Solvent Suppression with Symmetrically-Shifted Pulses

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Abstract: Symmetrically-shifted shaped pulses are used to suppress large solvent resonances such as water while allowing the observation of protons exchanging with, or buried under, the water resonance. Symmetrically-shifted pulses form a family of pulses that have been optimized for non-excitation of the water resonance and can be seen as having historical precedence in, and many of the properties of, long rectangular pulses and binomial sequences. Linear prediction software coupled with data shifting is used to eliminate the undesirable frequency-dependent phase and baseline problems normally associated with pulses or excitation schemes of this length. Zero-frequency solvent subtraction is also described. Results are illustrated for DNA and protein samples in 90% water.

Introduction

A great number of solvent suppression schemes for pulsed FT NMR of biopolymers in H_2O solution have been proposed in the last 15 years.[1,2] Few are in widespread use today. Presaturation[3] is the oldest and simplest method, and for many situations, it is generally considered the preferred method. The major disadvantage of presaturation is that besides saturating the water protons it also saturates protons that are in chemical exchange with the water and/or protons whose chemical shifts are near that of the water. This is particularly detrimental for spectra of nucleic acids where much of the interesting chemical and structural information comes from the exchangeable imino protons. This paper will also show the extent to which presaturation can reduce the intensity, and therefore the signal-to-noise, of virtually every peak in the NOESY spectrum of proteins.[4]

A majority of the alternative water suppression techniques that have been developed[1,2] are aimed at suppressing water by achieving a net non-excitation of the water while at the same time exciting as much of the rest of the spectrum as possible. The oldest non-excitation water suppression method is the long pulse approach where the frequency is carefully adjusted so that the water frequency falls at the first null in the $\sin(x)/x$ excitation profile created by the long rectangular pulse.[5] This approach allows observation of resonances either upfield or downfield of the water, but not both. Hore has used Bloch equation simulations to propose and analyze a number of "binomial" solvent suppression schemes consisting of equally spaced hard rectangular radio frequency pulses of lengths corresponding to the ratio of binomial coefficients.[6] The simplest binomial sequence, 1-$\bar{1}$, uses a hard 45° pulse represented by the 1 followed by a 45° pulse with a 180° phase shift represented by the $\bar{1}$ to produce a reasonably broad excitation profile on both sides of the water resonance, a rather sharp null at the water frequency, and a ~360° frequency-dependent phase shift. 1-$\bar{2}$-1 produces a much broader null at the water at the expense of a somewhat narrower excitation maximum and larger (~720°) frequency-dependent phase shift across the excitation bandwidth. These frequency-dependent phase shifts cause rolling baselines.[7] A number of further optimized binomial-based sequences have been proposed using additional pulses and/or non-uniform pulse widths and interpulse spacings.[8-10] Other techniques have involved a combination of soft and hard pulses.[11]

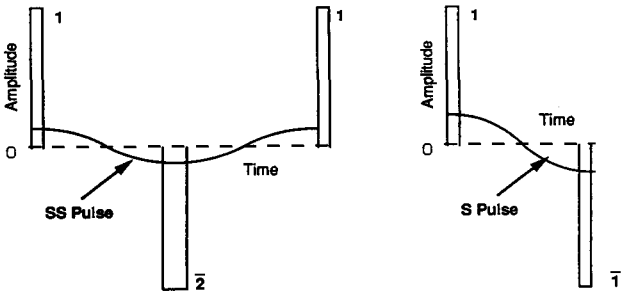

Figure 1. Comparison of the time domain characteristics of cosine amplitude modulated SS and S pulses with the binomial sequences 1-$\bar{1}$ and 1-$\bar{2}$-1.

Many desirable solvent suppression schemes suffer from larger frequency-dependent phase shifts and rolling baselines due to precession during the longer excitation periods.[2] This limits their use, particularly in two-dimensional spectroscopy. One approach to solving this problem is to use a spin echo[12] or to make the pulses self-refocusing.[13] The latter normally involves use of a 270° pulse or additional 180° elements before and after the starting 90° pulse shape.[14,15] The non-excitation water suppression scheme in greatest use today is jump-and-return[16] and its derivatives,[17] primarily because of its simplicity and flat baselines arising from the lack of a frequency-dependent phase shift.

Once the data have been acquired by one of the above methods, subtracting the residual solvent resonance from a spectrum or FID can greatly improve the spectral quality, especially of *n*D spectra where any "rocking" of the phase of the residual solvent resonance can modulate the nearby signals leading to apparent t_1 noise and streaks.[18,19]

(1) General review: Hore, P. J. *Methods in Enzymology* **1989**, *176*, 64–77.
(2) General review: Gueron, M.; Plateau, P.; Decorps, M. *Prog. NMR Spectrosc.* **1991**, *23*, 135–209.
(3) Hoult, D. I. *J. Magn. Reson.* **1976**, *21*, 337.
(4) Stoesz, J. D.; Redfield, A. G.; Malinowski, D. *FEBS Lett.* **1978**, *91*, 320.
(5) Redfield, A. G.; Kunz, S. D.; Ralph, E. K. *J. Magn. Reson.* **1975**, *19*, 114–117.
(6) Hore, P. J. *J. Magn. Reson.* **1983**, *55*, 283–300.
(7) Plateau, P.; Dumas, C.; Gueron, M. *J. Magn. Reson.* **1983**, *54*, 46.
(8) Smith, M.; Waterton *J. Magn. Reson.* **1987**, *68*, 526–532.
(9) Levitt, M. H.; Sudmeier, J. L.; Bachovchin, W. L. *J. Am. Chem. Soc.* **1987**. *109*, 6540–6541.
(10) Hall, M. P.; Hore, P. J. *J. Magn. Reson.* **1986**, *70*, 350–354.
(11) Sklenar, V.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1987**, *75*, 352–357.
(12) Sklenar, V.; Bax, A. *J. Magn. Reson.* **1987**, *74*, 469–479.
(13) Freeman, R.; Friedrich, J.; Xi-Li, W. *J. Magn. Reson.* **1988**, *79*, 561.
(14) Pocek, L.; Sklenar, V. *J. Magn. Reson.* **1990**, *86*, 376–383.
(15) Galloway, G. J.; Haseler, L. J.; Marsman, M. F.; Williams, D. H.; Doddrell, D. M. *J. Magn. Reson.* **1987**, *74*, 184–187.
(16) Plateau, P.; Gueron, M. *J. Am. Chem. Soc.* **1982**, *104*, 7310.
(17) Gueron, M.; Plateau, P.; Kettani, A.; Decorps, M. *J. Magn. Reson.* **1992**, *96*, 541–550.
(18) Plateau, P.; Dumas, C.; Gueron, M. *J. Magn. Reson.* **1983**, *54*, 46.
(19) Marion, D.; Ikura, M.; Bax, A. *J. Magn. Reson.* **1989**, *84*, 425–430.

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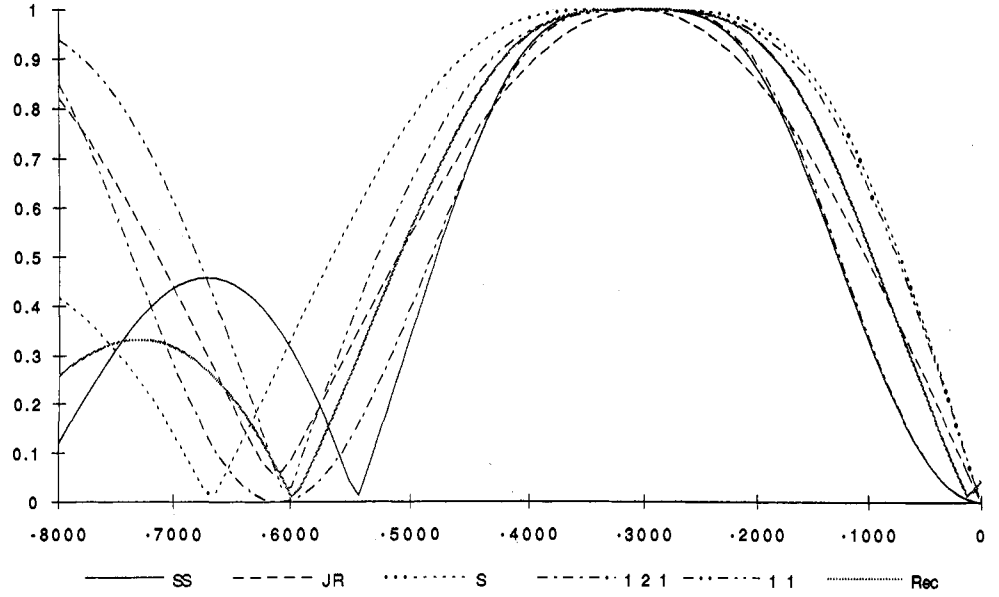

Figure 2. Comparison of the *XY* excitation profiles obtained by Bloch equation simulations of SS and S pulses with a simple rectangular pulse (rec), jump-and-return (JR), and the binomial sequences 1-$\bar{1}$ and 1-$\bar{2}$-1. All pulses are adjusted for maximum in the *XY* plane at ~3.08 kHz.

