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Extending the Excitation Sculpting Concept for Selective Excitation

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Nowadays, excitation sculpting is probably the most efficient way to achieve selectivity in an NMR experiment, since it associates very clean frequency selection with "user-friendliness." In the present report, it is shown that the excitation sculpting concept, originally based on a double pulse field gradient echo acting on a selected transverse magnetization, can be extended through new experiments designed to act on longitudinal magnetization. This leads to outstanding performances, especially when the transverse relaxation rate is a limiting factor as, for example, in the case of biological macromolecules. Several new sequences are proposed, aiming at the selection of magnetization aligned either/both on a transverse axis or/and on the *z*-axis. Their potentialities are illustrated in light of different applications including multiplet-selective excitation, band-selective excitation, and water suppression.

Key Words: **excitation sculpting; selective excitation; water suppression.**

Although multidimensional (*n*D) NMR spectroscopy has enjoyed unprecedented success for assignment purposes and structural determinations, limitations are encountered when very accurate measurements of both chemical shifts and coupling constants are required. This is due to intrinsic limitations in the achievable matrix sizes and to the necessity of keeping the measurement time of NMR experiments within acceptable limits regarding spectrometer stability. Most strategies aimed at circumventing this problem rely on the use of selective excitation at one or more steps in the NMR experiment and have been extensively reviewed (see, for example, *1–3*). The quality of the results of such experiments relies heavily on the performance of the selection scheme. Ideally, the selective pulse ("soft" pulse or DANTE train (*4*)) should homogeneously excite a well-defined spectral region, without spurious excitations outside this zone and without phase gradients arising from a mixing of the two transverse magnetization components. If the selective excitation yields a global flip angle of 90°, the two transverse magnetization components (M_x and M_y) are almost invariably created, leading to severe phase problems although some sophisticated shaping functions have been proposed to virtually suppress the dispersive component (see, for example, *5*). On the other hand, it has been demonstrated that selective schemes involving a 180° global flip angle (*6, 7*) yield intrinsically much better profiles, while appropriate phase cycling or the use of pulsed field gradients can eliminate all phase problems. Indeed, whereas a rectangular 90° soft pulse (or DANTE train (*4, 8*)) yields a central excitation peak flanked by an extensive pattern of sidelobes, a 180° selective pulse generates absorption (M_y) profiles free from oscillations far from resonance and with only weak positive spurious excitations around the selected region. This can be explained by the sinc^2 dependence of the 180° rectangular pulse profile as opposed to the sinc function dependence of the excitation profile when using a 90° rectangular pulse (*1*). Pioneering work in this field led to the well-known DANTE-Z (*6*) and SPIN-PINGING (*7*) sequences, based on 180° inversion or refocusing selective pulses, respectively.

Similarly, for a simple rectangular selective 180° pulse, the use of a double selective echo leads again to a substantially better excitation profile. This feature can be understood on the basis of the theory provided by Hwang and Shaka and colleagues (*9, 10*), who showed that the amplitude of the response in a single or double echo experiment is dictated by the probability of a spin flipping. As a result, the selectivity profile of a double selective echo method is the squared form of what is obtained with a single gradient echo involving the same inversion element. Thus, the selectivity of the refocusing procedure is considerably enhanced since a sinc^4 frequency response, virtually devoid of side-lobes, is obtained with a simple rectangular 180° soft pulse. The suppression of the dispersive component in a double echo experiment can be obtained with a 16-step EXORCYCLE-type (*11*) phase cycle derived from the one of the SPIN-PINGING experiments. However, the use of a double pulsed field gradient selective echo (DPFGSE sequence (*9, 10*)) is preferable since it reduces the selection procedure to 1 scan instead of 16. This method, also reported as excitation sculpting (*12, 13*), has recently enjoyed undeniable success although limitations are nevertheless encountered

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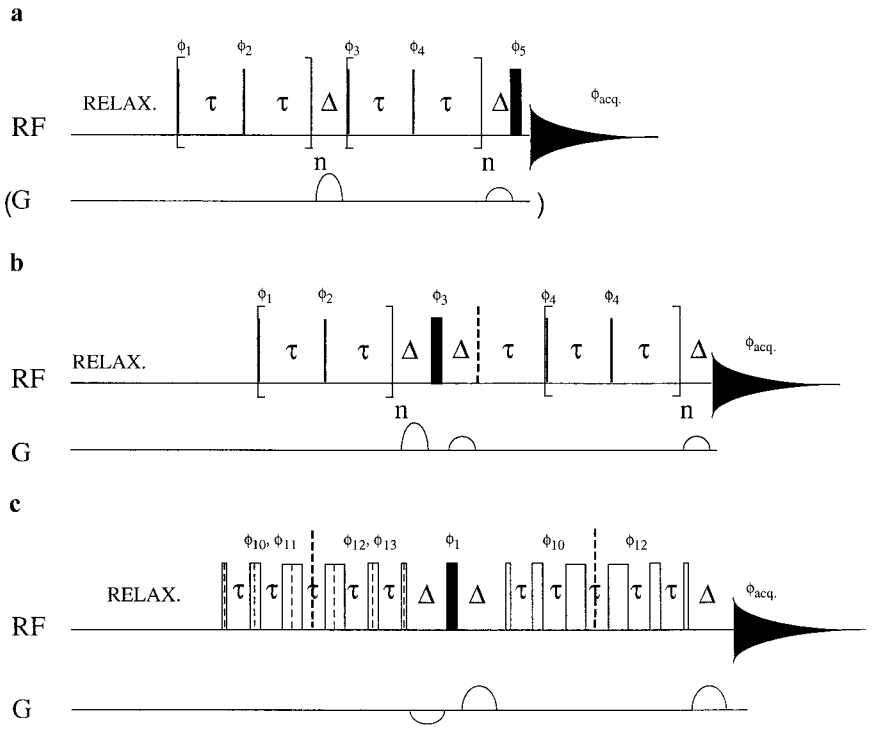

