Studies of the Chemical Structure of Gangliosides in Deer Antler, Cervus nippon

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The biological activity of deer antler has been considered to originate in the gangliosides, although the structures of gangliosides have not been well elucidated. The quality of deer antler as an Asian folk medicine has often been evaluated by the amount of gangliosides contained in the crude drug. We have completed the structural determination of five gangliosides isolated from deer antler in the present study.

Five ganglioside fractions were isolated and purified from deer antler, Cervus nippon, by the Folch–Suzuki partition method, DEAE–Sephadex A-25, and further by silica gel column chromatography. High field proton nuclear magnetic resonance spectroscopy, gas chromatography/mass spectrometry, and fast atom bombardment-mass spectrometry studies characterized the isolated ganglioside fractions. GM 3 and GD 3 were present in the isolated ganglioside fractions. Samples were hydrolyzed in trifluoroacetic acid for direct compositional analysis and analyzed for sialic acid and neutral sugar without prior derivatization. Separation of the monosaccharides was achieved by HPLC on a Dionex CarboPac column eluted at a high pH. The resolved monosaccharides were identified using standard monosaccharides by pulsed amperometric detection. N-Acetyl GM 3 (Neu5Ac), N-glycolyl GM 3 (Neu5Ga), and N-acetyl GD 3 (Neu5Ac) were present in the antler. The major ceramide moiety was composed of C 16:0 or C 22:6 fatty acids along with either C 18 sphingosine or C 24 eicosasphingosine.

Key words deer antler; ganglioside; N-acetyl GD 3 (Neu5Ac); N-acetyl GM 3 (Neu5Ac); N-glycolyl GM 3 (Neu5Ga)

Results and Discussion

Gangliosides are generally known to be potential candidates for the treatment of human disease. Although many scientists have been attracted by gangliosides for their biological activities, there has not been much success in developing gangliosides as a practical medicine because of their diversity and complexity of function. It is known that GM 3 appears in Purkinje neuron-related cells during the development period of the brain. GM 3 also affects the development of the brain. We report here the first results in regard to confirming the existence of five different gangliosides in deer antler.

Isolation of Gangliosides from Deer Antler by Column Chromatography

Crude gangliosides were obtained from antler extract by DEAE–Sephadex A-25 column chromatography eluted with a solution of ammonium acetate in

Natural products are of interest for their unique and diverse biological activities, as well as their structural elegance. Limitations in the development of synthesized medicines and the unexpected side effects of these medicines have led many researchers to focus on natural product chemistry in order to achieve a better quality of life. Among traditional Far East Asian medicines, a concoction of deer antler has been considered as one of the most effective and popular cure-all natural drugs. A 70% ethanol extract of deer antler, named Pantin or Pantocrin, has been on the Asian drug market for some time.1,2

It has been reported that there are many different lipids, peptides, carbohydrates, sterols and inorganic substances in deer antler.1,2 Deer antler extracts have been used in the treatment of liver damage, epilepsy, anemia, rheumatism and digestive stimulation. It has also been reported that, in general, the phosphates and gangliosides of deer antler can ease the symptoms of senility.3 Deer antler extract is composed of many compounds with different functional groups, such as proteoglycans with a hyaluronic acid core,4 epidermal growth factor,5 and others. Deer antler also contains inorganic substances, such as Ca, Pb, and Zn,6 as well as some water soluble hexosamines, uronic acids, sialic acids, carbohydrates, hydroxyprolines, mucopolysaccharides,7,8 and some water insoluble fatty acids, such as prostaglandins,9 phospholipids, glycolipids, and gangliosides.10

It is widely believed that the active component in antler is its gangliosides (GM 3, GM 1, GD 3), which are present in large quantities.9 However, to date, there has been no known method for the analysis of antler gangliosides other than TLC and HPLC. We have isolated five ganglioside fractions from antlers using the Folch–Suzuki partition method and further purified each component using DEAE–Sephadex A-25 and silica gel column chromatography.

Fast atom bombardment-mass spectrometry (FAB-MS), gas chromatography-mass spectrometry (GC-MS), and high field proton nuclear magnetic resonance (NMR) spectroscopy methods confirmed the structure of each component. Structural elucidation of the five isolated gangliosides, three in the GM 3 family and two in the GD 3 family, is reported herein.