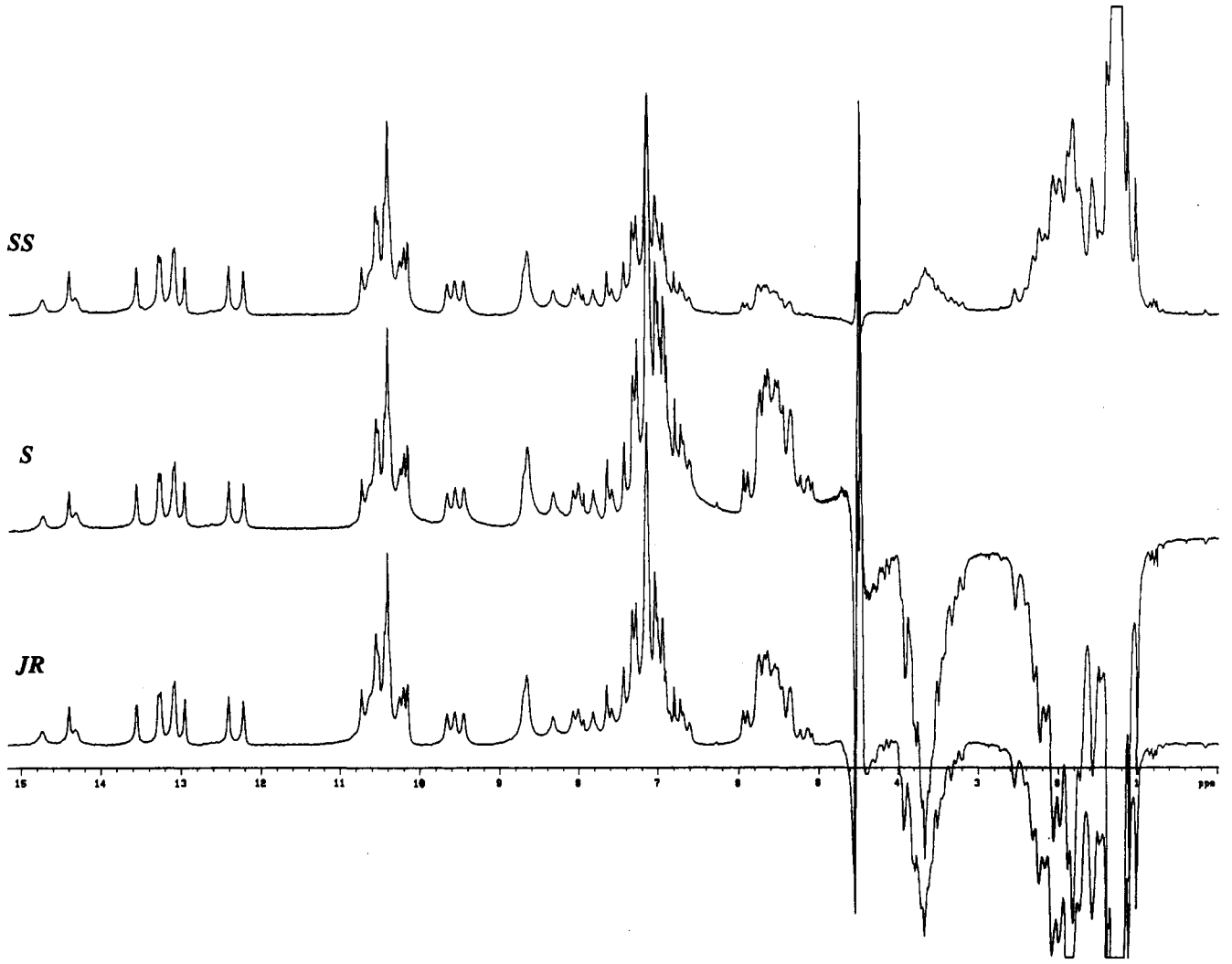

Figure 3. Comparison of the observed JR, S, and SS pulse excitation spectra of a 2 mM DNA sample. The pulses used were a 300-μs SS pulse, a 180-μs S pulse, and a jump-and-return sequence with 6.2-μs 90° pulses and 50-μs pulse spacing. The spectral width was 14 000 Hz. The frequency-dependent phase shift resulting from the use of the S and SS pulses has been corrected by the DS/LP technique with $N = 1$ and 2 points, respectively.

Theory

Symmetrically-Shifted Pulses. This paper represents several advances in water suppression techniques, the first of which involves the use of symmetrically-shifted shaped pulses as an alternative to other non-excitation water suppression pulses. A symmetrically shifted pulse is a shifted Laminar pulse[20] that contains an equal number of rectangular pulse components of the

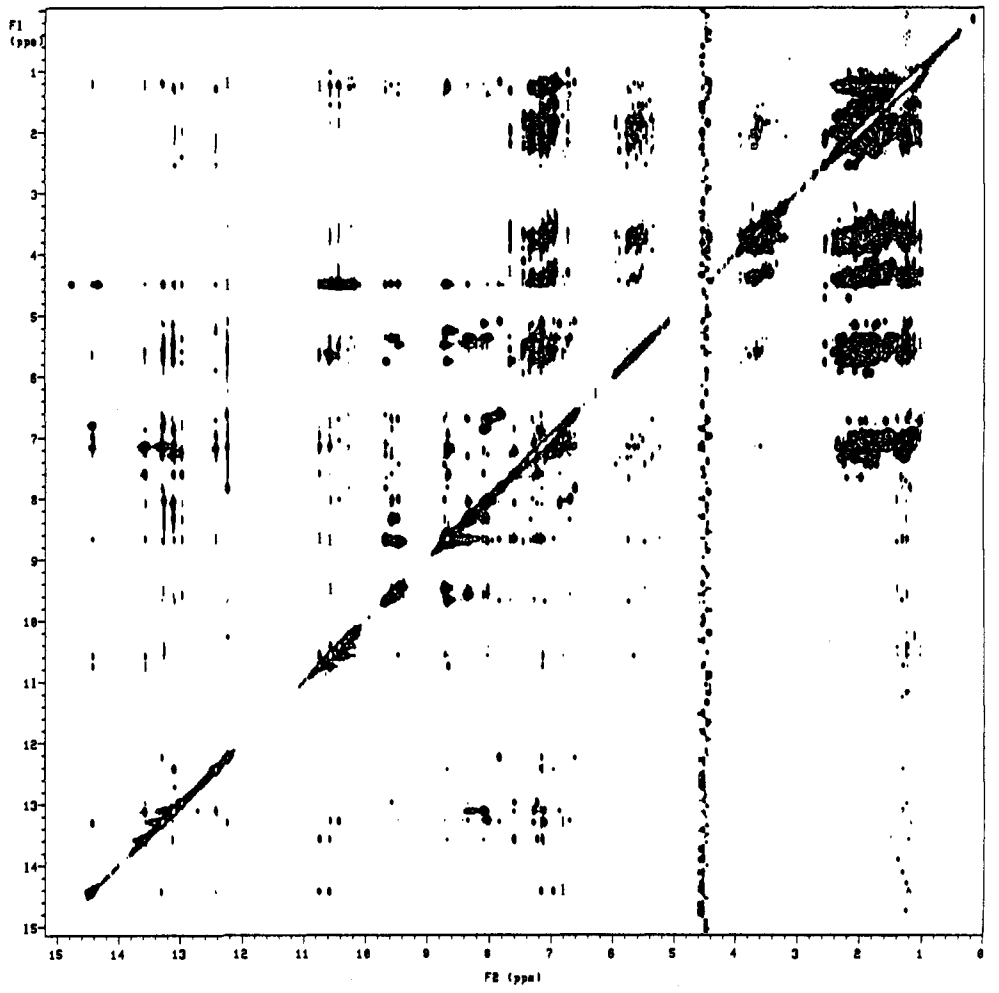

Figure 4. SS NOESY spectrum (250 μs SS read pulse) of a 2 mM DNA sample (contour plot of the full spectrum). The directly observed f_2 axis is horizontal. DS/LP ($N = 2$) was used to remove the frequency-dependent phase shift in F_2. Other significant parameters: 1.5 s relaxation delay, 32 transients per FID, with 256 complex increments collected in t_1.

same phase at ± an offset frequency.[21] If the offset frequency and the pulse length are carefully chosen so that the first null of each of the shifted rectangular components falls at the water frequency, a family of non-excitation water suppression pulses is achieved.

The SS pulse, the cornerstone of the symmetrically-shifted pulse family, is the frequency-symmetrical equivalent of the Redfield long-pulse technique. Conceptually it is equivalent to applying simultaneous 90° rectangular pulses with two separate, but in-phase, transmitters at ± an offset frequency from the water. While conceptually equivalent, the necessary constraint of the equal phase relationship between the ± frequency components makes it impractical to actually achieve with two physical NMR transmitters as the phase relationship between the two transmitters, which are necessarily at different frequencies, would be constantly changing. Symmetrically-shifted pulses, however, use cosine modulation of a single transmitter to achieve both frequency components with their necessary in-phase relationship.

