 Classification into a Novel Mollu-Series of Neutral Glycosphingolipids from the Lamp Shell, Lingula unguis

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Abstract: Seven neutral glycosphingolipids were isolated and purified from the lamp shell, Lingula unguis by successive column chromatography on ion exchange Sephadex (QAE-Sephadex), magnesium silicate (Florisil) and silicic acid (Iatrobeads). Their chemical structures were characterized as Glcβ1-1ceramide (CMS₁, CMS₂ and CMS₃), Manβ1-4Glcβ1-1ceramide (MIOse₄Cer, CDS), Manα1-3Manβ1-4Glcβ1-1ceramide (MIOse₃Cer, CTS), GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1ceramide (MIOse₂Cer, CQS) and GlcNAcβ1-4GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1ceramide (IVثيرGlcNAc-MIOse₂Cer, CPS) by compositional analysis, methylation analysis, exoglycosidase cleavage, gas-liquid chromatography, gas chromatograph-mass spectrometry and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Aliphatic constituents of CDS, CTS, CQS and CPS were virtually the same, with C16:0 and C18:0, and their 2-hydroxy homologues as the fatty acids, and octadecasphinga-4,8,10-trienine as the sole sphingoid. However, the ceramide compositions of CMS₁, CMS₂ and CMS₃ differed from those of the other four glycolipids. The fatty acid composition of CMS₁ consisted completely of C16:0 and C18:0, and their 2-hydroxy homologues as the fatty acids, and octadecasphinga-4,8,10-trienine as the sole sphingoid. However, the ceramide compositions of CMS₁, CMS₂ and CMS₃ differed from those of the other four glycolipids. The fatty acid composition of CMS₁ consisted completely of C16:0, C17:0 and C18:0 acids, but both CMS₂ and CMS₃ contained instead of their 2-hydroxy homologues. The sphingoid components of CMS₁ and CMS₂ were octadecasphinga-4-enine and octadecasphinga-4,8,10-trienine, but that of CMS₃ was entirely trihydroxysphingoid, 4-hydroxyoctadecasphiganine.

Key words: glycosphingolipid, mannolipid, marine tentaculata glycolipid (Lingula unguis)

1 Introduction

Glycosphingolipids, which consist of sugar and ceramide moieties, are amphipathic molecules present in the plasma membrane of eucaryotic cells, where they are involved in a variety of functions, including cell-cell recognition and interaction, differentiation, oncogene-sis, immunity and so on (5,6). Many structural variations have been reported in glycosphingolipids from the Mammalia and/or other vertebrate species (7,8). We have been interested in the structural relationship between the vertebrate and invertebrate glycosphingolipids and have so far found numerous unique glycosphingolipids in invertebrate species, especially from...
various protostomia phyla, Mollusca (9,10), Arthropoda (11-13), Annelida (4,14-20), Aschelminthes (21, 22) and Coelenterata (23).

We have initiated an investigation into the glycosphingolipids of the lamp shell, Lingula unguis, one of the oldest “living fossils”, which belongs to the class Brachiopoda, phylum Tentaculata, and have found that ceramide monosaccharide is the major component of their glycosphingolipids, minor ones being ceramide di-, tri-, tetra- and pentasaccharides together with much more complex glycosphingolipids. Isolation of ceramide mono-, di-, tri- and tetrasccharides has allowed their structural characterization as members of the Mollus-series glycosphingolipids. Furthermore, a novel Mollus-series glycosphingolipid, GlcNAcβ1-4GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1ceramide with an N-acetyl-chitobiose linkage, not previously described, was discovered in the ceramide pentasaccharide component.

In this communication, we describe the elucidation of the molecular structures of seven neutral glycosphingolipids, ceramide mono- (CMS1, CMS2 and CMS3), di- (CDS), tri- (CTS), tetra- (CQS) and pentasaccharides (CPS).

