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Analysis of NMR spectra of sugar chains of glycolipids by multiple relayed COSY and 2D homonuclear Hartman-Hahn spectroscopy

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We applied multiple relayed COSY and 2D homonuclear Hartman-Hahn spectroscopy to globoside, a glycolipid purified from human red blood cells. The subspectra corresponding to individual sugar components were extracted even from overlapping proton resonances by taking the cross sections of 2D spectra parallel to the F_2 axis at anomeric proton resonances, so that unambiguous assignments of sugar proton resonances were accomplished.

NMR; Globoside; Multiple relayed COSY; Sphingosine; 2D homonuclear Hartman-Hahn spectroscopy

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 and therapy of cancers [2]. Since the biological functions of glycolipids are closely connected to the structure of the sugar moieties, efficient and nondestructive methods for the structure determination of the sugar moieties of glycolipids are indispensable. Nuclear magnetic resonance (NMR) is suitable for this purpose and systematic analyses of glycolipids by one- and two-dimensional NMR have been reported to establish empirical rules for the structure determination of the sugar moieties [3–8]. However, at present, due to lack of enough NMR data, the structure of the sugar moieties of unknown glycolipids is difficult to determine in an empirical manner. Therefore, there is a great demand for non-empirical methods for the structure analysis of glycolipids.

The main difficulty encountered in the analysis of the NMR spectra of the sugar moieties of glycolipids is overlap of NMR resonances around 3–4 ppm, so that NMR methods for extracting the proton resonances of each sugar residue from the overlapping region are required. Multiple relayed COSY is useful for this purpose [9,10] and has been successfully applied to spectral analyses of oligosaccharides [11,12]. Another promising method is 2D homonuclear Hartman-Hahn spectroscopy [13,14]. In the present study, we applied both methods for the assignment of NMR resonances of globoside (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer, fig.1) and discussed their advantages and limitations.

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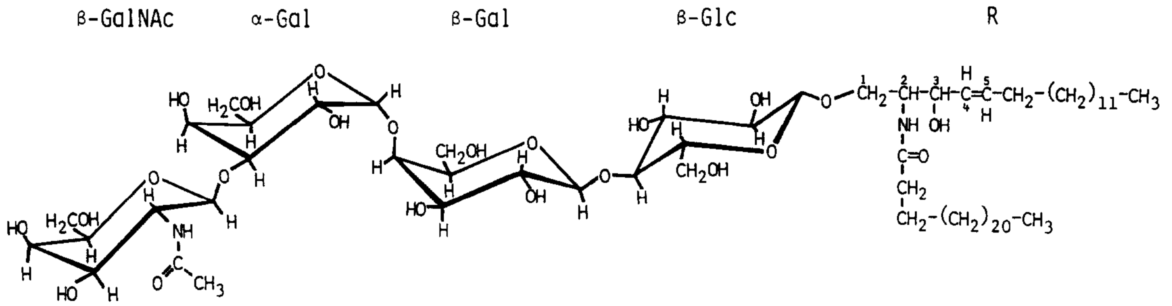


Fig.1. The structure of globoside.

2. EXPERIMENTAL

Globoside was purified from human red blood cells [15]. DMSO-d_6, ^2H_2O and TMS were purchased from Merck. A 4 mg sample of globoside was dissolved in a 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 0.5 ml of freshly prepared DMSO-d_6/^2H_2O (98:2, v/v). TMS was used as an internal reference for chemical shifts. NMR measurements were made on a JEOL GX-400 ^1H 400 MHz NMR spectrometer. All the measurements were made using 5 mm diameter sample tubes at 60°C. Multiple relayed COSY and 2D homonuclear Hartman-Hahn spectra were obtained by using the pulse sequences of Bax and Drobny [10] and Bax and Davies [14], respectively. All the 2D spectra were measured with 256 × 1024 data points and a spectral width of 2000 Hz. 64 scans were accumulated for each t_1 and the total measurement time for the 2D spectrum was about 12 h. After zero filling in the t_1 and t_2 dimension, a 512 × 2048 data matrix was processed. Sine-bell was applied for both t_1 and t_2 dimensions as an apodization function. The contour display of the spectrum is presented in the absolute-value mode.

