Isolation and structural analysis of three neutral glycosphingolipids from a mixed population of Caenorhabditis elegans (Nematoda: Rhabditida)

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The free-living nematode, Caenorhabditis elegans, has been proposed and analyzed as a prototypic model for parasitic nematodes. In order to study whether there is a structural basis for the proposed analogy with respect to nematode glycoconjugates, we have analyzed Caenorhabditis elegans glycosphingolipids. Three, simple neutral glycosphingolipid components of the neutral glycolipid fraction were isolated by high-performance liquid chromatography. Structural analysis was performed by methylation analysis, exoglycosidase cleavage, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and ceramide analysis. The chemical structures have been determined as Glcβ1Cer, Manβ4Glcβ1Cer and GlcNacβ3Manβ4Glcβ1Cer; that are characterized as belonging to the arthrodial series of protostomial glycosphingolipids. The ceramide moiety of the parent glycosphingolipid-ceramide mono-hexoside was dominated by 2-hydroxy fatty acids, and a d17:1 sphingoid-base with an iso- or anteiso-branched chain. The chemical composition of the three glycosphingolipids from Caenorhabditis elegans displayed close structural coincidence with the equivalent structures from the porcine parasitic nematode, Ascaris suum (G. Lochnit, R.D. Dennis, U. Zähringer, and R. Geyer, Glycoconjugate J., 1997), in support of this organism as a prototypic glycosphingolipid model for parasitic nematodes.

Key words: Arthro-series glycosphingolipids/Caenorhabditis elegans/d17:1 branched sphingoid-bases/MALDI-TOF-MS/nematode neutral glycosphingolipids

Introduction

Because of the anatomically and developmentally conserved phylogeny of the phylum Nematoda (Bird and Bird, 1991), Caenorhabditis elegans, as a free-living nematode, has been studied and analyzed as a prototypic model for parasitic nematodes (Ward, 1988). It has become recognized as a suitable object for the study of nematode basic biology at the cellular, molecular, and genetic level (Byerly et al., 1976a,b; Bazzicalupo, 1983) because of the characteristics of: small size, limited cell number, transparent cuticle, short life cycle, and mode of reproduction. The data obtained have been used to fill gaps in the knowledge of parasitic nematodes, such as, anatomy, biochemistry, physiology, development, and behavior. Particular emphasis has been placed on the cuticle of C. elegans, because of the parasitic nematode's surface as the interface of host-parasite interaction(s). The intrinsic properties of the nematode cuticle have been studied, as to the composition, developmental regulation, and dynamics of the cuticle; the function(s) of the surface coat; and relevance of the dauer larva to survival and dispersal in the life cycle of nematodes (Cassada and Russell, 1975; Blaxter et al., 1992; Politz and Philipp, 1992; Maizels et al., 1993).

Although C. elegans has become firmly established as a prototypic model for parasitic nematodes, little work has been performed on the lipids, in particular, the identity of the glyco(sphingo)lipids of this organism. Until recently, the sole analysis as to the presence of nematode glyco(sphingo)lipids (the use of parentheses is to indicate the non-establishment of a sphingoid moiety by chemical means) had often depended upon thin-layer chromatography (see review: Dennis and Wiegandt, 1993). Structural elucidation of the monoglycosylceramides has revealed the presence of unusually long-chained, hydroxylated fatty acids and a potentially nematode-specific, iso-branched C17:1 sphingoid base (Chitwood et al., 1995), the latter of which was also present in the sphingomyelin fraction. As the alkyl residues and fatty acids of ether- and ester-linked phospholipids did not correspond to that of the ceramide moiety (Satouchi et al., 1993), this would suggest a specific mechanism for the synthesis and incorporation of the long-chained, hydroxylated fatty acids.

