Carbon-13 nuclear magnetic resonance spectrometry of globotriaosylceramide

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Abstract Resonances in the carbon-13 natural abundance, proton-decoupled, 90.5 MHz nuclear magnetic resonance spectrum of globotriaosylceramide were assigned to specific carbon nuclei. The chemical shifts were rationalized in terms of the number of sugar residues, the sugar ring structures, the positions and anomeric configurations of the intersugar linkages, and the approximate degree of unsaturation of the alkyl chains of the ceramide moiety.—**Nunez, H. A., and C. C. Sweeley.** Carbon-13 nuclear magnetic resonance spectrometry of globotriaosylceramide. *J. Lipid Res.* 1982. **23:** 863–867.

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Glycosphingolipids are believed to be involved in functions related to intercellular communication, intercellular adhesion, cellular motility, regulation of cellular growth and differentiation, immune response, hormone reception, and internalization of macromolecular materials (1, 2). Structurally the glycosphingolipids contain a hydrophobic portion (formed by the alkyl chains of the fatty acyl and long-chain base moieties) that is imbedded in the plasma membrane, and a hydrophilic portion (formed by a variable number of sugar residues) that is generally assumed to be on the outer (extracellular) aspect of the membrane. Mammalian cells usually contain several kinds of neutral and acidic glycosphingolipids, whose structures contain subtle differences that need to be known for an eventual interpretation of their specific biological roles.

In this report, we analyze the high resolution, natural abundance ^{13}C nuclear magnetic resonance (NMR) spectrum of globotriaosylceramide ($GbOse_3Cer$) and show that this technique is a powerful tool for determining the number of sugar residues, the sugar ring structure, the position of the intersugar linkages, the sugar anomeric configuration, and the approximate degree of unsaturation. Although most of this information has already been obtained by chemical and enzymatic means, the sugar ring structure and the anomeric configuration of the glycopyranosidic form of the sugar residues can be uniquely determined by ^{13}C NMR spectrometry of the intact molecule. This study and other NMR data on glycosphingolipids (3–9) indicate that, as more spectra of different but related structures are analyzed, the technique will become an important method of structural characterization. Availability of structure-chemical shift relationships will be essential for future dynamic in vitro and in vivo studies of these substances in membranes by NMR.

EXPERIMENTAL

The spectrum of $GbOse_3Cer$ was obtained with 100 mg of sample dissolved in 3 ml of pyridine-d_5 in a 10-mm tube. The spectrometer was an NT-360 operating in the Fourier transform mode. Transients (4000) were accumulated in a NICOLET 1180 computer with 16K of memory using a sweepwidth of 5000 Hz. Under these conditions the acquisition time was 0.2 sec. No delay time between the pulses was used. The decoupling frequency was set at about 5 ppm in the proton spectrum scale. A two-level decoupling power was used in order to keep the probe temperature reading at 25°C. TMS was used as internal reference. The sample of $GbOse_3Cer$ was isolated from porcine intestine as previously described (10).

RESULTS AND DISCUSSION

The complete carbon-13 natural abundance, proton-decoupled, 90.5 MHz ^{13}C NMR spectrum of $GbOse_3Cer$ is shown in **Fig. 1** and Fig. 2. The chemical shift assignments are presented in **Table 1**. Fig. 1a contains the resonances corresponding to unsaturated carbons and that of the carbonyl carbon of the fatty acyl group (the

Abbreviations: ^{13}C NMR, carbon-13 nuclear magnetic resonance spectrometry; GL1 glucosylceramide; GL2, lactosylceramide; GL3, globotriaosylceramide; GL4, globotetraosylceramide; TMS, tetramethylsilance.

