Carbohydrate Research, 137 (1985) 244–252
Elsevier Science Publishers B V , Amsterdam – Printed in The Netherlands

Note

Determination of the position of acetic and sulfuric esters in glycosphingolipids by two-dimensional proton-nuclear magnetic resonance spectroscopy

SHINSEI GASA*, MITSURU NAKAMURA, AKIRA MAKITA, MITSUHIKO IKURA†, AND KUNIO HIKICHI‡
Biochemistry Laboratory, Cancer Institute, Hokkaido University School of Medicine, Sapporo 060 (Japan)

(Received November 11th, 1983, accepted for publication in revised form, April 25th, 1984)

Glycosphingolipids (GSLs), a group of glycoconjugates, are particularly abundant in animal cell-membranes. They play biological roles as antigens and as receptors for bacterial toxins.

Although, in the past, the carbohydrate structures of GSLs were analyzed almost exclusively by such destructive methods as methylation and hydrolysis with specific glycosidases[1], recent studies using intact GSLs[2,3] have established n.m.r. spectrometry as a useful nondestructive method for determination of GSL structure. From the results of n.m.r. analysis of a variety of GSLs, general rules were devised in terms of the chemical shift of anomeric and amide protons, the coupling constants, and quantitation of molar composition, including the lipid moiety (ceramide) of GSLs[3]. In one-dimensional proton-n.m.r. analysis, the spin-decoupling difference spectroscopy (SDDS) technique allows characterization of proton signals at anomeric and other carbon atoms of hexopyranosides in globo- and neolact-series GSLs[2] and identification of the linkage position of the *O*-acetyl group at the sialyl moiety of a ganglioside, 4-*O*-acetyl-*N*-glycolyl-α-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranosyl-ceramide (*O*-acetylhematoside)[4].

Dabrowski *et al.* introduced two-dimensional n.m.r. spectrometry for characterization of GSL carbohydrates. 2D-*J*-resolved spectrometry was used for analysis of couplings in the glucose residue of glycosyl-ceramide[5], and two-dimensional spin-echo correlated spectroscopy (2D-SECSY) was employed for partial structural determination of a pentadecaglycosylceramide[6]. The utility of 2D-SECSY and two-

*To whom correspondence should be addressed at Biochemistry Laboratory Cancer Institute, Hokkaido University School of Medicine, N15 W7, Sapporo 060, Japan.
†High-resolution NMR Laboratory, Department of Polymer Science, Faculty of Science, Hokkaido University.
‡Department of Polymer Science, Faculty of Science, Hokkaido University.

0008-6215/85/$ 03.30

dimensional correlation spectroscopy (2D-COSY) for assigning a proton to a specific carbon atom of a carbohydrate was confirmed by Bernstein *et al.*[7]. In fact, through the combination of homonuclear 2D-SECSY and two-dimensional nuclear Overhauser effect spectroscopy (2D-NOESY), Koerner *et al.*[8] and Prestegard *et al.*[9] elucidated the complete structure, including anomeric configuration, linkage position and sequence, of gangliosides.

In this report we demonstrate that the position of an acetic or sulfuric ester linkage to the saccharides of a GSL may be readily identified through 2D-SECSY or 2D-COSY.

EXPERIMENTAL

Materials. — Acetyl hematoside was prepared from equine erythrocyte stroma as described previously[4]. Cerebroside sulfate and galactosylceramide were isolated from equine kidney[10]. ($^2H_6$)Dimethyl sulfoxide (99.95%) was purchased from Aldrich (Milwaukee, WI).