Our own immunological studies have revealed the antigenicity, and most probable immunogenicity, of neutral glyco(sphingo)lipids derived from the adult, parasitic nematodes, Litomosoides carinii and Ascaris suum (Baumeister et al., 1994; Dennis et al., 1995). In addition, these and further serological studies on the neutral glyco(sphingo)lipid fraction of a larval cestode, Echinococcus granulosus (Dennis et al., 1993) and adult trematode, Schistosoma mansoni (Dennis et al., 1996), have indicated their potential in fulfilling the fundamental properties of serodiagnostic antigens in the detection of disease, namely, sensitivity and specificity. The next, rational step in the analysis of nematode glyco(sphingo)lipids would be to determine the structural basis for the general and restricted glycolipid antigens observed, with regard to their ultimate potential in the serodiagnosis of a nematodiasis and specific nematodiases, respectively. For this purpose, it is evident that the free-living nematode, C. elegans, is a relevant candidate as glyco(sphingo)lipid-model for parasitic nematodes. Perhaps, the ultimate advantage of this free-living nematode lies in its development and access to the various, larval stadia.

To establish C. elegans as a relevant glyco(sphingo)lipid-model for the analysis of parasitic nematodes, we have elucidated the structures of three, simple neutral glyco(sphingo)lipids detected and compared them to those of the parasitic nematode, A. suum (Lochnit et al., 1997). Because of the extraction procedure applied, as well as to simplify the nomenclature and avoid ambiguity, the components of the neutral glycolipid frac-
tion studied in detail will be termed, glycosphingolipids, throughout the text and prior to the description of their structural elucidation.

Results

Isolation and extraction of glycosphingolipids

The nematode *C. elegans* was cultivated on agar plates with the *Escherichia coli* strain OP-50 as food source. To obtain purified worms, a density gradient centrifugation with Percoll was performed. The Percoll-isolated worms were used as the source of nematode-derived glycosphingolipids. For isolation of the glycosphingolipids, 10 g of lyophilized worm material was used and extracted as described (Figure 1).

Following ion-exchange (DEAE-) chromatography, the neutral glyco(sphingo)lipid extract was subfractionated by silica-gel chromatography into two further fractions: glycosphingolipids containing simple, neutral carbohydrate residues and glycosphingolipids with more complex sugar moieties, differentiated by their migration behavior on high-performance thin layer chromatography (HPTLC; data not shown). In this report only the fast-migrating, simple species will be described.

The glycosphingolipids were separated by high-performance liquid chromatography (HPLC). Three different components were obtained, which were designated CMH (ceramide mono-hexoside), CDH (ceramide dihexoside), and CTH (ceramide trihexoside), according to their running properties when compared with standard glycosphingolipids (see Figure 2, lanes 2, 3, and 5).

**Neutral (simple and complex) glyco(sphingolipid)**

**Acidic glyco(sphingolipid)**

**Complex glyco(sphingolipid)**

**Simple, neutral glycosphingolipids**

**Individual neutral glycosphingolipid components**

Fig. 1. Protocol for the extraction, isolation, and fractionation of glycosphingolipids from *C. elegans*.

Structural analysis of the carbohydrate moieties of neutral glycosphingolipids

To determine the oligosaccharide moieties, the molecular masses of individual glycosphingolipids were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Figure 3), and of their constituent monosaccharides and linkage positions by methylation analysis (Table I).

For CMH, major pseudomolecular ions ([M+Na]+) of m/z 808, m/z 822, m/z 836, and m/z 850 could be detected (Figure 3A), which indicated a ceramide with bound hexose and heterogeneity in the former moiety. The hexose was confirmed by methylation analysis to be glucose (Table I). For CDH, major pseudomolecular ions ([M+Na]+) of m/z 970, m/z 984, m/z 998, and m/z 1012 could be measured (Figure 3B), which correlated with two hexose residues bound to the ceramide. The hexoses were determined as subterminal glucose and terminal mannose residues (Table I). For CTH, pseudomolecular ions ([M+Na]+) of m/z 1173, m/z 1187, m/z 1201, and m/z 1215 were detected (Figure 3C), which indicated two hexoses and one N-acetylhexosamine in the carbohydrate moiety. The methylation analysis revealed terminal N-acetylglucosamine, subterminal mannose, and internal glucose residues (Table I).

