear phase terms. For rectangular pulses at 10 and 20 kHz, the absolute phase errors evolve up to 72.3° and 40.3°, respectively, while phase errors for Ram-COOP$_{10\,kHz}$ and Ram-COOP$_{20\,kHz}$ pulses are within ±1°. All values are summarized in Table S1.

Pulse miscalibration and *rf* inhomogeneity are among the major obstacles to high spectral quality. Here, the robustness of pulses was probed by missetting the *rf* amplitude by ±5%. This is indicated in Figure 1A,B by dashed and dot-dashed lines. For rectangular pulses at 10 and 20 kHz the missetting affects the amplitude performance additionally up to about ±2.2% and contributes ±2.1° to the phase error, while for the Ram-COOP pulse at 20 kHz (10 kHz) the amplitude is impaired by a magnitude of up to -0.7% (-0.9%) and the absolute phase by 2.5° (3.9°), respectively (Table S1). We conclude that both rectangular and Ram-COOP pulses show similar contribution to the phase error upon a B_1 offset, whereas the Ram-COOP pulses display higher B_1 robustness in terms of amplitude performance compared to the corresponding rectangular pulses. Furthermore, the small B_1-dependent phase errors in individual Ram-COOP pulses cancel approximately for a realistic inhomogeneity distribution of the B_1 field, which however is not the case for rectangular pulses.

To benchmark the Ram-COOP pulses with currently available pulse elements we compared the performance with “plane rotation” and “adiabatic half-passage” pulses (AHP),[4c, 9] namely to BIR-4[4d] and CHIRP[10], which are commonly used in NMR and MRI applications. The total excitation performance is compared for the different excitation elements as shown in Figure 1C. Clearly, Ram-COOP pulses show superior performance both in terms of excitation and phase errors. A detailed analysis is given in the Supporting Information. We also note that the ultra-broadband excitiation of Ram-COOP pulses offers unique advantages for optimal folding and aliasing of resonances in multi-dimensional NMR spectroscopy (see Supporting Information).

