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# Pseudo-3D NMR spectroscopy: Application to oligo- and polysaccharides

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## SUMMARY

Several pseudo-3D NMR experiments are proposed for removal of overlaps in 1D $^1$H NMR spectra. A selective pulse and a chemical-shift-selective filter are used for double selection of the magnetization during the course of the pulse sequence. Different polarization transfer mechanisms are combined into pseudo-3D COSY-RELAY, COSY-TOCSY, COSY-NOESY, COSY-ROESY, RELAY-NOESY, RELAY-ROESY, RELAY-RELAY and RELAY-TOCSY experiments. The techniques are illustrated on oligo- and polysaccharide samples.

A major problem in the proton resonance assignment of saccharides is the presence of severe signal overlap in the spectral region between 3 and 4 ppm. In contrast, the anomeric proton resonances are usually well separated and therefore commonly used as a spectral window in the analysis of $^1$H NMR spectra of saccharides. With the aid of 2D relayed correlation spectroscopy (Eich et al., 1982) and/or 2D homonuclear Hartmann–Hahn spectroscopy (Braunschweiler and Ernst, 1983; Davis and Bax, 1985a), the link from anomeric to other ring protons can be established (Homans et al., 1984,1987). Equally important, NOEs from anomeric protons (Lemieux, 1980) provide the glycosidic linkage information in the molecule.

The special role of anomeric protons has been used in recent applications of a selective 2D NMR spectroscopy approach for targeting the anomeric region of saccharides. (Homans, 1990, Berthault et al., 1991; Bricher et al., 1991; Nuzillard and Maissiot, 1991). Alternatively, 2D NMR techniques converted into their 1D analogues (Bauer et al., 1984; Davis and Bax, 1985b; Kessler et al., 1986, 1989) have been used (Perly et al., 1987; Subramanian and Bax, 1987; Bazzo et al., 1990; Bircher et al., 1990). An obvious limitation of selective NMR techniques, especially their

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1D versions, is the signal separation needed for their selective excitation by soft pulses. Chemical-shift-selective filtration (CSSF) (Hall and Norwood, 1988a,b; Batta and Kövér, 1991; Uhrín et al., 1992), which relies exclusively on chemical shift differences, therefore provides a more versatile strategy. However, if complete degeneracy of chemical shifts occurs, this method also fails.

In principle, this degeneracy can be eliminated by means of 3D NMR spectroscopy. However, in most spectra overlaps of anomeric proton resonances usually do not involve more than two or three signals. The use of specially designed 1D experiments is therefore most appropriate for these cases.

A 1D experiment, which is designed to isolate spin systems involving completely overlapping signals at the beginning of the polarization transfer pathway, must incorporate at least two elements of selectivity. In the current application, a selective pulse and CSSF were used to achieve this goal. The pulse sequences proposed start with a partially selective COSY transfer from all overlapping anomeric protons to the neighbouring H-2 protons. If the chemical shift difference between H-2 protons is sufficient (approx. > 5Hz), the transverse magnetization is filtered through this spectral parameter prior to the next event in the sequence. In order to enable CSSF, the non-selective COSY pulse must be applied at one of the H-2 frequencies obtained from a preliminary 1D COSY. The necessary phase relationship between the selective and non-selective COSY pulses is maintained by shifting the phase of a soft pulse by $-2\pi\,(\nu_0 - \nu_2)\,\tau_0$, where $\nu_0$ and $\nu_2$ are the frequencies of the soft and hard pulse, respectively, and $\tau_0$ is the separation between these pulses in seconds. Filtration is achieved by a gradual displacement of a 180° $^1$H pulse from the middle of a successive delay which, according to the need of a particular experiment, is optimized to yield maximum anti or inphase magnetization of the H-2 protons. After filtration, an appropriate polarization transfer mechanism is used to yield combined experiments such as COSY-RELAY, COSY- TOCSY or COSY-NOESY. In accord with the nomenclature introduced by Boudot et al. (1990), the adjective pseudo-3D is used for these techniques, which contain two selectivity elements.