Symmetrically-shifted pulses can also be seen as having historical precedence in, and many of the properties of, the binomial sequences.[22] The time-domain profiles of two symmetrically-shifted pulses are illustrated in Figure 1: an SS pulse whose amplitude profile is a complete 2π cycle of a cosine, and an S pulse which is half of an SS pulse, or a half-cycle (π) of a cosine. Figure 1 also illustrates the similarity of SS and S pulses to the binomial sequences, 1-$\bar{2}$-1 and 1-$\bar{1}$. Figure 2 shows the down-field halves of the excitation spectrum or XY projection, from Bloch equation simulations[23] after a 360-μs SS pulse and a 220-μs S pulse (the up-field halves are the mirror images). Figure 2 also shows the equivalent excitation spectra from jump-and-return (JR), 1-$\bar{2}$-1, 1-$\bar{1}$, and long rectangular (rec) pulses for comparison. Parameters for all pulse simulation have been chosen to give maxima in the XY plane at ~3.08 kHz. From Bloch equation simulations, the appropriate length for an SS pulse is ~1.1 times the reciprocal of the frequency of the desired excitation maxima. An S pulse length is ~0.67 times the reciprocal of the desired excitation maxima.

As can be seen in Figure 2, the SS and S pulses, like their binomial equivalents, have broader excitation maxima compared to the sinusoidal profile of jump-and-return. Another differentiation is the shape of the profile near the water. The SS pulse (like 1-$\bar{2}$-1) has a second-order or U-shaped null at the water, whereas the S, 1-$\bar{1}$, long-pulse, and JR profiles are first-order or V-shaped. The SS pulse, therefore, can be expected to and does give much better water suppression at the cost of poorer excitation of resonances close to the water. An added bonus of the SS pulse is that both ± excitation profiles are in-phase or "up", i.e., there is no phase inversion at zero frequency as with JR, S, or 1-$\bar{1}$ responses.

The S pulse, so called because its amplitude profile is half of a SS pulse, has much better close-in excitation at the potential

(20) Patt, S. L. *J. Magn. Reson.* **1991**, *95*, 94–102.
(21) The use of a second symmetrically-placed pulse for cancelling unwanted nonresonance excitation has also been discussed by McCoy and Mueller and successfully applied to selective decoupling application. McCoy, M. A.; Mueller, L. *J. Magn. Reson. Ser. A* **1993**, *101*, 122–130.
(22) Hore, P. J. *J. Magn. Reson.* **1991**, *55*, 283–300.
(23) The Bloch equation simulations were run with "pulsetool" in Varian's VNMR software which allows simulation of the spin system response to an arbitrary pulse shape.
(24) Barkhuysen, H.; deBeer, R.; Bovee, W. M. M.; van Orondt, D. *J. Magn. Reson.* **1985**, *61*, 465–481.

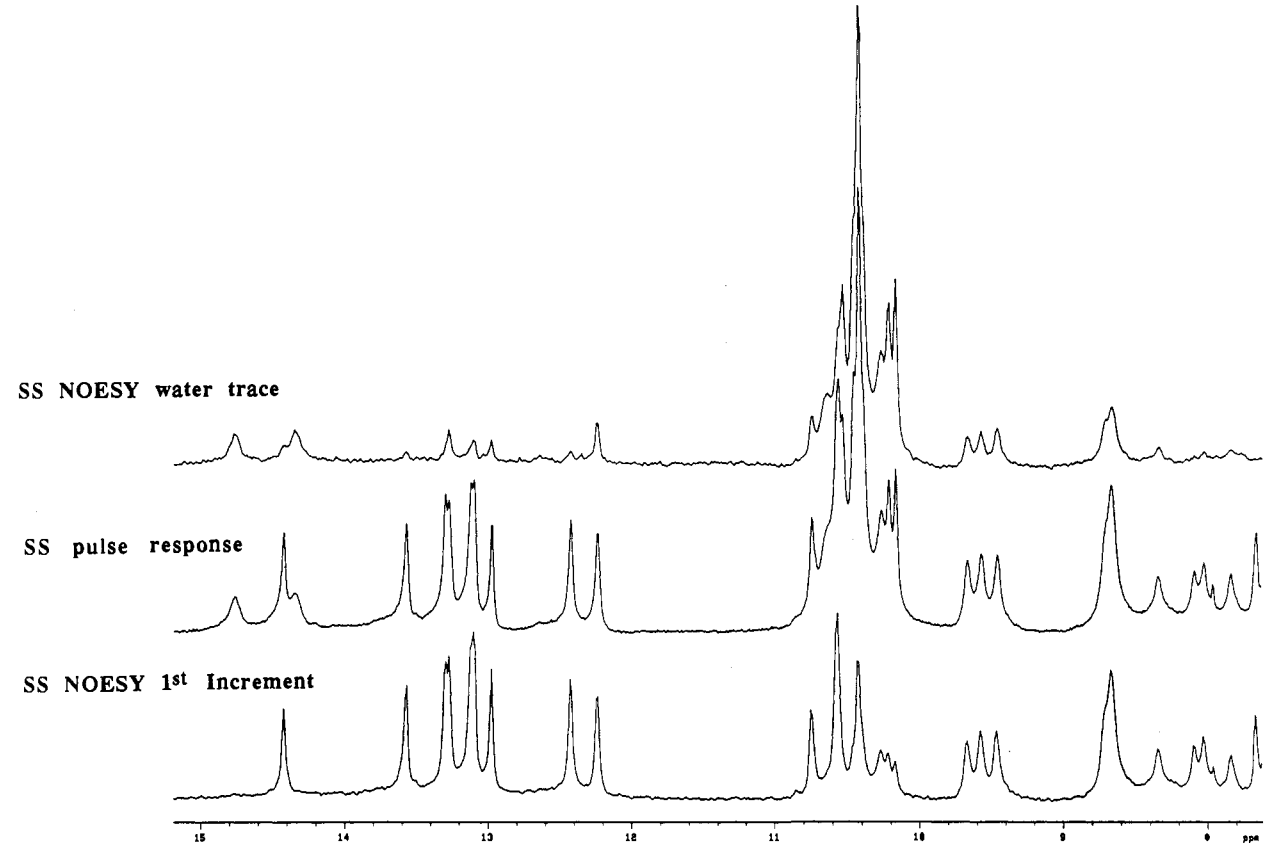

Figure 5. "Water trace" along F_2 from the SS NOESY spectrum in Figure 4 showing resonances detected during t_2 that are exchanging with, or relaxed by, protons at the chemical shift of the water during t_1. The SS pulse 1D spectrum and the SS NOESY first increment spectrum are included for reference.

cost of greater sensitivity to off-resonance water and therefore poorer suppression.

Data Shifting and Linear Prediction. The second advance in water suppression techniques concerns the use of data shifting and linear prediction[24] (DS/LP) for removing large frequency-dependent phase shifts from any pulse sequence, including binomials and symmetrically-shifted pulses, where data acquisition has been unavoidably delayed. Right shifting the data N points and then linear predicting the missing N points can be used to remove or subtract frequency-dependent phase shifts in multiples of 360°.

Zero-Frequency Subtraction (ZFS). Symmetrically-shifted pulses, JR, and binomial sequences tend to produce residual water signals that can be characterized by a single decaying sinusoid at or near zero frequency. The method used herein for removing the zero or low-frequency residual water from the FIDs includes the following: Pass the FID through a low pass filter of user-specified width so that the resultant filtered FID is dominated by the residual water signal. Fit the low-frequency filtered FID to an Nth order polynomial. Subtract the resultant polynomial function from each FID before Fourier transformation. This method removes the residual water resonance with minimal distortion of other resonances.

Experimental Methods

Symmetrically-Shifted Pulses. A symmetrically-shifted pulse of a given length is generated by starting with a rectangular waveform containing the appropriate number of 200-ns steps and then applying the either full or half-cycle cosine amplitude modulation in 1024 linear amplitude steps over a 60 dB range. (This process can be, and is in practice, accomplished by a complex vector multiplication of the starting rectangular waveform with sinusoids containing the ± offset frequencies). To assure rapid pulse rise-times, the calculated cosine modulated waveforms used herein were then padded with an additional 400 ns at the beginning and end in which the amplitude modulator was at the starting full value, but with the RF gate off. The transmitter frequency is set at the frequency of the water. The overall amplitude of the symmetrically-shifted pulses was controlled by the system programmable attenuator. Power levels for 90° symmetrically-shifted pulses can be obtained either by calculating the pulses from normal 90° pulse width calibrations for the desired pulse width with 6 dB added to account for the second frequency component or by varying the attenuator level and observing a response maximum at the frequency of the expected excitation maxima.

