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Analysis of NMR spectra of sugar chains of glycolipids by 1D homonuclear Hartmann-Hahn and NOE experiments

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We applied 1D homonuclear Hartmann-Hahn (1D-HOHAHA) and difference NOE experiments to determine the chemical structure of Forssman's antigen, a glycolipid purified from sheep red blood cells. The subspectra corresponding to the individual sugar components were extracted from overlapping proton resonances by selective excitation of the anomeric proton resonances, so that unambiguous assignments of the sugar proton resonances were accomplished. Then, difference NOE experiments were performed to determine the linkage of the sugar units. The present procedure was found to be useful for the structure determination of glycoconjugates and also reduces the amount of samples and machine time.

NMR; Forssman antigen; 1D homonuclear Hartmann-Hahn spectroscopy; Difference NOE; Coherence transfer

1. INTRODUCTION

Glycolipids are components of cell membranes and are thought to play important roles in higher order cell functions, such as cell-cell recognition [1]. Clinically, glycolipids are useful as specific markers of tumour cells for diagnosis of cancers [2]. Since the biological functions of glycolipids are closely connected to the structure of the sugar moiety, efficient and nondestructive methods for the structure determination of the sugar moiety are indispensable. NMR is suitable for this purpose [3–5]. However, the main difficulty encountered in the analysis of NMR spectra of sugar moieties is the overlap of NMR resonances, so that NMR methods for extracting the proton resonances of each sugar residue from the overlapping region are quite helpful. In the present study, we applied 1D homonuclear Hartmann-Hahn experiments (1D-HOHAHA) [6] to extract the subspectrum of each sugar component and then difference NOE experiments to determine the linkage of sugar residues of Forssman's antigen (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer).

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2. EXPERIMENTAL

Forssman's antigen was purified from sheep red blood cells [7]. DMSO-d_6 and ^2H_2O were purchased from CEA, and TMS from Merck. A 2 mg sample of Forssman's antigen was dissolved in mixed solvent of 400 μl DMSO-d_6 and 100 μl ^2H_2O and incubated at 60°C for 10 min to replace exchangeable protons with deuterons. Then, the sample solution was lyophilized. The resulting residue was dissolved in 500 μl of freshly prepared DMSO-d_6:^2H_2O (98:2, v/v) in 5 mm diameter NMR sample tubes. TMS was used as an internal reference of chemical shifts. NMR measurements were made on a Jeol GX-500 ^1H 500 MHz NMR spectrometer. 1D-HOHAHA spectra were obtained at 60° and 40° using the pulse sequence of

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Davis and Bax [6]. RF field was attenuated to 4 W to avoid damage of the power amplifier and the probe. Offset frequency was set to the center of the sugar proton region (4.0 ppm). Long pulse (51 ms for 180° pulse) was used for selective excitation. Difference NOE spectra were obtained at 40° with alternating accumulation of on-resonance and off-resonance irradiation.