In order to confirm the data of methylation analysis, purified CTH was treated with β-N-acetylhexosaminidase to release the terminal N-acetylglucosamine. The loss of the corresponding mass could be followed by MALDI-TOF-MS (Table II) and HPTLC (Figure 2), respectively. In the first case, pseudomolecular ions ([M+Na]+) of m/z 970 and m/z 998 could be detected which corresponded to the molecular masses observed for CDH. In addition, the migration behavior of CTH after treatment with β-N-acetylhexosaminidase was similar to that of CDH. The results obtained by digestion of CDH or pseudo-CDH (cleaved CTH) with β-mannosidase revealed the same pseudomolecular ions and migration properties as for CMH. By 360 MHz proton nuclear magnetic resonance spectroscopy, glucose was determined to be β-linked to the lipid moiety (preliminary data from U.Zahringer, Division of Immunochemistry, Center for Medicine and Biosciences, Research Center Borstel, Germany) in conformation of Chitwood et al. (1995).

Structural analysis of the ceramide substituent

Fatty acids, bound to CMH, were released as their fatty acid methyl esters under acidic conditions. They were extracted with n-hexane and identified by gas-liquid chromatography/mass spectrometry (GC/MS). The relative amounts and distribution of individual fatty acids, as determined from the pseudomolecular ions ([M+NH4]+) of corresponding methyl esters after chemical ionization with ammonia, are summarized in Table III. The data obtained demonstrated that the major part of the total fatty acids consisted of 2-hydroxylated and saturated fatty acids, namely, C21h:0, C22h:0, C23h:0, C24h:0, and C25h:0 as the major species. These results and the per cent distribution of the fatty acids in CMH corresponded with the detected native pseudomolecular ions of the glycosphingolipids CMH, CDH, and CTH (Figure 3, Table II). The branched hydroxylated and saturated fatty acids could be detected, too. The rest were non-hydroxy fatty acids, whereby C16:0, C17:0, and C18:1 represented 6.1%, 3.4%, and 6.2%, respectively.

The hydroxyl groups of the hydroxylated and saturated fatty acids were localized by GC/MS after electron-impact ioniza-
by the release of fatty acids under strong alkaline conditions and detection of the mass of the resulting product. The mass of the sphingoid moiety with bound glucose (lyso-CMH) was detected by MALDI-TOF-MS as a pseudomolecular ion ([M+Na]⁺) of m/z 470, which indicated a C17-sphingoid-base (Figure 5A). In another confirmatory experiment, the resultant free amino group of the sphingosine was acetylated. The expected pseudomolecular ion ([M+Na]⁺) of m/z 512 with an increased mass of 42 Da was shown by MALDI-TOF-MS (Figure 5B). Since only one mass could be detected after release of the fatty acids, the ceramide of the neutral glycosphingolipids was assumed to contain exclusively a C17-sphingosine.

The alkene chain of the sphingoid-base of CMH was analyzed by GC/MS, after oxidation of the double bond with NaOAc/KMnO₄ and derivatization of the released fatty acid as its picolinyl ester. In the total-ion chromatogram (see inset in Figure 6A) of the resulting products, two peaks (A and B) were detected after electron-impact ionization, which had a minor shift in their retention times, but yielded the same molecular ion ([M⁺]). The ion at m/z 305 corresponded to a derivatized C13 fatty acid. The mass spectra of the two peaks are shown in Figure 6A and 6B. Fragmentation of the fatty acid picolinyl esters led to a series of ions at m/z 290, 276, 248, 234, 220, 206, 192, 178, and 164. In Figure 6A, the fragment ion at m/z 276 was missing, whereas in Figure 6B, the fragment m/z 262 was present only in a small amount. These missing fragments indicated the presence of an iso-branched and an anteiso-branched C13 fatty acid, respectively. In agreement with these results, the sphingoid-bases of the simple, neutral glycosphingolipids of C. elegans were represented by the structures 15-methyl-2-aminohexadec-4-en-1,3-diol (Chitwood et al., 1995) and 14-methyl-2-aminohexadec-4-en-1,3-diol, respectively.

The data obtained by structural analysis of the carbohydrate moiety of CTH led to the conclusion that the carbohydrate-chain sequence of the studied, neutral glycosphingolipids belonged to the so-called arthro-series of insect glycosphingolipids (Table IV). The molecular mass detection of all three neutral glycosphingolipids by MALDI-TOF-MS allowed the extrapolation of results from the ceramide analysis of CMH to the ceramide constituents of CDH and CTH. Because we could detect the d17:1 sphingoid-base and fatty acids as components of the lipid moiety, these results confirmed the conclusion that these glycolipids could be characterized as glycosphingolipids. The presence of five major different hydroxylated fatty acid species and two possible branched forms of sphingosine allowed various permutations of ceramide to occur in these glycosphingolipids.