In the first application, coherent transfer via antiphase magnetization was applied after filtration, yielding a pseudo-3D COSY-RELAY experiment (Fig. 1a). The method is illustrated for the separation of the spin systems of two terminal β-glucopyranose residues of a modified LPS 1 containing a total of nine saccharide units (Masoud, H. and Richards, J.C., unpublished results). A partial structure of this oligosaccharide is given in the legend of Fig. 2. The anomeric proton resonances of the two β-glycopyranoses overlapped almost completely, with a chemical shift difference of only 1.9 Hz, while the corresponding H-2 resonances were separated by 55.0 Hz. The length of the filtration interval, $\tau_1$, was adjusted to yield a maximum antiphase magnetization of H-2s with respect to H-3s. Because the transverse magnetization of only two H-2 resonances was created by the initial selective COSY step, it was not necessary to run a complete CSSF for each spin system separately. Instead, more effectively, only two spectra were acquired which differed by 180° in the phase of one of the spin systems involved. In the first experiment, a 180° $^1$H pulse was applied in the middle of the $\tau_1$ interval and in the second this pulse was displaced by $0.25/(\nu_2-\nu_{2'})$ from the centre of $\tau_1$, yielding a 180° chemical shift evolution for the H-2′at $\nu_{2'}$, which was off the carrier frequency $\nu_2$. The sum and the difference of these two spectra leave the resonances of both glucose units separated. One- to four-step pseudo-3D COSY-RELAY experiments were performed in order to assign the resonances of both β-glucopyranose residues. The three-step RELAY spectra are shown before (Figs. 2b,c) and after (Figs. 2d,e) the editing. Because of higher order effects associated with the H-2 and H-3 resonances of Glc-II, the corre-