Methods. — Prior to measurement, GSLs (5–8 mg) were dried over phosphorus pentaoxide in an n.m.r. tube at 81° for 12 h under diminished pressure. The dried GSL was dissolved in 0.4 mL of ($^2H_6$)dimethyl sulfoxide by sonication. Cerebroside sulfate containing hydroxy and nonhydroxy fatty acid, and galactosylceramide containing nonhydroxy fatty acid, were analyzed in a JEOL FX-400 (400 MHz) spectrometer at 25°, whereas acetylhematoside was analyzed in a JEOL GX-500 (500 MHz) instrument operating in the Fourier-transform mode with quadrature detection. 1D spectra were obtained over a spectral range of 4000 Hz for cerebroside sulfate and galactosylceramide, and 5000 Hz for acetyl hematoside. Methylene protons from hydrocarbon (fatty acid and sphingosine) groups were set exclusively at 0 p.p.m. for the 1D spectrum of acetylhematoside. A 2D-SECSY spectrum[7,11] was observed over a spectral range of 3602 Hz on the F_2 axis with 2048 data points, and 2500 Hz on the F_1 axis with 256 data points. 2D-COSY spectra[7,12] were observed over a range of 3333 Hz on both the F_2 and F_1 axes, with the same data points used in SECSY for acetylhematoside and a stacked plot of COSY for cerebroside sulfate and galactosylceramide.

RESULTS

2D-SECSY of acetyl hematoside. — The 1D-spectrum and 2D-SECSY spectrum of acetylhematoside are shown in Fig. 1 (upper and lower parts, respectively). Peaks 1 and 2, and the peaks at δ 3.5–3.1 and 2.89 in the 1D spectrum, disappeared when deuterium oxide was introduced into the sample solution, indicating that these peaks correspond to exchangeable protons present at amide and hydroxyl groups. The contours arrayed on the horizontal half-line on the 2D spectrum represent the normal spectrum, and the other contours above or below the line signify cross-peaks that denote interaction between two protons. In Fig. 1, cross-peaks *a*

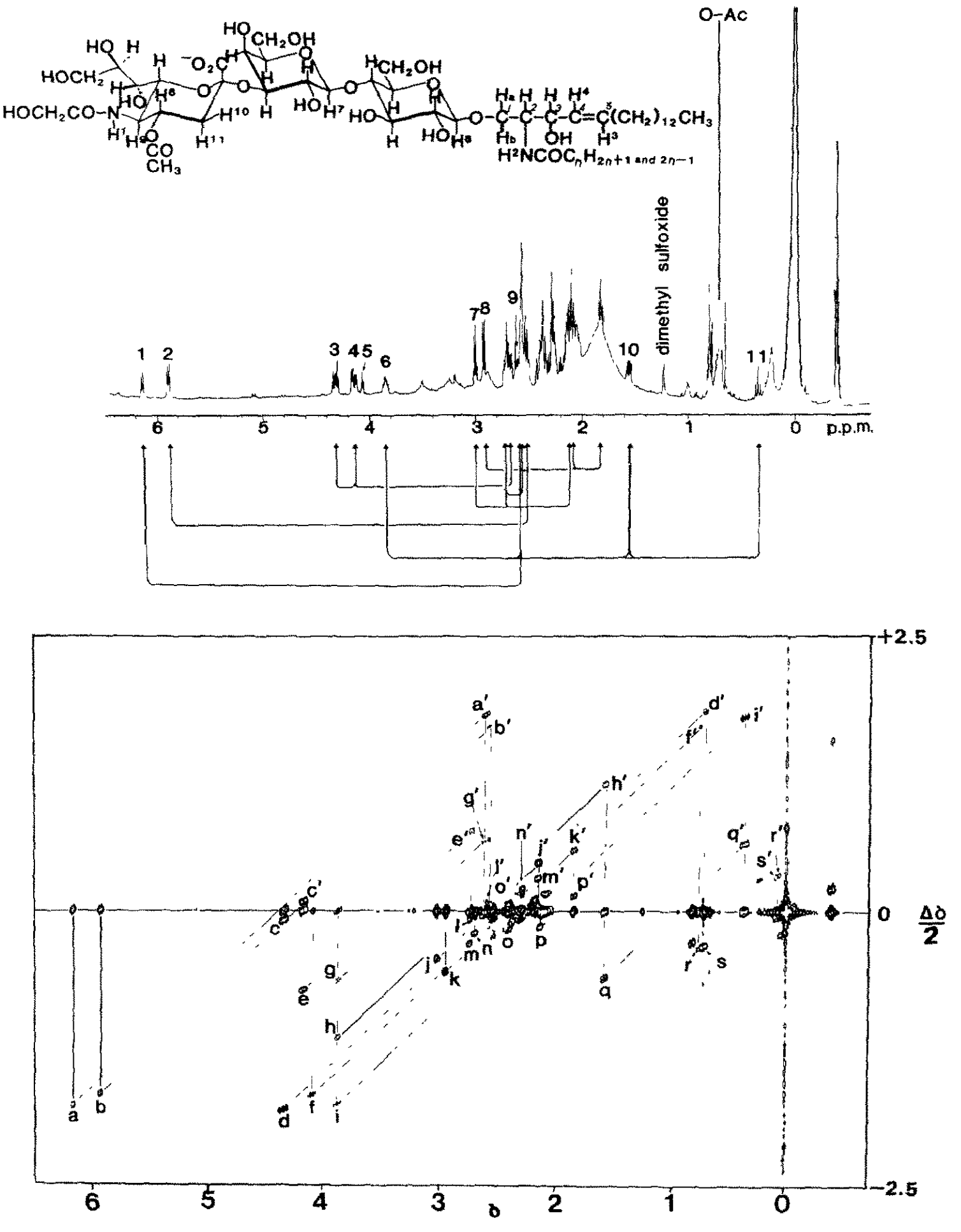
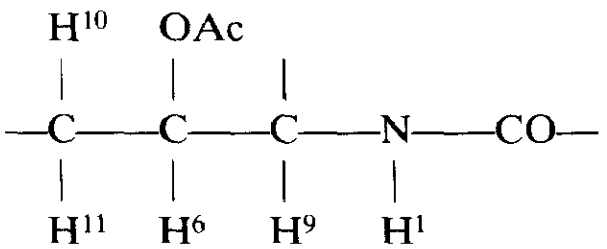