### Table I. Methylation analysis of the carbohydrate moieties of CMH, CDH, and CTH

<table>
<thead>
<tr>
<th>Linkage</th>
<th>Glycosphingolipid</th>
<th>Alditol acetate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMH</td>
<td>CDH</td>
</tr>
<tr>
<td>Glc(1-)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>-4Glc(1-)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Man(1-)</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>GlcNAc(1-)</td>
<td>1.1</td>
<td>2,4,6-ManOH</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>3,4,6-GlcN(Me)AcOH</td>
</tr>
</tbody>
</table>

*3,4,6-GlcN(Me)AcOH, 2-deoxy-2-(N-methyl)acetamido-3,4,6-tri-O-methylglucitol, etc.

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**Discussion**

Because of their accessibility, the cuticular surface glycoconjugates of free-living and plant parasitic nematodes, in particular C. elegans, have been analyzed as to the outer body wall-distribution of sialyl, galactosyl, mannosyl, and N-acetylglucosaminy1 residues (McCleare and Zuckerman, 1982; Zuckerman and Kahane, 1983; Jansson et al., 1986). Surface-located mannosyl and sialyl residues of C. elegans and Panagrellus redivivus were implicated in the chemotactic response towards E. coli (Jansson et al., 1984; Jeyaprakash et al., 1985), while the non-enzymatic removal of sialyl and galactosyl residues from the surface coat of the plant parasitic nematodes,
Fig. 3. Analysis of the glycosphingolipids by MALDI-TOF-MS. (A) CMH, (B) CDH, and (C) CTH were analyzed in the positive-ion reflectron mode using 2,5-dihydroxybenzoic acid as matrix. Corresponding pseudomolecular ions ([M+Na]+) are marked in the inserts.
Caenorhabditis elegans neutral glycosphingolipids

Helicotylenchus multicinctus and Meloidogyne javanica (Spiegel et al., 1982), implied the presence of glycolipid- and/or proteoglycan-bound glycoconjugates. Structural analyses carried out on glycoconjugates of nematodes so far include, for example, N-glycans of TSL-1 antigens from L1 larvae of Trichinella spiralis (Reason et al., 1994), O-glycans of Toxocara spp. excretory-secretory glycoprotein antigens (Khoo et al., 1991) and the molecular species of monohexosylceramide from C. elegans (Chitwood et al., 1995). The specific objective of this investigation was to structurally characterize simple neutral glycosphingolipids from a mixed-population of C. elegans and to compare these components to the neutral glycosphingolipids isolated from adults of the prototypic, pig parasite, A. suum (Lochnit et al., 1997), in order to test the relevance of C. elegans as a glyco(sphingo) lipid-model for the analysis of parasitic members of the phylum Nematoda.

Three glycosphingolipids from the neutral glycolipid fraction of C. elegans have been structurally characterized based on methylation analysis, exoglycosidase cleavage, MALDI-TOF-MS, and ceramide analysis. Their chemical structures have been determined as (Table IV): Glcβ1Cer, Manβ4Glcβ1Cer and GlcNacβ3Manβ4Glcβ1Cer. The most distinctive hallmarks of these neutral glycosphingolipid components were virtual agreement in the structural traits of the carbohydrate chain and ceramide moiety with equivalent structures of the parasitic nematode, A. suum (Lochnit et al., 1997); homologous identity of the oligosaccharide chain with the carbohydrate, arthro- series of insect glycosphingolipids (Dennis et al., 1985a,b; Table II. MALDI-TOF-MS measured molecular masses of glycosphingolipids

<table>
<thead>
<tr>
<th>Enzyme used</th>
<th>CMH</th>
<th>CDH</th>
<th>CTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>808, 836</td>
<td>970, 998</td>
<td>1173, 1201</td>
</tr>
<tr>
<td>β-N-Acetylgalactosaminidase (jack beans)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Mannosidase (H. pomatia)</td>
<td>808, 836</td>
<td>970, 998</td>
<td>808, 836</td>
</tr>
</tbody>
</table>

