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Relayed HOHAHA, a Useful Method for Extracting Subspectra of Individual Components of Sugar Chains

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The main difficulty encountered in the analysis of NMR spectra of sugar chains is the overlap of most sugar proton resonances except for reporter groups such as anomeric proton resonances (*1*). Therefore, NMR methods for extracting the proton resonances of each sugar component from an overlapping region are very useful. In previous studies, we applied multiple relayed COSY (*2–5*) and homonuclear Hartmann–Hahn spectroscopy (HOHAHA) (*6, 7*) to extract the subspectrum of each sugar component of glycolipids (*8, 9*), utilizing magnetization transfer from well-resolved anomeric proton resonances. In contrast to homonuclear multiple relay experiments, the HOHAHA experiment does not generate large amounts of multiple-quantum coherence during the mixing period and it redistributes the entire integrated intensity of one proton among all N protons in the same spin system. Hence, besides relaxation during the mixing time, the sensitivity of a 1D HOHAHA spectrum is reduced only by a factor N relative to a conventional one-dimensional ^{1}H spectrum. Magnetization transfer in the HOHAHA experiment is especially efficient when all couplings are of a similar order of magnitude. For example, for sugar residues such as Glc and GlcNAc which have couplings of 6–9 Hz all around the ring, complete subspectra can be obtained very efficiently.

If one of the couplings around the sugar ring is very small, it essentially blocks the Hartmann–Hahn flow of magnetization. For example, in Gal, GalNAc, and Fuc residues, magnetization is rapidly transferred among protons H1 through H4, but because of the typically very small coupling constant between H4 and H5 protons (1–1.5 Hz), HOHAHA transfer to H5 is not very efficient. Moreover, any magnetization transferred to H5 is rapidly "diluted" because of further HOHAHA transfer to the usually fast relaxing H6 protons. Here we demonstrate that a combination of HOHAHA and the conventional ^{1}H–^{1}H relay mechanism can be used effectively to circumvent this problem.

The pulse sequence of 1D-relayed HOHAHA is shown in Fig. 1, where magnetization is first transferred from H1 to H4 via H2 and H3 by HOHAHA using MLEV-

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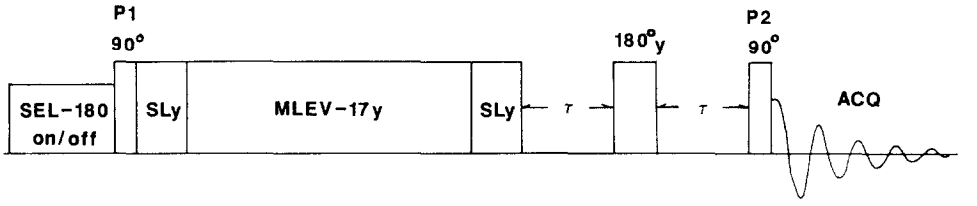

FIG. 1. Pulse sequence for the 1D-relayed HOHAHA experiment. P1, P2, and acquisition phases are cycled as follows; P1 (0°, 180°, 0°, 180°), P2 (90°, 90°, 270°, 270°), and acquisition phase (90°, 270°, 270°, 90°).