FIG. 1. Pulse sequences for (a) the DDANTE-Z (ZEST) experiment and the BEST experiment used either for (b) selective excitation or (c) water suppression. Solid rectangles represent radiofrequency hard pulses, where the pulse lengths are differentiated by the rectangle widths: the largest ones correspond to 90° pulses whereas the thinnest ones correspond to small flip angle pulses in the DANTE train. In (c), the open rectangles represent the 3–9–19 pulse train used as the selective procedure; the pulse lengths are differentiated by the rectangle widths, and the dotted lines dividing each pulse in the first 3–9–19 train indicate that these pulses are halved and have different phases (see text). The other symbols, along the row labeled G, correspond to pulsed field gradients (PFG) (brackets indicate that these PFGs are optional), τ stands for the nutation delay used in the pulse trains, and Δ is a small delay necessary for switching instrumental parameters (offset frequency, amplifier output power . . .). When no PFG are used, the phase cycling of DDANTE-Z is: $\phi_1 = 2x, 2(-x)$; $\phi_2 = x, -x, -x, x$; $\phi_3 = 8x, 8(-x)$; $\phi_4 = 4x, 8(-x), 4x$; $\phi_5 = x$; $\phi_{acq} = 2(x, -x), 2(-x, x)$. When using PFG, the phase cycling reduces to: $\phi_1 = \phi_3 = x$; $\phi_2 = x, -x$; $\phi_4 = 2x, 2(-x)$; $\phi_{acq} = x, -x$. The gradient-enhanced BEST experiment in (b) needs only the two-step phase cycling of the purged version of the DANTE-Z experiment (*17*): $\phi_1 = \phi_3 = \phi_4 = x$; $\phi_2 = x, -x$; $\phi_{acq} = x, -x$. The basic phase cycling for the water-suppression BEST sequence is: $\phi_1 = \phi_{10} = x$; $\phi_{12} = -x$; $\phi_{11} = x, -x$; $\phi_{13} = -x, x$; $\phi_{acq} = x, -x$. An additional CYCLOPS (*28*) phase cycling can be used to suppress quadrature artifacts.

when applied to large molecules such as biological macromolecules: short transverse relaxation times yield obviously non-negligible sensitivity losses during sequences involving multiple echoes.

In the present paper, we demonstrate that the excitation sculpting concept is not limited to gradient echo techniques, acting necessarily on transverse magnetization, but can be extended to other selective schemes, acting possibly on longitudinal magnetization. Our aim is to reduce sensitivity losses in the case of application to large biological macromolecules.

RESULTS AND DISCUSSION

In case of a symmetric time-domain soft pulse, the absorption selectivity profile in excitation sculpting techniques is only determined by the global flip angle, the suppression of the dispersive component being a virtue of the gradient echo, and must be independent of the axis along which the pulses are applied. Thus, if selective refocusing pulses are used in selective echo techniques, schemes involving inversion pulses are conceivable. This statement leads to the experiment depicted in Fig. 1a. We named this experiment DDANTE-Z (for double DANTE-Z), since it can be considered an extension of the DANTE-Z sequence (*6*), using two selective inverting 180° DANTE pulse trains coupled through a 16-step phase cycling aimed at suppressing all unwanted magnetization components. Selectivity profiles obtained with this experiment have been simulated and experimentally checked (Fig. 2) when using either a rectangular or a pulse-width-modulated (RE-BURP (*5*)) 180° DANTE train (*14*) as the selective procedure: they are virtually identical to those obtained with the DPFGSE sequence. As 180° pulses are applied on longitudinal magnetization, all sequences related to this scheme will be termed with the generic name ZEST (for *Z*-axis excitation sculpting techniques). In this scheme, phase cycling is the only way to select the inverted magnetization, since gradient echoes do not affect longitudinal magnetization.