Molecular masses of the neutral glycosphingolipids were measured before and after exoglycosidase-treatment. The masses were detected by MALDI-TOF-MS in the positive-ion reflectron mode using 2,5-dihydroxybenzoic acid as matrix.
Table III. Fatty acid composition of CMH

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Fatty acid</th>
<th>[M + NH$_4$]$^+$</th>
<th>Relative amounts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C16:0</td>
<td>288</td>
<td>6.1</td>
</tr>
<tr>
<td>2</td>
<td>C18:1</td>
<td>314</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>C18:0</td>
<td>316</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>C21h:0</td>
<td>374</td>
<td>4.4</td>
</tr>
<tr>
<td>5</td>
<td>C22h:0</td>
<td>388</td>
<td>40.0</td>
</tr>
<tr>
<td>6</td>
<td>C23h:0</td>
<td>402</td>
<td>7.0</td>
</tr>
<tr>
<td>7</td>
<td>C24h:0 iso</td>
<td>416</td>
<td>3.3</td>
</tr>
<tr>
<td>8</td>
<td>C24h:0</td>
<td>416</td>
<td>17.5</td>
</tr>
<tr>
<td>9</td>
<td>C25h:0 iso</td>
<td>430</td>
<td>1.2</td>
</tr>
<tr>
<td>10</td>
<td>C25h:0</td>
<td>430</td>
<td>2.9</td>
</tr>
<tr>
<td>11</td>
<td>C26h:0</td>
<td>444</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Fatty acids were analyzed as their fatty acid methyl esters by GC/MS and identified by their retention times and pseudomolecular ions ([M + NH$_4$]$^+$) after chemical ionization with ammonia. Peak numbers refer to Figure 4. Relative amounts are based on peak ratios of individual fatty acid derivatives normalized to 100%; components of <1% have not been assigned. C16:0, saturated fatty acid with 16 carbon atoms; C21h:0, saturated hydroxy fatty acid with 21 carbon atoms, etc. Hydroxyl groups were assigned to C2 in all hydroxy fatty acids. The presence of iso-branched hydroxy fatty acids was confirmed by [M-15]$^+$ fragment ions in corresponding electron-impact mass spectra.

Dennis and Wiegandt, 1993); and a CMH-derived ceramide moiety with docosanoic and tetracosenoic acids, as the major 2-hydroxylated C22:0 and C24:0 fatty acids extant, as well as d17:1 sphingoid-bases with iso- and anteiso-branched chains. Hence, their structural features link them to a common biogenetic pathway (arthro-series), in which synthesis proceeds by stepwise addition of the specified monosaccharide.

Comparison of the glycosphingolipids from monoxenically cultivated C.elegans (this publication) with the molecular species of monohexosylceramide from axenically propagated worms (Chitwood et al., 1995) revealed that the latter demonstrated decreased microheterogeneity in the sphingoid-base and an equivalent, but different, microheterogeneity among the straight-chained, saturated, 2-hydroxylated fatty acids and their iso-branched analogs. Similarly, the structural differences between neutral glycosphingolipids of C.elegans (this publication) and A.suum (Lochnit et al., 1997) were restricted to the ceramide moiety, in that, the latter manifested decreased microheterogeneity among the fatty acids.

As glyco(sphingo)lipids have not been detected in the axenic culture medium (Chitwood et al., 1995) or in E.coli (data not shown) of the monoxenic propagation medium for the free-living nematode, C.elegans, in the first instance, it is not necessary to invoke alternative mechanism(s) to their de novo biosynthesis from precursor molecules. Ultimately, only incorporation experiments performed with appropriate, labeled precursors, for example, serine and suitable fatty acids, will confirm or disprove the present assumption of de novo glycosphingolipid biosynthesis by nematodes. In terms of the iso-anteiso-branched d17:1 sphingoid-bases, when present in organisms, their occurrence is either in trace amounts or of restricted distribution, for example, certain atypical bacteria and protozoa (Karlsso, 1970a,b). Thus far, respective glyco(sphingo)lipids have been isolated from Bacteroides spp., obligate anaerobes of the gastrointestinal tract (Stoffel et al., 1975), and Sphingomonas spp. aerobic opportunistic pathogens.

(Yamamoto et al., 1978; Kawahara et al., 1991), but not from E.coli.