Data Shifting and Linear Prediction. In practice, linear prediction[25] of $N+1$ points is necessary where N is the number of complex data points the data has been right-shifted, as the first "actually collected" point in the FID is distorted by the step response of the spectrometer's analog filters. Normally, the amplitude of the first complex point is restored by multiplication by a correction factor, and any residual error is further reduced by zero-order baseline correction of the spectra. The results to date suggest that after data shifting, this same error is best corrected by linear prediction.

To achieve the desired data point timing, i.e., to ensure that the frequency-dependent phase shift is a multiple of 360°, one can either increase or decrease the normal pre-acquisition delays used by the softwre to compensate for the group delay of the analog filter. Most data shown here were obtained using a slightly longer pre-acquisition delay time and $N = 2$ for SS pulses. It is also possible to considerably shorten the pre-acquisition delay and use $N = 1$ for an SS pulse. As long as the second actually acquired point is not too distorted, this seems the preferable technique as one fewer point needs to be linear predicted. If after the fact the baseline distortion from the second actually acquired point is found to be too great, the second actually acquired point can still be linear predicted.

Zero-Frequency Subtraction (ZFS).[26] Typical values of the low pass filter function are ±300 Hz. An 11th order polynomial was then fit and then subtracted from each FID. The residual water in all FIDs, except for the data obtained using presaturation, and as otherwise noted, was reduced by zero-frequency subtraction. All spectra were baseline corrected

(25) The linear prediction used herein is a standard part of Varian's VNMR software and is based on the methods in ref 24.

(26) ZFS software is a standard part of Varian's VNMR software package.

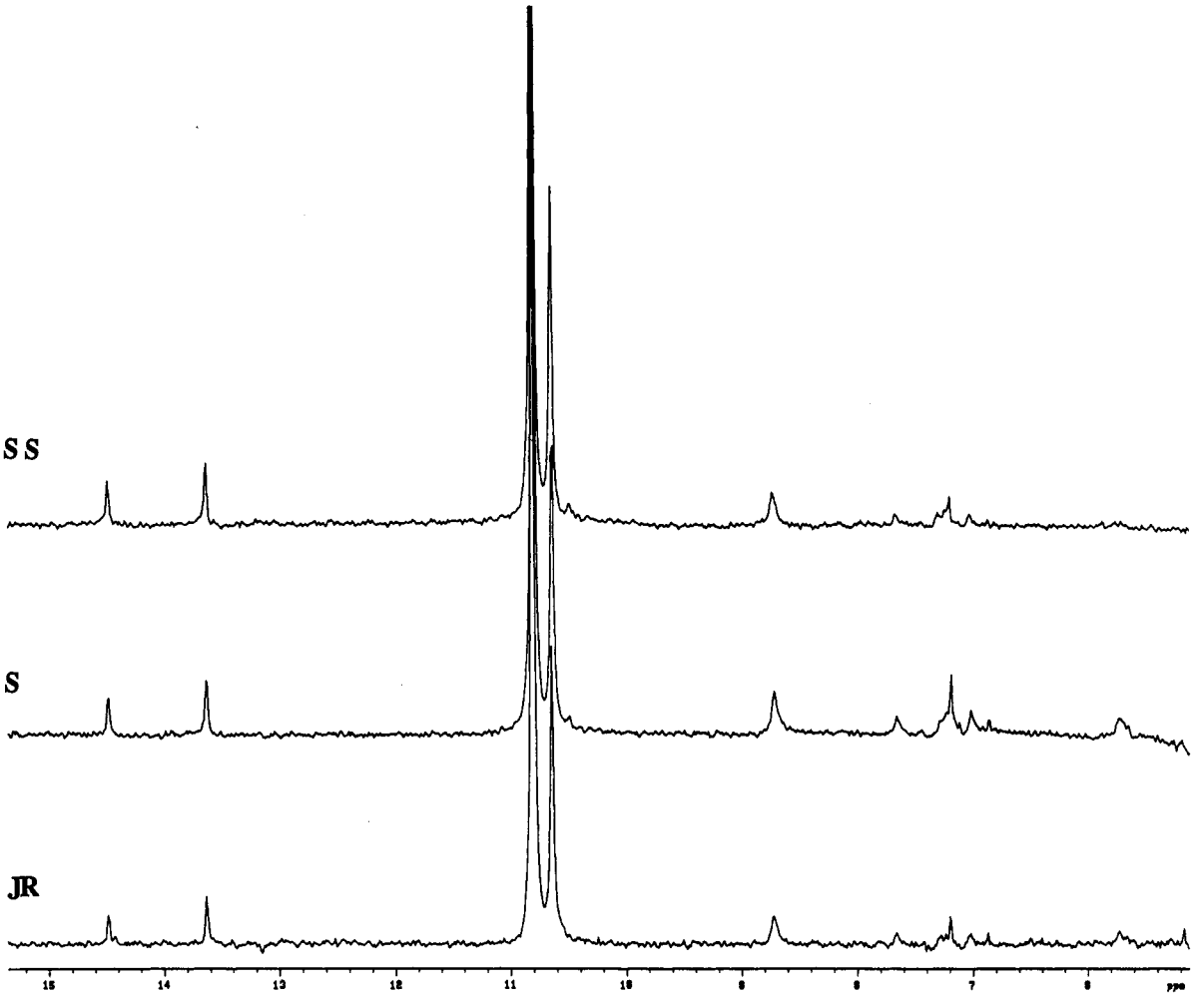

Figure 6. Comparison of F_2 traces from three NOESY spectra of the DNA sample obtained using the SS, S, and JR read pulses from Figure 3. The traces show the effect of the read pulse excitation profiles in F_2 for protons observed in F_1 at a chemical shift of 10.4 ppm.

in F_2 using a spline correction[27] (multiple connected straight lines) between baseline regions that were appropriate for correcting the whole spectrum. 2D spectra were baseline corrected in F_1 using spline correction, but with only two baseline regions defined at the edges of the spectra.

SS NOESY. An SS or S pulse can be used as the read pulse in a "hard-soft" NOESY sequence.[28] The first two pulses surrounding the t_1 period are hard and fully excite the 90% water resonance. Accordingly, the phase of the first pulse was shifted by ±45°, rather than 0°, 90°, in acquiring complex pairs in t_1 to equalize the effects of radiation damping.[29] Phase cycling: first pulse, 315° (−45°), 135°; second pulse, x (8), $-x$ (8); read pulse, x, $-x$, y, $-y$, $-x$, x, $-y$, y; receiver, x, x, y, y, $-x$, $-x$, $-y$, $-y$, $-x$, $-x$, $-y$, $-y$, x, x, y, y. F_1 axial displacement[30] was not used. While not necessary, a homospoil pulse[31] can be used in the center of the mixing period to aid water suppression and minimize artifacts.

Instrumentation. 600-MHz spectra were recorded on a standard UNITY 600 spectrometer equipped with Programmable Pulse Modulators for waveform generation. The probe used was a UNITY proton observe triple resonance probe. 500-MHz spectra were obtained on a standard UNITY 500 equipped with the Ultra-NMR shim system and a 10-mm proton observe probe.

Samples: 2 mm DNA sample of C-GC, pH 4.6, 100 mM phosphate buffer (5 mm diameter);[32] 2 mM TF1 sample in 90% H_2O, pH 6.8, 100 NaCL, 100 mM PO_4 (5 mm diameter),[33] 1 mM lysozyme sample in 90% H_2O, pH 4.8 (10 mm diameter), from a commercial preparation (Sigma).

Results and Discussion

Figure 3 compares the observed excitation profiles of a 300-μs SS pulse, a 180-μs S pulse, and a jump-and-return sequence and shows the expected differences in excitation of the excitation profiles near the water.

A comparison of the relative water suppression (without ZFS) in the absolute intensity, absolute value display mode suggests that S and SS pulses both give better suppression than JR under these conditions by a factor of 2.7 and 35, respectively. Thus as can be seen by examining either the time domain (Figure 1) or the frequency domain excitation profiles (Figures 2 and 3), the S and SS pulses are indeed the soft continuous equivalents of the 1-$\bar{1}$ and 1-$\bar{2}$-1 hard pulse binomial sequences.

Why use symmetrically-shifted pulses rather than the hard pulse binomial equivalent? They suppress water very well and very easily. Suppression factors of 10^4 to 10^5 have been realized with SS pulses without using multitransient cancellation. On the other hand 1-$\bar{2}$-1 is rarely used, as its theoretically excellent second order water suppression is often limited by a number of practical considerations, such as pulse rise-time which is not self-compensating in 1-$\bar{2}$-1, as it is with sequences containing identical positive and negative pulses, e.g. 1-$\bar{1}$ and 1-$\bar{3}$-3-$\bar{1}$.[1] Based on Figure 2, one would expect poorer water suppression with an S pulse than with jump-and-return because of the S pulse's steeper excitation profile near the water. In practice the S pulse has

(27) Spline correction is the standard baseline correction, "bc" command, in Varian's VNMR software. It subtracts a baseline correction function consisting of multiple straight line segments, between user defined baseline regions.

