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Gradient-Enhanced One-Dimensional Proton Chemical-Shift Correlation with Full Sensitivity

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High-quality spectra were obtained by implementing pulsed field gradients (PFGs) as part of 1D selective experiments. The use of PFGs for coherence rejection rather than coherence selection ensures that there is no loss of signal and the sensitivity of these experiments is the same as that of their phase-cycled predecessors. The excitation scheme chosen ensures that these experiments are highly resistant to spin–spin relaxation. The following techniques are described: 1D ge-TOCSY, 1D ge-NOESY, 1D ge-TOCSY–TOCSY, 1D ge-NOESY–NOESY, 1D ge-TOCSY–NOESY, and 1D ge-NOESY–TOCSY. Their applications, for the separation of overlapping spin systems, tracing spin-diffusion signals, and extending the transfer of magnetization beyond an individual spin system, are illustrated using oligo- and polysaccharide samples.

INTRODUCTION

Proton chemical-shift correlation was greatly facilitated by the introduction of two-dimensional NMR spectroscopy (*1, 2*). Although feasible, adding a third dimension (*3–5*) did not prove as beneficial for homonuclear experiments as it did for heteronuclear NMR spectroscopy. Reduced digital resolution in indirectly detected dimensions and increased measurement times are the principal limitations of homonuclear 3D methods. On the other hand, one-dimensional methods which use some sort of selective excitation for the simplification of spectra have attracted considerable attention. Numerous 1D analogs of 2D and 3D techniques as well as novel 1D methods have been designed to date (*6–19*).

Relying purely on phase cycling for the selection of coherence pathways, previous 1D selective methods were liable to suffer from cancellation artifacts. This has changed by the introduction of pulsed field gradients (PFGs) (*20, 21*). Spectra in which coherence pathways are selected by PFGs do not contain cancellation artifacts since signals which would give rise to them are not digitized at all. Most of the modifications of phase-cycled 1D selective techniques which have appeared recently in the literature followed this approach (*22–29*). On the negative side, such spectra usually contain only half of the signal, which is likely to be further attenuated by incomplete gradient rephasing due to molecular diffusion. An alternative approach is to implement PFGs for coherence rejection (*30*). Methods which employ PFGs for coherence rejection do not suffer from sensitivity losses associated with most of the coherence selection methods. Although coherence rejection methods still rely on phase cycling for the final coherence selection, the intensity of their cancellation artifacts is greatly reduced since the unwanted coherences have been attenuated significantly by the action of PFGs. The double pulsed-field-gradient spin-echo (DPFGSE) sequence, originally developed for water suppression (*31*), is the basis of 1D coherence rejection selective methods published to date (*32–34*).

The loss of sensitivity due to gradients is particularly severe in 1D selective methods which combine several polarization-transfer periods, each containing a gradient-selection step (*29*). Combined losses from concatenation of two steps results in the reduction of the signal by a factor of four, without taking into account the loss of magnetization due to molecular diffusion. In the present work we demonstrate how PFGs can be implemented as part of 1D selective techniques which concatenate several polarization-transfer steps without compromising their sensitivity. The methods do not contain the DPFGSE module and were designed to keep the T_2 relaxation losses at a minimum. The techniques discussed include 1D ge-TOCSY and 1D ge-NOESY as building blocks, and their combinations 1D ge-TOCSY–TOCSY, 1D ge-NOESY–NOESY, 1D ge-TOCSY–NOESY, and 1D ge-NOESY–TOCSY.

EXPERIMENTAL

All spectra were acquired using a Varian INOVA 600 MHz NMR spectrometer equipped with a waveform generator for the generation of selective pulses and a 5 mm *z*-axis pulsed-field-gradient triple-resonance probe. The temperatures used were 25°C for the model trisaccharide **1,** 30°C for the polysaccharide **2** isolated from *Proteus mirabilis*