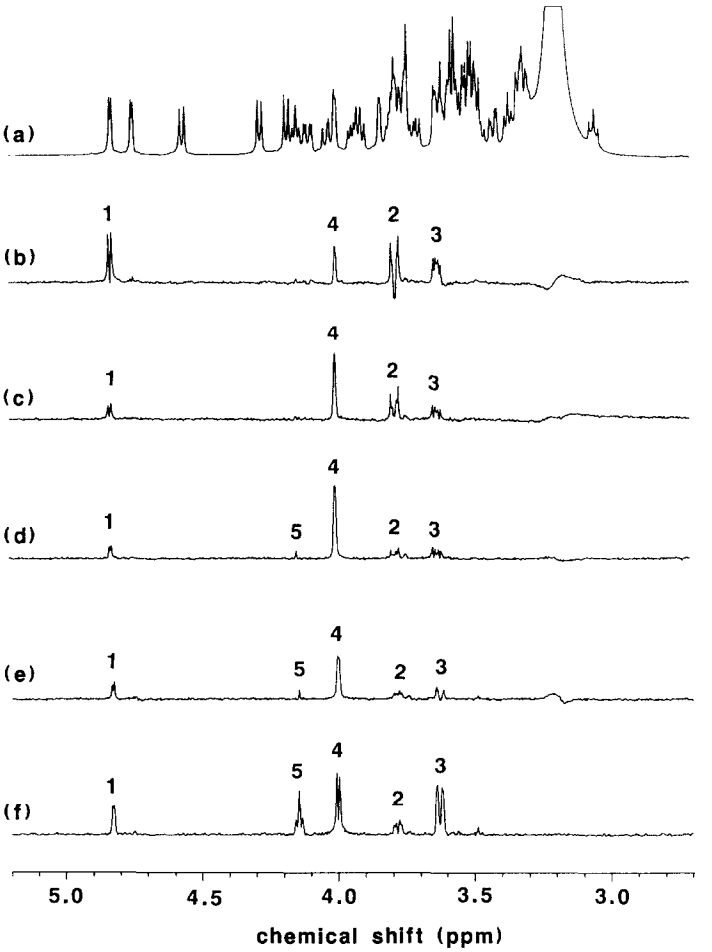

FIG. 2. Comparison of 1D HOHAHA and 1D-relayed HOHAHA spectra of Forssman antigen at 500 MHz. These spectra were obtained on a JEOL JNM-GX500 NMR spectrometer. The pulse sequence was generated using the PGX 200 pulse programmer. A 5 mg sample of Forssman's antigen was dissolved in DMSO/D_2O (98/2) solution. The spectra were obtained at 60°C. (a) Normal spectrum, 1D HOHAHA spectra with a mixing time of (b) 128 ms, (c) 192 ms, (d) 224 ms, (e) 320 ms, and (f) absolute-value mode 1D-relayed HOHAHA spectrum with a mixing time of 224 ms and a delay time of 100 ms (τ = 50 ms).

17 type mixing (*6*); magnetization of H4 is subsequently relayed to H5 by the conventional pulse-interrupted free-precession process. Similar to 1D HOHAHA (*7, 10*), the sequence starts with selective spin inversion of a preselected proton resonance in odd-numbered scans. The homonuclear decoupler is used for generating this 180° pulse. In even-numbered scans, the decoupler is switched off. This ensures that in the difference spectrum only resonances from protons directly or indirectly coupled to the inverted proton will be present.

In the present study, we have applied relayed HOHAHA to extract the subspectra of the individual sugar components of Forssman antigen (GalNAcα1–3GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–Cer) (*11, 12*). Comparison of the efficiency

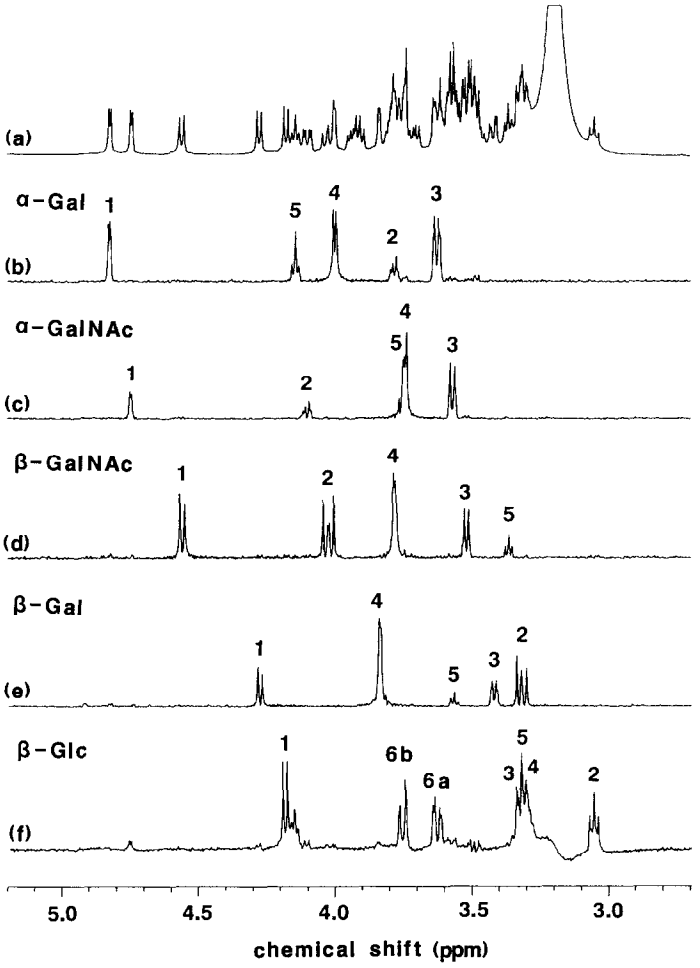

FIG. 3. Subspectra of individual sugar components of Forssman antigen. (a) Normal spectrum, 1D-relayed HOHAHA spectra of (b) α-Gal, (c) α-GalNAc, (d) β-GalNAc, (e) β-Gal, and (f) 1D HOHAHA spectrum of β-Glc, where the anomeric proton of each sugar component was selectively inverted. The 1D-relayed HOHAHA spectra are displayed in the absolute-value mode.