ZEST experiments appear particularly well suited to studying large biomolecules because they partly avoid the sensitivity

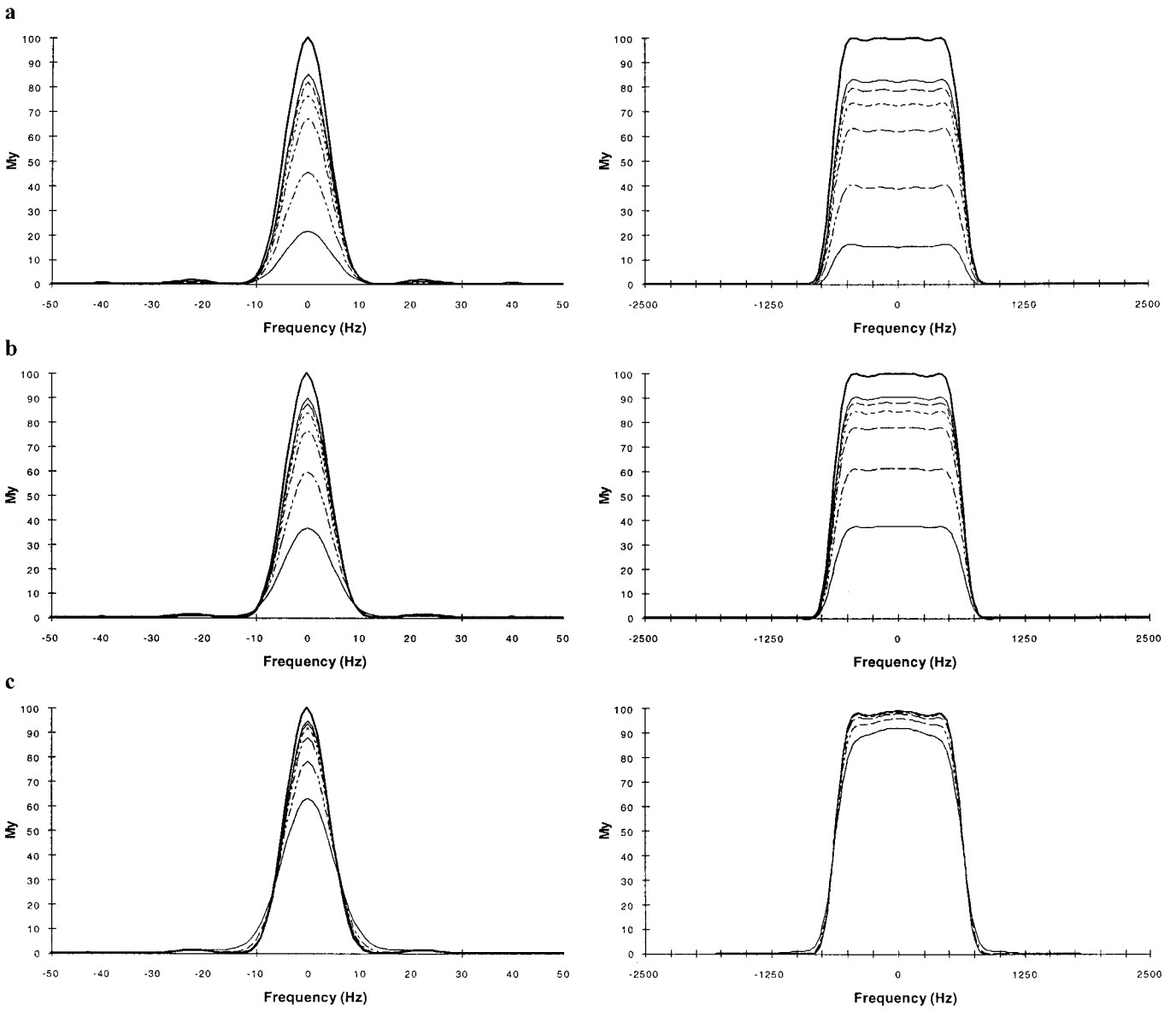

FIG. 2. Overlaid selectivity profiles computed for (a) DPFGSE, (b) BEST, and (c) ZEST experiments with various values of T_2 (infinite T_1). A 60-ms rectangular DANTE train (left) or a 4-ms RE-BURP 32-pulse-width-modulated DANTE train (right) were chosen as the 180° selective pulse. In these numerical simulations, gradient echoes were replaced by spin echoes, with the appropriate phase cycling, in order to keep a constant duration in all sequences. The T_2 values used for calculation were 1, 2, 4, 6, 8, and 10 times the duration for the 180° selective pulse (the solid plot correspond to a "theoretical" profile, i.e., for infinite values of T_2 and T_1). All selectivity profiles in this report have been computed using a program described elsewhere (*29*).

losses due to short transverse relaxation times that would occur in sequences involving multiple echoes. This point has already been discussed in detail (*14*) in the case of pulse-width-modulated DANTE trains, the conclusions being applicable to any kind of inversion or refocusing scheme, and is illustrated in Fig. 2. It shows the effects of short T_2 (infinite T_1) on the selectivity profiles when using either a rectangular or a pulse-width-modulated RE-BURP 180° DANTE train as the selective scheme. For DDANTE-Z, the decrease of the on-resonance magnetization is considerably more limited when compared to the original excitation sculpting sequence described by Hwang and Shaka. A very slight broadening can be observed for DDANTE-Z when extreme conditions are reached ($T_2 \approx$ duration of the 180° pulse): such an effect has been reported for excitation versus inversion pulses (*15*) and has the same origin in the present situation.