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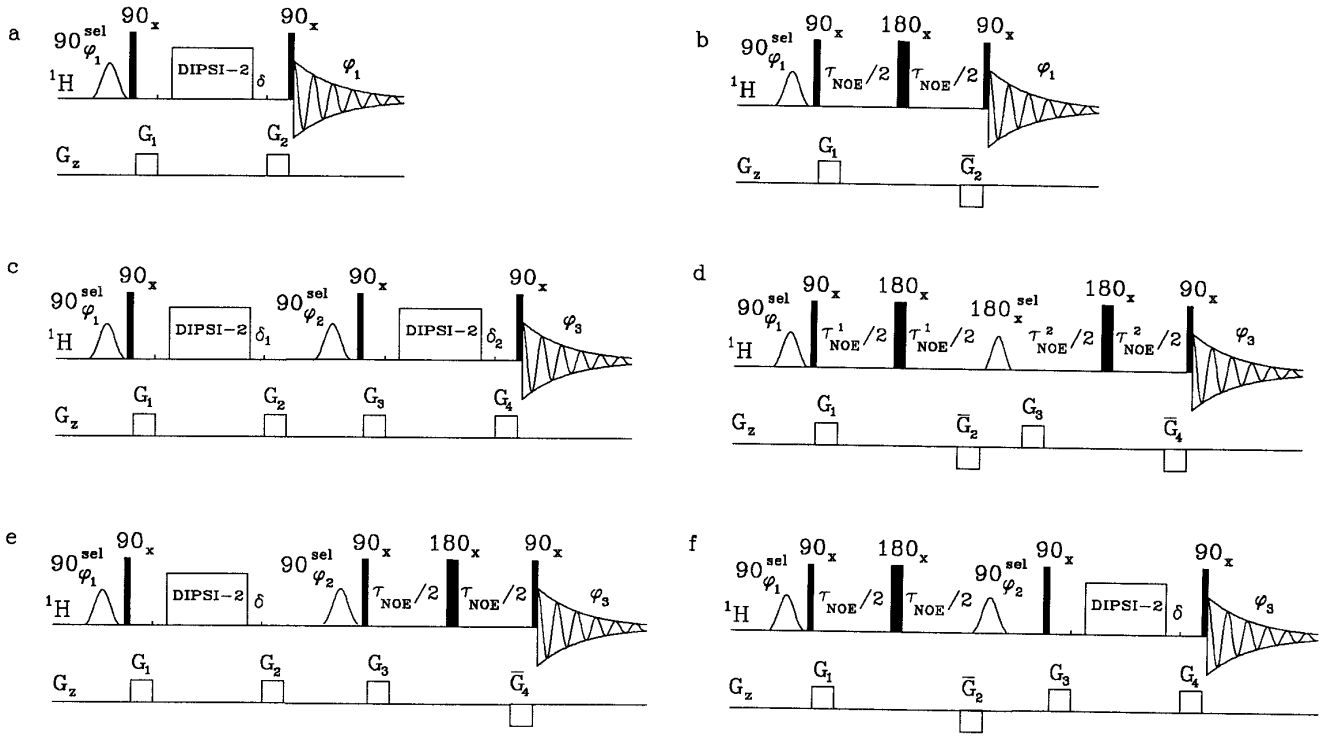

FIG. 1. Pulse sequences of 1D selective gradient-enhanced chemical-shift-correlated experiments: (a) 1D ge-TOCSY, (b) 1D ge-NOESY, (c) 1D ge-TOCSY–TOCSY, (d) 1D ge-NOESY–NOESY, (e) 1D ge-TOCSY–NOESY, and (f) 1D ge-NOESY–TOCSY. The pulsed field gradients were 1 ms in length and had the following strengths: $G_1 = 8.0$ G/cm, $G_2 = 6.4$ G/cm, $G_3 = 11.0$ G/cm, and $G_4 = 8.8$ G/cm. The following phase cycling was applied: $\varphi_1 = x, -x$, $\varphi_2 = 2x, 2(-x)$, $\varphi_3 = x, 2(-x), x$. The 180° selective pulse in (d) is applied for two scans on-resonance and for two scans off-resonance. The delay, δ, in TOCSY experiments can be optimized for suppression of ROESY peaks in macromolecules or made variable for removal of antiphase components of multiplets.

strain 7570 (*35*), and 42°C for polysaccharide **3** isolated from *Proteus mirabilis* O:57 (*36*). The sample quantities of **1, 2,** and **3** were 5, 8 , and 10 mg, respectively, dissolved in 0.6 ml of D_2O. The RF strength for the DIPSI-2 sequence (*37*) was 12.8 kHz.

RESULTS AND DISCUSSION

The reduction in the intensity of cancellation artifacts by the use of PFGs as a means of coherence rejection is illustrated by comparison of gradient-enhanced and purely phase-cycled 1D TOCSY spectra, acquired using the pulse sequence of Fig. 1a. In this experiment the magnetization of a selected proton is restored along the *z* axis by the action of the initial 90° selective and nonselective pulses applied along the same axis. This leaves the magnetization of all other protons in the *xy* plane where it is dephased by PFGs before and after the DIPSI-2 pulse train. Only the magnetization spin-locked along the *z* axis remains unaffected by the gradients. A two-scan phase cycle is required to complete the selection process and to cancel any signals transferred to the *z* axis before the final read pulse due to either relaxation or pulse imperfections.

Four 1D TOCSY spectra were acquired using the trisaccharide **1** which contains three Me groups. Protons from these groups yield sharp, intense singlets which usually leave visible cancellation artifacts in difference spectra. Even larger residual signals might appear at the resonance frequency of the solvent, in this case from the HOD protons. One- and two-scan spectra of **1** acquired according to the pulse sequence of Fig.1a using zero and nonzero gradient strengths are shown in Fig. 2. The intensity of residual signals in the one-scan gradient spectrum (Fig. 2c) is comparable to that of the cancellation artifacts detected in the two-scan phase-cycled spectrum (Fig. 2b). Clearly, the two-scan gradient spectrum (Fig. 2d) is superior to its two-scan phase-cycled analog (Fig. 2b). The intensity of the signals is identical in both spectra, whereas the cancellation artifacts are barely visible in the former spectrum. A 4000-fold suppression of the HOD signal achieved between spectra (a) and (d) is modest by comparison with water-suppression techniques (*31, 32*) used for H_2O samples, but nevertheless is sufficient for the current application.