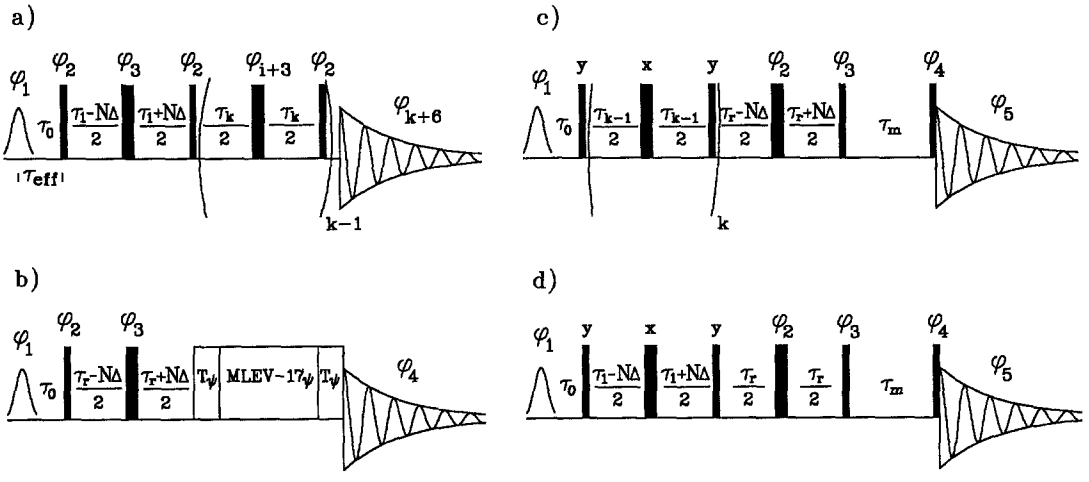


Fig. 1. Pulse sequences of double-selective pseudo-3D NMR experiments. Thin bars represents 90° pulses and thick bars 180° pulses. In all sequences a 90° Gaussian pulse of duration $\tau_{sel}$ was used for the initial selection of magnetization. $\tau_{eff} = \tau_{sel}/2 + \tau_0$, for a doublet $\tau_{eff} = 1/2J_{HH}$, k marks the number of relay steps, i = 1,...k−1, $\tau_k$ intervals are optimized in order to yield maximum antiphase magnetization for individual polarization transfer steps; Δ is an increment of the CSSF and N goes from 0 to n, n being the number of increments of the filter. $\tau_r$ is optimized in order to refocus the magnetization prior to the TOCSY or NOESY (ROESY) steps: $\tau_m$ is the NOE mixing interval. The $\phi_1$ phase programs given below must be corrected for the frequency change that occurs between the soft and the hard COSY pulses (see text). (a) pseudo-3D COSY-RELAY. The following phase programs have been used: $\phi_1$ = 2(x,−x), 2(y,−y); $\phi_2$ = 4y, 4(−x); $\phi_3$ = 2x, 2(−x), 2y, 2(−y); $\phi_4$ = x; $\phi_5$ = 8x, 8y; $\phi_6$ = x; $\phi_7$ = 2(y,−y), 2(−x,x); $\phi_8$ = 2(y,−y), 2(x,−x); $\phi_9$ = 2(y,−y), 2(−x,x), 2(−y,y), 2(x,−x); $\phi_{10}$ = 2(y,−y), 2(x,−x), 2(−y,y), 2(−x,x). (b) pseudo-3D COSY-TOCSY; $T_\psi$ denotes trim pulses of phase ψ. The following phases have been used: $\phi_1$ = 4(x,−x), 4(−x,x); $\phi_2$ = 16y; $\phi_3$ = 8x, 8(−x); ψ = 2(y,−y), 2(−y,y), 2(y,−y), 2(−y,y); $\phi_4$ = 4(−x,x), 4(x,−x). In addition all the phases can be shifted by 90°, making a 32 item phase cycling. (c) pseudo-3D COSY-NOESY (k = 0) and COSY-RELAY (k ≥ 1). (d) pseudo-3D RELAY-NOESY with CSSF on H-2s and back transfer to H-1 protons. The following phases have been used in (c) and (d) pulse sequences: $\phi_1$ = x,−x; $\phi_2$ = 16x, 16(−x), 16y, 16(−y); $\phi_3$ = 8x, 8(−x); $\phi_4$ = 2x, 2(−x), 2y, 2(−y); $\phi_5$ = 2(x,−x,−x,x,y,−y,−y,y,−x,x,x,−x,−y,y,y,−y), 2(−x,x,x,−x,−y,y,y,−y,x,−x, −x,x,y,−y,−y,y). All spectra were recorded on a Bruker AMX-600 spectrometer with a two- channel interface. A selective excitation unit was used to generate a Gaussian pulse composed of 1024 points and truncated at the 1% intensity level. The transmitter channel was used for water suppression, selective and non-selective pulses.

sponding spectrum shows a worse signal-to-noise ratio than the Glc-I subspectrum. A partial transfer of magnetization beyond H-5, to H-6′, is observed for the same reason.

The possibility for spectral editing in CSSF pseudo-3D experiments improves their overall sensitivity when compared to similar techniques that use selective pulses for both selection steps (Boudot et al., 1990; Poppe and van Halbeek, 1992). In the later techniques, only one subspectrum can be obtained per experiment, causing a twofold increase in total experiment time.

In the next example, CSSF was followed by a TOCSY step, resulting in a pseudo-3D COSY-TOCSY experiment (Fig. 1b). In this experiment, the length of the filter, being the $\tau_r$ interval, was adjusted to give maximum inphase magnetization for the H-2 protons. The same procedure for separation of two subspectra was followed as in the previous pseudo-3D COSY-RELAY experiment. The results after the summation and subtraction of the original spectra are shown in Figs. 3b,c. The efficiency of a TOCSY transfer, unlike a RELAY experiment, does not depend on