Scrutiny of distantly related members of the Protostomia for potential phylogenetic relationships, according to the neutral glycosphingolipid composition, indicated no substantial structural relatedness between the described neutral glycosphingolipids from C.elegans and those found in the Cestoda (Nishimura et al., 1991; Dennis et al., 1992; Persat et al., 1992; Kawakami et al., 1993, 1994), Trematoda (Levery et al., 1992; Makaaru et al., 1992), or Annelida (Sugita et al., 1992). The observation as to the frequent appearance of ceramide moiety-
Fig. 5: Analysis of lyso-CMH (A) and N-acetylated lyso-CMH (B) by MALDI-TOF-MS in the positive-ion reflectron mode using 2,5-dihydroxybenzoic-acid as matrix. Pseudomolecular ions ([M+Na]⁺) are marked.
derived, hydroxylated fatty acids in the glycosphingolipids of nonparasitic and parasitic helminths would appear to support the evolutionary contention that they possess a common, functional property relevant to the helminth plasma membrane and/or outer body wall. The closest glycosphingolipid structural homology perceived for *C. elegans* was with the diplopodan arthropod, *Parafontaria laminata armigera* (Sugita et al., 1994). Among more distantly related members of the Protostomia, the neutral glycosphingolipid-composition of this millipede most closely resembled that of the three neutral glycosphingolipids of *C. elegans* studied here. The ceramide moiety was distinguished by sphingoid-bases of nonbranched and branched species of d17:0, d17:1, and d18:1, and fatty acids of nonhydroxylated as well as 2- and 3-hydroxylated fatty acids of C22:0, C23:0, and C24:0. The carbohydrate chain belongs fundamentally to the arthro-series, although it is modified, in part, by fucosylation. This speculative conclusion based on glycosphingolipids is corroborated by studies on the molecular evolution of the Metazoa (Field et al., 1988; Bergström, 1991). Based on the molecular evolution of RNA polymerase II (Sidow and Thomas, 1994), the metazoan evolutionary tree would now indicate that the phylogenetic position of the Nematoda-lineage is closest to that of the Arthropoda.

**Materials and methods**

**Cultivation of *C. elegans***

In this study mixed populations of *C. elegans* (wild-type strain N2, var.Bristol) were used. Growth, maintenance on agar-plates and food source were as described previously (Brenner, 1974; Sulston and Hodgkin, 1988). To harvest the...
chloroform/methanol (98/2, v/v)-suspended silica-gel 60 (70-250 mesh; Merck, Darmstadt, Germany) and equilibrated with chloroform/methanol (98/2, v/v). The neutral fraction was applied and fractionated by a stepwise gra-

The dried, desalted (glyco)lipid extract (550 mg) was redissolved in chloroform/methanol (98/2, v/v). Simple neutral glycosphingolipids, including the neutral (simple and complex) glyco(sphingo)lipids, were obtained by washing the column with 50 ml of chloroform/methanol/water (70/10/25, v/v/v); dried, redissolved in chloroform/methanol/water (10/10/1, v/v/v), and stored at 4°C.

The worms were thawed slowly and sonified for 30 min at 4°C with a 0.5 s periodic impulse at an intensity of 50% (Cell Disrupter B15; Branson, Danbury, CT). After sonification, the homogeneous suspension was lyophilized and the dried material (10 g) ground to a powder in a mortar and pestle. The extraction was performed consecutively as follows and described previously (Dennis et al., 1995): the first extraction step was carried out threefold with 200 ml chloroform/methanol/water (10/10/1, v/v/v) for 30 min at 50°C, after 10 min sonification; followed by extraction twice with 200 ml chloroform/methanol/water (30/60/8, v/v/v) at 4°C for 60 min and at 4°C overnight, after 10 min sonification; and twice with 200 ml 2-propanol/ln-hexane/water (55/20/25, v/v/v) for 30 min at 50°C, and 10 min sonification. All steps were interspersed with a centrifugation of 10 min at 2000 x g. The supematants were pooled and rotary evaporated to dryness under reduced pressure at 40°C. For desalting of the sample, the dried extract (3.6 g) was dissolved in 120 ml chloroform/methanol/aqueous 0.1 M KCl (3/48/47, v/v/v) and mixed with 40 g Chromabond C18-ec (Macherey and Nagel, Düren, Germany) in a reversed-phase batch procedure. Salts were removed by washing three times with 160 ml methanol/water (8/92, v/v) and the bound, hydrophobic components were eluted twice with 120 ml MeOH and three times with 120 ml chloroform/methanol (2/1, v/v). The desalted fraction was dried by rotary evaporation under reduced pressure at 40°C. During further extraction and isolation procedures, any desalting step of the glycolipid fractions was performed on 1-2 ml prepacked columns of Chromabond C18-ec (see below).