A prerequisite for the application of selective 1D methods is the possibility of selectively exciting one proton. As pointed out earlier (*16*), this condition can be relaxed to

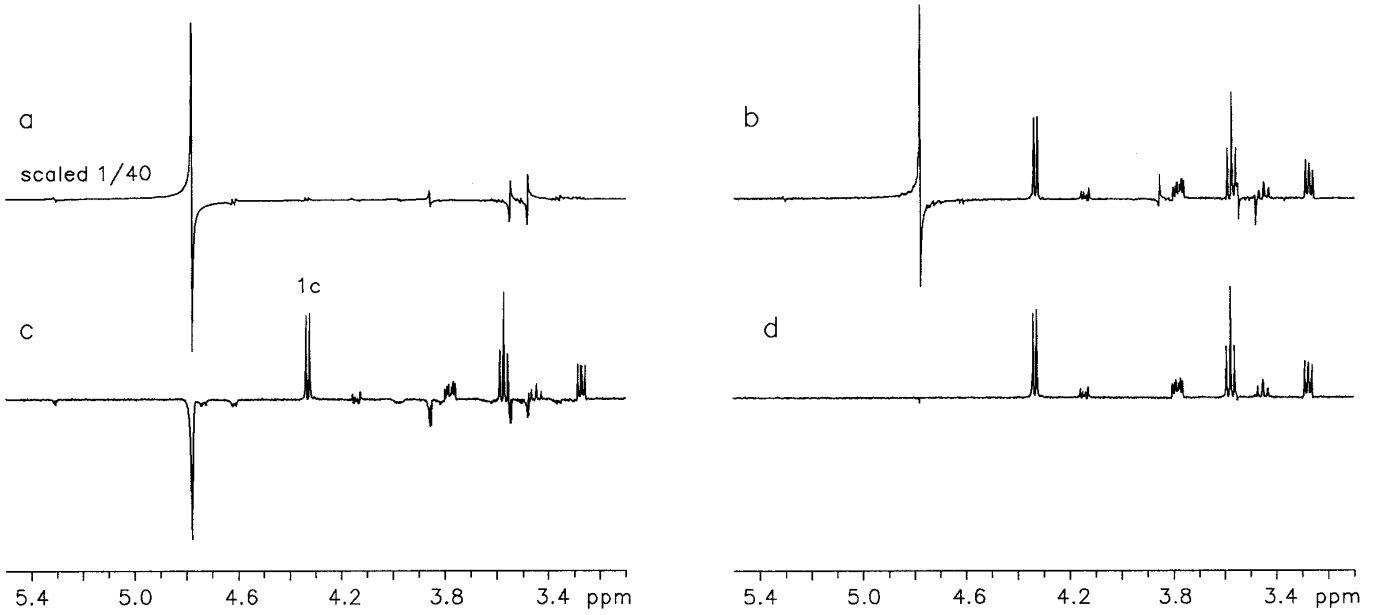

FIG. 2. Effect of PFGs on the quality of 1D TOCSY spectra of **1.** Spectra were acquired with a 79.0 ms spin-lock time using the pulse sequence of Fig. 1a and selective excitation of H-1c by a 104 ms quiet-SNEEZE pulse. The pulsed-field-gradient strength was $G_1 = 8.0$ G/cm and $G_2 = 6.4$ G/cm for (c,d) and zero for (a,b). The number of scans was one (a,c) and two (b,d). Spectra are plotted in absolute intensities with (a) scaled down 40 times.

some extent, for methods containing more than one selection step. Initial overlaps not eliminated during the first selective steps can be removed during the second selective transfer. It should be noted, however, that complications might arise if two coupled spins are excited at the same time by the first selective pulse (*38*). In the following example the concatenation of two TOCSY steps is illustrated. A semiselective TOCSY transfer initiated from overlapping resonances, followed by a selective TOCSY transfer from one of the resulting well-resolved spins, yields the desired separation of individual spin systems. Even if two or more protons have identical chemical shifts, a potential problem for 2D methods, their spin systems can be separated by a 1D TOCSY–TOCSY experiment.

It is straightforward to construct a 1D ge-TOCSY–TOCSY pulse sequence (Fig. 1c) by replacing the read pulse of the 1D ge-TOCSY by the same sequence. The second selective 90° pulse is applied to an isolated proton revealed by the first transfer. This can be done either by imposing a phase ramp (*39, 40*) on the second selective pulse or simply by changing the carrier frequency. Since this change is made when relevant magnetization is along the *z* axis, no phase adjustment to either pulse is needed. A minimum four-scan phase cycle needs to be applied in order to select for the spin system in which two protons have experienced selective pulses. PFGs are again used only for dephasing the transverse magnetization.