DDANTE-Z can be used in place of DANTE-Z in any situation (*3*), keeping its robustness and its "user-friendliness." As an example, we present a more specific application of ZEST techniques for multiplet selection in the case of extremely overcrowded spectra: when $|\nu_A - \nu_X| < |J_{AM} + J_{XN}|$, where X (coupled to spin N) has the closest chemical shift to the

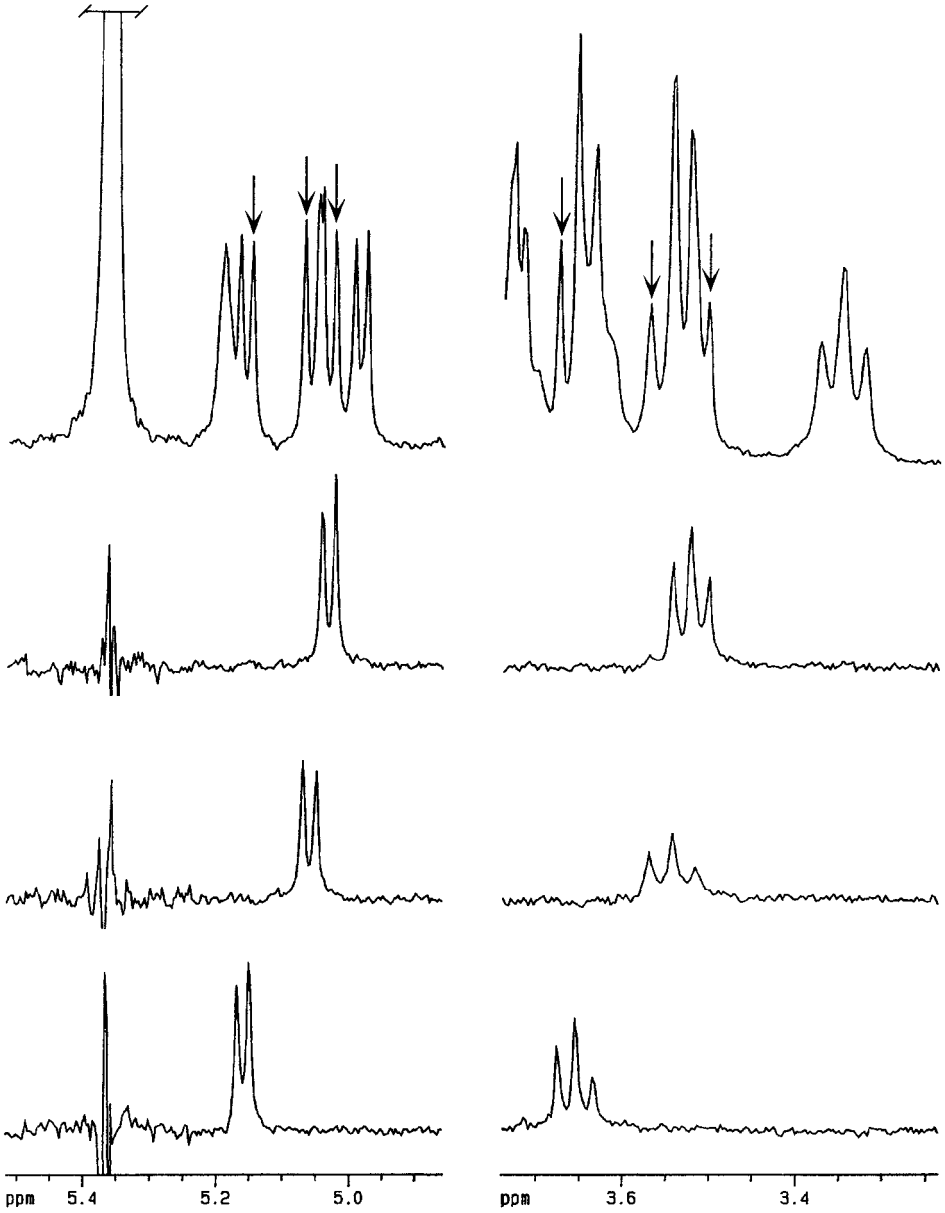

FIG. 3. Application of the DDANTE-Z sequence to multiplet selection in the case of overcrowded spectra. Upper trace: zooms in the anomeric region (left) and in the nonanomeric region (right) of the sugar resonances of the proton spectrum of the saponin SAPO30 (*30*), a heterosidic compound containing seven sugar residues attached to a terpenic (gypsogenin) moiety. The other traces show the result of line selection (indicated with arrows in the conventional proton spectrum) on different multiplets in these regions. Rectangular DANTE train of 600 pulses ($n = 300$, according to Fig. 1) were used for selective inversion, with a nutation delay τ of 100 μs. Note the good suppression of the residual HDO signal, although about 100 times greater than the sugar signals in the proton spectrum. All experiments involved the accumulation of 16 scans (8K complex data points) with a recycle time of 1 s and without the use of PFG. Spectra were recorded at 30°C on a Bruker AMX400 spectrometer equipped with a ^{1}H–^{13}C–^{15}N triple-resonance probe head. The sample concentration was approximately 2 mM in a mixture of D_2O/d_6-pyridin (50:50). No apodization was applied before Fourier transform, and the spectra were not baseline corrected.