2 Experimental

2.1 Preparation of the Neutral Glycolipids from a Marine Tentaculata, Lingula unguis

The soft tissues (omitting shells and peduncles) obtained by boiling the lamp shell, Lingula unguis (5 kg) collected at Ariake Bay in Kyusyu Island in June were homogenized with acetone in a blender to remove neutral fatty substances and water, and then filtered. The residue (440 g) was extracted at 25°C two times with 2.5 L of a chloroform-methanol mixture, 2:1 (v/v) and with 2 L of 1:1 (v/v). The combined chloroform-methanol extracts were subjected to mild alkaline hydrolysis in 0.5 M KOH in methanol-water, 95:5 (v/v) at 37°C for 6 h to eliminate glycerolipids. The hydrolysate was acidified (pH 1.0) with conc. HCl, kept for 1 h at 20°C in that condition, and dialyzed against tap-water for 2 d. The inner fluid was concentrated to near dryness in vacuo at 40°C, and precipitated by addition of acetone (alkali-stable product: yield, 7.1 g). The alkali-stable product was taken up in chloroform-methanol-water, 30:60:8 (v/v) and applied to a column (2.5 × 40 cm) packed with OH- form of QAE-Sephadex A-25 (Pharmacia LKB Biotechnology Inc.) equilibrated with the same solvent. The column was eluted successively with the same solvent (five column volumes) and with pure methanol (two volumes) as neutral solvents, and with 0.45 M ammonium acetate in methanol (five volumes) as a polar solvent. The separation of neutral and acidic glycolipids was monitored by thin-layer chromatography (TLC) as described below. The eluates obtained from this column using the neutral solvents were pooled and evaporated to dryness (neutral glycolipid fraction : yield, 2.4 g). The neutral glycolipid fraction was acetylated and then fractionated on a column (2.2 × 70 cm) packed with Florisil, 60~100 mesh (US Silica Co.) (24). The column was eluted successively with 300 mL of hexane-dichloroethane, 4:1 (v/v), 300 mL of pure dichloroethane, 900 mL of dichloroethane-acetone, 1:1 (v/v), 900 mL of dichloroethane-methanol, 9:1 (v/v), 900 mL of dichloroethane-methanol, 3:1 (v/v) and 900 mL of dichloroethane-methanol-water, 2:8:1 (v/v). The fractions of acetylated glycolipids eluted by dichloroethane-acetone (1:1) and dichloroethane-methanol (9:1) were combined and evaporated to dryness, and reacted with 0.5 M KOH in methanol at 37°C for 6 h. The reaction mixture was dialyzed against tap-water, and lyophilized (yield: 670 mg). The deacylated product was applied to a column (2 × 60 cm) of Iatrobeads 6RS-8060 (Iatron Lab.) equilibrated with chloroform-methanol-water, 80:20:1 (v/v). Chromatography was carried out by seven stepwise elutions with 200 mL each of the following chlo-
Neutral Glycosphingolipids from the Lamp Shell, Lingula unguis

Roform-methanol-water mixtures; 80:20:1 (v/v) (Fr. 1, yield: 265.4 mg), 70:30:2 (Fr. 2, 24.7 mg), 60:40:3 (Fr. 3, 9.2 mg), 50:50:5 (Fr. 4, 6.4 mg), and 40:60:6, 30:70:7 and 20:80:10 (Fr. 5, 252.8 mg) (Fig. 1). Fractions 1 to 4 are used in this study, and work with Fr. 5 is in progress.

2.2 Thin-Layer Chromatography

The following solvent systems were employed: chloroform-methanol-water, 80:20:1, 60:45:10 and 60:35:8 (v/v); and 1-propanol-water-28% ammonia water, 75:15:5 (v/v). Glycolipids on thin layer plates of silica gel 60 (E. Merck) were visualized by spraying with orcinol-H2SO4 reagent followed by heating at 110°C.