The method is illustrated for polarization transfer from two overlapping resonances in the high-field region of the proton spectrum of **1.** Protons at 3.3 ppm were selectively excited by a 90° quiet-SNEEZE pulse (*41*) (Fig. 3b) and a 35.9 ms mixing time 1D ge-TOCSY spectrum was acquired (Fig. 3c). In two subsequent 1D ge-TOCSY–TOCSY experiments, the second selective pulse was applied to isolated resonances at 4.35 and 3.99 ppm, respectively. These protons belong to different spin systems; consequently two subspectra (Figs. 3d and 3e) were obtained by these experiments.

It should be noted that alternative methods using selective spin-lock fields (*42, 43*) can be applied, in principle, for separating spin systems where initial overlap exists. The attractive feature of this approach is the possibility of suppressing the leakage of magnetization caused by passive coupling constants by means of additional selective RF fields (*44, 45*).

Selecting for the transfer of magnetization along the *z* axis in the 1D ge-TOCSY and 1D ge-TOCSY–TOCSY experiments allows *z* filtration (*46*) to be used for the removal of antiphase magnetization without additional pulses. This is achieved by varying the lengths of intervals surrounding the spin-lock period (*47*). A regular incrementation of the δ_2 interval preceding the read pulse of the 1D ge-TOCSY–TOCSY was applied to acquire a *z*-filtered 1D ge-TOCSY–TOCSY spectrum of **1** (Fig. 3f) using parameters otherwise identical to those for the spectrum of Fig. 3e.

A fixed δ interval can serve a different purpose when acquiring TOCSY spectra of macromolecules. By setting δ equal to one-half of the mixing time, ROE peaks created during the spin lock are canceled out by opposite phase NOE peaks originating during the δ interval.

Besides concatenating two coherent transfers in a 1D ge-

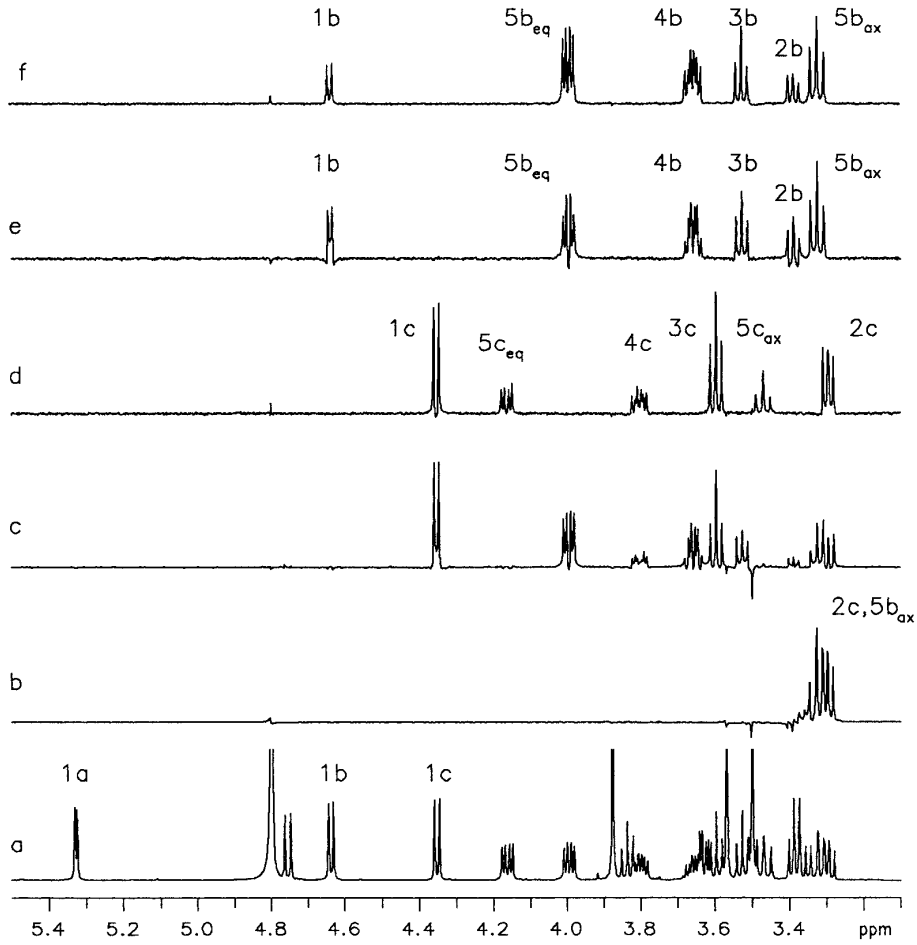

FIG. 3. Illustration of the 1D ge-TOCSY–TOCSY technique on compound **1.** (a) An eight-scan ^{1}H spectrum of **1;** (b) selective excitation of H-2c and H-5b$_{ax}$ by a 104 ms quiet-SNEEZE pulse. The number of transients (NT) was eight. (c) 1D ge-TOCSY spectrum of **1** with selective excitation as in (b) followed by a 35.9 ms mixing time, NT = 4; (d) 1D ge-TOCSY–TOCSY spectrum of **1** obtained using the pulse sequence of Fig. 1c and selective excitation by two quiet-SNEEZE pulses at H-2c/H-5b$_{ax}$ and H-1c, respectively. The second mixing time was 93.4 ms, NT = 16; In (e), the second selective pulse was applied to H-5b$_{eq}$. The *z*-filtered spectrum (f) was acquired using parameters similar to those for spectrum (e) except that the δ_2 delay was incremented 16 times in steps of 1 ms. The total number of transients was 64.