nucleus *A* (coupled to spin *M*) under investigation. By matching accordingly the length of the 180° selective pulse, DDANTE-Z can be applied to an individual line: as a result, the whole multiplet will be selected, regardless of the degree of selectivity of the 180° pulse (Fig. 3). The origin of this striking feature has been explained by Bauer and Freeman (*16*) and can be summarized by the following considerations. Depending on the phase cycle step, the two concatenated 180° pulse trains of DDANTE-Z yield either a magnetization state where one of the lines of the multiplet has been inverted (global flip angle of 180°) or an unaffected state of the magnetization (global flip angle of 0 or 360°). When applied to one of the two lines in a doublet, for instance, flipping the on-resonance magnetization to $-z$ leads to a longitudinal order represented by $2I_zS_z$. The

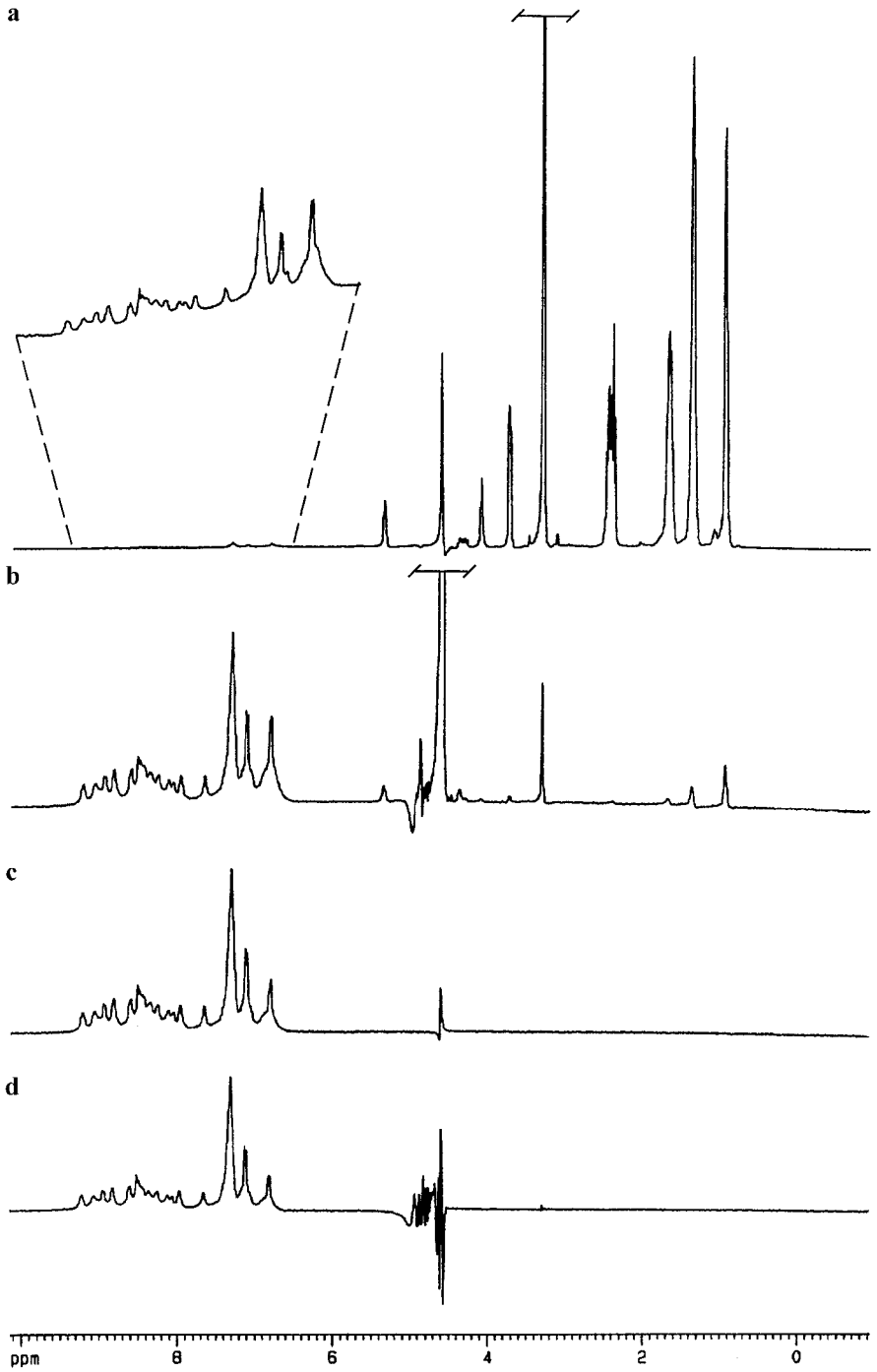

FIG. 4. ^{1}H NMR spectra of protegrin in the presence of DHPC micelles (ratio, 1:50). Spectra were recorded at 25°C on a Bruker AMX400 spectrometer equipped with a *z*-gradient ^{1}H–^{13}C–^{15}N triple-resonance probe head. The peptide concentration, in H_2O/D_2O (90:10) solvent at pH 3.5, was 2 mM. (a) Conventional 1D spectrum. Water suppression was performed by the WATERGATE sequence using a 3–9–19 refocusing pulse (*25*). The insert shows a zoom of the amide–aromatic peptide region with a vertical expansion by about a factor of 100. (b) Selection of the amide region of protegrin using the SPFGSE sequence. (c) Selection of the amide region of protegrin using the BEST sequence described in Fig.1b. (d) Selection of the amide region of protegrin using the DPFGSE sequence; the artifact at the water resonance is due to parasitic refosusing by the second gradient echo of the water magnetization defocused by the first gradient echo. In all of the selective experiments, a 4.8-ms RE-BURP pulse (implemented as a 32-pulse-width-modulated DANTE-train) was used for selective refocusing, without any water-suppression technique. A 3-Hz line broadening was applied before Fourier transform of the FIDs. All spectra result from the coaddition of 128 transients.