Fig 1. One-dimensional n.m.r. spectrum (upper) and contour plot by 2D-SECSY (lower) of acetylhematoside. The spectra were taken in ($^2H_6$)dimethyl sulfoxide solution at 500 MHz in a range of 0–6 5 p.p.m The peak numbers on the 1D spectrum correspond to the proton numbers given in the structural formula. The arrows at the bottom of the 1D spectrum show the couplings between protons

and a' in the 2D spectrum indicate that there is a coupling between peaks 1 (an amide proton) and 9 in the 1D spectrum, as shown at the bottom of the 1D spectrum. This coupling arises from the linkage of the amide group to the carbon atom to which the proton of peak 9 is attached. Similarly, contours g and g' show a coupling between peaks 9 and 6; h and h' between peaks 6 and 10; i and i' between peaks 6 and 11; and q and q' between peaks 10 and 11. Peaks 10, 11, 6, 9 and 1 therefore fall into a relation of the ABMPX spin-system for which the following partial structure is required:



This structure is present only in the sialyl moiety of acetyl hematoside, confirming the presence of an *O*-acetyl group at C-4 of the *N*-glycolylneuraminosyl moiety, as proposed previously[4]. The shift to lower field of the acetoxy methine proton (H-4 of sialic acid) is due to the deshielding effect of the acetic ester group.