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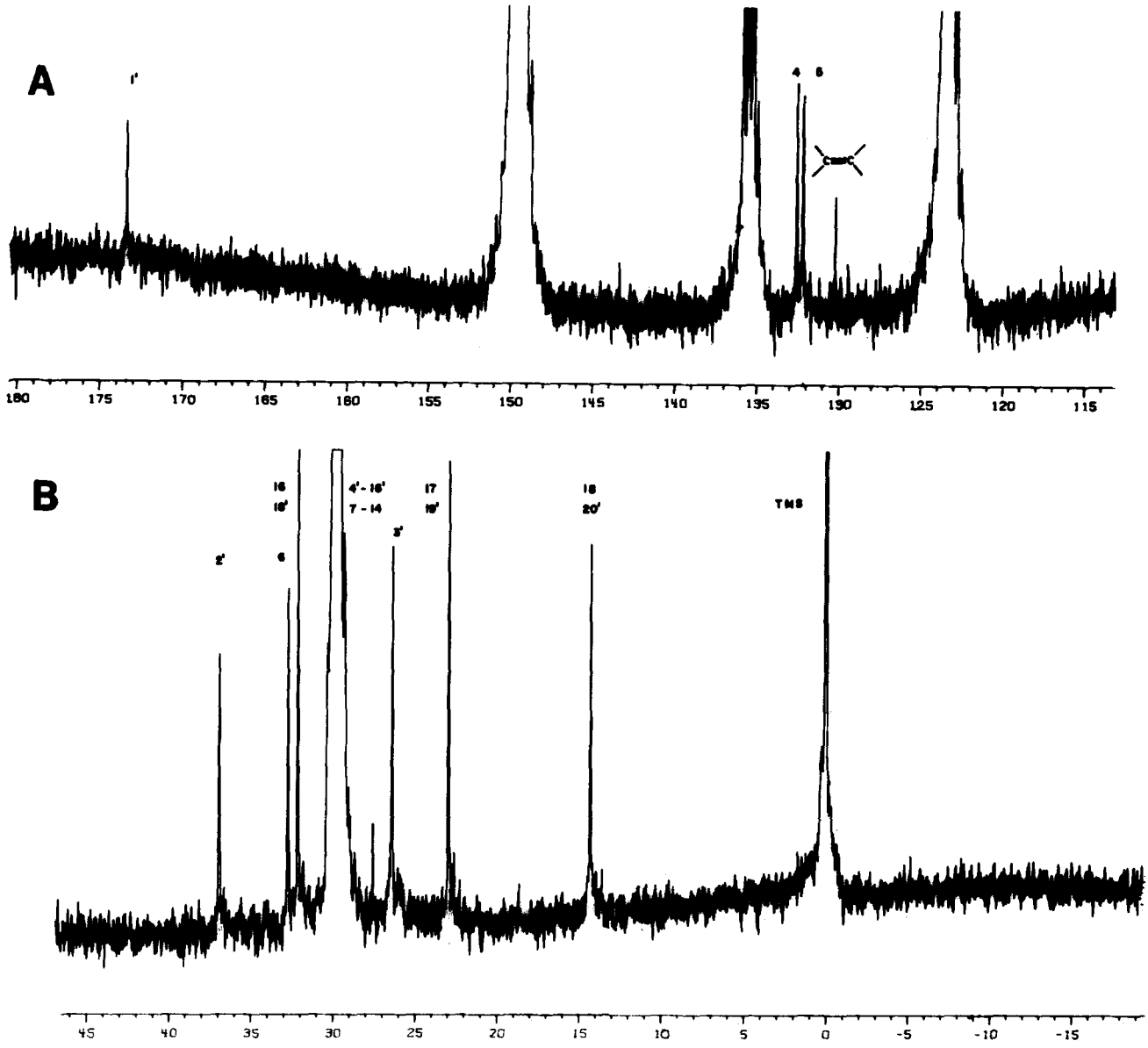

Fig. 1. Partial proton-decoupled, 90.5 MHz ^{13}C-NMR spectrum of globotriaosylceramide ($GbOse_3Cer$) in pyridine-d_5. A) Region at which the solvent (three large envelopes), the unsaturated and the amide carbon of globotriaosylceramide resonate. B) Region at which the saturated carbons of the sphingosine and the fatty acyl moieties resonate. The numbering of the peaks corresponds to the structure of globotriaosylceramide shown in Fig. 2.

three large envelopes are from the pyridine solvent). These sp^2 hybridized carbon nuclei invariably have their resonances in the region of 120 to 180 ppm. In the case of $GbOse_3Cer$, the peak at 173.4 ppm can be assigned to the only carbonyl carbon present in the molecule, the amide linkage of the ceramide moiety. The spectrum shows three more peaks in this region. Those at 132.6 and 132.2 ppm have approximately the same intensities and are assigned to the vinyl carbons of sphingosine. At this high magnetic field they are magnetically non-equivalent, as expected from their chemical structure; C4 (linked to a hydroxy-containing carbon) should be deshielded with respect to C5 (3–8). Consequently, C4 is assigned to the 132.6 ppm resonance and C5 to the 132.2 ppm peak. The chemical shift difference between these two peaks is 0.4 ppm, whereas in glucosylceramide from human liver this difference is 4.9 ppm (4). The chemical shift difference between these two vinyl carbon nuclei peaks of the sphingosine moiety is highly dependent upon the solvent (3), the nature of the saccharide (6, 8), and the fatty acid (8) moieties.