TOCSY–TOCSY, two incoherent polarization transfers can be combined to form a 1D ge-NOESY–NOESY experiment. This 1D analog of the 3D NOESY–NOESY experiment (*48*) can be used for tracing the spin-diffusion pathways in macromolecules.

The NOESY building block of this sequence, 1D ge-NOESY (Fig. 1b), is similar to the 1D DPFGSE–NOESY sequence (*32*). They differ only in the initial selective-excitation scheme. In both sequences, PFGs and one or more nonselective inversion pulses strategically placed during the mixing interval are used for the reduction of unwanted signals (*49*). In contrast to the DPFGSE-based methods (*32–34*) where all spins are flipped into the *xy* plane before the selection of the magnetization, in the 1D ge-NOESY, the purging by means of PFGs starts after the magnetization of the selected spin was returned to the *z* axis. This is a feature common to all pulses sequences proposed here. In the DPFGSE method, it is predominantly spin–spin relaxation which determines the relaxation-related losses, while in the proposed pulse sequences these can be minimized by a proper choice of a 90° selective pulse which is resistant to relaxation (*50*). Although not a uniformly exciting pulse, the half-Gaussian pulse (*51*) combines high selectivity with high resistance to spin–spin relaxation and was therefore used during this study for selective excitation of signals in polysaccharides. In order that the large dispersive component created by the half-Gaussian pulse be removed by PFGs and phase cycling, any phase difference between the selective and nonselective pulses must be eliminated (*8*). We have found the half-Gaussian pulse sufficient when a qualitative interpretation of the data was required. For quantitative interpretation of NOE spectra, the use of a uniformly exciting pulse is recommended.