The cross-peaks and the assignment of their protons to components of acetylhematoside are summarized in Table I. Peaks 3 and 4 (H-4 and -5 due to the *trans*-alkenic bond of sphingosine, respectively, in Table I) are demonstrated to be coupled to each other by contours c and c'. Peaks 3 and 4 are coupled to respective adjacent methylene and methine protons, as is evidenced by the presence of d, d' and e, e'. An array of contours, $f \rightarrow f' \rightarrow r \rightarrow r'$, in the fatty acid moiety shows a coupling sequence in which *cis*-alkenic protons (peak 5) are coupled with methylene protons near the alkenic bond to yield f and f', and with other methylene protons (~0.05 p.p.m.) at a position distant from the alkenic bond to yield r and r'. Similarly, an array of $j \rightarrow j' \rightarrow m' \rightarrow m \rightarrow l \rightarrow l'$ in a galactose moiety indicates sequential couplings from the α-anomeric proton to the protons at C-2→C-3→C-4; $k \rightarrow k' \rightarrow p' \rightarrow p$ in a glucose moiety shows sequential couplings from the α-anomeric proton to the protons at C-2→C-3. Coupling of protons attached to the following carbon atoms of the respective monosaccharide moieties could not be assigned, because their cross-peaks were mutually overlapping and the threshold used to present contours was too high in this study: H-5 and -6 in galactose; H-4, -5, and -6 in glucose; and H-6, -7, -8, and -9 in *N*-glycolylneuraminic acid. However, these protons were assigned by Koerner *et al.* in *N*-acetylneuraminyllactosylceramide (ordinary hematoside)[8].

2D-COSY of cerebroside sulfate and galactosylceramide. — The stacked-plot presentations by 2D-COSY of cerebroside sulfate and galactosylceramide are

*The numbering system used in Table I and Figs. 1–3 is for convenience in the present context; it does not correspond to conventional, systematic numbering.

TABLE I

CONNECTIVITIES BETWEEN PROTONS IN ACETYLHEMATOSIDE AS REVEALED BY 2D-SECSY

Cross-peaks	*GSL component*	*Coupling pairs and assignment*[a]
a,a'	NeuGc[b]	amide (1) and H-5 (9)
b,b'	Sph[c]	amide (2) and H-2 (δ 2.58)
c,c'	Sph	H-5 (3) and H-4 (4)
d,d'	Sph	H-5 (3) and H-6 (δ 0.70)
e,e'	Sph	H-4 (4) and H-3 (δ 2.71)
f,f'	FA[d]	alkenic and allylic (δ 0.74)
g,g'	NeuGc	H-4 (6) and H-5 (9)
h,h'	NeuGc	H-4 (6) and Heq-3 (10)
i,i'	NeuGc	H-4 (6) and Hax-3 (11)
j,j'	Gal	H-7 (7) and H-2 (δ 2.13)
k,k'	Glc	H-1 (8) and H-2 (δ 1.82)
l,l'	Gal	H-3 (δ 2.74) and H-4 (δ 2.57)
m,m'	Gal	H-3 (δ 2.74) and H-2 (δ 2.13)
n,n'	Sph	Ha-1 (δ 2.68) and Hb-1 (δ 2.26)
o,o'	Sph	Hb-1 (δ 2.26) and H-2 (δ 2.50)
p,p'	Glc	H-3 (δ 2.10) and H-2 (δ 1.82)
q,q'	NeuGc	Heq-3 (10) and Hax-3 (11)
r,r'	FA	allylic (δ 0.74) and the adjacent (δ 0.05)
s,s'	Sph	H-6 (δ 0.70) and H_2-7 (δ 0.08)

[a]Numerical assignments are given according to the sequential position of the carbon atom to which the proton is attached on the carbohydrate or lipid moiety. The number or δ value in parentheses corresponds to the number assigned to the proton in the structural formula in Fig. 1 or to the chemical shift of the proton, respectively. [b]*N*-Glycolylneuraminic acid. [c]Sphingosine. [d]Fatty acid.