The resonance at 130.3 ppm could belong to a pair of magnetically equivalent unsaturated carbons present in the internal part of the fatty acyl chain. Its relative

TABLE 1. Carbon-13 chemical shift assignments for globotriaosylceramide from porcine intestine[a]

Carbon Number	Structural Moiety				
	Sphingosine	N-Acyl	Glcβ-	Galβ-	Galα
1	70.5[e]	173.4	105.4	105.7	103.1
2	55.5	36.8	74.8[c]	72.6[d]	70.7[e]
3	72.7[d]	26.4	76.6[b]	74.7[c]	71.1[e]
4	132.6		81.9	79.8	71.0[e]
5	132.2		76.5[b]	76.2[b]	71.1[d]
6	32.7		60.5[f]	61.9[f]	62.7[f]
7	|	28–30			
—	28–30	|			
15	|				
16	32.0				
17	22.9				
18	14.3	32.0			
19		22.9			
20		14.3			

[a] Chemical shifts in parts per million (ppm) downfield from internal tetramethylsilane. Approximate accuracy is ±0.1 ppm.
[b,c,d,e,f] Within each group (see text and Fig. 2) the assignments can be exchanged.

size indicates that not all of the $GbOse_3Cer$ molecules contain this fatty acyl chain, a form of heterogeneity that was also found in glucosylceramide of human origin (4). Using the integrated values of the resonances corresponding to the unsaturated carbons, an estimation of the total unsaturation present in the fatty acyl chain can be obtained. There are, however, several factors (relaxation time of the observed nuclei, nuclear Overhauser enhancement, waiting time between the spectrometer pulses) intrinsic to the structure and to the NMR experiment that influence the integrated value (11). Therefore, unless the experiment is specially designed with these factors in mind, the results must be interpreted with some caution. A recent ^{13}C NMR study indicated that glucosylceramide derived from liver of a patient with Gaucher's disease contained approximately 15% of unsaturation (4). From the $GbOse_3Cer$ spectrum (Fig. 1) a comparable amount of unsaturation could be estimated.

Fig. 1b shows the spectrum between 10 and 40 ppm, a region in which only the alkyl carbons of the ceramide moiety resonate (3–9). The large peak at approximately 30 ppm is actually an envelope containing the resonances of numerous methylene carbons, including C7 to C15 of the sphingosine moiety and C4 to C17 of the fatty acyl moiety. All of these carbon nuclei are nearly equivalent from the magnetic point of view and are not resolved at this magnetic field. The remainder of the peaks in this part of the spectrum have been assigned as follows: C2′, 36.8; C6, 32.7; C16 and C18′, 32.0; C3′, 26.4; C17 and C19′, 22.9; C18 and C20′, 14.3 ppm. These assignments are in agreement with those reported from other laboratories (3–9) and with our reports on the ^{13}C NMR of globoside (6, 12).

Fig. 2 shows the resonances that appear between 50 and 110 ppm. In this region of the spectrum only the carbohydrate carbons and C1 to C3 of sphingosine are present (3–8). All the 21 carbons can be accounted for and are resolved or nearly resolved at 90.5 MHz. This part of the spectrum provides convincing information regarding the number of sugar residues, the anomeric configurations, the ring structure, and the intersugar linkages present in an oligosaccharide structure. In the following paragraphs this portion of the spectrum is analyzed in that context.