It is often prescribed for frail children. When the right dosage is given to a week child it can be effective, but an excess dosage, in some cases, can cause retardation. The gangliosides in deer antler probably play a major role in the development of the brain. We report here the first results in regard to confirming the existence of five different gangliosides in deer antler.

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methanol. After removing the solvent, the ganglioside fraction was dialyzed with cellulose ester membranes (molecular weight cut-off 500 daltons) to eliminate ammonium acetate. Silica gel column chromatography of the resulting crude ganglioside mixture was performed using chloroform : methanol : water (60 : 35, v/v/v) as eluents. The preliminary result on the separation of gangliosides showed that at least two major classes of gangliosides were present in deer antler. The GM₁ class and the GD₃ class, with lower Rf values than the standard GM₁ were detected by spraying the HPTLC plates of the crude material with either an orcinol–sulfuric acid or resorcinol–hydrochloric acid reagent, and were separated. They are G₁-a, G₁-b, G₂, G₃-a, and G₃-b, with a decreasing order of Rf value. The purity of the isolated gangliosides was confirmed by HPTLC.

**Structural Characterization of Gangliosides by FAB-MS** Samples in methanol were subjected to negative ion MS analysis. According to a previous report by Hemling, gangliosides exhibited a characteristic negative ion mass fragmentation pattern in FAB-MS. Therefore, a negative ion FAB-MS may be useful for the identification of structurally similar compounds (Fig. 1).

The molecular ion (M–H)⁻ of G₁-b appears at m/z 1263. The fragment ion at m/z 972 (z₃, M–H−291) results from the loss of a sialic acid moiety which can undergo subsequent fragmentation to yield z₂ (m/z 810, M−H−291−162, loss of a hexose) and z₁ (m/z 648, M−H−291−hexose−162, loss of a hexose).

The molecular ion of G₂ appears at m/z 1167. The fragment ion at m/z 860 (z₂, M−H−307) results from the loss of a sialic acid moiety which can undergo subsequent fragmentation to yield z₁ (m/z 698, M−H−307−162, loss of a hexose). Compound G₂ only differs from G₁-b in a sialic acid moiety where G₁-b contains N-acetylgalactosaminic acid (Neu5Ac, mass difference 291 daltons) instead of N-glycolylneuraminic acid (Neu5Gc, mass difference 307 daltons), as in G₂.

The molecular ion of G₃-a appears at m/z 1555 (M−H)⁻ and its sodium adduct (M−2H+Na⁻) at 1577. Consecutive fragment ions which appear at m/z 1263 (z₂, M−H−291, loss of a sialic acid), 972 (z₃, M−H−sialic acid−291, loss of a sialic acid), 810 (z₄, M−H−291−162, loss of a hexose) and 648 (z₅, M−H−291−162−162, loss of a hexose) are characteristic. Compound G₃-b shows a molecular ion at m/z 1464 (M−2H+Na⁻) and m/z 1442 (M−H)⁻ in the negative ion FAB-MS. The fragment ion at m/z 1151 (z₆, M−H−291) results from the loss of a sialic acid moiety. This fragment ion can undergo subsequent fragmentation to yield z₅ (m/z 860, M−H−sialic acid−291, loss of a sialic acid), z₂ (m/z 698, M−H−291−162−loss of a sialic acid), z₁ (m/z 536, M−H−291−hexose−162, loss of a hexose). These results indicate that compounds G₁-a, G₁-b, and G₂ belong to the GM₁ family of gangliosides. The sialic acid of G₂ is composed of Neu5Gc, while the sialic acid of G₁-a and G₁-b is Neu5Ac. Compounds G₃-a and G₃-b also appear to belong to the GD₃ family of gangliosides, but both differ in their fatty acid portion of the ceramide.