3. RESULTS AND DISCUSSION

NMR spectra of glycolipids have been analyzed using SECSY [4]. However, even the spectral resolution obtained by this method does not seem to be sufficient to trace cross peaks for correlating chemical shifts of coupled spins in a complex spectral region. Peracetylation of glycolipids alleviates this problem due to dispersion of resonances [16]. However, the structural analysis of intact glycolipids is greatly preferable. Sequential magnetization transfer, starting from the anomeric proton to other protons of each sugar residue by multiple relayed COSY or 2D homonuclear Hartman-Hahn spectroscopy, is quite promising for analyses of complex sugar NMR spectra. The subspectrum of each sugar residue can be extracted by taking the cross section parallel to the F_2 axis at diagonal peaks of anomeric protons. This permits an unambiguous assignment of the sugar proton resonances, since the anomeric proton resonances are well separated from the rest of other sugar proton resonances and are also well resolved from each other. Fig.2 shows a double relayed COSY spectrum of globoside where delay times, τ_1 and τ_2 were each set to be 25 ms. Taking COSY, relayed COSY and double relayed COSY, we can assign H1, H2, H3 and H4 proton resonances of each sugar residue sequentially as is shown in fig.2. In the case of double relayed COSY, the efficiency of the magnetization transfer from H1 to H4 via H2 and H3 protons depends on the magnitude of the vicinal coupling constants, J_{12}, J_{23} and J_{34}, which then determine the delay times. Since hexose and hexosamine exist in chair conformations and thus vicinal coupling constants take relatively fixed values, the optimization of delay times is possible for individual types of sugars but not for all. For example, the efficiency of magnetization transfer is optimized when τ_1 and τ_2 are set to be 30 ms and 80 ms, respectively, for β-galactose and β-galactosamine. However, magnetization decay occurs during long delay times, so that as a compromise we used 25 ms for τ_1 and τ_2, in the present