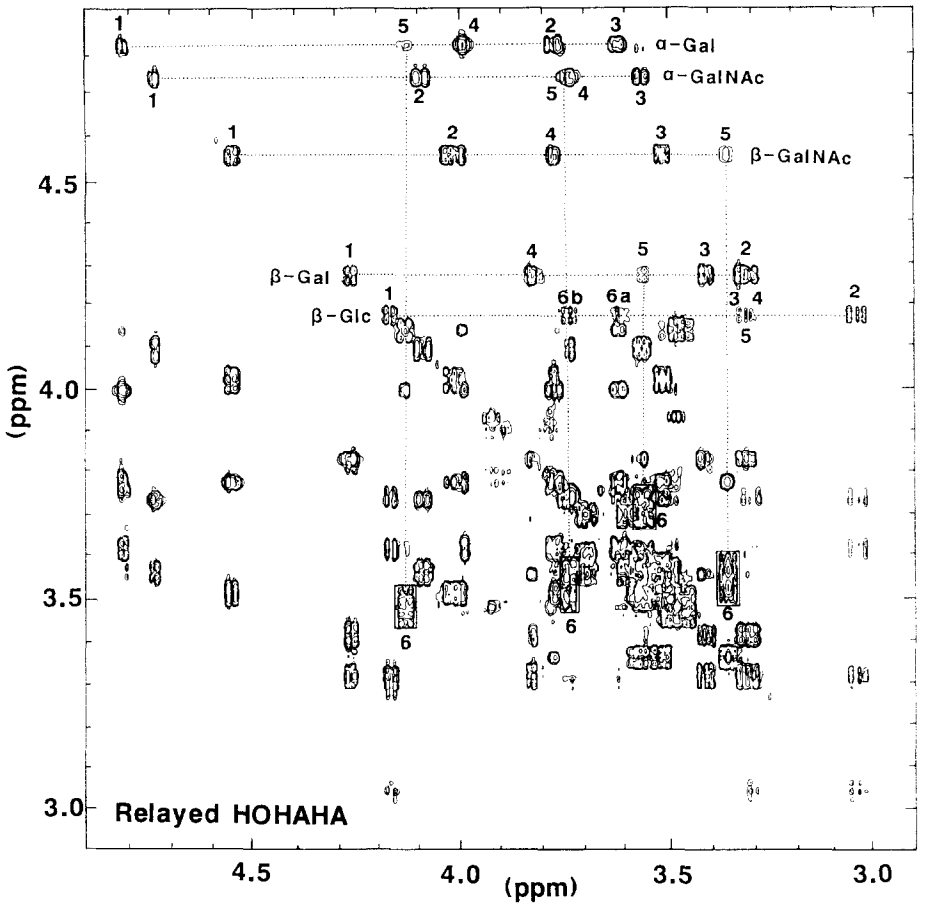

FIG. 4. Absolute value mode 2D-relayed HOHAHA spectrum of Forssman antigen.

of magnetization transfer by 1D HOHAHA and 1D-relayed HOHAHA is shown in Fig. 2, where the anomeric proton resonance of α-Gal unit was selectively inverted. In 1D HOHAHA, the efficiency of magnetization transfer from H1 to H2–H4 was very good (Fig. 2b, 2c). The accumulated H4 magnetization transferred from H1 was maximal at a mixing time of 224 ms (Fig. 2d). However, with this mixing time, H5 proton resonance did not appear. Even with a longer mixing time of 320 ms, only a very small signal of H5 appeared (Fig. 2e). Figure 2f shows a 1D-relayed HOHAHA spectrum with a mixing time of 224 ms for HOHAHA and a delay time of 100 ms for a relayed magnetization transfer step. In contrast to 1D HOHAHA (Fig. 2e), H5 shows up clearly in Fig. 2f, showing that the efficiency of magnetization transfer from H4 to H5 is much better for 1D-relayed HOHAHA than that for 1D HOHAHA, although the same total mixing time was used for both experiments. A delay time longer than 100 ms did not improve the results. S/N was worse due to magnetization decay during the long delay time. Therefore, in subsequent experiments, we set the delay time to 100 ms. Figure 3 shows 1D-relayed HOHAHA spectra of Forssman

antigen, where the anomeric proton resonance of each sugar component was selectively inverted. The mixing times were set to 224 ms for α anomers and 144 ms for β anomers, respectively. In contrast to using the original 1D HOHAHA experiment, all the proton resonances from H1 to H5 of Gal and GalNAc in Forssman antigen were extracted showing that relayed HOHAHA is a useful method for extracting the sugar proton resonances. Of course, the 1D-relayed HOHAHA can also be performed in a 2D version. Figure 4 shows a 2D-relayed HOHAHA spectrum of the sugar proton region of Forssman antigen. At the chemical shift of the anomeric proton resonance of each sugar component, cross peaks of (H1, H2), (H1, H3), (H1, H4), and (H1, H5) were developed. Once the chemical shifts of H5 proton resonances of Gal and GalNAc were identified, H6 proton resonances were unambiguously assigned from the cross peaks of (H5, H6). Since spin couplings between those protons are usually large, we can observe relatively large cross peaks in the 2D-relayed HOHAHA spectrum as marked in Fig. 4. Thus, all the sugar proton resonances can be assigned to the individual sugar components. Once the sugar proton resonances are extracted, those protons can be used as structural probes to determine the connectivity between individual sugar residues.

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