(28) Cutnell, J. D. *J. Am. Chem. Soc.* **1982**, *104*, 362–363.

(29) Torchia, D. A.; Sparks, S. W.; Bax, A. *Biochemistry* **1988**, *27*, 5135–5141.

(30) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393–399.

(31) Vold, R. L.; Waugh, J. S.; Klein, M. P.; Phelps, D. E. *J. Chem. Phys.* **1968**, *48*, 3821.

(32) DNA sample supplied by Dinshaw Patel, Columbia Presbyterian Hospital/Memorial Sloan Kettering.

(33) TF1 sample provided by Joseph Parello, La Jolla Cancer Institute.

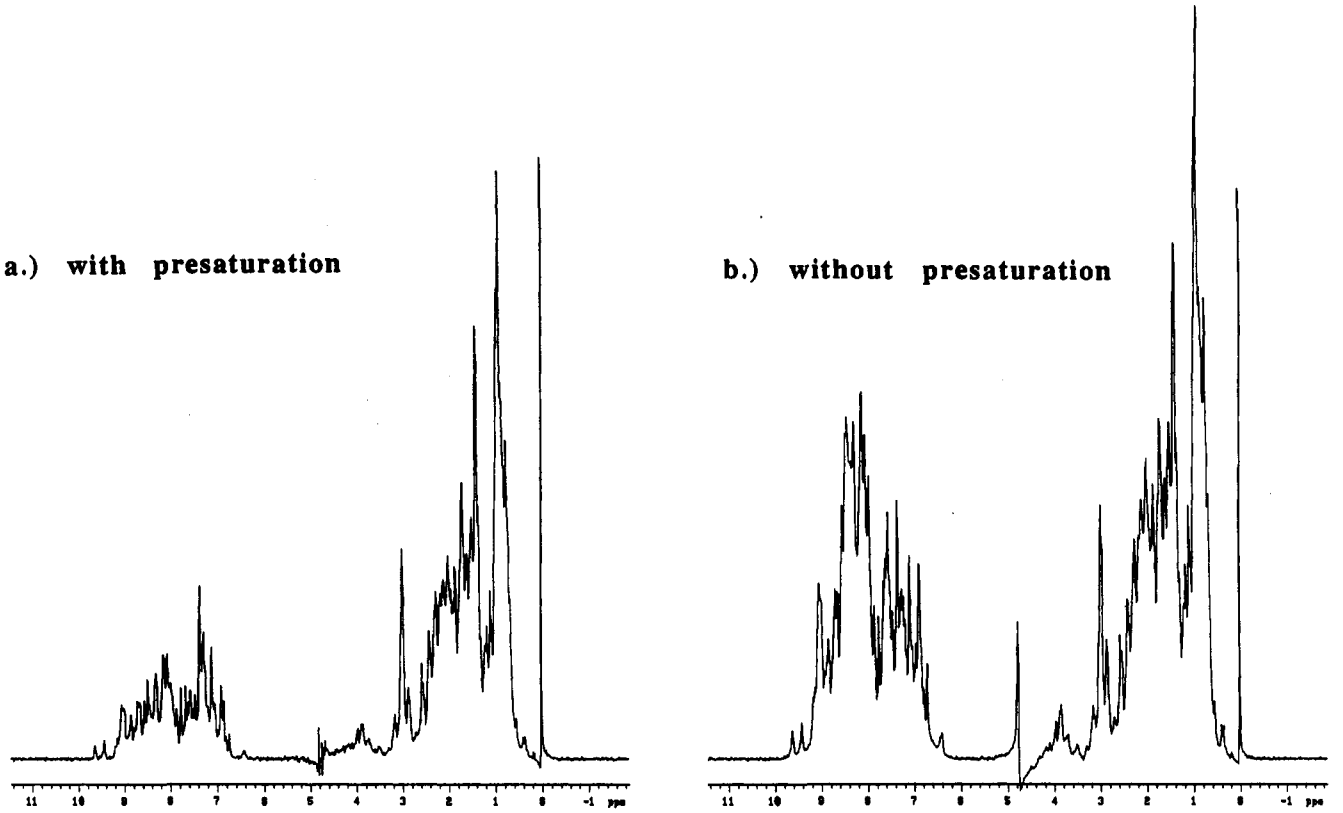

Figure 7. 1D spectra of a 2 mM (dimer) sample of TF1-2H[FYSV] (see text), with and without presaturation, obtained back-to-back using a 1.5 s relaxation delay followed by 475 μs SS pulse for observation. Figure 7a was obtained using a 50-Hz presaturation field during the relaxation delay. No presaturation was used in Figure 7b. DS/LP ($N = 2$) was used to remove the frequency-dependent phase shift in F_2.

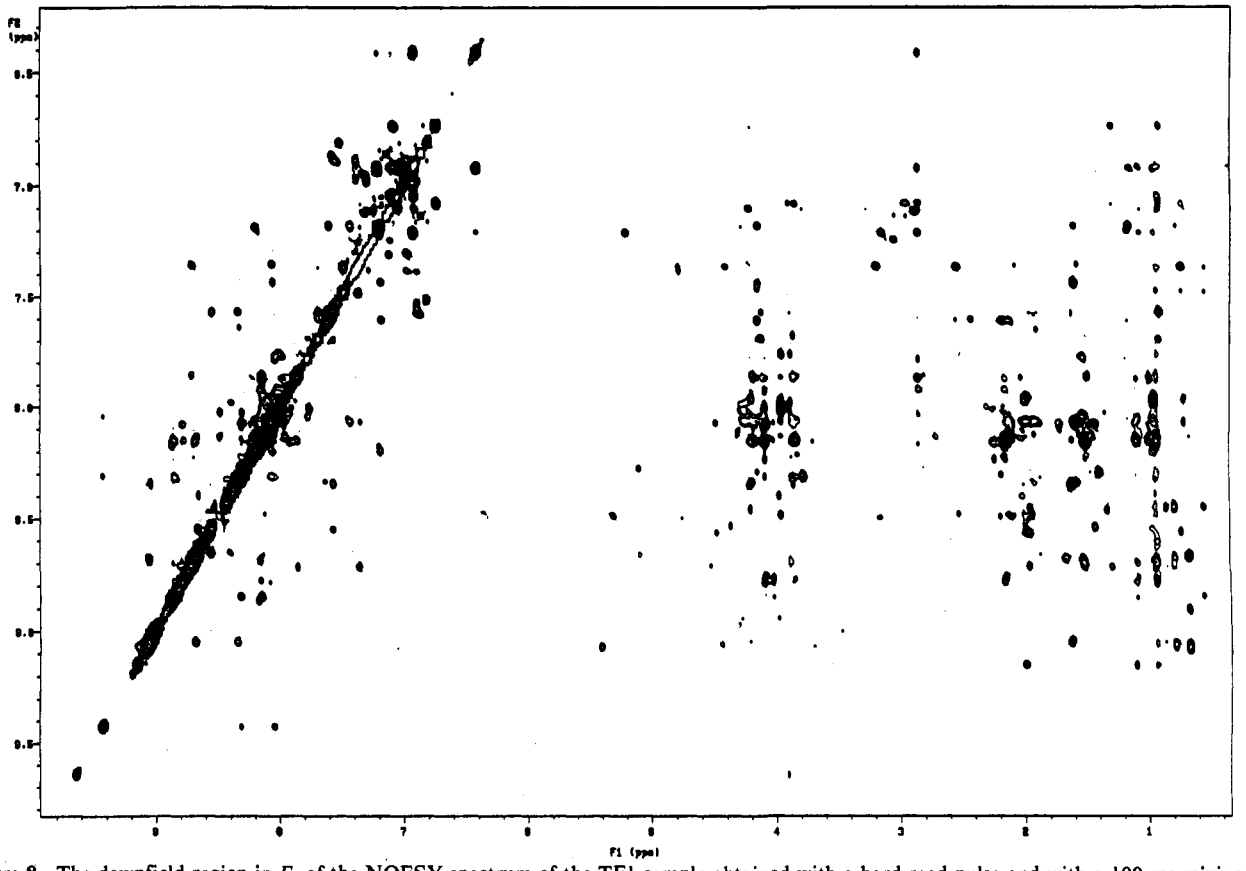

Figure 8. The downfield region in F_2 of the NOESY spectrum of the TF1 sample obtained with a hard read pulse and with a 100-ms mixing time. A 50-Hz presaturation field was used during the 1.5-s relaxation delay. Other parameters: 64 transients per FID with 200 complex increments in t_1. Linear prediction was used to extend the data set to 512 complex increments in t_1.

been seen to give somewhat better suppression than jump-and-return even though it has a much wider excitation profile. For 1D spectroscopy where direct observation of peaks near the water may be important, the S pulse would seem to be the method of