Within the broad range from 50 to 110 ppm, three distinct narrower windows have been established. These are approximately 90 to 110 ppm, 65 to 85 ppm, and 60 to 65 ppm for the anomeric, the ring, and the exocyclic carbons, respectively (6, 13). Carbon atoms that are attached to N or S are exceptional, as in the case of N-glycosylated residues, sugar amines, and sulfonated carbohydrates (14). The $GbOse_3Cer$ "anomeric window" shows three peaks (Fig. 1). Using the chemical shifts of the anomeric carbons of model glycosides and the lower members of the globo series, glucosylceramide and lactosylceramide (3–8, 12–21), these peaks can be assigned to GalβC1 (105.7 ppm), GlcβC1 (105.4 ppm), and GalαC1 (103.1 ppm). The resonance of GlcβC1 is at lower field than that reported by Koerner et al. (4) for GlcβC1 in glucosylceramide. Koerner et al. (4) obtained the spectrum in a chloroform–methanol solution whereas ours was recorded in pyridine. This solvent effect was also observed by Sillerud et al. (5), who obtained equivalent chemical shifts for glucosylceramide and lactosylceramide in pyridine–benzene solutions. These anomeric chemical shifts provide good evidence that the three major

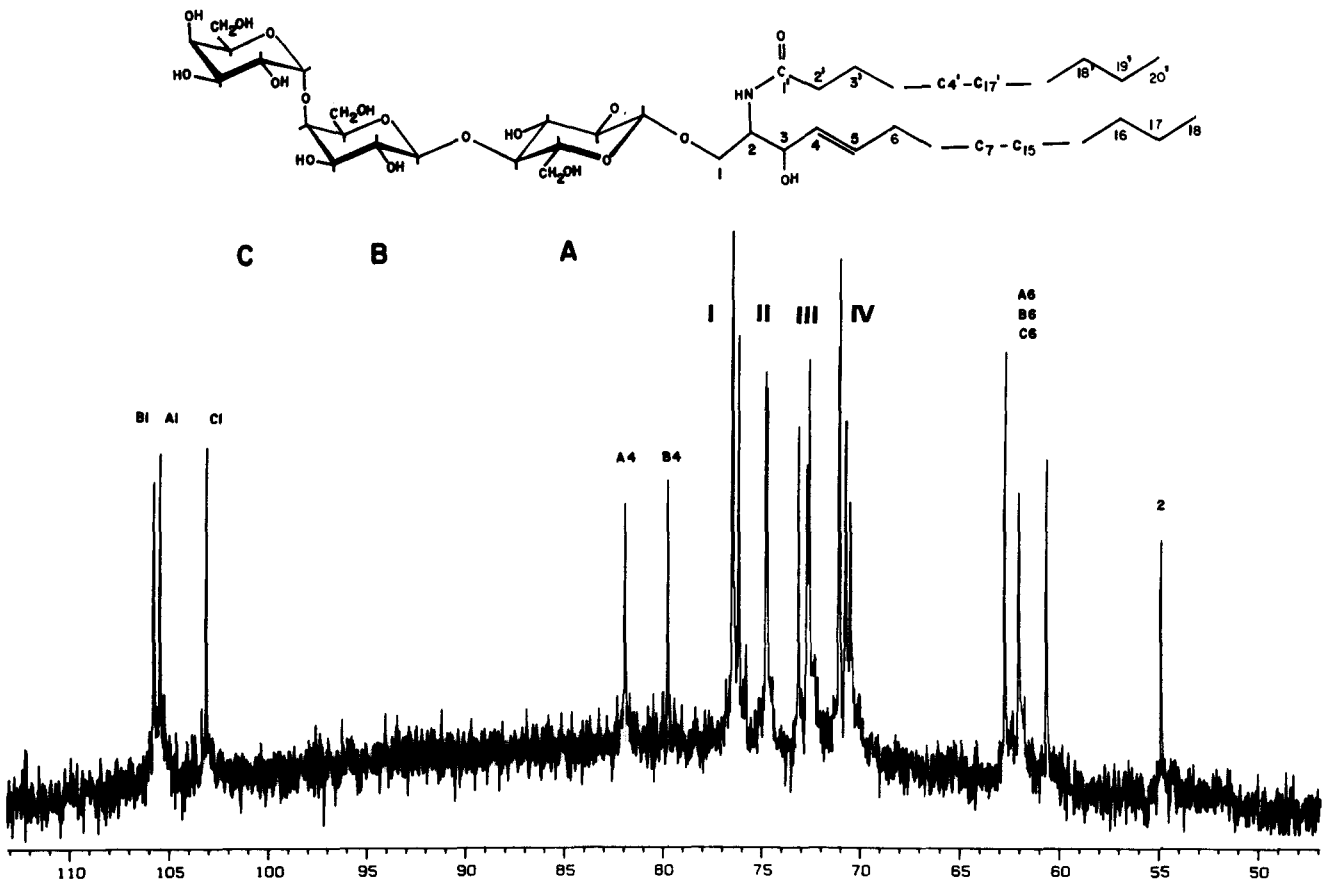

Fig. 2. Partial proton-decoupled, 90.5 MHz ^{13}C-NMR spectrum of globotriaosylceramide ($GbOse_3Cer$) in pyridine-d_5. This region of the spectrum contains all the resonances of the oligosaccharide moiety and those corresponding to C1, C2, and C3 of the sphingosine moiety. The assignments are discussed in the text.

residues of $GbOse_3Cer$ are in the pyranosyl form, since C1 of glycofuranosyl residues resonate at lower fields (16). This conclusion is confirmed by the observation that the $GbOse_3Cer$ spectrum lacks resonances in the 83 and 85 ppm region where C4 of α and β galactofuranosides resonate (16). Additionally, Koerner et al. (4) have shown that the glucosyl moiety of glucosylceramide is in the β-glucopyranosyl form.

It is well established that O-derivatization of a glycopyranosyl carbon induces an 8 to 10 ppm downfield chemical shift displacement of the carbon directly involved in the linkage and usually a displacement of less than 1 ppm of the adjacent carbons (6, 13, 16). Using this argument, the presence of three peaks in the 60 to 63 ppm region (Fig. 2) indicates that the three hydroxymethyl carbons of the glycopyranosyl residues of $GbOse_3Cer$ are underivatized, and consequently the intersugar linkages must involve glycopyranosyl ring carbons, as chemically demonstrated for $GbOse_3Cer$ (2, 6) and discussed below in terms of the ^{13}C NMR spectrum.