3. RESULTS AND DISCUSSION

Fig.1a shows the 500 MHz ^{1}H NMR spectrum of the lower field region of Forssman's antigen at 60°. Except for the anomeric proton resonances (shown with asterisks), most of the sugar proton resonances overlap within the region from 3 to 4 ppm, which makes analysis of the NMR spectrum of glycolipids difficult. In the previous study [8], we have shown the usefulness of multiple relayed COSY and 2D-HOHAHA experiments for extracting the subspectrum of each sugar residue by taking cross sections parallel to the F2 axis at the anomeric proton resonances. However, as an inherent drawback of 2D methods, these methods essentially require a large amount of machine time and samples. So, their application to the structural analysis of a small amount of glycolipids is not practical. The 1D method equivalent to 2D-HOHAHA is more promising, where the subspectra of sugar residues can be obtained by selective excitation of the anomeric proton resonances (H1). Fig.1b–e shows the dependence on mixing time of the subspectra of α-galactose of Forssman's antigen obtained by 1D-HOHAHA at 60°. Efficiency of the coherence transfer depends on the magnitude of the vicinal coupling constants and the duration of the mixing time. With a short mixing time (fig.1b), the coherence of H1 transfers to H2 and H3 protons. Upon increase in mixing time, the coherence transfers to H4 through H2 and H3 (fig.1c). However, the coherence transfer to H5 requires a much longer mixing time due to the small vicinal coupling constant between H4 and H5. With a mixing time longer than 200 ms, H5 proton resonance can be identified. Since hexose and hexosamine exist in the chair conformation, we can tune the mixing time a priori to the individual type of sugars. For example, we can extract all proton resonances of the β-glucose residue with a mixing time of 200 ms (fig.2f). The choice of mixing time depends on efficiency of the coherence transfer and decay of the coherence in the rotating frame. In the following experiment, we used 200 ms as the mixing time. Fig.2 shows the subspectra of the sugar residues of Forssman's antigen. Compared to the 2D methods, the spectral resolution by 1D-HOHAHA is enough to resolve the spin-spin couplings of each resonance. From the analysis of the chemical shifts and splitting patterns, the type and the anomeric configuration of the individual sugar residues as well as the assignment of the proton resonances can be accomplished as is shown in fig.2. The time required for observing each subspectrum is about 6 min for 64 scans of on-resonance and off-resonance irradiation. The total time required for the assignment of all sugar residues is less than 1 h. In contrast to the overnight accumulation for the measurement of multiple relayed COSY or 2D-HOHAHA, 1D-HOHAHA significantly reduces the experimental time.

Once the assignments of the proton resonances are made, the linkage of each sugar residue can be elucidated by difference NOE experiments. Fig.3 shows the difference NOE spectra of Forssman's antigen, where the anomeric proton resonances are selectively irradiated. Since the NOE effect was small and could not be detected at 60°, NOE experiments were made at 40°. Fig.3b shows the difference NOE spectrum where H1 of α-galactose is selectively irradiated. With reference to the subspectra obtained by 1D-HOHAHA, the NOE signals are readily assigned to H4 of β-galactose and H2 of α-galactose, thus permitting the linkage of Galα1-4Galβ. Upon selective irradiation of H1 of β-*N*-acetylgalactosamine, the NOE effects are observed for H3 of α-galactose and H3 and H5 of β-*N*-acetylgalactosamine (fig.3d). Subsequent irradiation on H1 of β-galactose gives interresidue NOE at H4 of β-glucose as well as intraresidue NOE (fig.3e). Thus, the sugar linkage of GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer is confirmed with reference to the 1D-HOHAHA and difference NOE spectra. However, upon irradiation of H1 of α-*N*-acetylgalactosamine, we observe NOE at H3 and H4 of β-*N*-acetylgalactosamine, suggesting the linkage of either GalNAcα1-3GalNAcβ or GalNAcα1-4GalNAcβ. Model building of Forssman's antigen supports the dihedral angle around the sugar linkage of