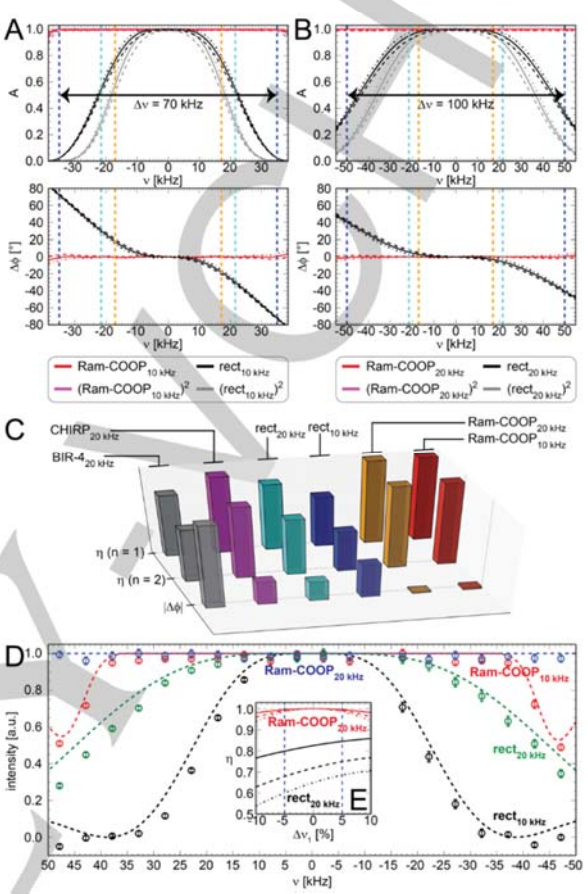

Figure 1. Simulations and experimental verification of Ram-COOP broadband excitation pulses. Numerical simulations for signal amplitude $A(\nu)$ and phase errors $\Delta\phi(\nu)$ (cf. SI Equation (1,2)) are shown for Ram-COOP (red) and rectangular pulses (black) at a maximum *rf* field of (A) 10 kHz and (B) 20 kHz, respectively. Solid, dashed, dot-dashed lines refer to the simulation with ν_1, $0.95 \times \nu_1$, $1.05 \times \nu_1$ (ν_1 = 10 kHz, 20 kHz), considering no or ±5% B_1 inhomogeneity. The vertical line (blue, dashed) depicts the target excitation bandwidth for the generation of the respective Ram-COOP pulse. The required ^{13}C bandwidth for excitation of aromatic and aliphatic resonances is 143 ppm (Figure S9-10). The dashed vertical lines in orange and cyan denote this limit at a ^{1}H Larmor frequency of 950 MHz ($\Delta\nu$ = ±17 kHz) and 1.2 GHz ($\Delta\nu$ = ±21.6 kHz), respectively. Simulations, when applying the $S^{(1)} - \tau - S^{(2)}$ pulse element a second time as required in multidimensional experiments, are shown in magenta and gray for the Ram-COOP and the rectangular pulse, respectively. (C) Comparison of the total excitation performance η (with n = 1,2 occurrences of the $S^{(1)} - \tau - S^{(2)}$ pulse element; η is defined in the footnote of Table S1) and the maximum absolute phase error $|\Delta\phi|$ for BIR-4, CHIRP, rectangular and Ram-COOP pulses. Pulse parameters correspond to the first sub-row of Table S1 for each shape. (D) Experimental excitation profiles were determined by offset-dependent $^{1}H,^{13}C$ HMQC experiments (see Supporting Information). Experimental and simulated data are shown as circles and dashed lines, respectively. (E) The performance of the Ram-COOP$_{20\,kHz}$ and rect$_{20\,kHz}$ pulse are compared as a function of the B_1 inhomogeneity $\Delta\nu_1$ at a target bandwidth of 100 kHz. The solid, dashed, dot-dashed lines refer to the simulations, when applying the pulse element one, two or three times, respectively.

The performance of Ram-COOP pulses was experimentally verified with $^{1}H,^{13}C$ HMQC experiments, as shown in Figure 1D. We recorded these spectra at varying ^{13}C transmitter frequencies to cover approximately ±50 kHz. The aromatic spectral region was integrated as a function of the offset. The simulations agree well with experiment. Note, that a small deviation from the experimental points observed for rectangular pulses reflects the significant phase errors and the smaller B_1 robustness of rectangular pulses compared to Ram-COOP pulses. Sensitivity losses due to limited excitation bandwidth and *rf* inhomogeneities are a major concern in higher dimensional experiments, where the excitation and flip-back scheme is applied multiple times. As shown in Figure 1E, the Ram-COOP pulses are largely unaffected by missetting the *rf* field and applying multiple evolution periods, while rectangular pulses are significantly impaired by both. This is further discussed in the Supporting Information.

We next implemented the pulses into a set of standard as well as advanced biomolecular NMR experiments to demonstrate the benefit of broadband excitation with Ram-COOP pulses in $^{1}H,^{13}C$ HMQC, ^{13}C-edited $^{1}H,^{1}H$ NOESY-HMQC and HMQC-NOESY-HMQC, and ^{13}C-detected $^{13}C,^{13}C$ NOESY experiments. The corresponding pulse sequences are shown in Figure S1.