HPTLC

To resolve the glycosphingolipids into single species, the neutral fraction was fractionated by fastatom-HPLC (6RS-S-1010, 10 µm, 4.6 x 50 mm; Macherey and Nagel). Column chromatography was performed at 30°C with a flow rate of 1 ml/min. After equilibration with 100% A (2-propanol/ln-hexane/water; 55/44/1, v/v/v) and injection of the sample, the proportion of solvent B (2-propanol/ln-hexane/water; 55/30/10, v/v/v) was linearly increased to 100% in 30 min and held isocratically for 20 min. Fractions of 1 ml were collected and controlled for their glycosphingolipid content by HPTLC.

Enzymatic digestion

For determination of the monosaccharides, their sequence and anomic configuration, neutral glycosphingolipids with the running properties of ceramide dihexoside (CDH) and ceramide trihexoside (CTH) were treated with B-N-acetylhexosaminidase (only CTH) from jack beans (Sigma, Deisenhofen, Germany) and α-mannosidase (CDH, CTH) from jack beans (Sigma, Deisenhofen, Germany) and equilibrated with chloroform/methanol (98/2, v/v). The neutral fraction was applied and fractionated by a stepwise gra-

Table IV. Structures proposed for the analyzed neutral glycosphingolipids of C. elegans

<table>
<thead>
<tr>
<th>Component</th>
<th>Carbohydrate structure</th>
<th>Sphingoid base</th>
<th>Characteristic hydroxylated fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMH</td>
<td>Glc(B1-1)</td>
<td>di:7:1</td>
<td>C21h:0, C22h:0, C23h:0</td>
</tr>
<tr>
<td>CDH</td>
<td>Man(B1-4)Glc(B1-1)</td>
<td>iso-olefinic-branched*</td>
<td>C24h:0, C25h:0</td>
</tr>
<tr>
<td>CTH</td>
<td>GlcNAc(B1-3)Man(B1-4)Glc(B1-1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The structures were deduced from the analytical results obtained by methylation, positive-ion MALDI-TOF-MS, exoglycosidase cleavage and ceramide analysis.

*15-methyl-2-arabinohexadec-4-en-1,3-diol/14-methyl-2-arabinohexadec-4-en-1,3-diol.

dient of increased polarity. The highly hydrophilic lipids were eluted with chloroform/methanol (98/2, v/v). The simple, neutral glycosphingolipids and phospholipids were eluted in chloroform/methanol/water (65/25/4, v/v/v) from the column. Complex glycosphingolipids (20 mg) were eluted with chloroform/methanol/water (70/10/25, v/v/v); dried, redissolved in chloroform/methanol/water (10/10/1, v/v/v), and stored at 4°C. The simple, neutral glycosphingolipid fraction was rotary evaporated to dryness under reduced pressure at 40°C and dried over P2O5. To remove saponifiable and non-saponifiable contaminant lipids, the neutral fraction was peracetylated for 18 h in the dark at room temperature with 10 ml pyridine and 6 ml acetic anhydride. The reaction mix was rotary evaporated to dryness three times with toluene, dried under N2, and redissolved in 1,2-dichloroethane/n-hexane (4/1, v/v). To obtain only neutral glycosphingolipids, column chromatography with Florisil (10 x 0.8 cm, 60–100 mesh; Merck) was carried out. The column was equilibrated with 1,2-dichloroethane/n-hexane (4/1, v/v), the per-

The worms were washed with 0.9% aqueous NaCl solution to remove the remnants of Percoll by gentle centrifugation. The cleaned worms were stored at -30°C until required.
25 μl of β-mannosidase and/or 160 μl of β-hexosaminidase were incubated with dried aliquots of 5–10 μg CDH or CTH. After cleavage, most of the detergent could be removed by chromatography using a pre-packed column of Chromabond C18-ec as described above. The resultant glycosphingolipids were analyzed by HPTLC and MALDI-TOF-MS.