2.3 Gas-Liquid Chromatography (GC) and Gas Chromatograph-Mass Spectrometry (GC-MS)

For determination of the composition of the fatty acids and sugars, 50~200 μg of glycolipids were methanolyzed in thick glass test tubes (16 × 125 mm with Teflon-lined screw caps: Pyrex, Iwaki Glass Co.) with 200 μL of freshly prepared 1 M anhydrous methanolic HCl using a microwave oven (Sharp RE-Z3W6, 100 V, 60Hz: Sharp Electric Co.). The samples were exposed to the maximum power (500 W) of the microwave oven for 1 min (25, 26). After methanalysis, the samples were cooled to room temperature inside the microwave oven. The fatty acid methyl esters produced were extracted three times with 400 μL each of n-hexane and analyzed by using a Shimadzu GC-18A gas chromatograph and a capillary column of Shimadzu HiCap-CBP 5 (0.22 mm × 25 m) programmed at 2°C /min from 170°C to 230°C. The methanolic phase that remained was evaporated to dryness for deacidification under a nitrogen stream. The residue containing methylglycosides was trimethylsilylated with a mixture of pyridine, hexamethyldisilazane and trimethylchlorosilane, 9:3:1 (v/v). The reaction mixture was injected into the same capillary column programmed at 2°C /min from 140°C to 230°C. Sphingoids prepared from each glycolipid by methanalysis with 1 M aqueous methanolic HCl at 70°C for 18 h were converted to their O-trimethylsilyl (N-free) derivatives (27) and analyzed with the same capillary column at 2°C/min from 200°C to 240°C. For the methylation study, permethylation of the samples was performed, using 300 μg each of the purified glycolipids, with NaOH and CH3I in dimethyl-sulfoxide (28). The permethylated glycolipids were acetylated and hydrolyzed with 300 μL of a mixture of HCl-water-acetic acid, 0.5:1:5:8 (v/v) by exposing to the maximum power of the microwave oven for 1 min (26, 29), reduced with NaBH4, and then acetylated with a mixture of acetic anhydride-pyridine, 1:1 (v/v) at 100°C for 15 min. The partially methylated alditol acetates thus obtained were analyzed by GC and GC-MS on the same HiCap-CBP 5 capillary column as described above. Electron impact (EI) and chemical ionization (CI) mass spectra were taken using a Shimadzu GCMS-QP 5050 gas chromatograph-mass spectrometer under the following conditions: oven temperature, 80°C (2 min) → 180°C (20°C/min) → 240°C (4°C /min); interface temperature, 250°C; injection port temperature, 240°C; helium gas pressure, 100 kPa; ionizing voltage, 70 eV (EI) and 100 eV (CI); ionizing current, 465 J.

Fig. 1 Thin-Layer Chromatogram of the Neutral Glycolipids from the Lamp Shell, L. unguis. 
Lane 1, neutral glycolipid fraction obtained by QAE-Sephadex A-25 and Florisil column chromatography; lanes 2, 3, 4, 5 and 6, glycolipid (Fr. 1~5) fractionated by Iatrobeads column chromatography using step-wise elution system with chloroform-methanol-water: 80:20:1 (Fr. 1), 70:30:2 (Fr. 2), 60:40:3 (Fr. 3), 50:50:5 (Fr. 4), and 40:60:6, 30:70:7 and 20:80:10 (Fr. 5), respectively. a~e, The positions corresponding to ceramide monosaccharides; f, the positions corresponding to more complex glycolipids. The plate was developed with chloroform-methanol-water, 60:45:10 (v/v). The spots were visualized by orcinol-H2SO4 reagent.

2.4 Sugar Cleavage by Exoglycosidases

α-Mannosidase [EC 3.2.1.24] from jack beans (Boehringer Mannheim), β-mannosidase [EC 3.2.1.25] from Achatina fulica (Seikagaku Corp.) or β-N-acetylhexosaminidase [EC 3.2.1.52] from jack beans (Seikagaku Corp.) were used. Samples of 10 μg-30 μg were suspended in 0.1 mL of 0.05 M citrate buffer (pH 4.5) for α-mannosidase, of 0.1 M citrate buffer (pH 4.5) for β-mannosidase or of 0.1 M citrate buffer (pH 5.0) for β-N-acetylhexosaminidase, containing 0.1 mg of sodium taurodeoxycholate. The reaction was carried out with 0.1 unit of α-mannosidase, with 0.01 unit of β-mannosidase or with 0.1 unit of β-N-acetylhexosaminidase at 37°C for 12 h. Each incubation was stopped by adding 0.5 mL of chloroform-methanol, 2:1 (v/v). The hydrolysates, after extraction into the lower phase, were dried under a nitrogen stream and then analyzed by TLC.

2.5 Matrix-assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS analysis of purified glycolipids was performed using a Shimadzu/KRATOS KOMPACT MALDI I mass spectrometer equipped with a Workstation SPARC station, operating in positive-ion linear mode. Ions were formed by a pulsed ultraviolet laser beam (N2 laser, 337 nm; 3-nanosecond-wide pulses/s). The matrix used was 7-amino-4-methylcoumarin (Sigma). External mass calibration was provided by the [M+H]+ ions of ceramide mono-~hexasaccharides (M.W . 699~1633) prepared from the green bottle fly, Lucilia caesar (30).

