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\(_3\)Cer) 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\(_3\)Cer 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\(_3\)Cer 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\(_3\)Cer 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, tetramethylsilanide.

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Fig. 1. Partial proton-decoupled, 90.5 MHz $^{13}$C-NMR spectrum of globotriaosylceramide (GbOse$_3$Cer) 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$_3$Cer, 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

<table>
<thead>
<tr>
<th>Carbon Number</th>
<th>Sphingosine</th>
<th>N-Acyl</th>
<th>Galβ-</th>
<th>Galα-</th>
<th>Galβa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70.5'</td>
<td>173.4</td>
<td>105.4</td>
<td>105.7</td>
<td>103.1</td>
</tr>
<tr>
<td>2</td>
<td>55.5</td>
<td>36.8</td>
<td>74.8'</td>
<td>72.6a</td>
<td>70.7'</td>
</tr>
<tr>
<td>3</td>
<td>72.7'</td>
<td>26.4</td>
<td>76.6a</td>
<td>74.7'</td>
<td>71.1'</td>
</tr>
<tr>
<td>4</td>
<td>132.6</td>
<td>81.9</td>
<td>79.8</td>
<td>71.0'</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>132.2</td>
<td>76.5'</td>
<td>60.5'</td>
<td>61.9'</td>
<td>62.7'</td>
</tr>
<tr>
<td>6</td>
<td>32.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>28-30</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>16</td>
<td>32.0</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>17</td>
<td>22.9</td>
<td></td>
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</tr>
<tr>
<td>18</td>
<td>14.3</td>
<td>32.0</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>19</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
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</tbody>
</table>

* Chemical shifts in parts per million (ppm) downfield from internal tetramethylsilane. Approximate accuracy is ±0.1 ppm.

Within each group (see text and Fig. 2) the assignments can be exchanged.

- size indicates that not all of the GbOse3Cer 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 13C NMR study indicated that glucosylceramide derived from liver of a patient with Gaucher's disease contained approximately 15% of unsaturation (4). From the GbOse3Cer 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 13C 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 GbOse3Cer "anomeric window" shows three peaks (Fig. 1). Using the chemical shifts of the anomeric carbons of model glycosides and the lower members of the glob0 series, glucosylceramide and lactosylceramide (3–8, 12–21), these peaks can be assigned to GalgC1 (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 anomic chemical shifts provide good evidence that the three major
residues of GbOse₃Cer are in the pyranosyl form, since
C1 of glycofuranosyl residues resonate at lower fields
(16). This conclusion is confirmed by the observation
that the GbOse₃Cer spectrum lacks resonances in the 83
and 85 ppm region where C4 of α and β galacto-
furanosides resonate (16). Additionally, Koerner et al.
(4) have shown that the glucosyl moiety of glucosylcer-
amide is in the β-glucopyranosyl form.

It is well established that O-derivatization of a gly-
copyranosyl carbon induces an 8 to 10 ppm downfield
chemical shift displacement of the carbon directly in-
volved 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 hydroxy-
methyl carbons of the glycopyranosyl residues of
GbOse₃Cer are underivatized, and consequently the in-
tersugar linkages must involve glycopyranosyl ring car-
bons, as chemically demonstrated for GbOse₃Cer (2, 6)
and discussed below in terms of the ¹³C NMR spectrum.

Glycopyranosides of Gal and Glc do not show reso-
nances 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 car-
bons 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 res-
ides of GbOse₃Cer were underivatized: GalaC4 (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 re-
mainder of the GbOse₃Cer 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 unambigu-
ously rationalized even though its structure is known.
The major difficulties in completing the structure-chemi-
cal 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 GbOsesCer 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 (GalaC5, 73.1; ceramide C3, 72.7; GalβC2, 72.6), and IV (GalaC3, 71.1, GalaC4, 71.0; GalaC2, 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-acetamidoglyco-pyranosides, 55.5 ppm (19).

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REFERENCES