Methylation analysis
To determine the mono-saccharide components and their linkage positions, the glycosphingolipids were permethylated (Paz-Parra et al., 1985) and hydrolyzed. Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by gas chromatography/ mass spectrometry (GC/MS) using the instrumentation and microtechniques described earlier (Geyer et al., 1983; Geyer and Geyer, 1994). For the separation of fatty acid species, a fused-silica capillary column (DB-1, 0.25 mm ID, 60 m; 60°C) was used. The column temperature was increased from 80°C at 7°C/min, to a final temperature of 320°C and held isothermally for 10 min. Spectra were recorded either after chemical ionization with ammonia or electron-impact ionization at an electron energy of 2.4033*10^-17 J or 1.1215*10^-17 J, respectively.

Analysis of lyso-CMH
Because of the fatty acid heterogeneity of the ceramide moiety, the fatty acids were released from 60 μg CMH by treatment with 500 μl 1 M KOH for 18 h at 100°C in order to simplify the detection and analysis of the sphingoid-base. After this time, the reaction mix was neutralized and desalted as described above. The eluate was dried under N2 and redisolved in chloroform/methanol/water (10/10/1, v/v/v). The molecular mass of lyso-CMH (monosaccharide-bound sphingoid-base) was determined by MALDI-TOF-MS. For acetylation of the resulting free amino group, 30% of the monosaccharide-bound sphingoid-base was dried under N2 and treated with 190 μl H2O, 10 μl pyridine, and 20 μl acetic anhydride for 30 min at room temperature. The mass of the acetylated sphingoid-base was measured by MALDI-TOF-MS.

Analysis of sphingoid-base
To analyze the sphingosine species present, corresponding alkane chains were released by oxidation of the double bond with KMnO4/NaIO4 (Hayashi and Matsubara, 1971). CMH (50 μg) was incubated in 200 μl butanolv, 600 μl 0.02 M Na2CO3, and 440 μl H2O (containing 5 mg NaIO4 and 0.4 mg KMnO4). The reaction was carried out overnight at room temperature in the dark. The mixture was treated with HCl to obtain a low pH. Protonated fatty acids were extracted with n-hexane and analyzed on a fused-silica capillary column (DB-1, 0.25 mm ID, 60 m; 60°C) was used. The column temperature was increased from 80°C, at 7°C/min, to a final temperature of 320°C and held isothermally for 10 min. Spectra were recorded either after chemical ionization with ammonia or electron-impact ionization at an electron energy of 2.4033*10^-17 J or 1.1215*10^-17 J, respectively.

MALDI-TOF-MS
For the molecular mass determination of glycosphingolipids (Hillelkenkamp et al., 1991; Bahr et al., 1994; Harvey, 1995) a MALDI-TOF mass spectrometer (Vision 2000, Finnigan-MAT, Bremen, Germany) was used in the positive-ion mode. Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by gas chromatography/ mass spectrometry (GC/MS) using the instrumentation and microtechniques described earlier (Geyer et al., 1983; Geyer and Geyer, 1994). The sample was applied to the MALDI-TOF-mass spectrometer (Vision 2000, Finnigan-MAT, Bremen, Germany) a MALDI-TOF-MS (Vision 2000, Finnigan-MAT, Bremen, Germany) was used in the positive-ion mode. Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by gas chromatography/ mass spectrometry (GC/MS) using the instrumentation and microtechniques described earlier (Geyer et al., 1983; Geyer and Geyer, 1994). For the separation of fatty acid species, a fused-silica capillary column (DB-1, 0.25 mm ID, 60 m; 60°C) was used. The column temperature was increased from 80°C, at 7°C/min, to a final temperature of 320°C and held isothermally for 10 min. Spectra were recorded either after chemical ionization with ammonia or electron-impact ionization at an electron energy of 2.4033*10^-17 J or 1.1215*10^-17 J, respectively.

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Abbreviations
CDH, ceramide dihexoside; Cer, ceramide; CMH, ceramide monohexoside; CTH, ceramide trihexoside; GC/MS, gas-liquid chromatography/ mass spectrometry; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

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