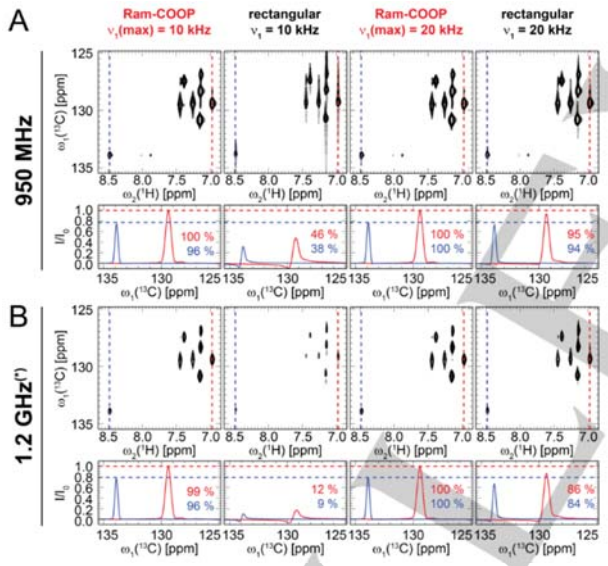

Figure 2. Experimental comparison of rectangular and Ram-COOP pulses in $^{1}H,^{13}C$ HMQC spectra, using the pulse sequence in Figure S1A. All experiments were performed at a ^{1}H Larmor frequency of 950 MHz (22.3 T). The external magnetic field labeled with an asterisk (*) was simulated by adjustment of the transmitter frequency (Figure S5E). The spectra were normalized by the global maximum at each external magnetic field and plotted at the same contour levels. The dashed vertical lines depict the position of the 1D slice in the $\omega_1(^{13}C)$ dimension, shown below each 2D correlation plot.

Figure 2 shows experimental 2D $^{1}H,^{13}C$ HMQC spectra with Ram-COOP or rectangular pulses at an *rf* field of 10 and 20 kHz, respectively. Following scenario (ii) introduced in the Supporting Information (Figure S5), the region displayed is centered on the aromatic signals, while the transmitter has been placed in the middle of the aliphatic resonances at 36 ppm. This enables detection of aromatic and aliphatic resonances in a single spectrum using optimized folding of ^{13}C resonance in the indirect dimension.

Already at 950 MHz (Figure 2A), the Ram-$COOP_{10\ kHz}$ pulses significantly outperform the $rect_{10\ kHz}$ pulses. The latter yield only about 40-50% excitation performance and show large phase distortions, as visible in the slice through the $\omega_1(^{13}C)$ dimension, plotted at the bottom of the figure. Ram-COOP shows perfect excitation and no phase errors. At 20 kHz the Ram-COOP and rectangular pulses show similar excitation performance. It should be noted, however, that small phase distortions are detectable for rectangular pulses, although an excitation of up to 95% was achieved. This was predicted by simulations (Figure 1B). At the same time, the corresponding Ram-COOP pulse creates signal with negligible phase error and about 100% amplitude.

At a simulated external magnetic field corresponding to 1.2 GHz proton Larmor frequency (Figure 2B) Ram-COOP pulses remain at 100% performance in excitation with zero phase error, while rectangular pulses at 10 and 20 kHz perform only up to 12% and 86%, respectively, and suffer from notable phase distortions.

Furthermore, we implemented Ram-COOP pulses into a 3D ^{13}C-edited $^{1}H,^{1}H$ NOESY-HMQC and a 2D $^{13}C,^{13}C$ NOESY pulse sequence (Figure S1B,D), respectively. The spectra comparing Ram-COOP to rectangular excitation at 950 MHz are shown in Figure S7. Clearly, Ram-COOP excitation enhances the sensitivity, while minimizing the phase distortions in both experiments, and thereby allowing for detection of even very weak cross-peaks.

To demonstrate the benefit of Ram-COOP in experimental setups with multiple ^{13}C evolution periods, we implemented Ram-COOP pulses into a 4D ^{13}C-edited $^{1}H,^{1}H$ HMQC-NOESY-HMQC experiment (Figure S1C). Experiments were applied to the A39V/N53P/V55L triple mutant of the Fyn SH3 domain[11] and a DNA representing a deoxyribozyme (*vide infra*) (Figure 3A). Notably, the gains in signal-to-noise are around 15-20%. Therefore, to achieve the sensitivity obtained with Ram-COOP, but using rectangular pulses at an *rf* field of 20 kHz, the experimental time increases by ~30-45%, which is significant, when considering limited sample lifetimes and solubility as well as material expenses.