**Analysis of Sugar Moiety by Anion Exchange HPLC** An anion exchange HPLC with a CarboPac PA-1 column was performed to determine the structures of sialic acids and hexoses in antler gangliosides. First, gangliosides G₁, G₂, and G₃ were hydrolyzed with 100 mM trifluoroacetic acid and analyzed by anion exchange HPLC using a CarboPac PA-1 column.
(TFA) to release free sialic acids. In order to confirm the existence of sialic acid in the hydrolyzed product of gangliosides, HPLC analysis was performed. The standard Neu5Ac retention time was found in hydrolyzed products of G1 and G3, while the standard Neu5Gc retention time was found in the hydrolyzed product of G2 as the sialic acid moiety. In the next step, gangliosides G1, G2, and G3 were treated with 5 M aqueous TFA solution to determine the existence of a hexose moiety. The HPLC analysis of three samples from G1, G2, and G3 consistently showed that two different hexoses were present. Comparing their retention times, these two hexoses were proven to be galactose and glucose.

Thus, it is certain that compounds G1 and G3 contain Neu5Ac, galactose, and glucose, while compound G2 contains Neu5Gc, galactose, and glucose.

Analysis of Fatty Acid Moiety by GC-MS To determine the structures of fatty acids in the ceramide moiety of antler gangliosides, GC-MS was performed with methyl esters of fatty acid which were obtained from isolated gangliosides by methanalysis. The mass spectrum of each GC component was studied, and the molecular ion for methyl esters of fatty acids appeared at \( m/z \) 270 of G1-b, G3-b, G2 and at \( m/z \) 354 of G1-a, G3-a. Therefore, the corresponding fatty acids present \([M−14]^+\) were found in the form of \( C_{16:0} \) (256) and \( C_{22:0} \) (340).

The mass difference of 112 (648 – 536), as seen in the G1-a and G1-b FAB-MS spectra, explains that there are eight methylene unit \((\text{CH}_2)\) differences in each ceramide moiety. The same observation can be made in the case of G3-a and G3-b. According to the molecular ions, two ceramide moieties correspond to \( C_{42} \) and \( C_{34} \). Therefore, the analysis of the GC-MS spectral data of the gangliosides, combined with FAB-MS data, confirmed that the fatty acid of G1-a and G3-a is made of \( C_{22:0} \) and the fatty acid of G1-b, G3-1, and G2 is made of \( C_{16:0} \). The data also support the findings that the long-chain bases of the gangliosides are either eicosasphingosine \((C_{20})\), in the cases of G1-a and G3-a, or sphingosine \((C_{18})\) in the cases G1-b, G3-b, and G2.

Structural Analysis of Gangliosides by NMR Spectroscopy In order to confirm the result obtained from FAB-MS and HPLC and to investigate the structure of G-1b, \(^1\)H-NMR analysis was performed. A two-dimensional COSY spectrum of G1-b in 100% DMSO-\( d_6\) at 303 K referenced to tetramethylsilane (TMS) is shown in Fig. 3. On the top of the COSY spectrum, a 1D–\(^1\)H spectrum in the same region is shown, with assignment. Also in the small box, the 1D spectrum of the down field is shown, too.

Most of the resonances of the ring protons appear between \( \delta \) 3.0 and 3.8 and show severe spectral overlap. However, a good dispersion of chemical shifts of exchangeable hydroxyl and amido protons allowed us to complete the \(^1\)H assignment of G1-b. By the addition of 20 \( \mu l \) of D\(_2\)O in the sample, all of...
the hydroxyl protons and amido protons are exchanged with deuterium, and these exchangeable protons were easily assigned. A COSY experiment has made it possible to confirm the G1-b structure as GM3. In the downfield shown in Fig. 3, the NH and OH8 of neuraminic acid and the NH of ceramide appear at δ 7.98, 6.18, and 7.43, respectively. Anomeric protons of Glc(I) and Gal(I) are well resolved and assigned at δ 4.12 and 4.15, respectively. The spin systems of each ring were traceable from H1 to H6 in the COSY spectrum. Based on the spectroscopic analysis, the structure of G1-b was identified as shown in Fig. 4.