Glycopyranosides of Gal and Glc do not show resonances at fields lower than 77 ppm (15). Therefore, the two resonances at 81.9 and 79.8 ppm in Fig. 2 must belong to the two O-derivatized glycopyranosyl ring carbons involved in the intersugar linkage. Existing data (13, 15) indicate that there should be six resonances in the 69 to 71 ppm region if the three glycopyranosyl residues of $GbOse_3Cer$ were underivatized: GalαC4 (70.2 ppm), GalαC2 (71.0 ppm), GalαC3 (71.3 ppm), GalβC4 (70.0 ppm), GlcβC4 (70.7 ppm), and C1 of the ceramide moiety (70.5 ppm). In this region, however, there are only four peaks (Fig. 2). This observation, together with the excellent correlation between the established chemical shifts for gluco- and galacto-pyranosides (15) in the remainder of the $GbOse_3Cer$ spectrum, indicates that the two displaced resonances belong to GlcβC4 and to GalβC4.

The spectrum of this complex oligosaccharide moiety cannot, at the present time, be entirely and unambiguously rationalized even though its structure is known. The major difficulties in completing the structure-chemical shift correlation are the proximity of some of the chemical shifts and the relatively high sensitivity of the chemical shifts to the sugar substituent and conforma-

tional effects. To overcome or minimize this problem, the availability of assigned chemical shifts of closely related structures is essential. In some cases a conclusive chemical shift assignment has to be obtained by specific isotopic substitution as previously described for monosaccharides (15, 17, 18) and disaccharides (13, 19, 20, 21). In other cases NMR techniques can be used (13). In this study, using the chemical shifts from model compounds and the lower members of the globo series (3–5, 12), the remainder of the resonances in the $GbOse_3Cer$ spectrum have been assigned at two levels of confidence, the highest being for groups I (GlcβC3, 76.6; GlcβC5, 76.5; GalβC5, 76.2), II (GlcβC2, 74.8; GalβC3, 74.7), III (GalαC5, 73.1; ceramide C3, 72.7; GalβC2, 72.6), and IV (GalαC3, 71.1, GalαC4, 71.0; GalαC2, 70.7; ceramide C1, 70.5) and the lowest for the individual carbon nuclei within the groups. Finally, the resonance at 54.8 ppm can be assigned to C2 of sphingosine which, because of its linkage to the amide nitrogen, resonates in the same region as C2 of 2-deoxy-2-acetamidoglycopyranosides, 55.5 ppm (19).

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