TABLE II

CONNECTIVITIES BETWEEN PROTONS IN CEREBROSIDE SULFATE REVEALED BY 2D-COSY

Cross-peaks	*GSL component*	*Coupling pairs and assignment*[a]
a,a'	Sph[b]	H-4 (1) and H-5 (2)
b,b'	HFA[c]	OH-2 (δ 5.67) and H-2 (6)
c,c'	Sph	H-4 (2) and H-3 (5)
d,d'	Gal	H-1 (3) and H-2 (7)
e,e'	Gal	H-3 (4) and H-2 (7)

[a]Protons are numbered according to their sequential positions. The number or δ value in parentheses is the peak number or the chemical shift assigned in Fig. 2, respectively. [b]Sphingosine. [c]Hydroxy fatty acid.

shown in Figs. 2 and 3, respectively, together with their 1D spectra for comparison. The data from the 2D spectra and their assignment are summarized in Table II for cerebroside sulfate and in Table III for galactosylceramide. In Fig. 2, contour *d* indicates coupling between an anomeric proton (peak 3) and H-2 (peak 7) of galactose, as shown at the bottom of the 1D spectrum. Similarly, the presence of contour *e*, which is on the same line as *d*, shows the interaction between H-2 and H-3 (peak 4). The chemical shift of H-3 consequently is deduced to be δ 4.05. Cross-peak *a*