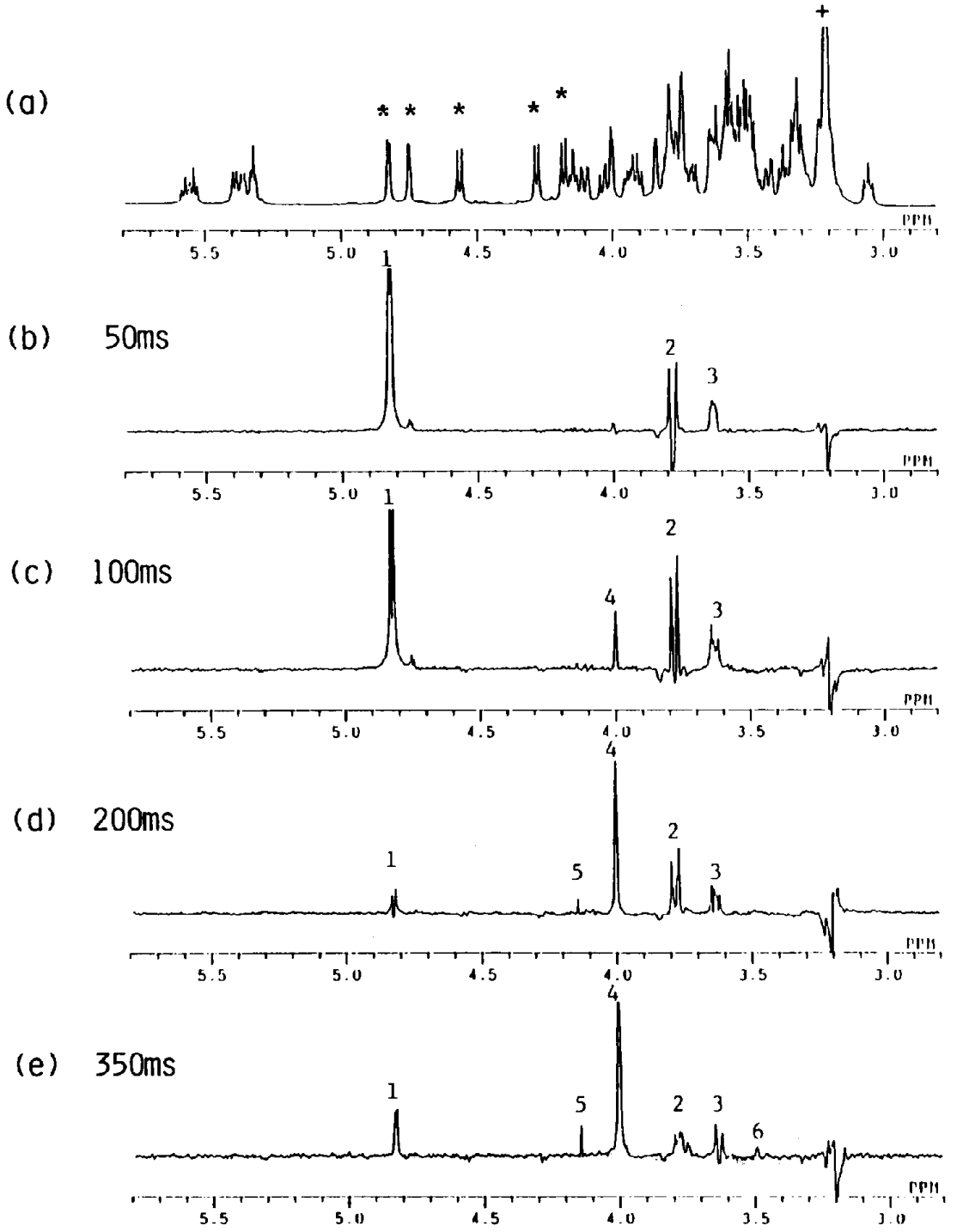

Fig.1. Dependence of 1D-HOHAHA spectra of the α-galactose of Forssman's antigen on the mixing time. The normal spectrum (a) and 1D-HOHAHA spectra with mixing times of (b) 50 ms, (c) 100 ms, (d) 200 ms and (e) 350 ms. *, anomeric proton resonances; +, residual water signal.