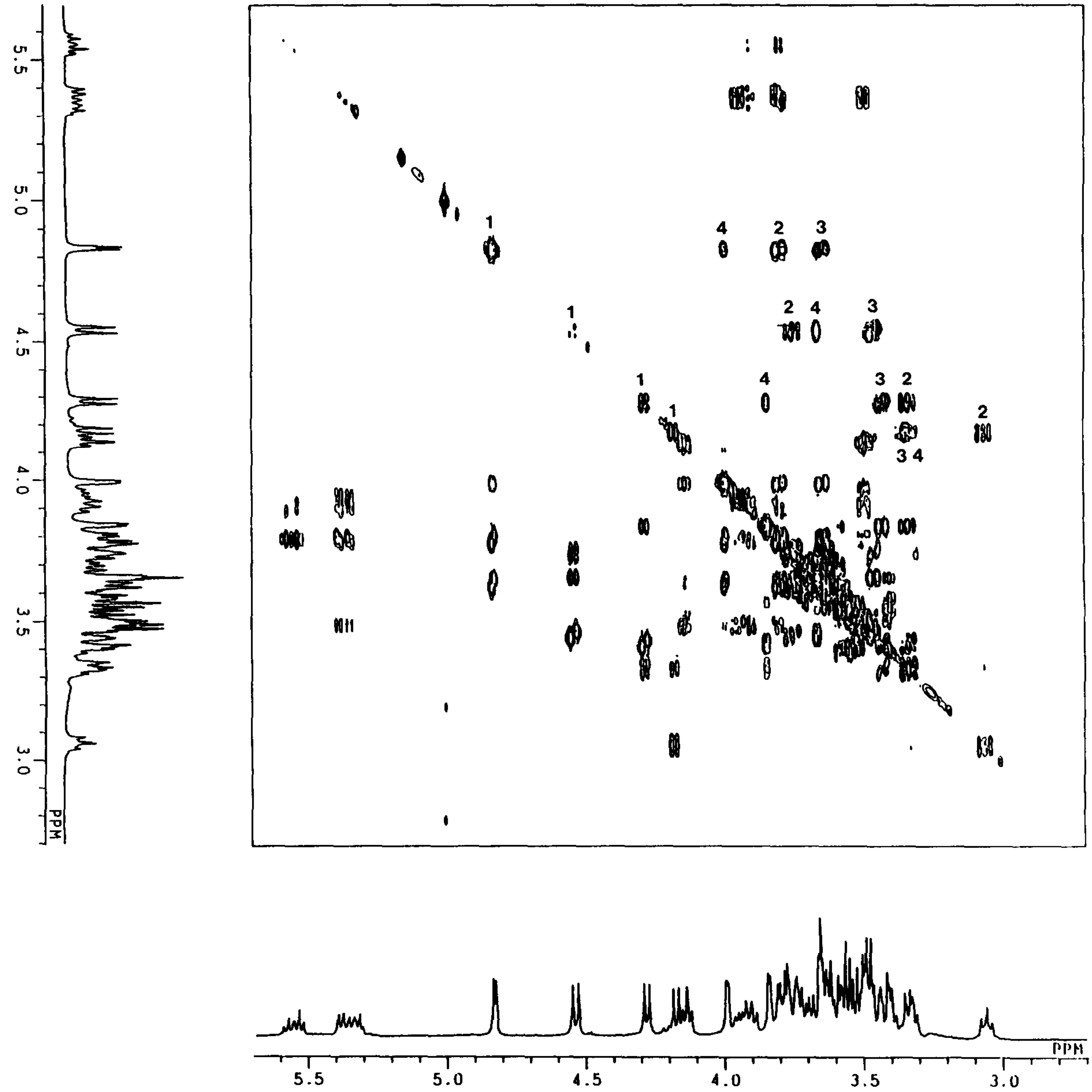