3 Results

3.1 Isolation and Purification of Seven Neutral Glycosphingolipids

The neutral glycolipid fraction from the lamp shell, Lingula unguis fractionated by QAE-Sephadex, Florisil and Iatrobeads column chromatography was separated on TLC into two major and several minor orcinol-H2SO4 positive fractions (Fig. 1), corresponding to ceramide mono- (Fr. 1), di- (Fr. 2), tri- (Fr. 3), tetra- and pentasaccharide fractions (Fr. 4) and a much more complex glycolipid fraction (Fr. 5). Further purification of each fraction (Fr. 1~4) was carried out by means of a second Iatrobeads column chromatography (1 × 60 cm). The columns were severally eluted with a linear gradient elution system of chloroform-methanol-water, 90:10:0.5 (400 mL)~80:20:1 (420 mL) for Fr. 1, and with a solvent mixture of 1-propanol-water-28% ammonia water, 75:5:5 for Fr. 2 and 75:15:5 for Frs. 3 and 4, in consideration of polarities in each glycolipid fraction. TLC of the further purified Frs. 1~4 are shown in Fig. 1. From Fr. 1, migrating as ceramide monosaccharide, three sub-fractions CMS1, CMS2 and CMS3 were purified (Fig. 2, Panel A). It is apparent from Fig. 1 that each of Frs. 2, 3 and 4 contained more than one substance; further purification of each fraction with the help of Iatrobeads chromatography using the solvent mixtures described above, followed by TLC in an alkaline solvent system, demonstrated this quite clearly. From Frs. 2 and 3, substances (CDS and CTS) migrating to positions corresponding to ceramide di- and trisaccharides were purified; and from Fr. 4, two substances (CQS and CPS) migrating to positions corresponding to ceramide tetra- and pentasaccharides were isolated (Fig. 2, Panel B). Their yields were CMS1, 30 mg; CMS2, 150 mg; CMS3, 6 mg; CDS, 1.5 mg; CTS, 2.2 mg; CQS, 1 mg; and CPS, 1.5 mg, respectively.

3.2 Sugar Constituents, Sequences and Linkages in Purified Neutral Glycosphingolipids (CMS1, CMS2, CMS3, CDS, CTS, CQS and CPS)

Each glycolipid was methanolyzed by micro-wave mediated method and the methanolysates were converted to trimethylsilyl derivatives for GC analysis. The gas chromatograms defined the sugar component, of which appropriate molar ratios were determined after correction for their relative molar responses, of CMS1, CMS2, and CMS3, as only glucose, CDS as glucose and mannose (molar ratio, 1:1), CTS as glucose and mannose (1:2), CQS as glucose, mannose and N-acetylglucosamine (1:2:1) and CPS as glucose, mannose and N-acetylgalactosamine (1:2:2), respectively (Table 1). Subsequently, in order to determine the sugar linkages, the partially methylated alditol acetate derivatives from each glycolipid were prepared and analyzed by GC and GC-MS. The identification was accomplished according to the data of Björndal et al. (31), Jansson et al. (32), Stellner et al. (33) and Tai et al. (34). The methyl-
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The neutral glycosphingolipids were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). TLC analysis demonstrated the presence of 1-substituted glucose (1,5-di-(O-acetyl)-2,3,4,6-tetra-(O-methyl)glucitol, 1Glc) for CMS₁, CMS₂, and CMS₃; 1-substituted mannose (1,5-di-(O-acetyl)-2,3,4,6-tetra-(O-methyl)mannitol, 1Man) and 1,4-substituted glucose (1,4,5-tri-(O-acetyl)-2,3,6-tri-(O-methyl)glucitol, 1,4Glc) for CDS, respectively; 1Man, 1,3-substituted mannose (1,3,5-tri-(O-acetyl)-2,4,6-tri-(O-methyl)mannitol, 1,3Man) and 1,4Glc for CTS, respectively; 1-substituted N-acetylgalactosamine (1,5-di-(O-acetyl)-3,4,6-tri-(O-methyl)-N-acetylglucosaminitol, 1GlcNAc), 1,2-substituted mannose (1,2,5-tri-(O-acetyl)-3,4,6-tri-(O-methyl)mannitol, 1,2Man), 1,3Man and 1,4Glc for CQS, respectively; and 1GlcNAc, 1,4-substituted N-acetylgalactosamine (1,4,5-tri-(O-acetyl)-3,6-di-(O-methyl)-N-acetylglucosaminitol, 1,4GlcNAc), 1,2Man, 1,3Man and 1,4Glc for CPS, respectively (Fig. 3).