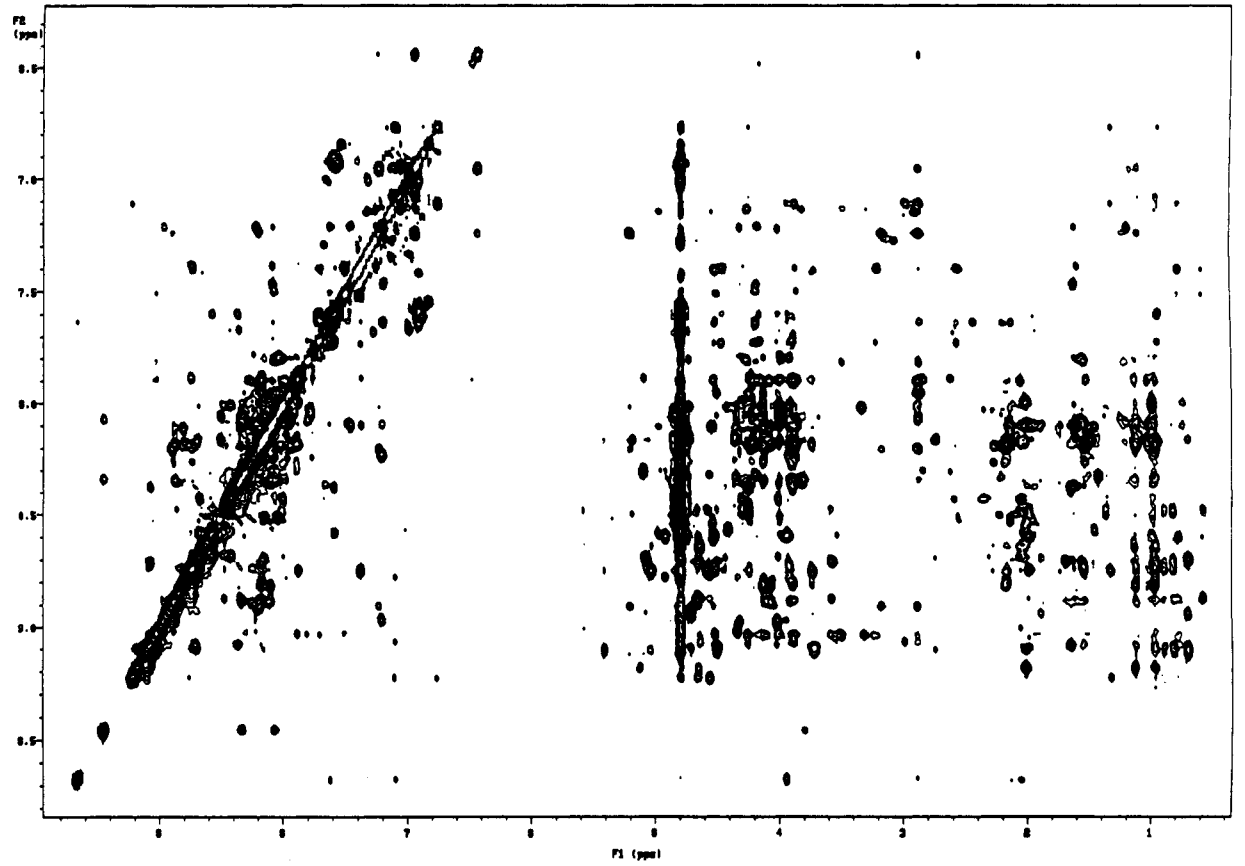

Figure 9. A contour plot of the downfield region (in F_2) of the SS NOESY spectrum of the 2 mM TF1 sample with a 100-ms mixing time. A 400-μs SS read pulse was used in place of the NOESY read pulse, and therefore no presaturation was needed. Other parameters: 64 transients per FID with 200 complex increments in t_1. Linear prediction was used to extend the data set to 512 complex increments in t_1. DS/LP (N = 1) was used to remove the frequency-dependent phase shift in F_2 and the residual water was reduced by ZFS.

choice, while for nD spectroscopy the better suppression of the SS pulse may well prove advantageous.

It has been obvious for some time that some shaped pulse, tailored excitation,[34] or more complex scheme would be better for non-excitation water suppression than the long pulse in terms of excitation profile and phase characteristics. Unfortunately most of the proposed schemes have been more complicated than a simple 90° pulse. In the real world of high-resolution probes with their high Q values and high filling factors, radiation damping and phase glitch[35] can very much limit the performance of complex schemes, particularly those using hard pulses, and especially if the scheme depends on inverting the bulk water. Applying two simultaneous 90° pulses smoothly, continuously, and at the desired frequencies seems a better approach. The trajectory of the water during an SS pulse stays near the $+Z$ axis (within 29°), never going near (or below) the XY plane. With high-resolution probes, this approach of using a simpler pulse, followed by removal of the frequency-dependent phase shift by data processing may prove more practical than a more complex self-refocussed pulse where radiation damping and other practical issues may degrade actual performance.

More complex symmetrically-shifted pulses with multiple offset frequencies, different power levels, and different timings have been seen to work theoretically via Bloch equation simulations. Whether they will work as well in practice as the simpler SS and S pulses remains to be seen.

NOESY Spectra of DNA. Symmetrically-shifted pulses seem very appropriate for examining the 2D spectra of nucleotides in H_2O. Since the first two pulses in the SS NOESY sequence are hard, i.e. ~10 μs, all resonances including the water and protons under the water are excited and their frequencies labeled during the t_1 evolution time. The read pulse can then observe these protons via their NOESY cross peaks to protons in the regions excited by the read pulse and detected during t_2. Since not all peaks are excited by the final read pulse that returns magnetization to the observable XY plane, the spectrum will be asymmetric with peaks near the water in F_2 missing. In many cases the added information about exchangeable and buried protons will more than compensate for the loss of the often redundant symmetrical information.

Based on Figure 3, the H_1' region at ~5.5 ppm and the H_4, H_5' H_5'' region at ~3.8 ppm are better excited in F_2 by the S pulse than the SS pulse, but this is likely not important in 2D spectroscopy as the whole spectrum is excited in F_1, and interactions between these protons may be studied in D_2O. The better water suppression and the resultant ease-of-use are therefore likely to make the SS pulse the better choice for multidimensional spectroscopy.

Figure 4 shows a contour plot of an SS-NOESY (250 μs SS read pulse) of the same sample run with a homospoil pulse in the center of the 150 ms mixing period. The homospoil pulse actually degraded water suppression if applied at the beginning of the mixing period, but it helped considerably when placed in the middle. The exchangeable imino (~10–15 ppm) and amino (~8–9 ppm) protons and their interaction with other protons are easily observed as cross peaks in Figure 4.

Figure 5 shows the water trace along F_2 at the chemical shift of the water in F_1 from the SS NOESY spectrum in Figure 4. For comparison purposes, Figure 5 also includes the first increment SS NOESY spectrum and the SS pulse 1D spectrum from Figure 3. The water trace in Figure 5 clearly shows that most, if not all, of the exposed imino protons between 10 and 11 ppm and the faster exchanging H-bonded imino protons at 14.2 and 14.4 ppm

(34) Tomlinson, B. L.; Hill, H. D. W. *J. Chem. Phys.* **1973**, *59*, 1775.
(35) Gerstein, B. C.; Dybowski, C. R. *Transient Techniques in NMR of Solids*; Academic Press: New York, 1985; see section on p 175 on the Treatment of Nonideal rf Perturbations.