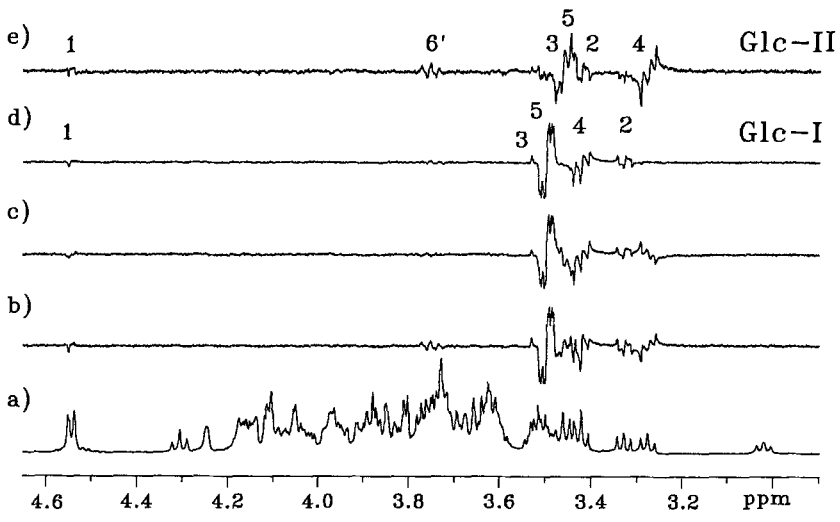


Fig. 2. Pseudo-3D COSY-RELAY spectra of two terminal glucoses of oligosaccharide **1**. a) Partial $^1$H spectrum of **1** at 600 MHz and 27 °C (b) and (c) spectra were acquired using the pulse sequence in Fig. 1a (k = 3) and an initial polarization transfer from overlapping anomeric protons of terminal glucoses. The duration of the Gaussian pulse was 50 ms, $\tau_0$ = 39 ms, $\tau_1$ = 50 ms, $\Delta$ = 9.09 ms, $\tau_2$ = 50 ms, $\tau_3$ = 40 ms; the number of scans was 64; the relaxation delay and acquisition times were 2 and 1.4 s, respectively. N = 0 for the first and N = 1 for the second spectrum. (d) is the sum of (a) and (b). (e) is the difference between (a) and (b). A partial structure of oligosaccharide **1** is as follows:

β-Glc*p*(I) → Glc*p*(III) → Hep*p* → Hep*p*(I) → KDO → Glc*p*N → GlcNol
↑ ↑
Hep*p* β-Glc*p*(II)

chemical shift differences between the spins involved, hence the same signal-to-noise ratio was observed in pseudo-3D COSY-TOCSY spectra of both Glc-I and Glc-II.

Another possible combination, a pseudo-3D COSY-NOESY experiment (Fig. 1c, k = 0), is illustrated with a bacterial polysaccharide **2**, which contains a terminal 3,6-dideoxy-4-C(1-hydroxyethyl)-D-xylohexose (see inset of Fig. 4) (Pavliak, V., Brisson, J.R., Uhrín, D., Tzinabos, A.O., Kasper, D.L. and Jennings, H.J., unpublished results). In an effort to solve the absolute configuration of the hydroxyethyl substituent, a series of 1D NOESY experiments (Kessler et al., 1989) with different mixing times was performed. This was possible for most of the protons of the xylose ring but not for the CH proton of the hydroxyethyl (H-7) itself, because it overlaps other resonances in the $^1$H NMR spectrum. A pseudo-3D-COSY-NOESY spectrum was therefore acquired with an initial COSY transfer from the $CH_3$ (H-8) protons of the hydroxyethyl group. Since it overlapped another $CH_3$ resonance and was also close to additional $CH_3$ signals, it was necessary to apply a complete CSSF at H-7, prior to NOE mixing, by shifting the 180° pulse within a $\tau_r$ interval in small steps instead of acquiring only two spectra as shown in previous examples. The length of the $\tau_r$ interval was at the same time optimized to yield a maximum inphase magnetization of H-7. The resulting spectrum is very clean (Fig. 4b) and shows NOEs from H-7 to the neighbouring $CH_3$ group and several ring protons of xylose. Complete results, including a polysaccharide structure together with the conformational analysis, will be presented elsewhere.