For determination of anomeric configurations of the sugar residues, CDS, CTS, CQS and CPS were subjected to specific sequential enzymatic hydrolysis: CDS was degraded to ceramide monosaccharide (CMS₁) by β-mannosidase; CTS to CMS₂ in a stepwise fashion through the consecutive actions of α- and β-mannosidases; and the both CQS and CPS to CMS₁ in a stepwise fashion using β-N-acetylationhexosaminidase, and α- and β-mannosidases (Fig. 4).

Furthermore, components of the enzymatic hydrolysates in each step were confirmed by MALDI-TOF MS analysis. MALDI-TOF MS spectra of ceramide monosaccharides produced from CDS, CTS, CQS and CPS, ceramide disaccharides from CTS, CQS and CPS, ceramide disaccharides from CTS, CQS and CPS,

Table 1  Sugar Components and Their Molar Ratios in the Purified Neutral Glycolipids from the Lamp Shell, L. unguis.

<table>
<thead>
<tr>
<th></th>
<th>Glc</th>
<th>Man</th>
<th>GlcNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMS₁</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMS₂</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CMS₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDS</td>
<td>1.00</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>CTS</td>
<td>1.00</td>
<td>1.73</td>
<td></td>
</tr>
<tr>
<td>CQS</td>
<td>1.00</td>
<td>1.93</td>
<td>0.65</td>
</tr>
<tr>
<td>CPS</td>
<td>1.00</td>
<td>2.00</td>
<td>1.21</td>
</tr>
</tbody>
</table>

Fig. 2  Thin-Layer Chromatograms of Seven Purified Glycolipids, CMS₁, CMS₂, CMS₃, CDS, CTS, CQS and CPS.
Panel A: lane 1, Fr. 1 (the same as lane 2 in Fig. 1); lanes 2 to 4, CMS₁, CMS₂ and CMS₃ obtained from Fr. 1 by the second Iatrobeads column chromatography using a chloroform-methanol-water gradient elution system. Panel B: lanes 1, 3 and 5, Frs. 2, 3 and 4 (the same as lanes 3 to 5 in Fig. 1); lanes 2, 4, 6 and 7, CDS, CTS, CQS and CPS obtained from Frs. 2 to 4 by the second Iatrobeads column chromatography using 1-propanol-water-28% ammonia system. The plates were developed with chloroform-methanol-water, 80:20:1 (v/v) for Panel A and with 1-propanol-water-28% ammonia water, 75:15:5 (v/v) for Panel B. The spots were visualized by orcinol-H₂SO₄ reagent.
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and ceramide trisaccharides from CQS and CPS revealed pseudomolecular ions [M+H]+ at m/z 696 and 724 (main molecular species of ceramide: fatty acid-sphingoid, 16:0-d18:3; and 18:0-d18:3), at m/z 858 and 886, and at m/z 1020 and 1048, which were consistent with Hex-Cer, Hex2-Cer and HexNAc-Hex2-Cer, respectively (data not shown). From these findings, the structures of seven glycolipids were identified as Glcβ1-1Cer for CMS1, CMS2, and CMS3 (glucocerebrosides), Manβ1-4Glcβ1-1Cer (MIOse2Cer) for CDS, Manα1-3Manβ1-4Glcβ1-1Cer (MIOse2Cer) for CTS, GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1Cer (MIOse2Cer) for CQS, and GlcNAcβ1-4GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1Cer (IV4βGlcNAc-MIOse2Cer) for CPS, respectively.