Using the 1D ge-NOESY as a building block, the mixing

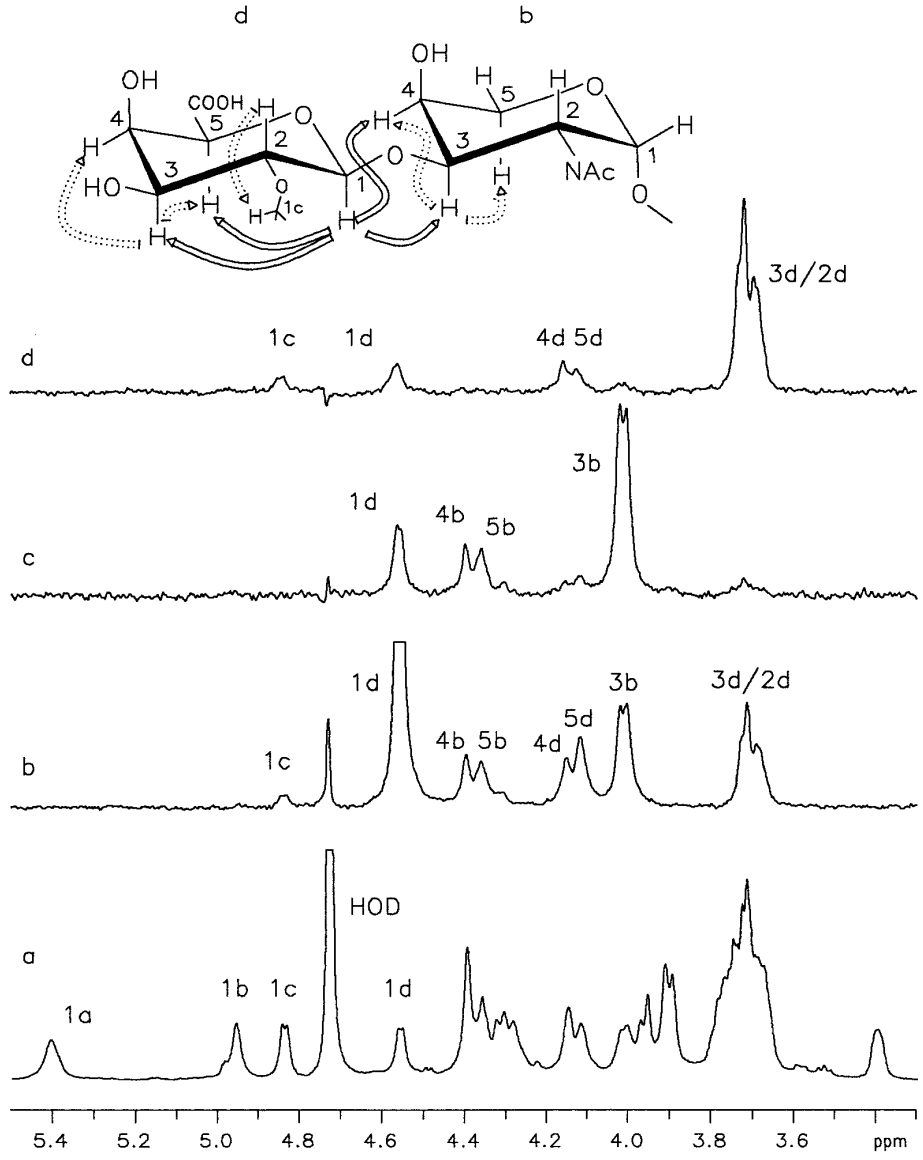

FIG. 4. Tracing the spin diffusion in NOESY spectra of **2** by the 1D ge-NOESY–NOESY technique. (a) ^{1}H spectrum of **2,** NT = 4; (b) 1D ge-NOESY spectrum of **2.** H-1d was selectively excited by a half-Gaussian pulse of 43.5 ms. A mixing time of 200 ms was used, NT = 16. (c) 1D ge-NOESY–NOESY spectrum (NT = 128) of **2** acquired using the pulse sequence of Fig. 1d. The 90° and 180° selective pulses were a 43.5 ms half-Gaussian and a 65 ms i-SNOB-2 (*50*) pulse applied at H-1d and H-3b frequencies, respectively. In spectrum (d) the 180° selective pulse was applied to H-3d/2d protons. The first and the second mixing times were 100 and 50 ms. A partial structure of **2** is given in the inset with direct and mediated NOESY transfers indicated by solid and dotted lines, respectively.

interval of a 1D ge-NOESY–NOESY experiment is divided into two periods, which generally are not equal, separated by a selective inversion pulse (Fig. 1d). This pulse is applied in two of every four scans to a proton which shows an intense NOE with the selectively excited proton in a 1D ge-NOESY experiment. The NOE buildup continues during the second mixing interval, but only NOE signals from the selectively inverted proton created during this interval are selected for by the phase cycling.

The 1D ge-NOESY–NOESY technique is illustrated by tracing the spin diffusion observed in the 1D NOESY spectra of the polysaccharide **2.** Usually three or four protons show NOE contact with an anomeric proton of a nonterminal β-D-hexopyranose residue. These are H-3 and H-5 protons from the same residue, a proton across the glycosidic linkage and, sometimes, one of the vicinal neighbors of this proton, especially if equatorial. Significant spin diffusion was therefore suspected when seven signals were detected in a 200 ms mixing time 1D ge-NOESY spectrum with selective excitation of H-1d (Fig. 4b). The two most intense signals ap-