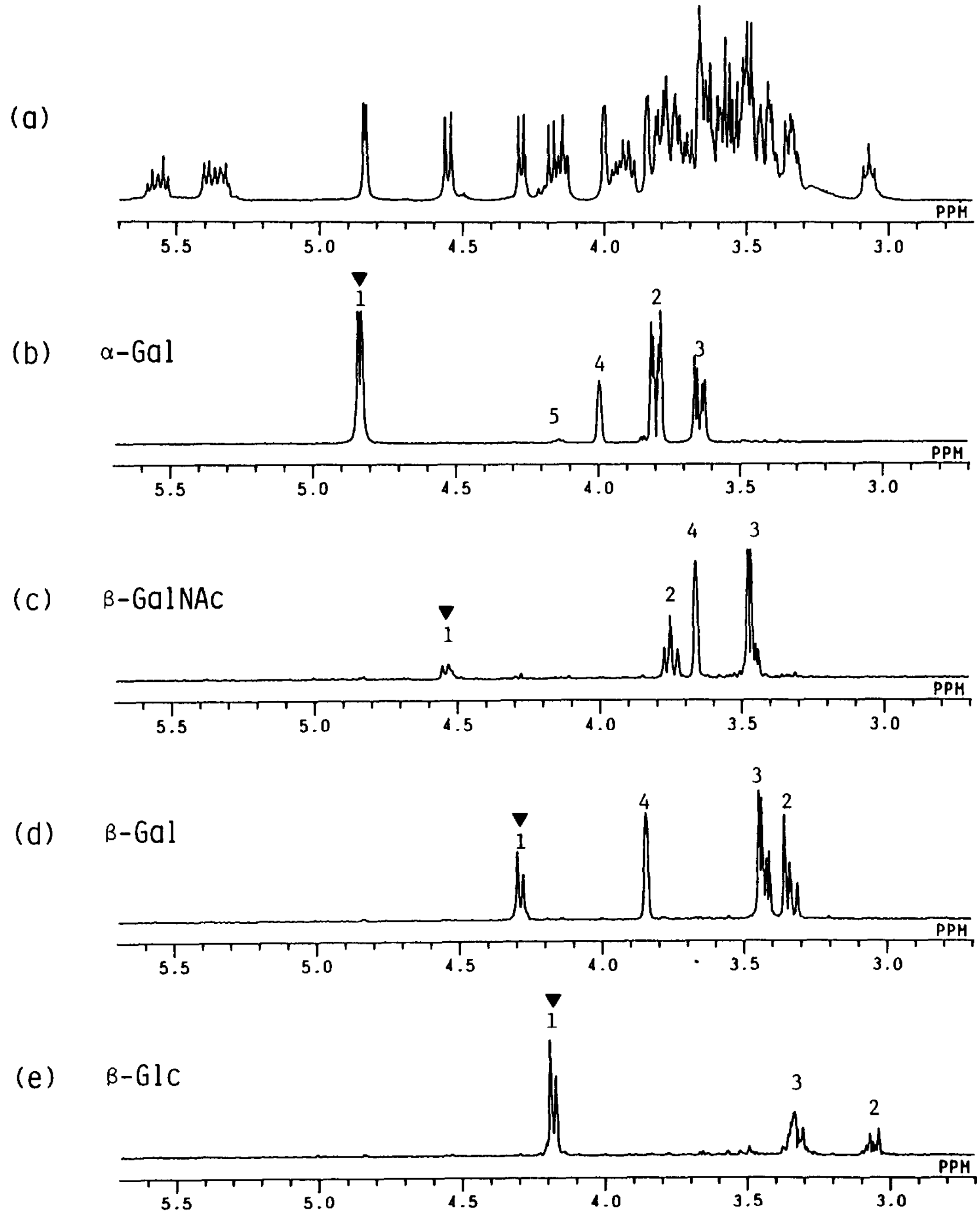