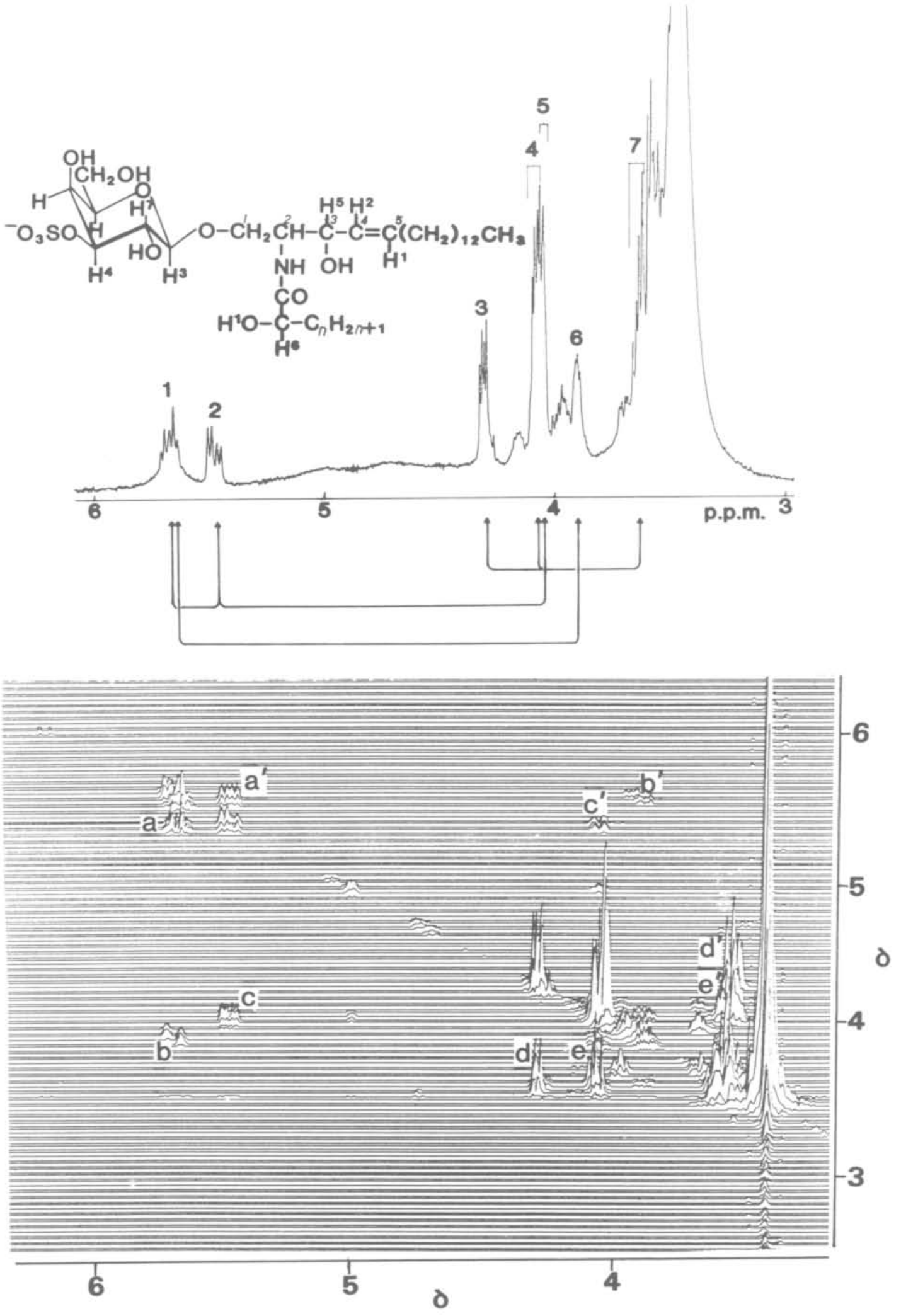


Fig. 2. One-dimensional n.m.r. spectrum (upper) and 2D presentation (lower) by 2D-COSY of cerebroside sulfate containing hydroxy and nonhydroxy fatty acids. The spectra were recorded in a ($^2H_6$)dimethyl sulfoxide solution at 400 MHz in the range of 3.1–6.0 p.p.m. The numerical labels in the structural formula correspond to the peak numbers on the spectrum, and arrows demonstrate couplings between protons.