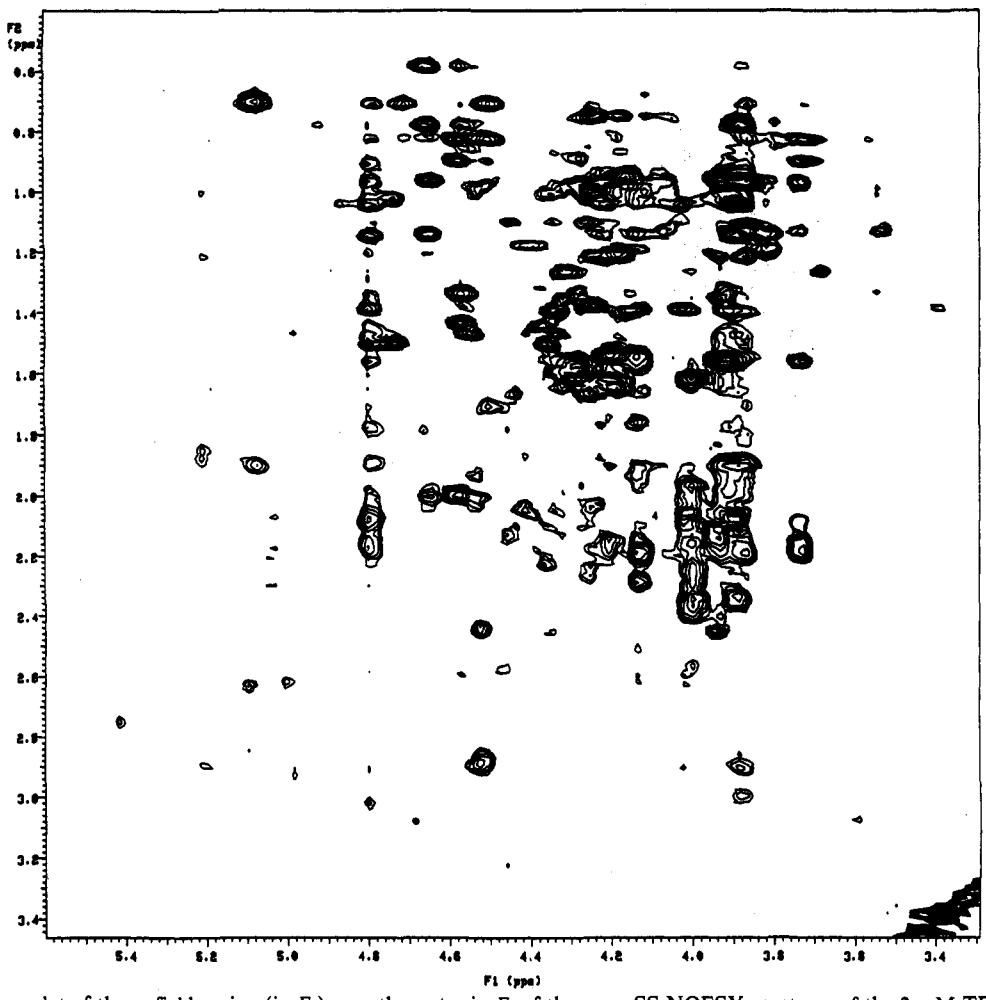

Figure 10. A contour plot of the upfield region (in F_2) near the water in F_1 of the same SS NOESY spectrum of the 2 mM TF1 sample as Figure 9.

are exchanging with bulk water. Note that not all of the faster exchanging peaks that occur in the water trace or 1D spectrum, e.g. the H-bonded imino protons at 14.4 and 14.8 ppm, show up in the first increment NOESY spectrum or on the diagonal of the full SS NOESY spectrum in Figure 4.

Figure 6 compares the F_2 traces from three NOESY spectra of the DNA sample obtained using the SS, S, and JR read pulses from Figure 3 for protons observed in F_1 at a chemical shift of 10.4 ppm. Again the expected effects of the F_2 excitation profiles are seen.

The use of a non-excitation read pulse such as an SS pulse or JR has distinct advantages for observing exchangeable protons over saturating read pulses such as those using spin lock pulses in that the bulk water and therefore the exchangeable protons are not saturated by the read pulse. The use of a spin lock water suppression read pulse[36] in the NOESY experiment caused a loss of half the intensity of the exchangeable protons with this DNA sample, even when the relaxation delay was increased to 3.2 s to allow additional recovery! Since the relaxation delay in most experiments is considerably shorter than the T_1 of the bulk water, keeping the bulk water unsaturated is of primary importance in the observation of exchangeable protons.

Application of SS Pulses to Protein Spectra. Two comparisons of SS NOESY vs presaturation in NOESY are illustrated with a type 2 DNA binding protein, the transcription factor 1 (TF1) from *Bacilius subtilis* phage SB01, a 22 kDa homodimer, and lysozyme, both at 30°C. In the case of the TF1 sample, a selectively deuterated recombinant protein sample was used (TF1-^{2}H[FYSV]) in which all non-exchangeable protons were substituted with deuterium (to a mean isotope ratio of 80%) with the exception of Phe, Try, Ser, and Val residues (30). The advantages of using selectively deuterated high molecular weight protein samples have been recently discussed.[37,38] Figure 7 compares two 16 transient 1D spectra of the TF1 sample, with and without presaturation, obtained back-to-back using a 1.5 s relaxation delay followed by 400 μs SS pulse for observation. Figure 7 clearly shows that presaturation saturates not only the exchangeable protons in this experiment but every other resonance as well, via saturation transfer or spin diffusion.[39] Except for the DSS peak at 0 ppm for which the 1.5 s relaxation delay is inadequate, every peak in Figure 7a shows a decrease with presaturation when compared with Figure 7b where no presaturation was used.

Figures 8 and 9 compare the downfield (F_2) region of two NOESY spectra of the TF1 sample, presaturation and SS, respectively, run with a 100-ms mixing time. Figures 8 and 9 are plotted at the same level. At pH 6.8 and with the relatively open structure of the DNA binding protein, TF1, the effect of presaturation on the amide protons is particularly dramatic as can be seen by comparing either the downfield half of the spectra in Figure 7, or the NOESY spectra in Figures 8 and 9. Not only are there many new peaks in Figure 9 at the chemical shift of water, ~4.8 ppm, but almost every peak in the fingerprint or amide region of the spectrum is considerably more intense in the SS-NOESY spectrum. Most of the intensity at F_1 = 4.8 ppm

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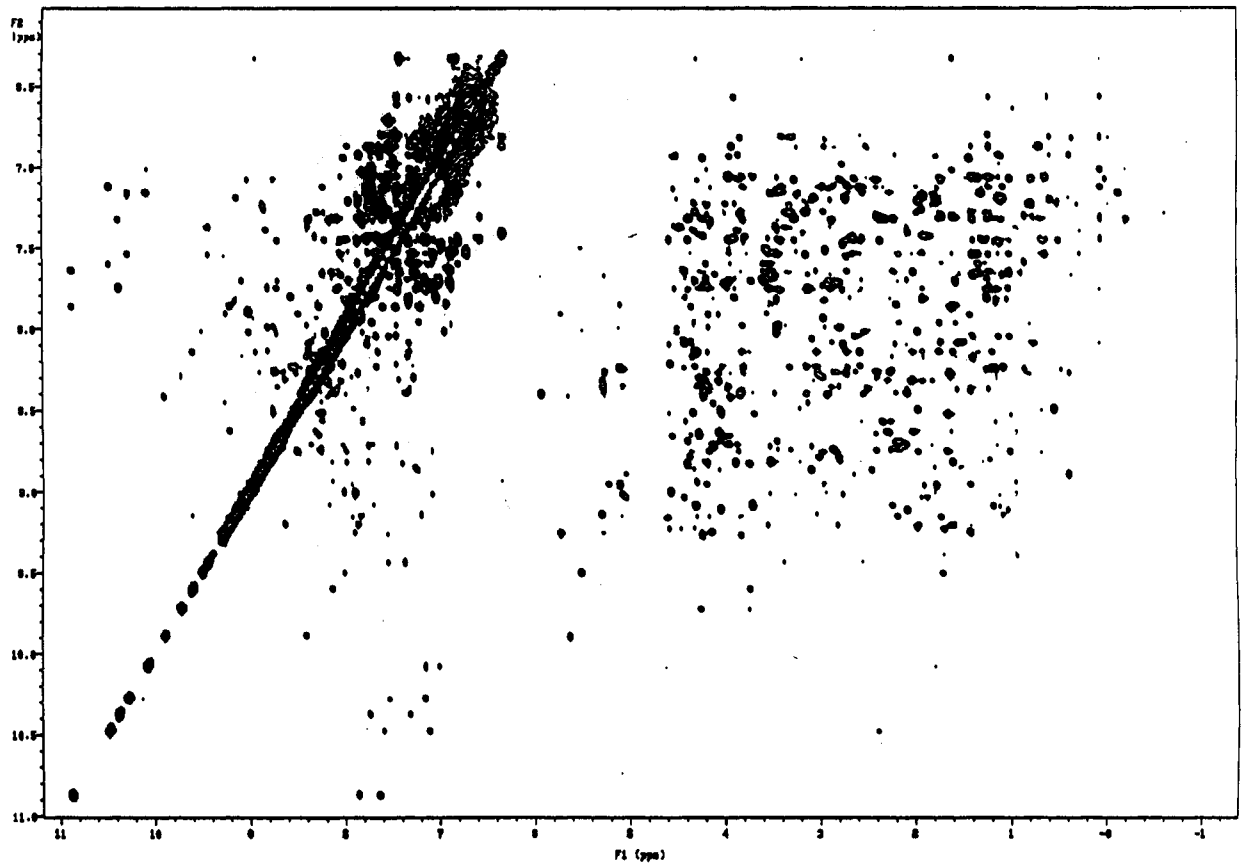

Figure 11. A contour plot of the downfield region in F_2 of the NOESY spectrum of a 1 mM 10-mm lysozyme sample at 30 °C with a 150-ms mixing time. A 50-Hz presaturation field was used during the 1.5-s relaxation delay. Sixteen transients per FID were acquired with 300 complex increments in t_1.

in Figure 9 is likely to be a result of chemical exchange between the bulk water and the amide protons. Other intensity differences between Figures 8 and 9 arise from a variety of factors as will be discussed for the lysozyme results. Figure 10 shows the upfield (F_2) NOESY correlations in the TF1 sample near the water chemical shift in F_1. Clearly there are several resonances at the water frequency in F_1 that show NOESY cross peaks to various aliphatic resonances. These could either be bound water molecules or 3-spin correlations that also include an exchangeable amide proton. The quality of the water suppression provided by SS pulses may well prove to be a valuable tool in examining bound water molecules. (Note that the results in Figures 9 and 10 were obtained by using $N = 1$ for the DS/LP procedure, despite the 475-μs SS pulse.)