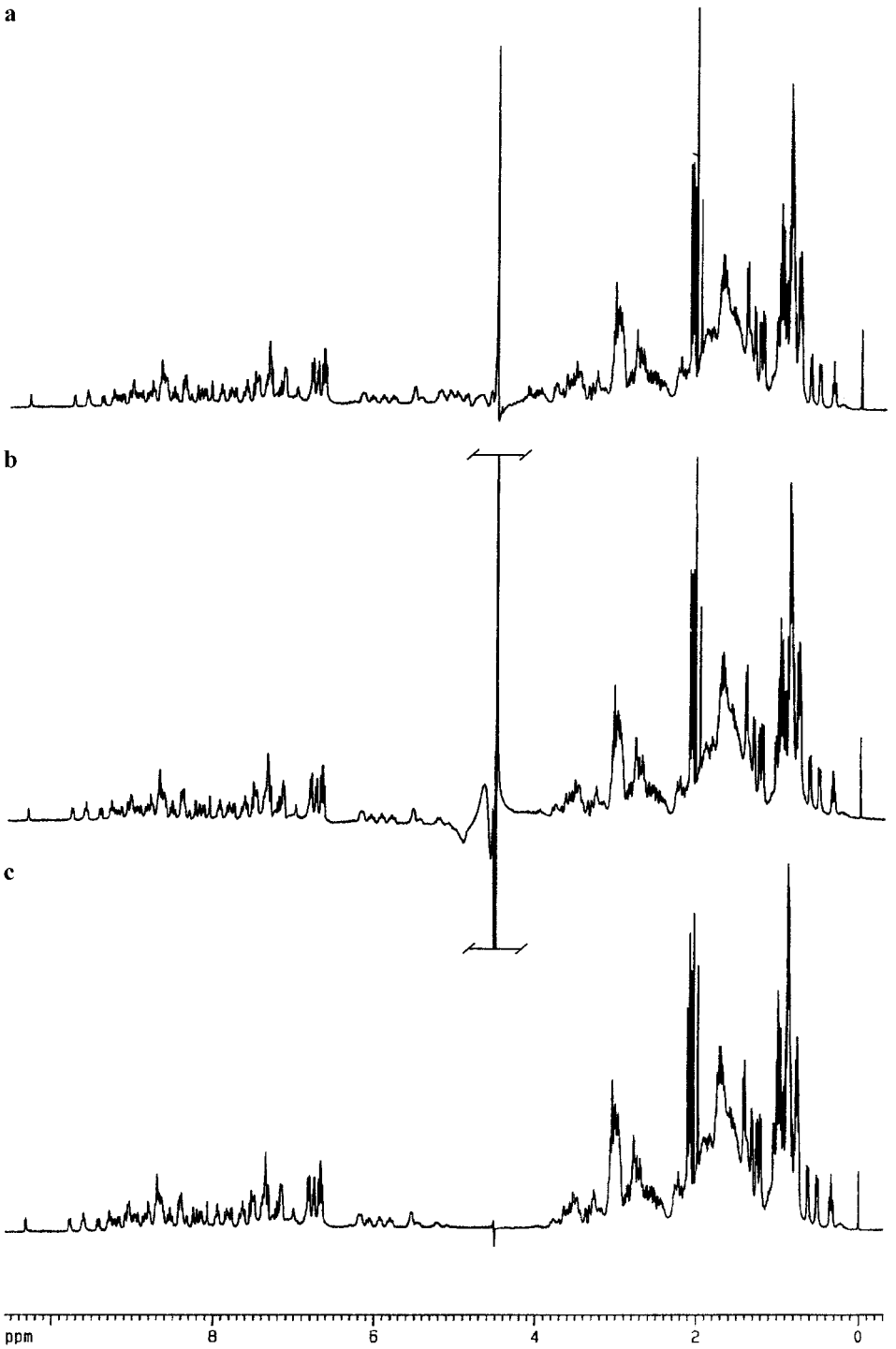

FIG. 5. 1D spectra obtained on a 4 mM sample of γ-cardiotoxin, a 60-residue small protein from *Naja nigricollis* venom (*45*), dissolved in water (10% D_2O, for the lock), at pH 3.5 and 35°C. All spectra (32 transients) were recorded on a Bruker AMX400 spectrometer equipped with a *z*-gradient $^{1}H–^{13}C–^{15}N$ triple-resonance probe head. Water suppression was performed using (a) the WATERGATE sequence, (b) the original double gradient echo excitation sculpting technique proposed by Hwang and Shaka, and (c) the BEST sequence. A W5 pulse train was used as the selective procedure in the three experiments, with τ = 350 μs.