In recent year, RNA and DNA molecules are emerging as key mediators of biological function and biomedical applications. NMR studies of isotope-labeled nucleic acids are challenging due to a large ^{13}C chemical shift range. In particular, DNA, containing the methyl-bearing nucleobase thymine, shows an inherently large spectral range. Here, we applied the Ram-COOP pulses to a recently established class of self-hydrolyzing deoxyribozymes, named I-R3.[12] The $^{1}H,^{13}C$ correlation spectrum in Figure S8 illustrates that an excitation bandwidth of ~144 ppm is required for covering resonances from adenine C2 to thymine C7. The bandwidth is almost the same as for proteins (Table S1), therefore we expect very similar gains when employing Ram-COOP instead of rectangular pulses.

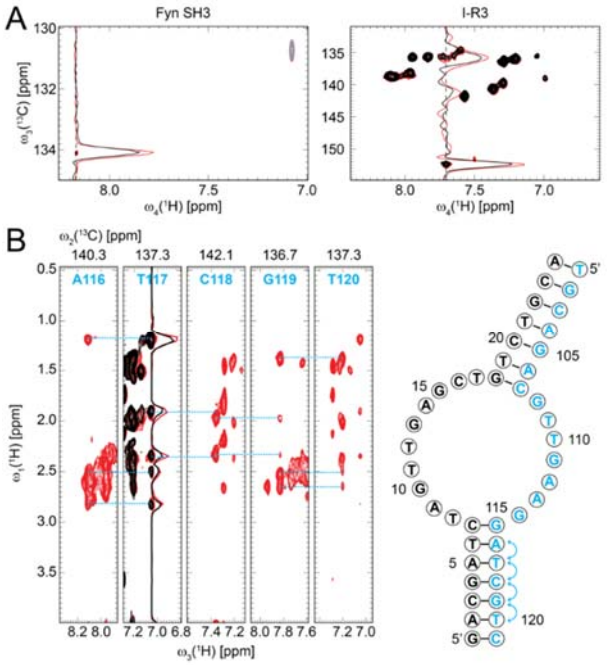

Figure 3. (A) Application of Ram-COOP pulses in 4D spectroscopy. 2D (ω_3, ω_4) projections of a 4D ^{13}C-edited ^{1}H,^{1}H HMQC-NOESY-HMQC experiment (Figure S1C) are shown for (*left*) Fyn SH3 and (*right*) deoxyribozyme I-R3 recorded at 950 MHz. The sensitivity gain of Ram-COOP over rectangular pulses was about 15-20 %. (B) Ram-COOP pulses applied to the deoxyribozyme I-R3. Only one strand (colored cyan) was uniformly [^{13}C,^{15}N] labeled. NOE strips for H6/H8 protons from a 3D ^{13}C-edited ^{1}H,^{1}H NOESY-HMQC spectrum, recorded with non-uniform sampling using Ram-COOP$_{20\ kHz}$ are shown in red. For comparison, the H6 strip of T117 is shown for the experiment employing rectangular pulses at an *rf* field of 20 kHz (black contours). Sequential connectivities are indicated by dashed cyan lines.

Signal-to-noise in NOESY experiments used for structural analysis is critical as especially less intense NOE correlations can provide crucial long-range distance information. In particular, non-uniformly sampled spectra benefit from a high signal-to-noise ratio by reduced processing artifacts and the detection of weak NOE correlations.[13] The connection of five residues in the double-stranded region of the I-R3 DNA is shown in Figure 3B for ^{1}H,^{1}H strips taken from a non-uniformly sampled 3D ^{13}C-edited NOESY-HMQC spectrum, recorded at 950 MHz. We observe sensitivity gains of 5-10%, corresponding to a reduction in experimental time of ~10-20%. At higher fields, larger gains will be obtained due to the increased bandwidth required. The higher sensitivity enables the efficient detection of inter-residual cross peaks, and thus thereby greatly aids resonance assignment.

In conclusion, we present numerically optimized broadband Ram-COOP pulses, which allow the excitation and flip-back over a bandwidth of 70 to ~120 kHz, applying *rf* amplitudes on the order of only 10 to 20 kHz. In contrast, the respective rectangular pulses show significant phase distortions and fail to fully excite this target bandwidth. In this manner, the cooperative pulses presented here allow to significantly extend the excitation and flip-back of resonances beyond the bandwidth limit of rectangular pulses, imposed by hardware restrictions. The low-power Ram-COOP pulses with 10 kHz *rf* amplitude already allow uniform excitation over 70 kHz and will prove critical for probes with limited *rf* power, and for samples with high salt concentrations in cryoprobes, as an alternative to using tubes with smaller diameters.