Figures 11 and 12, which are plotted at the same level, illustrate the effects of presaturation with a 1 mM lysozyme sample at pH 4.6. Under these conditions proteins are generally assumed not to have many exchangeable protons. Indeed, only a few NHs can be seen exchanging with the water at 4.8 ppm. The greater intensity and number of peaks in Figure 12 arises for three additional reasons. First, there are some peaks near the water that are bleached by presaturation prior to t_1 and are therefore missing from the bleached area at $F_1 = \sim 4.8$ ppm in Figure 11. Second, there also are several peaks at 9.2–9.3 ppm in F_2 that are saturated in Figure 11 by chemical exchange. The third and major factor, however, is that almost every peak in Figure 11 is increased in amplitude in Figure 12 by, on the average, 60%. If the vertical scale of Figure 11 was increased by 60%, the spectra would look similar except for those few peaks lost due to bleaching or chemical exchange and, of course, the 60% difference in the signal-to-noise ratio!

SS NOESY. The use of SS or any non-excitation read pulse in NOESY rather than presaturation has its own set of problems because of the large residual water signal at the beginning of the mixing time. The two hard pulses, with a ±45° phase shift between them, leave the water with a large projection on the XY plane and on $\pm Z$ axis on alternate transients. Normal NOESY phase cycling will cancel this XY magnetization, but the poor water suppression with each transient potentially leads to dynamic range problems. During the mixing time, radiation damping is an ally, rotating the net water magnetization back toward $+Z$ within a few tens of milliseconds.[40,41] With mixing times of ~100 ms, the central component of the water will be completely removed by the combination of radiation damping and cancellation via phase cycling. Unfortunately, small-field modulations such as those from line frequencies, helium boil-off, and/or floor vibrations may create artifacts that will not cancel with phase cycling.

One answer to this problem is a homospoil pulse to destroy the unwanted XY magnetization during the mixing period, since the desired NOESY information is being developed on the Z axis. If the water XY magnetization is destroyed at the beginning of the mixing time period, radiation dampling is also eliminated and the water magnetization on alternate transients will remain on $-Z$, relaxing with the much longer water T_1, and will be stimulated into radiation damping during acquisition by the read pulse. Placing the homospoil in the middle of the mixing time allows radiation damping to destroy the central water component while the homospoil removes the artifacts around the residual water created by the unwanted field modulations.

Applications of SS Pulses for Indirect Detection of Labeled Proteins. There are several potential applications of SS pulses in the indirect detection of carbon and nitrogen in labeled proteins using double, triple, or quadruple resonance techniques.[42] First,

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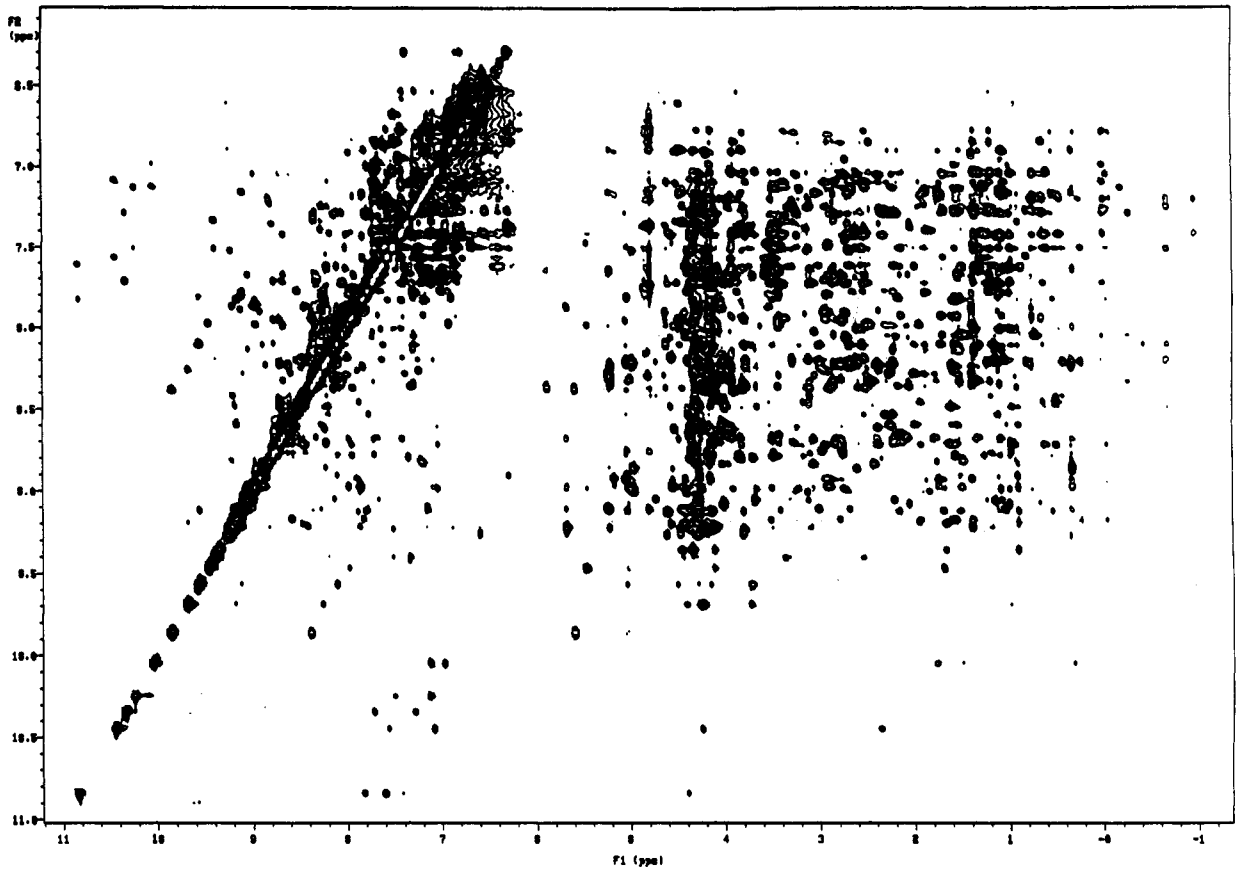

Figure 12. A contour plot of the downfield region in F_2 of the SS NOESY spectrum of a 1 mM 10-mm lysozyme sample at 30 °C with a 150-ms mixing time. A 475-μs SS read pulse was used for the NOESY read pulse, and therefore no presaturation was needed during the 1.5-s relaxation delay. Sixteen transients per FID were acquired with 300 complex increments in t_1.

many of these techniques involve detection via the amide proton in H_2O and presaturation of the water is normally used. On the basis of the evidence presented herein, SS pulses should give better sensitivity for most, if not all, of the amide protons. Secondly, SS pulses should be preferable to the rectangular pulses used to semiselectively excite the α and carbonyl carbons, e.g. the second-order null from an SS pulse on the carbonyls should cause much less unwanted perturbation of the α carbon resonances by the "carbonyl" pulses. Furthermore, the equivalent SS pulses are essentially no longer than the rectangular pulses they replace so they can be easily used in and around carbon evolution times. To date it would seem that either S or SS pulses make reasonable 90° pulses but that S pulses are preferred for 180° pulses. Furthermore, the combination of SS and S pulses combined with z-gradient pulses gives very good water suppression. This is an area of current ongoing research.

Conclusions

Symmetrically-shifted pulses are clearly viable and superior alternatives to other non-excitation water suppression sequences now in use such as jump-and-return or the binomial series. The SS pulse in particular gives remarkably efficient water suppression. The S pulse, on the other hand, has the widest excitation profile of any of the commonly used techniques with somewhat better suppression than the more commonly used jump-and-return. The practically of symmetrically-shifted pulses is greatly enhanced by usage of DS/LP to remove frequency-dependent phase shifts, a technique that will also substantially help binomial water suppression as well. By using more complex shaped pulse and data processing techniques, better results are obtained compared to current practice, since fewer demands are placed on the high-Q, high-sensitivity NMR probe with its inherent problems in dealing with the 90% water signal.

SS NOESY has also been illustrated as a superior technique for proteins compared with presaturation, a water suppression method which through saturation transfer can result in a significant loss in sensitivity, especially in the critical fingerprint region. It is likely that for many proteins the overall gain in sensitivity in the fingerprint region with nonsaturating methods such as SS NOESY, as well as the ability to observe exchangeable protons, may increasingly overcome the loss of symmetry due to the lack of direct α proton excitation in F_2 and become the preferred approach. The advantage of SS pulses over previous approaches, including presaturation and jump-and-return, is that it gives much better water suppression and is easier to use.

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