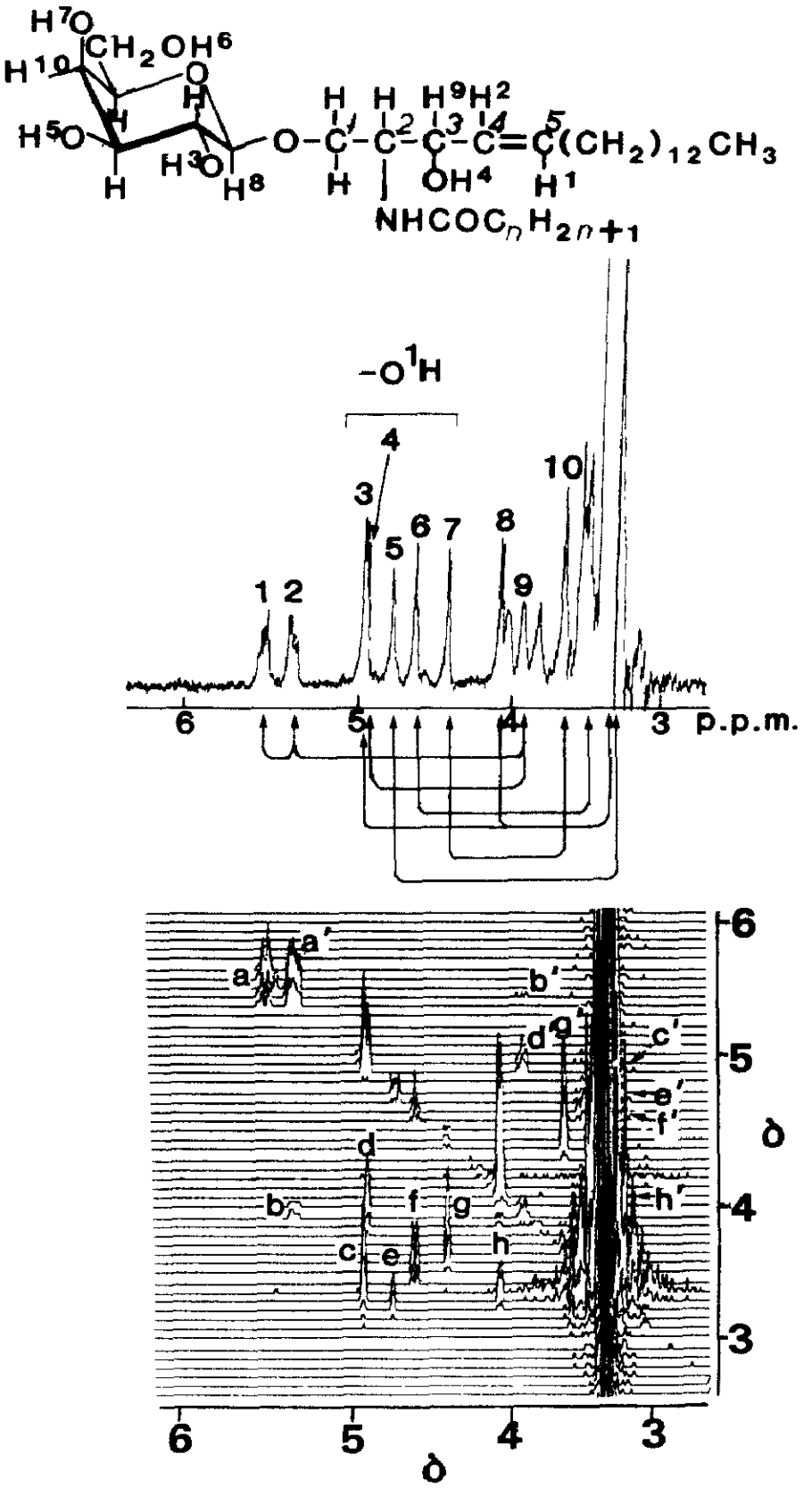


Fig. 3. One-dimensional n.m.r. spectrum (upper) and 2D presentation (lower) by 2D-COSY of galactosylceramide containing nonhydroxy fatty acids. The spectra were recorded in a ($^2H_6$)dimethyl sulfoxide solution at 400 MHz in the range of 3.0–6 0 p.p.m. The numbers given in the structural formula correspond to the peak numbers on the 1D spectrum, and arrows at the bottom of the 1D spectrum show the couplings between protons.

indicates a connectivity between H-4 and -5 of sphingosine, as described in the case of acetyl hematoside. Peaks 5 and 6 in the 1D spectrum are assigned to H-3 of sphingosine and H-2 of hydroxy fatty acid, respectively.

After assignment of the other peaks (see Table III), the chemical shift of H-3 of the galactose moiety of galactosylceramide is deduced to be δ 3.37, which is

TABLE III

CONNECTIVITIES BETWEEN PROTONS IN GALACTOSYLCERAMIDE CONTAINING NONHYDROXY FATTY ACID REVEALED BY 2D-COSY

Cross-peaks	*GSL component*	*Coupling pairs and assignment*[a]
a,a'	Sph	H-5 (1) and H-4 (2)
b,b'	Sph	H-4 (2) and H-3 (9)
c,c'[c]	Gal	OH-2 (3) and H-2 (δ 3.35)
d,d'	Sph	OH-3 (4) and H-3 (9)
e,e'[c]	Gal	OH-3 (5) and H-3 (δ 3.37)
f,f'[c]	Gal	OH-6 (6) and H-6 (δ 3.35 and δ 3.43)
g,g'	Gal	OH-4 (7) and H-4 (10)
h,h'[c]	Gal	H-1 (8) and H-2 (δ 3.35)

[a]Assignments are given according to the sequential positions of the protons in Fig. 3. The number or δ value in parentheses is the peak number or the chemical shift assigned in Fig. 3, respectively. [b]Sphingosine. [c]Hidden by the H_2O peak.

hidden by the H_2O peak, from the connectivity (contour *e*) between H-3 and the 3-hydroxyl proton (peak 5 in the 1D spectrum, Fig. 3). The connectivities shown by other contours, as assigned in Table III, support the proposed structure of galactosylceramide. Judging from the difference ($\Delta\delta$ 0.68) in the chemical shift of H-3 on the galactose moiety between cerebroside sulfate and galactosylceramide, the sulfate group is deduced to be linked at O-3 in cerebroside sulfate. As the electronegative sulfate group has a deshielding effect, H-3 in cerebroside sulfate would be shifted to lower field than H-3 in galactosylceramide. The position of the sulfate group be also verified by the absence of a 3-hydroxyl proton in the 1D-spectrum of cerebroside sulfate (data not shown).