### 3.3 Aliphatic Components of CMS1, CMS2, CMS3, CDS, CTS, CQS and CPS

The aliphatic components of CMS1, CMS2, CMS3, CDS, CTS, CQS and CPS were determined by GC and GC-MS, and summarized in Table 2. CMS1, CMS2 and CMS3 were constituted of three typical aliphatic com-

![Fig. 3](image_url)

Gas Chromatograms of Partially Methylated Alditol Acetates Derived from CMS2, CDS, CTS, CQS and CPS for Glycosidic Linkage Analysis (Substituted Positions of Sugars).
components, which differed however in the nature of the fatty acid and sphingoid components. The fatty acid composition of CMS$_1$ consisted entirely of C16:0, C17:0 and C18:0, but CMS$_2$ and CMS$_3$ were composed instead of the 2-hydroxy homologues of these acids. The sphingoid compositions of CMS$_1$ and CMS$_2$ were octadecasphinga-4-enine and octadecasphinga-4,8,10-trienine (35), whereas that of CMS$_3$ was entirely trihydroxysphingoid, 4-hydroxyoctadecasphinganine. It may therefore be suggested that their migration differences on TLC in Fig. 2 were owing to their aliphatic components. On the other hand, the aliphatic constituents of CDS, CTS, CQS and CPS were virtually the same, with C16:0 and C18:0, and their 2-hydroxy homologues as the fatty acids, and octadeca-4,8,10-trienine as the sole sphingoid.

Table 2. Chemical Compositions of the Ceramide Portions of the Lamp Shell Neutral Glycolipids.

<table>
<thead>
<tr>
<th></th>
<th>CMS$_1$</th>
<th>CMS$_2$</th>
<th>CMS$_3$</th>
<th>CDS</th>
<th>CTS</th>
<th>CQS</th>
<th>CPS</th>
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<tr>
<td>Nonhydroxy acid (%)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>16:0</td>
<td>49.5</td>
<td>26.9</td>
<td>25.9</td>
<td>37.0</td>
<td>24.0</td>
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<td></td>
</tr>
<tr>
<td>17:0</td>
<td>9.8</td>
<td>5.8</td>
<td>2.5</td>
<td>5.8</td>
<td>4.5</td>
<td></td>
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<tr>
<td>18:0</td>
<td>40.7</td>
<td>26.0</td>
<td>27.9</td>
<td>33.5</td>
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<td>19:0</td>
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<td>2.6</td>
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<td>6.5</td>
<td>3.4</td>
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<td>21:1</td>
<td>2.0</td>
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<td>1.9</td>
<td>1.0</td>
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<td>22:0</td>
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<td>23:0</td>
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<td>24:0</td>
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<td>12.6</td>
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<td>Hydroxy acid (%)</td>
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<td>h16:0</td>
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<td>h18:0</td>
<td>24.2</td>
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<td>5.8</td>
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<td>Sphingoid (%)</td>
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<tr>
<td>d18:1</td>
<td>55.0</td>
<td>38.1</td>
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<tr>
<td>d18:3</td>
<td>45.0</td>
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<td>—</td>
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<tr>
<td>t18:0</td>
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<td>100</td>
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<tr>
<td>a, 2-hydroxy.</td>
<td>b, dihydroxy sphingoid; t, trihydroxy sphingoid.</td>
<td>tr, trace</td>
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3.4 MALDI-TOF MS Analysis of CMS$_1$, CMS$_2$, CMS$_3$, CDS, CTS, CQS and CPS

MALDI-TOF MS has recently been shown to be a convenient technique for glycolipid analysis. The putative structures of seven purified glycolipids were confirmed by positive-ion mode MALDI-TOF MS analysis as shown in Fig. 5. Their mass spectra have two–four different pseudomolecular species, [M+H]$^+$ ions in good accordance with the values calculated from the
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Proposal structures: that is, CMS 1, [M+H]+ ions at m/z 696 and 724 with one mole each of glucose, fatty acid (assigned as 16:0 or 18:0) and sphingoid (assigned as d18:1) (see Table 2 for results of fatty acid and sphingoid analyses by GC and GC-MS); CMS 2, at m/z 712 and 740 with one mole each of glucose, fatty acid (as 2-hydroxy 16:0 or 18:0) and sphingoid (as d18:3); CMS 3, at m/z 734 and 762 with one mole each of glucose, fatty acid (as 2-hydroxy 16:0 or 18:0) and sphingoid (as t18:0); CDS, at m/z 858 and 886 with one mole each of glucose, mannose, fatty acid (as 16:0 or 18:0) and sphingoid (as d18:3); CTS, at m/z 1020 and 1048 with one mole of glucose, two moles of mannose, and one mole each of fatty acid (as 16:0 or 18:0) and sphingoid (as d18:3); CQS, at m/z 1223 and 1251 with one mole of glucose, two moles of mannose, one mole of N-acetylglucosamine, and one mole each of fatty acid (as 16:0 or 18:0) and sphingoid (as d18:3); and CPS, at m/z 1426, 1454, 1510 and 1538 with one mole of glucose, two moles each of mannose and N-acetylglucosamine, and one mole each of fatty acid (as 16:0, 18:0, 22:0 or 24:0) and sphingoid (as d18:3), respectively.