Table 1. 1H-NMR Data (δ) for G1-b in DMSO-d6 at 303 K

<table>
<thead>
<tr>
<th>Residue</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>NAc</th>
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<tbody>
<tr>
<td>Glcβ (I)</td>
<td>H</td>
<td>4.12</td>
<td>3.04</td>
<td>3.66</td>
<td>3.54</td>
<td>3.55</td>
<td>3.77(a)</td>
<td>3.83(b)</td>
<td>4.27</td>
<td></td>
</tr>
<tr>
<td>Glcβ (II)</td>
<td>OH</td>
<td>4.15</td>
<td>3.24</td>
<td>3.96</td>
<td>3.88</td>
<td>3.38</td>
<td>3.39(a)</td>
<td>3.45(b)</td>
<td>4.56</td>
<td></td>
</tr>
<tr>
<td>Neuα</td>
<td>OH</td>
<td>1.31(a)</td>
<td>2.86(e)</td>
<td>4.37</td>
<td>4.24</td>
<td>3.34</td>
<td>3.40</td>
<td>3.13</td>
<td>3.56</td>
<td>3.40(a)</td>
</tr>
<tr>
<td>Cer (R)</td>
<td>OH</td>
<td>3.97(b)</td>
<td>7.43(b)</td>
<td>7.98(b)</td>
<td>4.52</td>
<td>6.18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| a) Assigned by the 1H–1H COSY spectrum. b) Amido protons.

Table 2. Structural Determination of the Five Isolated Gangliosides

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ganglioside</th>
<th>Sphingosine</th>
<th>Fatty acid</th>
<th>Sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1-a</td>
<td>GM3</td>
<td>Eicosasphingosine (C20)</td>
<td>C22:0</td>
<td>Neu5Ac</td>
</tr>
<tr>
<td>G1-b</td>
<td>GM3</td>
<td>Sphingosine (C18)</td>
<td>C16:0</td>
<td>Neu5Ac</td>
</tr>
<tr>
<td>G2</td>
<td>GM3</td>
<td>Sphingosine (C18)</td>
<td>C16:0</td>
<td>Neu5Ac</td>
</tr>
<tr>
<td>G3-a</td>
<td>GD3</td>
<td>Eicosasphingosine (C20)</td>
<td>C22:0</td>
<td>Neu5Ac</td>
</tr>
<tr>
<td>G3-b</td>
<td>GD3</td>
<td>Sphingosine (C18)</td>
<td>C16:0</td>
<td>Neu5Ac</td>
</tr>
</tbody>
</table>

In conclusion, the structural analysis of isolated antler gangliosides using FAB-MS, HPLC, GC-MS, and NMR spectroscopy made the structural determination of gangliosides GM3 and GD3 possible. Gangliosides GM3 and GD3 were composed of long-chain bases of either eicosasphingosine(C20) or sphingosine(C18), as well as C16:0 and C22:0 fatty acids. The GM3 was composed of sialic acid in the form of either N-acetylneuraminic acid or N-glycolylneuraminic acid, while GD3 was composed of N-acetylneuraminic acid. Structural determination of the five isolated gangliosides is summarized in Table 2.

**Experimental**

**Materials** All solvents and reagents used were purchased at the highest available commercial quality. All aqueous solutions were prepared using deionized and distilled water. Spectrograde methanol and chloroform were used for HPLC. HPTLC and silica gel column chromatography were performed using a Merck Kieselgel 60 F254 (silica gel) TLC plate and Kieselgel 60, 230—400 mesh ASTM (silica gel), respectively. Deer antler (Cervus nippon) was purchased at an oriental drug market. A ganglioside mixture, purified from bovine brain, was purchased from Sigma Chemical. Anhydrous orcinol was obtained from BDH Chemical and resorcinol was purchased from Wako Pure Chemical Industries. Cellulosic membranes for dialysis (molecular weight cut-off: 500) were purchased from Spectrum.

**TLC** TLC was performed on a silica gel 60 HPTLC plate (Merck, Kieselgel 60 F 254) with either solvent eluent system A (n-propyl alcohol : 0.1% aq. CaCl2, 4 : 1, v/v) or system B (chloroform : methanol : 2.5 M NH4OH, 65 : 35 : 8, v/v/v). After development, TLC spots were visualized by spraying the plates with either an orcinol–ferric chloride–sulfuric acid solution or resorcinol solution, followed by heating the plates at 120 °C for 20 min.