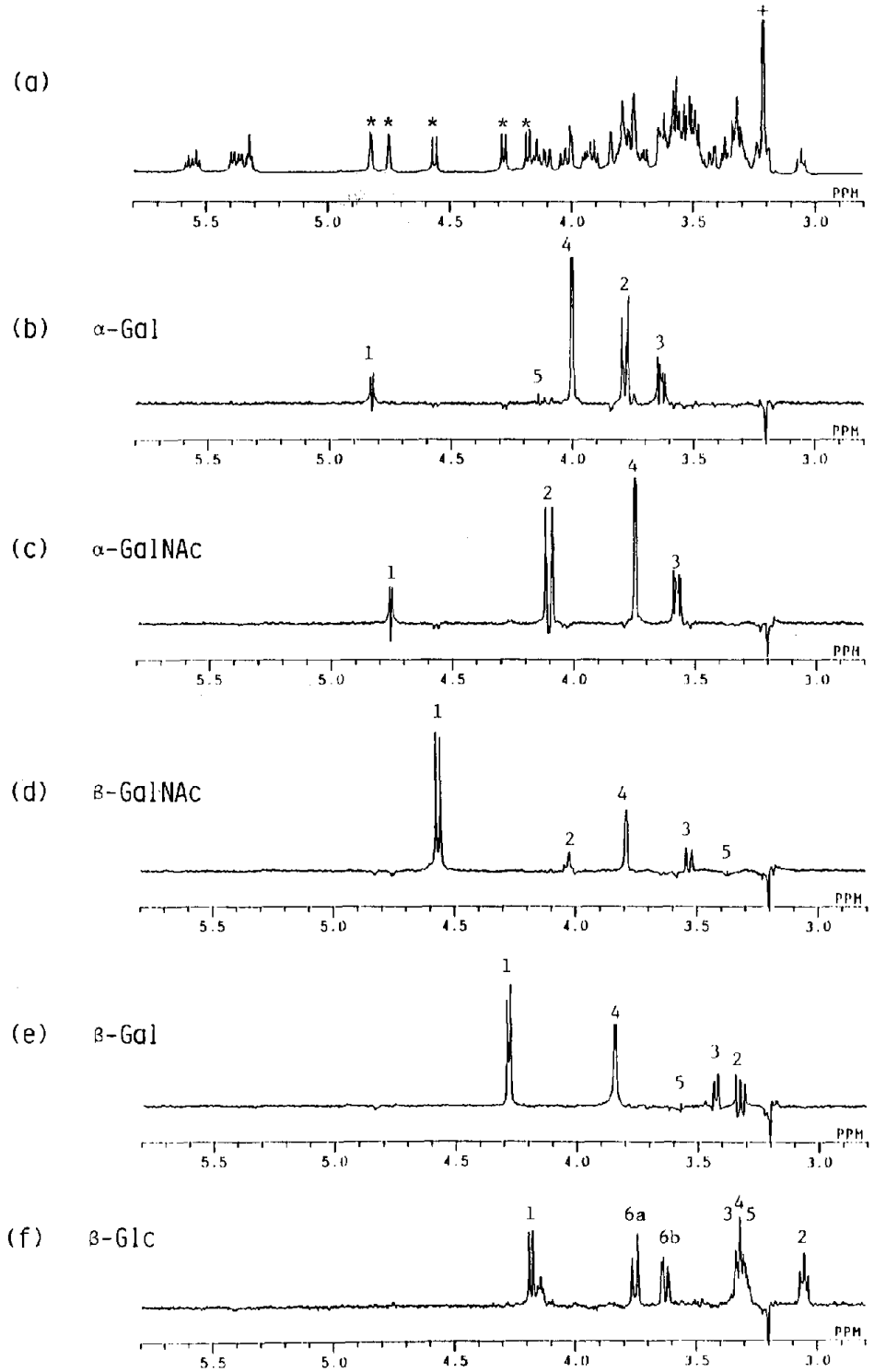

Fig.2. 1D-HOHAHA spectra of Forssman's antigen. The normal spectrum (a) and selective excitation of the anomeric proton resonances of (b) α-galactose, (c) α-*N*-acetylgalactosamine, (d) β-*N*-acetylgalactosamine, (e) β-galactose and (f) β-glucose. The assignment of the sugar proton resonances is also given. *, anomeric proton resonances; +, residual water signal.