This example illustrates that anomeric protons are not the only spins suitable as the starting

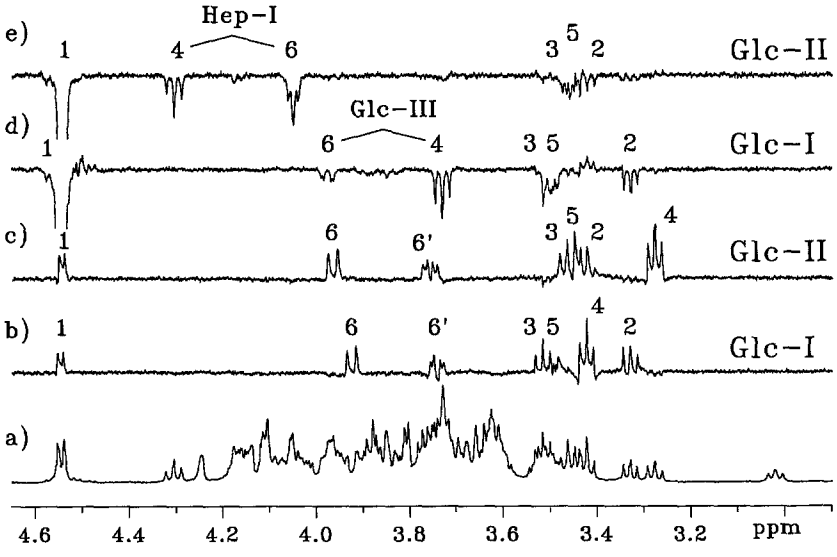


Fig. 3. Pseudo-3D COSY-TOCSY and RELAY-NOESY spectra of the oligosaccharide **1**, using an initial polarization transfer from overlapping anomeric protons of terminal glucoses. The following parameters were identical for both pseudo-3D COSY-TOCSY and RELAY-NOESY experiments: $\tau_{sel}$ = 50 ms, $\tau_0$ = 39 ms, $\Delta$ = 9.09 ms. N = 0 in the first and N = 1 in the second experiment, the relaxation delay was 2 s and the acquisition time was 1.4 s. (a) Partial $^1$H spectrum of **1** at 600 MHz after 8 scans. The summation (b) and subtraction (d) of two pseudo-3D COSY-TOCSY spectra acquired using the sequence in Fig. 1b with the following parameters for the original spectra (not shown): $\tau_r$ = 28 ms, 2.5 ms trim pulse and the total mixing time of 87 ms. Number of scans was 128 in both spectra. The summation (d) and subtraction (e) of two pseudo-3D RELAY-NOESY spectra acquired using the pulse sequence in Fig. 1d with the following parameters for the original spectra (not shown): $\tau_1$ = 120 ms, $\tau_r$ = 64 ms, $\tau_m$ = 400 ms. Number of scans was 1024 in both spectra.

point for pseudo-3D experiments in saccharides. Deoxy protons, or more generally other partially overlapped resonances, e.g., signals located at the edges of the resonance envelope between 3 and 4 ppm, offer convenient spectral windows.

The NOEs in the previous pseudo-3D COSY-NOESY experiment were obtained after single coherent transfer of magnetization to their direct neighbours. However, it is also possible to prolong the chain of relay steps and to build up the magnetization of a more remote proton, before the application of a final NOE step (pulse sequence in Fig. 1c, $k \geq 1$). CSSF is again performed during the refocusing interval preceding the NOE step. This experiment, called pseudo-3D RELAY-NOESY, is an alternative to the recently proposed pseudo-3D TOCSY-NOESY experiment (Boudot et al., 1990; Poppe and van Halbeek, 1992), in which a TOCSY mechanism was used to obtain the magnetization of a remote proton along with two selective pulses used to direct the magnetization transfer pathway.