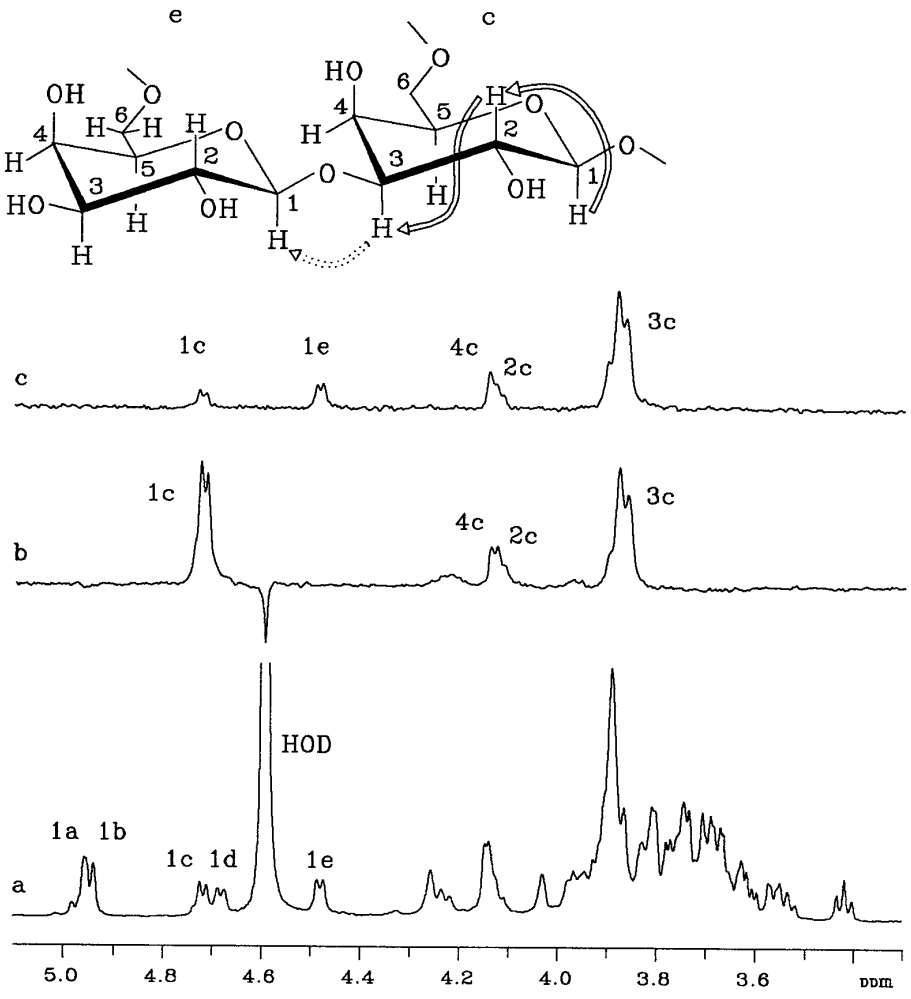

FIG. 5. Illustration of the 1D ge-TOCSY–NOESY technique on polysaccharide **3.** (a) ^{1}H spectrum of **3,** NT = 4; (b) 1D ge-TOCSY spectrum of **3,** NT = 8; H-1c was selectively excited by a 57 ms half-Gaussian pulse, the mixing time was 57.5 ms, and the δ delay was 28.8 ms; (c) 1D ge-TOCSY–NOESY spectrum (NT = 64) of **3** acquired using the pulse sequence of Fig. 1e. Selective pulses preceding the TOCSY and the NOESY periods were half-Gaussian pulses of 57 and 43.5 ms applied to H-1c and H-3c protons, respectively. Mixing times were 57.5 ms for the TOCSY and 250 ms for the NOESY transfer; the δ delay was 28.8 ms . A partial structure of **3** is given in the inset with TOCSY and NOESY pathways indicated by solid and dotted lines, respectively.

peared at 4.00 ppm (H-3b) and 3.70 (strongly coupled H-3d/2d). These were selectively inverted in two consecutive 1D ge-NOESY–NOESY experiments. The lengths of the first and the second mixing times were set to 100 and 50 ms, respectively. Signals of H-1d, H-4b, and H-5b were observed in the spectrum (Fig. 4c) after selective inversion of H-3b. The presence of the resonance H-5b in this spectrum implies that this proton appeared in the 1D ge-NOESY spectrum of Fig. 4b because of spin diffusion rather than due to the direct NOE between H-1d and H-5b. This is partially true for the H-4b signal, but in this case, because of 1d–3b glycosidic linkage, there is a high probability of a direct NOE between H-1d and H-4b. Consequently, signal of H-4b in the 1D NOESY spectrum is a mixture of direct and indirect transfer. A 1D ge-NOESY–NOESY spectrum of **2** with selective inversion of H-2d/3d (Fig. 4d) shows signals of H-1c, H-1d, H-4d, and H-5d. Based on this experiment, it can be concluded that the H-5d signal in the 1D NOESY spectrum is a mixture of direct NOE from H-1d and spin diffusion mediated by H-3d. The other two signals of H-4d and H-1c are pure spin-diffusion signals mediated by the H-3d proton. The presence of the H-1c signal in the NOESY–NOESY spectrum of Fig. 4d was initially interpreted as indicative of the 1c–3d linkage. However, when higher-order effects between the H-3d and H-2d protons were established, the presence of a 1c–2d linkage was eventually confirmed by heteronuclear experiments.

This example illustrates the fact that interpretation of 1D NOESY spectra of polysaccharides may be complicated by spin-diffusion and higher-order effects. The 1D NOESY–