hard 90° read pulse converts this longitudinal order into the unobservable quantity $2I_yS_y$, yielding a spectrum where the corresponding whole multiplet has disappeared. The steps where on-resonance magnetization is unaffected are used only for subtraction purposes and yield an unaffected spectrum. Therefore, the final result, after subtraction, shows up in the

form of an unaffected doublet (with halved intensity). Such a demonstration is valid for any kind of multiplet, but, depending on the inverted line in a triplet, quartet . . . , different types of longitudinal orders are created which may yield different fractions of the magnetization after the 90° read pulse. The possibility of exciting a whole multiplet with DDANTE-Z from the selection of only one line is advantageous when dealing with overcrowded spectra, and immediate applications are 1D selective experiments (1D COSY, 1D *z*-TOCSY, 1D NOESY . . .) which become feasible in the case of entangling multiplets. The design of these ZEST 1D experiments from the original DDANTE-Z is very simple and can be deduced from the corresponding sequences using DANTE-Z (*3*).

The main drawback of the ZEST sequences is the necessity of an extensive phase cycling (16 steps) for eliminating all unwanted magnetization, which is obviously contrary to one of the main goals of selection schemes: the reduction of measuring time. Nevertheless, the phase cycling of the inversion procedures can be significantly decreased using pulse field gradients as a purge, as has already been reported for DANTE-Z (*17*). This has the advantage of reducing the phase cycle of the DDANTE-Z sequence to 4 steps. Another point to be noted is that the phase-cycled techniques only discriminate the selected magnetization from the rejected one by an opposite sign, the selection of the desired magnetization occurring through a subtraction process associated with the phase cycling. As a consequence, the *whole magnetization* evolves in the transverse plane during the acquisition process. Thus, when a huge solvent resonance is present, the ADC dynamic range limits would impose a solvent suppression scheme combined with the selectivity procedure. On the contrary, the gradient echoes in the DPFGSE sequence refocus only the selectively inverted magnetization, all other components being destroyed without the help of a phase cycling. This property pleads in favor of gradient-enhanced sequences for all studies in a protonated solvent.

We thus designed an "hybrid" experiment (Fig. 1b) in order to accommodate the insensitivity to short T_2 of the DDANTE-Z sequence and the advantages conferred by the use of pulsed-field gradient echoes to the DPFGSE sequence. In this experiment, the first 180° DANTE train is applied to longitudinal magnetization, whereas the second one is applied to transverse magnetization. For this reason, we refer to all sequences using this scheme with the generic term BEST (for both axes excitation sculpting techniques). The use of a selective gradient echo confers the water-suppression capability to this experiment, while the use of a pulsed-field gradient as a purge at the end of the 180° inverting DANTE train allows us to reduce its phase cycle to only two steps. As demonstrated in Fig. 2, transverse relaxation during this sequence has an intermediate effect between what is observed for DDANTE-Z and for DPFGSE.

To illustrate the performances of this experiment, we present results obtained on protegrin, an 18-residue antimicrobial peptide (*18*), solubilized in water containing dihexanoic phosphatidylcholine (DHPC) micelles, at high detergent-to-peptide ratio (protegrin/DHPC, 1:50). Detergent solubilization is a common procedure for studying membrane proteins and peptides by means of methods designed for soluble biopolymers. Deuterated detergents are used in most of these studies (for a review, see *19*) in order to fully observe the protein ^{1}H resonances. This limits their scope by the current availability of only a few (and expensive!) detergents in deuterated forms. By an approach based on band-selective excitation, Seigneuret and Lévy (*20*) have demonstrated that protonated detergents can be advantageously used in such an investigation. This approach rests on the fact that the amide and aromatic resonances region in a peptide or protein spectrum is almost invariably free of detergent lines. From the selection of this region, the structure of the solubilized peptide can be solved using f_2 band-selective homonuclear techniques. If the use of DANTE-Z has been originally proposed to achieve this goal, excitation sculpting methods can considerably improve this strategy. Figure 4a shows the nonselective ^{1}H NMR spectrum of the DHPC–protegrin mixture at 25°C: it is dominated by the detergent contribution that obscures the aliphatic and α-proton resonances. The amide–aromatic resonances can be observed but appear very weak and over a strongly distorted baseline. Figure 4b shows the same spectrum, recorded with the SPFGSE (single pulsed-field gradient selective echo) (*21*) sequence applied on the amide region. A 180° RE-BURP pulse-width-modulated DANTE train was used with the aim of a constant excitation over the whole amide resonance spectral range. This sequence allows for a better suppression of both the solvent and the detergent signals than the DANTE-Z sequence originally proposed. The peptide resonances appear now without baseline distortion. Nevertheless, the nonnegligible excitation observed in the suppression region, which is inherent to the RE-BURP pulse selectivity profile, leads to the presence of intense artifacts along a folded diagonal in the 2D f_2 band-selective correlation spectra (NOESY not shown). Figure 4c shows the same spectrum recorded now using the BEST sequence: excitation sculpting affords a perfectly artifact-free spectrum of the amide resonances, with negligible intensity losses compared with the spectrum recorded with a single selective gradient echo. As a consequence, the 2D f_2 band-selective NOESY spectrum recorded with this sequence used in place of the usual read pulse is completely free of skewed diagonal peaks (not shown). Due to the very short T_2 of the peptide amide protons in the protegrin–DHPC comicelles (molecular weight $\approx 30-40$ kDa (*22, 23*)), the use of the DPFGSE sequence entails, for a similar selectivity, a significant loss in signal-to-noise ratio (about 25–30%) (Fig. 4d).