Fig.2. Double relayed COSY spectrum of globoside in the sugar proton resonance region.

experiment (fig.2). Fig.3b–e shows the cross sections taken parallel to the F_2 axis at anomeric proton resonances of the sugar residues. The resolution in the cross section is sufficient to resolve the splitting pattern of each resonances. Since each proton resonance of the hexose and hexosamine residues has a characteristic splitting pattern, the subspectra obtained by the cross section of the double relayed COSY are enough to identify the types and anomeric configurations of the sugar residues. The singlet resonances in fig.3b–d are readily assigned to H4 resonances of the galactose or galactosamine residues. Considering chemical shifts and splitting patterns of proton resonances, the subspectra shown in fig.3b–e correspond to α-galactose, β-galactosamine, β-



galactose and β-glucose, respectively. Thus, the sugar components of globoside can be identified from the analysis of the subspectra. We applied triple relayed COSY for the assignment of H5 proton resonances. However, the magnetization transfer from the anomeric proton resonance beyond H4 was difficult due to small spin coupling constants between H3 and H4 and H4 and H5 protons for galactose and galactosamine. However, by taking the cross sections of the double relayed COSY at H4 proton resonances rather than the anomeric proton resonances, we could assign H5 and H6 proton resonances (fig.3f).

The proton resonances of the sphingosine moi-

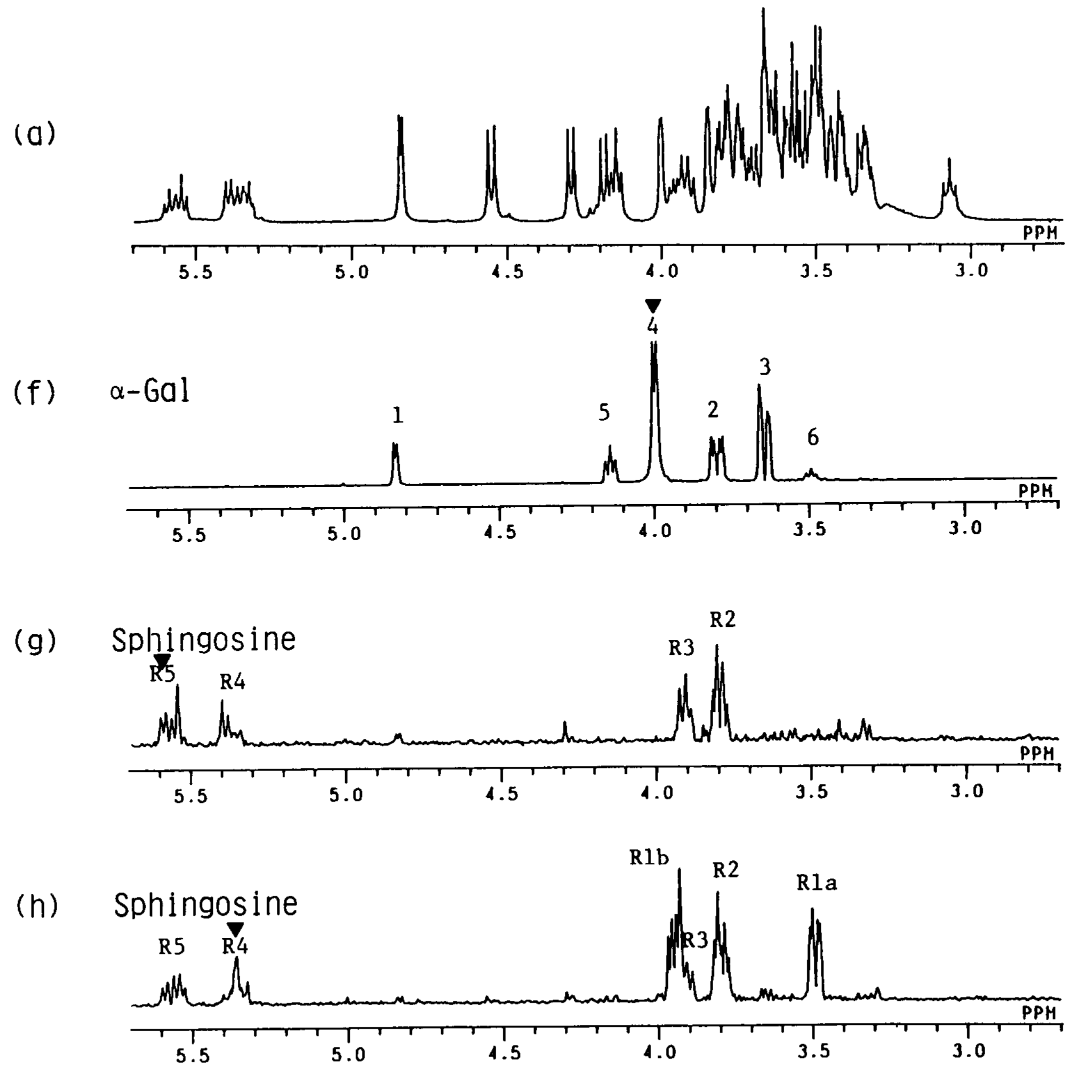


Fig.3. The cross sections of the double relayed COSY spectrum taken parallel to the F_2 axis at the diagonal peaks of the anomeric proton resonances (b–e), H4 proton resonance (f), and R5 and R4 proton resonances of the sphingosine moiety (g,h) with the normal spectrum (a). Assignments of the resonances are also shown.

ety adjacent to the hydroxyl or the amide groups overlap the sugar proton resonances as well, which complicates the spectral analysis of glycolipids. Double relayed COSY was also useful for the assignment of these proton resonances. Fig.3g,h shows the cross sections taken parallel to the F_2 axis at the diagonal peaks of R5 and R4 proton resonances of the sphingosine moiety. We can see the magnetization transfer from R5 to R2 via R4 and R3 and from R4 to R1a and R1b via R3 and R2 (fig.1). Thus, the proton resonances of each sugar component and the sphingosine moiety can be extracted and assigned unambiguously by taking the cross section parallel to the F_2 axis of the double relayed COSY spectrum. In this study, we did not use an incremented mixing time which was applied to the oligosaccharide from ovomucoid to 'tune' the coupling constants [11]. However, the results show sufficient efficiency of the magnetization transfer for different types of sugars.

2D homonuclear Hartman-Hahn spectroscopy is another useful method to extract the spin systems