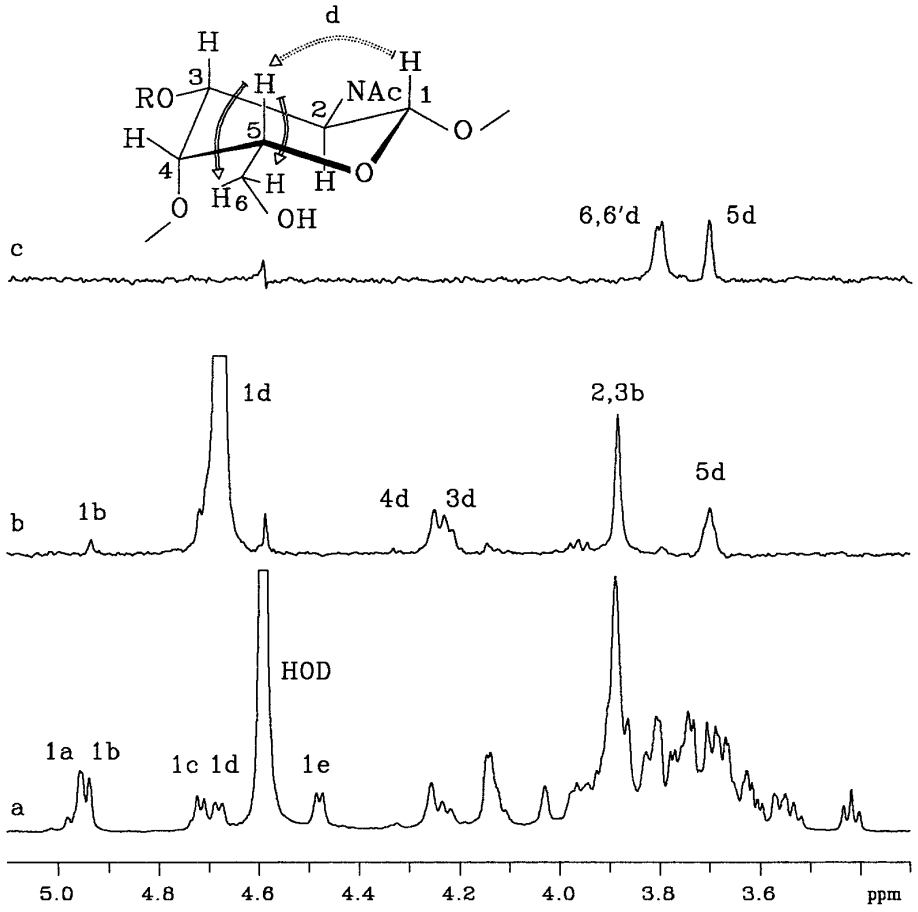

FIG. 6. Illustration of the 1D ge-NOESY–TOCSY technique on compound **3.** (a) ^{1}H spectrum of **3,** NT = 4; (b) 1D ge-NOESY spectrum of **3** (NT = 16); H-1d was selectively excited by a 57 ms half-Gaussian pulse; the mixing time was 250 ms. (c) 1D ge-NOESY–TOCSY spectrum (NT = 128) of **3** acquired using the pulse sequence of Fig. 1f. Selective pulses preceding the TOCSY and the NOESY period were half-Gaussian pulses of 57 and 43.5 ms applied to H-1d and H-5d protons, respectively. Mixing times were 250 ms for the NOESY and 48 ms for the TOCSY transfer; the δ delay was 24 ms. A partial structure of **3** is given in the inset with NOESY and TOCSY transfers indicated by dotted and solid lines, respectively.

NOESY technique can provide assistance in identification and assignment of spin-diffusion signals.

The transfer of magnetization can be extended beyond individual spin systems when coherent and incoherent polarization-transfer mechanisms are combined (*14–19*). The 1D ge-TOCSY and 1D ge-NOESY sequences may be used as building blocks: thus the 1D ge-TOCSY–NOESY and 1D ge-NOESY–TOCSY (Figs. 1e and 1f) are obtained by replacing the last read pulse of a 1D ge-TOCSY by a 1D ge-NOESY sequence and vice versa. Once again the second 90° selective pulse is applied at the frequency of the proton chosen for the second polarization transfer when the relevant magnetization is along the *z* axis. Both methods are illustrated using polysaccharide **3** consisting of five monosaccharide residues per repeating unit.

The use of the 1D ge-TOCSY–NOESY is illustrated by the unambiguous identification of the 1e–3c linkage in **3.** The 1D ge-NOESY spectrum with selective excitation of the H-1e proton (data not shown) reveals its NOE contact with a proton at 3.89 ppm, but as five protons resonate within a range of $\pm$0.01 ppm, it is not possible to assign this signal unambiguously. The 1D ge-TOCSY experiment with selective excitation of H-1c yields a similarly shaped signal at 3.89 ppm (Fig. 5b). Based on experiments with different mixing times, this signal is assigned to H-3c. When a selective NOESY transfer is initiated from this proton in a 1D ge-TOCSY–NOESY experiment, an intense signal of H-1e is observed (Fig 5c), which proves the existence of the 1e–3c linkage.

The use of the 1D ge-NOESY–TOCSY method is illustrated by assignment of signals of residue **d.** The 1D ge-NOESY spectrum of **3** with selective excitation of proton H-1d (Fig. 6b) shows among others the signal of H-5d. This proton is used for the consecutive TOCSY transfer in a 1D ge-NOESY–TOCSY experiment. Regardless of the length of the TOCSY mixing time used, only signals of H-6,6′d are observed (Fig. 6c). This is due to a very small coupling constant $J_{4d,5d} < 1$ Hz which created a ''bottleneck'' for the

TOCSY transfer. For the same reason, a 1D ge-TOCSY spectrum with selective excitation of H-1d shows only signals up to proton H-4d (data not shown). Combination of the above 1D experiments identified the **d** residue as β-D-galactopyranose and allowed assignment of all its protons.

In conclusion, we have shown a simple way for implementing pulsed field gradients as a part of the phase-cycled 1D analogs of *n*D experiments. These methods increase the quality of spectra at no expense to their sensitivity and are designed to keep relaxation-related losses at a minimum. Although concatenation of only two polarization steps was illustrated, it is straightforward to extend the chain of polarization transfers further (*18*).

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