For diamagnetic proteins, a ^{13}C excitation bandwidth of ≈200 ppm is required to excite all resonances ranging from carbonyls to methyls. Therefore, the bandwidth of the Ram-COOP pulses will easily suffice for the next generations of NMR magnets, even beyond an external magnetic field of 47 T (2 GHz ^{1}H Larmor frequency). Paramagnetic proteins typically already require larger bandwidths at state-of-the-art magnetic fields and can be more efficiently tackled by Ram-COOP pulses, even when taking account of strong relaxation effects during the Ramsey-sequence as found in paramagnetic systems (Figure S12A).[14] Here, we applied Ram-COOP pulses for excitation of ^{13}C resonances, we note, however, that these broadband pulses can clearly be applied to any other nucleus of interest, such as ^{1}H, ^{15}N or ^{19}F.[15]

The duration of the presented pulses is on the order of tens of microseconds, thus insignificant compared to relaxation times in small and also large biomolecules. Furthermore, Ram-COOP pulses can be easily extended up to excitation bandwidths of >150 kHz, although with a trade-off in B_1 robustness or pulse duration. Here, we considered Ram-COOP pulses with $R > 0$, allowing for an effective evolution time during the pulse duration, requiring back-prediction of the acquired signal. Note that it is also possible to optimize Ram-COOP pulses with $R = 0$, albeit at the cost of reduced pulse performance or longer pulse duration.[6b]

In basic experiments performed at state-of-the-art magnetic fields with a single evolution period, Ram-COOP performs slightly better than rectangular pulses. However, for complete excitation of the full range of ^{13}C resonances Ram-COOP outperforms rectangular pulses even at currently available magnetic fields. Moreover, multidimensional experiments comprising multiple evolution elements at currently available magnetic fields greatly benefit from cooperative pulses. We also demonstrate that Ram-COOP can be readily combined with non-uniform sampling utilizing synergetic effects of improved resolution and sensitivity, while reducing spectral artifacts.

Ram-COOP pulses are easily implemented by simply replacing the pair of rectangular with the pair of Ram-COOP pulses and considering the relative offset evolution period of 0.529 instead of $2/\pi = 0.64$. In principle, pulses with smaller values of *R* can be generated with little compromise of the excitation efficiency owing to a flat plateau around the maximum quality factor.[6b]

We note that COOP building blocks are also expected to greatly enhance the performance of other pulse sequence building blocks, such as the echo element. Further extensions of the COOP principle will be of great utility for many NMR applications at highest magnetic fields and for spins with inherently large chemical shift dispersions, e.g. ^{19}F in biomolecular and small molecule applications.[16]

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Keywords: High-field NMR Spectroscopy • Cooperative Broadband Excitation Pulses • Optimal Control Theory • Ramsey • 1.2 GHz • 28 T • 4D HMQC-NOESY-HMQC

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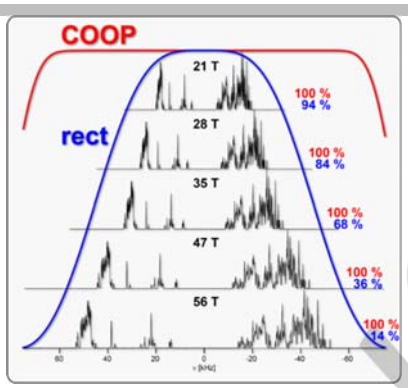
COMMUNICATION

The development of ultra-highfield NMR magnets requires robust radio frequency pulses to cover the increasing frequency spectral bandwidth. We present cooperative excitation pulses with pulse durations on the order of tens of microseconds, which provide ultrabroadband excitation for present and future magnetic field strengths well beyond 28 T (1.2 GHz ^{1}H). The performance is demonstrated for proteins and nucleic acids.



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