Finally, we present an improvement in excitation sculpting techniques for the purpose of water suppression. Among the numerous techniques proposed and reviewed (*24*), the most popular is nowadays the WATERGATE method (*25, 26*). This sequence is clearly homologous to the SPFGSE (*21*) scheme

used for selective excitation purpose: the only difference arises from the selective 180° pulse which, in the case of WATERGATE, must refocus all resonances except that of water. This selective pulse can be a hard 180° pulse sandwiched by two soft 90° pulses or a frequency selective pulse train of form 3α–τ–9α–τ–19α–τ–19α–τ–9α–τ–3α (denoted 3–9–19 or W3 pulse (*27*)). The delay τ (usually a few hundreds of microseconds) is used to control the null-inversion points, with $62\alpha = 180°$. These two methods give comparable results, although for an equivalent total pulse length the W3 pulse train entails a broader peak elimination region (*27*). Nevertheless, this drawback is largely counterbalanced by its easy implementation, as it does not require any calibration other than the one of a conventional 90° hard pulse.

In one of the very first reports dealing with excitation sculpting applications, Hwang and Shaka advocated a double (instead of a single) selective gradient echo with the goal of improving the WATERGATE sequence (*9*). When using the W3 pulse train, for example, a better water suppression is in principle obtained due to the fact that the resulting frequency-domain profile is just the square of that of W3, leading to a flatter suppression region around zero frequency. Despite its theoretical performances, excitation sculpting is seldom used for water suppression, at least in the context of biological macromolecule studies. In our opinion, there are at least three explanations for that. First, the flat profile at zero frequency is obtained at the expense of a wider suppression region, yielding a substantial reduction of the intensities of peaks not too far from the water resonance, such as those of the α protons in peptides and proteins. Nevertheless, it should be easy to overcome this drawback by using W4 or W5 pulse trains (*27*), which provide considerably narrower noninversion and, moreover, information from these nonexchangeable protons is better obtained from spectra recorded in heavy water. Second, it appears to be rather tricky—at least in our hands—to avoid a partial refocusing by the second gradient echo of the strong water signal defocused by the first gradient echo, even if the gradient levels and durations are under computer control. This is particularly true if all the pulse field gradients are applied on the same axis (z-gradient probes) and/or if the B_1 homogeneity of the probe is not perfect, thus leading to variable values for the nominal pulse flip angles along the sample volume. This partial refocusing of the water signal produces an artifact at zero frequency that deteriorates the quality of the water suppression. Third and more importantly, the duration of the water-suppression scheme by excitation sculpting is twice that of the standard WATERGATE. Indeed, the important duration of gradient echoes (usually a few milliseconds, including the selective(s) pulse(s), the pulse field gradients, and their recovery delays) may render this water-suppression scheme questionable for the study of large biological macromolecules for which the transverse relaxation time is a limiting parameter.

In order to reduce the effects of transverse relaxation, we propose to use the gradient-enhanced BEST sequence where the 180° DANTE pulse trains are replaced by a W3 pulse train (or possibly by a W4 or W5 pulse train, if more selectivity is desired). Each pulse of the first pulse train is divided into two pulses (with halved length), and the phase of each second pulse is alternated every other scan so as to deal with a subtraction process (Fig. 1c). An efficient purging of transverse components existing at the end of the first pulse train (by mean of a single pulsed field gradient) allows us to restrict the phase cycling to only a twofold increase compared with the WATERGATE sequence. Since the first pulse train is applied along the z-axis, the magnetization losses due to T_2 effects are considerably reduced, and the resulting peak intensities are comparable to those obtained with the conventional WATERGATE sequence when using a gradient echo of similar characteristics. On the other hand, the resulting suppression profile is considerably enhanced (Fig. 5c) and virtually identical to what was predicted for the excitation sculpting scheme of Hwang and Shaka (*9*), but without the artifacts arising from parasitic refocusing (Fig. 5b).

CONCLUSION

The use of a double gradient echo for selective excitation through excitation sculpting techniques involves some limitations due to severe sensitivity losses by short transverse relaxation times. In the present paper, we have demonstrated that excitation sculpting is not limited to transverse magnetization, but can be transposed to longitudinal magnetization (ZEST experiments), where it becomes governed by the generally much longer longitudinal relaxation times. Excitation sculpting of longitudinal magnetization implies, however, phase cycling and, by contrast with gradient-enhanced experiments, ZEST experiments must be appended by a water-suppression scheme when used in a protonated solvent. We thus designed the hybrid (BEST) experiments, where both longitudinal and transverse magnetization are "sculpted." Such experiments are relatively immune to transverse relaxation effects and include the capability of water suppression.

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