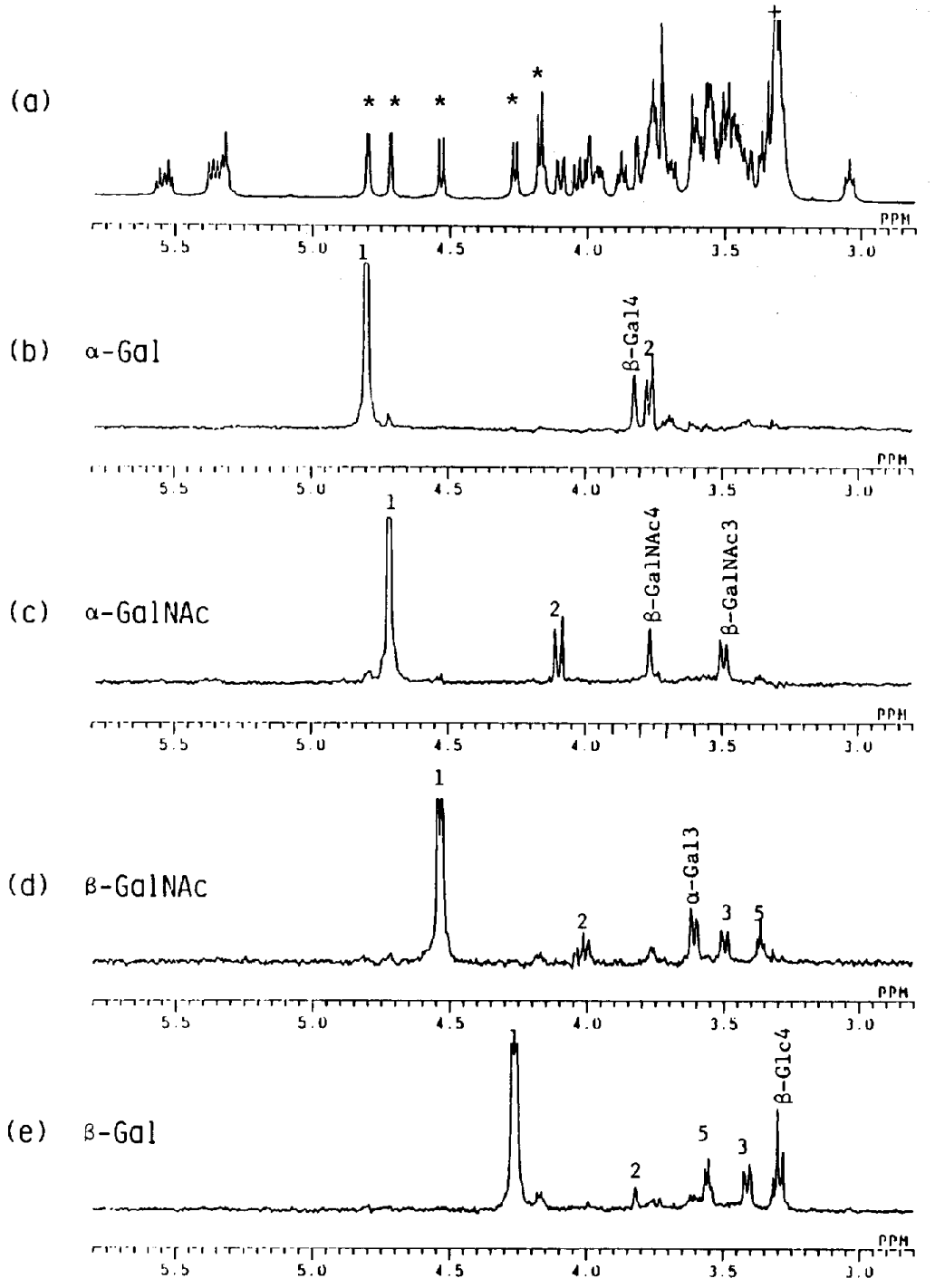

Fig.3. NOE difference spectra of Forssman's antigen. The normal spectrum (a) and NOE difference spectra upon selective irradiation of the anomeric proton resonances of (b) α-galactose, (c) α-*N*-acetylgalactosamine, (d) β-*N*-acetylgalactosamine, (e) β-galactose. Intra- and interresidue NOE effects are also shown. *, anomeric proton resonances; +, residual water signal.

GalNAcα1-3GalNAcβ being distorted due to steric hindrance so that H1 of α-*N*-acetylgalactosamine is located in close proximity to H3 and H4 of β-*N*-acetylgalactosamine.

In spite of the difficulty encountered in the interpretation of the NOE difference spectrum, the present procedure is useful for structural analysis of the glycolipids in a non-empirical manner.

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