DISCUSSION

Prior analyses of the carbohydrate structures of GSLs have used methylation analysis to locate the position of such ester groups as the sulfate group of cerebroside sulfate[13], but this procedure is not convenient for locating the (alkali-labile) *O*-acetyl ester group. The position of the *O*-acetyl group in acetyl hematoside was therefore determined by a spin-decoupling technique, as well as by acetalation followed by methylation[4]. In the present study, we have demonstrated that the linkage positions of sulfate and *O*-acetyl esters in GSLs may be readily determined by 2D n.m.r. spectrometry, either by the COSY or SECSY techniques, as cerebroside sulfate and acetyl hematoside have been established to possess sulfate[13] and *O*-acetyl[14] groups, respectively. This finding is based on the observation that protons at carbon atoms bearing an ester group shift to lower field. An *O*-acetyl group causes a greater shift than a sulfate group.

2D Spectrometry affords structural information similar to that obtainable with 1D-SDDS in that both types of spectrometry provide proton signals at C-2 to

-6 of the hexopyranosides in GSLs, as demonstrated previously[2] and also in the present study. Proton coupling may also be detected by the SDDS technique through irradiation of selected proton pairs that display peaks in a conventional 1D spectrum[4]. However, 2D techniques are superior to SDDS analysis in that the former allow interpretation of signals due to proton couplings, contours present in the field above and below the half center line in SECSY (Fig. 1), and 2D peaks present other than on a 45-angled line in COSY (Figs. 2 and 3), from which much information can be obtained. 2D-SECSY and -COSY techniques are prime choices for structural analysis of a GSL containing an ester group. A shortcoming of a stacked plot is that some cross-peaks are hidden by mutual overlapping.

REFERENCES

1 C. C. SWEELEY AND B. SIDDIQUI, in M. I. HOROWITZ AND W. PIGMAN (Eds.), *The Glycoconjugates*, Vol. 1, 1977, pp. 459–540.
2 J. DABROWSKI, P. HANFLAND, AND H. EGGE, *Biochemistry*, 19 (1980) 5652–5658.
3 S. GASA, T. MITSUYAMA, AND A. MAKITA, *J. Lipid Res.*, 24 (1983) 174–182.
4 S. GASA, A. MAKITA, AND Y. KINOSHITA, *J. Biol. Chem.*, 258 (1983) 876–881
5 A. YAMADA, J. DABROWSKI, P. HANFLAND, AND H. EGGE, *Biochim Biophys. Acta*, 618 (1980) 473–479.
6 J. DABROWSKI AND P. HANFLAND, *FEBS Lett.*, 142 (1982) 138–142.
7 M. A. BERNSTEIN, L. D. HALL, AND S. SUKUMAR, *Carbohydr. Res.*, 103 (1982) c1–c6.
8 T. A. W. KOERNER, JR., J. H. PRESTEGARD, P. C. DEMOU, AND R. K. YU, *Biochemistry*, 22 (1983) 2676–2687.
9 J. H. PRESTEGARD, T. A. W. KOERNER, JR., P. C. DEMOU, AND R. K. YU, *J. Am. Chem. Soc.*, 104 (1982) 4993–4995.
10 S. GASA AND A. MAKITA, *J. Biochem. (Tokyo)*, 88 (1980) 1119–1128.
11 K. NAGAYAMA, K. WUTHRICH, AND R. R. ERNST, *Biochim. Biophys. Res. Commun.*, 90 (1979) 305–311.
12 A. BAX, R. FREEMAN, AND G. MORRIS, *J. Magn. Reson.*, 42 (1981) 164–170.
13 T. YAMAKAWA, N. KISO, S. HANDA, A. MAKITA, AND S. YOKOYAMA, *J. Biochem (Tokyo)*, 52 (1962) 226–227
14 S. HAKOMORI AND T. SAITO, *Biochemistry*, 8 (1969) 5082–5088.