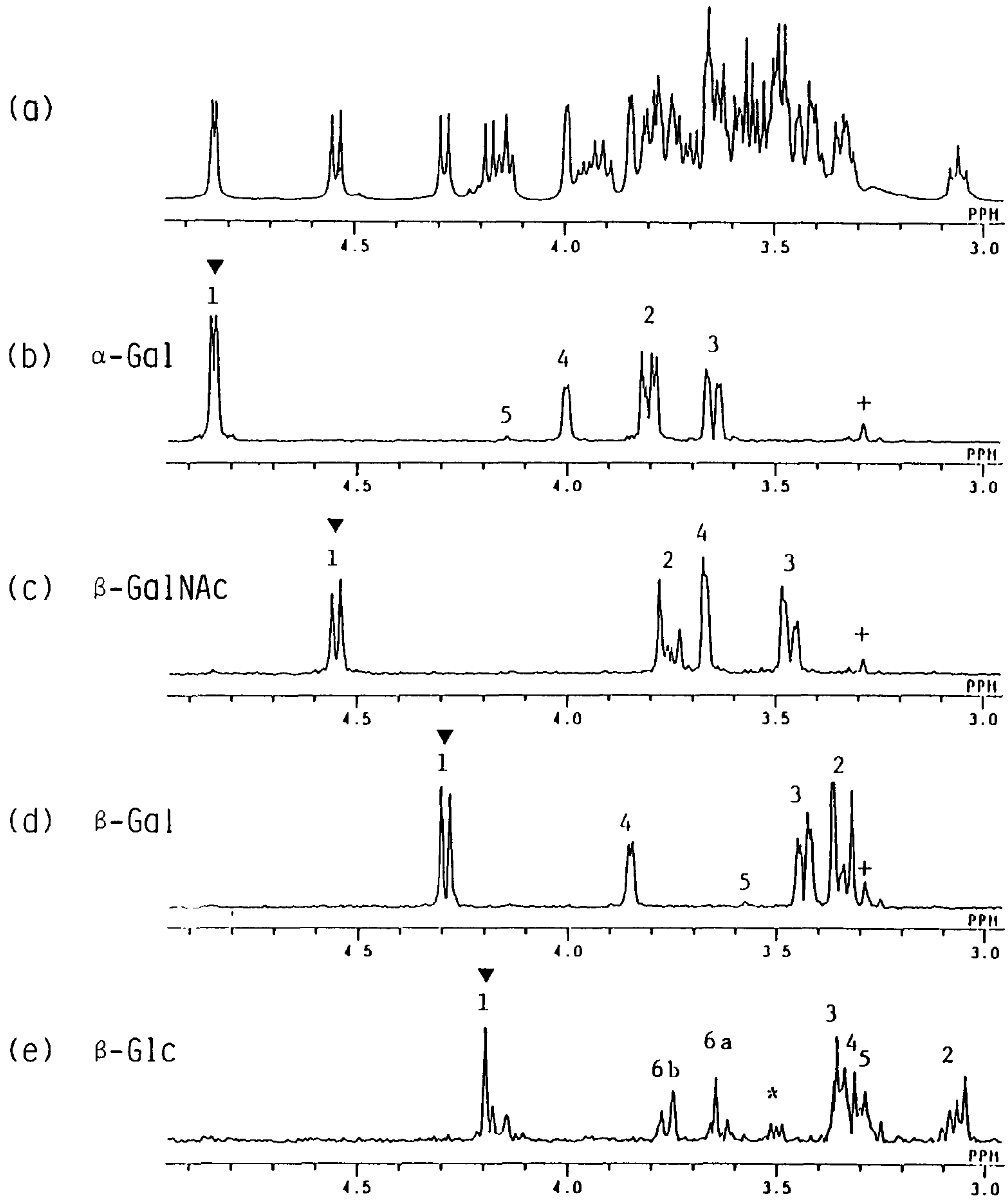


Fig.4. The cross sections of the 2D homonuclear Hartman-Hahn spectrum taken parallel to the F_2 axis at the diagonal peaks of the anomeric proton resonances of globoside (b–e) with the normal spectrum (a). Assignment of the resonances are also shown. (+) Residual HDO proton resonance.

of the sugar residue, where mixing of the magnetization occurs through spin-spin coupling during locking time in the rotating frame. Fig.4b–e shows the cross sections of 2D homonuclear Hartman-Hahn spectra of globoside at the diagonal peaks of the anomeric proton resonances, where the mixing time was set to be 85 ms. The subspectra are similar to those of the double relayed COSY. However, it should be noted that all the proton resonances in the glucose residue can be extracted (fig.4e). This is due to the large spin coupling constants between vicinal protons, which make efficient mixing of magnetization during the locking time. Moreover, we observe H5 proton resonances of galactose and galactosamine residues in the spectra. Compared with double relayed COSY, 2D homonuclear Hartman-Hahn spectroscopy is more efficient for mixing of magnetization. However, under the present experimental conditions, the subspectra obtained by the 2D Hartman-Hahn method are less suitable for the analysis of splitting patterns of proton resonances as are shown in figs 3 and 4. For this purpose, 1D homonuclear Hartman-Hahn spectroscopy is more useful [17].

These methods are helpful for the assignment of the sugar proton resonances of globoside, as shown here, and are also useful for glycolipids with much longer sugar chains, since the spectral resolution in the anomeric proton region is usually good. Once the assignments of the sugar proton resonances are made, these resonances are used to identify the linkage of the sugar residues through NOE experiments. In a forthcoming paper we shall report the structural determination of an unknown glycolipid using multiple relayed COSY and 2D homonuclear Hartman-Hahn spectroscopy combined with the NOE method.

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