**Extraction and Isolation of Glycosphingolipids** A mixture of glycosphingolipids was isolated from deer antler according to the procedure previously reported by Folch14) and Suzuki.15) Slices of deer antler (1.2 kg) were extracted two times with ten volumes of both chloroform and methanol mixtures (2 : 1 and 1 : 2, v/v) at ambient temperature. Chloroform was added to the combined chloroform : methanol extracts and the total volume ratio of chloroform : methanol became 2 : 1. The solution was shaken with a 0.2 volume of 0.88% aqueous potassium chloride (KCl) for 1 h, then was left standing for an additional 12 h. After separation of the aqueous layer, the organic layer was extracted again with an equal volume of chloroform : methanol : 0.88% aqueous KCl solution mixture (3 : 48 : 47, v/v/v). The third extraction was performed without KCl. The combined aqueous layer was concentrated under reduced pressure, and the residue was dialyzed in water to eliminate KCl and residual carbohydrates. The resulting solution was

![Fig. 4. Chemical Structure of Isolated Ganglioside G1-b from Deer Antler](image-url)
freeze-dried and stored in a chloroform: methanol solvent mixture (1:1, v/v).

Extracted crude gangliosides were placed on an ion exchange column (DEAE–Sepharose A-25 gel, 2×40 cm) and sequentially eluted with a chloroform: methanol: water (30:60:8, v/v/v) solvent mixture, 100% methanol, 0.05 M ammonium acetate in a methanol solution, and 0.45 M ammonium acetate in methanol. The collected fractions were concentrated and the resulting product mixtures were subjected to silica gel column chromatography (1×22 cm) first using chloroform: methanol (60:35), then chloroform: methanol: water (60:45:8) for further purification. HPTLC confirmed the purity of each band by visual inspection with either I2 vapor or resorcinol–hydrochloric acid spray.

**HPLC** To search for the presence of sialic acid and hexose in the gangliosides, anion exchange HPLC was performed with Dionex pumps, a Spectra system P2000 injector, a pulsed amperometric detector with a gold working electrode, and a detector integrator. A CarboPac PA-1 column (4×250 mm) was used with an adequate eluent system at a flow rate of 1 ml/min. The ganglioside solution in chloroform: methanol (1:1, v/v) was concentrated, dissolved in water, and treated with 2 volumes of 100 mM TFA at 80 °C. After stirring for 1 h, the hydrolyzed product was concentrated, diluted, dissolved in water, and treated with 2 volumes of 100 mM TFA at 150—230 °C (10 °C/min temperature increments). Spectra were obtained within a scan range of 55—550 m/z. Ions were produced by fast atom bombardment (FAB) mass spectra were taken with the first (MS-1) of the two mass spectrometers of a JMS-HX110A/110A tandem mass spectrometer using a cesium ion gun operated at 22 kV. Approximately 10 μg of sample dissolved in methanol was mixed with 10 μl of triethanolamine (BDH) as the matrix in the negative-ion mode on the FAB probe tip.

**GC-MS** Ganglioside fractions (200 μg) were placed in screw-capped tubes and treated with methanol: 10 M HCl (9:1, v/v) at 76 °C for 16 h. The resulting fatty acid ester was extracted 3 times with hexane and directly analyzed by GC-MS spectrometry using a Hewlett Packard HP 5890 GC connected with an HP 5989B MS engine mainframe. The column used for GC-MS was an Omegawax-320 (30 m×0.32 mm) with a helium carrier gas flow rate at 1 ml/min, and a temperature range of 150—230 °C (10 °C/min increments). Spectra were obtained within a scan range of m/z 55—550.

**1H-NMR and 2D 1H−1H COSY** For the spectral assignment of all protons, including exchangeable protons, the sample was dissolved in 100% DMSO-d6. In order to distinguish non-exchangeable protons from the exchangeable protons, the sample was dissolved in 0.4 ml of 98:2 DMSO-d6–D2O solvent for NMR analysis. Chemical shifts of 1H resonances were expressed relative to an internal TMS signal at 0 ppm. For the COSY experiment, 300 transients with 2 k complex data points were collected for each of the increments, with a relaxation delay of 1.2 s between successive transients. A high-resolution 1D spectrum (64 k) was acquired with a spectral width of 3937 Hz. All spectra were recorded on a 300 MHz spectrometer. NMR spectra were processed offline using the FELIX software package on an SGI workstation.

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**References and Notes**

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