4 Discussion

In the present study, the seven simplest neutral glycolipids, ceramide mono-, di-, tri-, tetra- and pentasaccharides including three kinds of ceramide monosaccharides from the lamp shell, *L. unguis* belonging to the class Brachiopoda, a member of the phylum Tentaculata, were isolated and characterized by GC, GC-MS and methylation analyses, exoglycosidase cleavage, and positive-ion MALDI-TOF MS. From the data obtained, the following structures for these seven components can be proposed: Glcβ1-1Cer (CMS 1, CMS 2 and CMS 3), Manβ1-4Glcβ1-1Cer (CDS), Manα1-3Manβ1-4Glcβ1-1Cer (CTS), GlcNacβ1-2Manα1-3Manβ1-4Glcβ1-1Cer (CQS) and GlcNacβ1-4GlcNacβ1-2Manα1-3Manβ1-4Glcβ1-1Cer (CPS). Furthermore, the presence of CDS, CTS and
CQS suggested that the glycolipid series in the lamp shell are categorized to the Mollu-series as MIOse₃Cer, MIOse₄Cer and MIOse₅Cer, respectively. However, we found another glycolipid (CPS, IV²βGlcNAc-MIOse₂Cer), which has a unique sugar linkage, N-acetylgalactosamine-chitobiose characterized by an additional N-acetylgalactosamine residue attached to the terminal N-acetylgalactosamine of MIOse₄ oligosaccharide through a β1-4 linkage. Moreover, this glycolipid has so far not been reported in any other organisms of the animal kingdom. Additionally, when visualized on a TLC plate, the neutral glycolipid fraction of the lamp shell showed the existence of much more complex glycolipids (Fig. 1). As examples of these glycolipids from the preliminary results, we have found five glycolipids containing a GlcNAcβ1-4GlcNAcβ1-2Manα1-3Manβ1-4Glcβ oligosaccharide core, namely GlcNAcβ1-4(Fuc-)GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1Cer, Man3Me-(GalNAc-)Fuc-GlcNAcβ1-4GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1Cer, Man3Me-(GalNAc-)Fuc-(Xyl-)GlcNAcβ1-4GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1Cer, Man3Me-(GalNAc-)Fuc-GlcNAcβ1-4(Fuc-)GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1Cer and Man3Me-(GalNAc-)Fuc-(Xyl-)GlcNAcβ1-4(Fuc-)GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-1Cer (unpublished data, the complete structural determination which is in progress).

Therefore, this new oligosaccharide structure, GlcNAcβ1-4GlcNAcβ1-2Manα1-3Manβ1-4Glcβ, could be characteristic of a core series of the lamp shell glycolipids in the Tentaculata. The phylum Tentaculata is divided into three classes as Brachiopoda, Phoronida and Bryozoa, which have a unique organ named “lophophore” as a common feature. It is also considered that the Brachiopods are among the most ancient bilaterians to appear in the Cambrian fossil record (36). Of them, more than 12,000 species are fossils, while about 350 species exist today. It is assumed that the Brachiopods are the most ancient in this phylum, and the Bryozoa is intermediate between Brachiopoda and Phoronida. However, the classification of these creatures is taxonomically incomplete as yet. Recently, gene sequence analysis in many animals has revealed that they can be categorized into three groups, that is, the “Deuterostomes”, the “Ecdysozoans” including insects and roundworms, and the “Lophotrochozoans” including molluscs and earthworms (37). From those results, furthermore, it was shown that the Brachiopoda was classified into the “Lophotrochozoans”. Our structural investigations have shown not only that lamp shell neutral glycolipids with GlcNAcβ1-2Manα1-3Manβ1-4Glcβ oligosaccharide core are classified into the Mollu-series, but that they may also comprise the beginning of a new Mollu-series of glycolipids. This would suggest that the class Brachiopoda are the highest animals in the phylum Tentaculata, because the lamp shell, L. unguis contains not only Mollu-series glycolipids but also ceramide phosphoethanolamine, which has been characterized in higher animals of the phylum Mollusc (38).

References