A slightly modified pseudo-3D RELAY-NOESY experiment (Fig. 1d) offers an interesting possibility for unambiguous assignment of NOEs from two or more anomeric protons, the signals of which overlap severely. In this experiment, the magnetization is transferred first from H-1s to H-2s and after CSSF at H-2s is sent back to H-1 protons. A complete refocusing of H-1s, due to their doublet character, can be achieved during the refocusing interval prior to NOE mixing. Similarly to the previous experiments on **1**, two pseudo-3D spectra were acquired using a different position of a 180° $^1$H pulse during the filtration interval $\tau_1$. Their sum and difference are shown

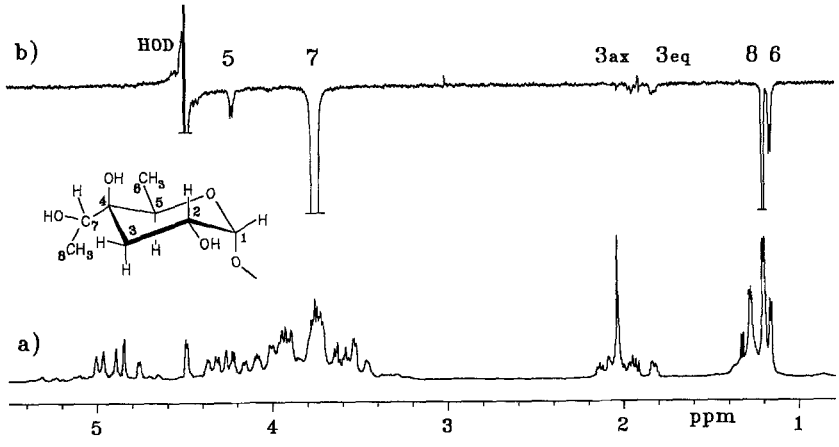


Fig. 4. Pseudo-3D COSY-NOESY experiment on polysaccharide **2**. The structure of a terminal 3,6-dideoxy-4-C-(1-hydroxyethyl)-D-xylohexose is shown in the inset. (a) $^1$H spectrum of **2** at 600 MHz and 50 °C. (b) Pseudo-3D COSY-NOESY spectrum of **2** acquired using the sequence in Fig. 1c; initial transfer of magnetization from H-8 and the following parameters: $\tau_{sel}$ = 100 ms. $\tau_0$ = 29 ms, k = 0, $\tau_r$ = 32 ms, $\Delta$ = 0.5 ms, N = 0,1,...64, $\tau_m$ = 250 ms. 32 scans were accumulated in each spectrum, using an acquisition time of 1s and a relaxation time of 4s. Water suppression was applied in spectrum (a) but not in spectrum (b).

in Figs. 3d,e. Based on this experiment, it was possible to establish the Glc-I → Glc-III and Glc-II → Hep-I sequence in oligosaccharide **1**.

For molecules where $\omega_0\tau_c \approx 1$, NOE mixing used in pseudo-3D COSY-NOESY and RELAY-NOESY experiments can be easily replaced by a ROE spin-lock period (Bothner-By et al., 1984), yielding pseudo-3D COSY-ROESY and RELAY-ROESY experiments.

Although in all the examples presented severely overlapping resonances of only two protons complicated the selection of the magnetization at the beginning of the experiment; it can be anticipated that even if three anomeric signals overlapped it would not be necessary to run a whole CSSF for each of them separately. Instead, the acquisition of three spectra would be sufficient. The first with a suppressed chemical shift evolution for all H-2 protons created in a COSY step and another two spectra with a 180° chemical shift evolution for each of the two H-2s. Individual spin systems or NOE connectivities could be then identified for all three saccharide residues by editing these three spectra.

In all the proposed experiments, except for the pseudo-3D RELAY-NOESY (Fig. 1c, k ≥ 1), CSSF was performed immediately after the COSY step. Should there not be sufficient chemical shift separation between H-2 protons, the magnetization could be further transferred along the spin system and filtered during one of the later spin echoes. This procedure, when applied to pseudo-3D RELAY-RELAY and RELAY-TOCSY experiments, would, however, require a full-scale CSSF, yielding only one subspectrum per experiment.

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