Glycoconjugate Journal (1997) **14:** 389–399

Structural analysis of neutral glycosphingolipids from *Ascaris suum* adults (Nematoda: Ascaridida)★

Günter Lochnit[1], Roger D. Dennis[1], Ulrich Zähringer[2] and Rudolf Geyer[1,*]

[1]*Institute of Biochemistry, University of Giessen, Friedrichstrasse 24, D-35392 Giessen, Germany*
[2]*Division of Immunochemistry, Center for Medicine and Biosciences, Research Center Borstel, D-23845 Borstel, Germany*

The neutral glycosphingolipid fraction from adults of the pig parasitic nematode, *Ascaris suum*, was resolved into four components on thin-layer chromatography. The high-performance liquid chromatography-isolated components were structurally analysed by: methylation analysis; exoglycosidase cleavage; gas-liquid chromatography/mass spectrometry; liquid secondary-ion mass spectrometry; and, in particular, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Their chemical structures were determined as: Glc(β1-1)ceramide, Man(β1-4)Glc(β1-1)ceramide, GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide and Gal(α1-3)GalNAc(β1-4)GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide; and were characterized as belonging to the arthro-series of protostomial glycosphingolipids. No glycosphingolipid component corresponding to ceramide tetrasaccharide was detected during these analyses. The ceramide composition of the parent glycosphingolipids was dominated by the 2-(*R*)-hydroxy C24 : 0 fatty acid, cerebronic acid, and C17 sphingoid-bases: 15-methylhexadecasphing-4-enine and 15-methylhexadecaphinganine in approximately equal proportions. The component ceramide monohexoside was characterized by an additional 15-methylhexadecaphytosphingosine.

***Keywords*: arthro-series glycosphingolipids, *Ascaris suum*, (*iso*-branched) C17 sphingoid-bases, neutral glycosphingolipids, parasitic nematodes**

***Abbreviations*: CDH, ceramide dihexoside; Cer, ceramide; CMH, ceramide monohexoside; CPH, ceramide pentahexoside; CTH, ceramide trihexoside; CTetH, ceramide tetrahexoside; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPTLC, high-performance thin-layer chromatography; LSIMS, liquid secondary-ion mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; N-, Nz- and A-glyco(sphingo)lipids, neutral, neutral zwitterionic and acidic glyco(sphingo)lipids, respectively**

Introduction

If the definition of a successful parasitic nematode of humans is the infection of the maximal number of people with the minimal amount of mortality and morbidity, then *Ascaris lumbricoides* must serve as a supreme example. Ascariasis is present in approximately 25% of the world population, with an estimated annual mortality of 20 000 and annual morbidity of 1 000 000 [1]. Man has acquired only partial protection to re-infection, whereby the main immune response is humoral and is directed to the migrating larvae, whilst a cellular reaction is directed against adult worms when located in abnormal organs, *ie*, not in the lumen of the small intestine [2]. A further aspect of this disease is the high level of serological cross-reactivity with antigens derived from other parasitic helminths, that makes immunodiagnosis of little relevance in detecting larval and/or adult infections of *A. lumbricoides* in man. Such antigenic cross-reactivity may be turned to advantage in nematode diseases of veterinary significance, where the same molecule(s) may serve as cross-protective vaccines against different parasitic helminths [3].

Our primary interest by the systematic analysis for the structural basis of the serological cross-reactivity of parasitic nematode-derived antigens is, as a potential detour around this immunodiagnostic impasse, detecting Nematoda-specific molecules of immunological relevance. Because we were particularly interested in the immunogenic, natural products of parasitic nematodes, we have concentrated our efforts on their glycoconjugates [3]. Using the porcine equivalent of *A. lumbricoides* as a

★ During the editing process of this manuscript, it was brought to the attention of the authors the existence of a publication in Japanese covering the same topic and reaching, essentially, the same conclusions: Sugita M, Mizunoma T, Hayata C, Hori T, Nakatani F, Aoki K (1994) Classification into arthro-series of neutral glycosphingolipids from porcine roundworm, *Ascaris suum* (Aschelminthes, Nematoda). *J Jpn Oil Chem Soc (Yukagaku)* 43: 495–501.

* To whom correspondence should be addressed. Tel and Fax: + 49-641-99-47400; E-mail: Rudolf.Geyer@biochemie.med.uni-giessen.de

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prototypic, parasitic nematode, we have examined the immunoreactivity of neutral fraction glycosphingolipids obtained from adult worms of *A. suum* [4]. Of the high-performance thin-layer chromatography (HPTLC)-resolved fast and slow migrating band groups, the former were found to contain neutral species that were non-antigenic, whilst the latter contained amphoteric species that were antigenic. In addition and of relevance to the phenomenon of non-specific immunodiagnosis of *A. lumbricoides* infections in humans, antigenic and serological cross-reactivity could be extended between the HPTLC-resolved, slow migrating, amphoteric glycosphingolipids of *A. suum* and the equivalent glycolipids of the parasitic nematodes, *Litomosoides carinii* and *Nippostrongylus brasiliensis*.

To further our analyses of the neutral fraction glycosphingolipids of adult *A. suum*, we have characterized the structures of the non-antigenic species as a prelude to determining the chemical basis for the immunoreactivity of the corresponding antigenic compounds; since the carbohydrate backbone of the amphoteric glycosphingolipids of *A. suum* is expected to correspond, at least in part, to the biogenetic series derived from the oligosaccharide chain sequences of the neutral species [5].

Materials and methods

Materials

Undamaged, washed adult male and female worms (800 g wet weight) were pulverized at $-20\,^{\circ}C$ in a precooled Waring blender and lyophilized.

Isolation, purification and fractionation of the neutral glycosphingolipids

Glycosphingolipids were isolated by consecutive extractions: twice with 3000 ml chloroform : methanol : water (10 : 10 : 1, by volume) at 50 °C for 30 min; once with 1500 ml chloroform : methanol : 0.8 M aqueous sodium acetate (30 : 60 : 8, by volume) overnight at 4 °C; and twice with 2000 ml 2-propanol : n-hexane : water (55 : 20 : 25, by volume) at 50 °C for 30 min. The raw extracts were pooled, centrifuged at $2500 \times \mathbf{g}$ for 10 min and the resultant supernatant evaporated to dryness before desiccation overnight over P_2O_5. To remove most of the contaminant triglycerides the residue was treated with 600 ml acetone for 2 h at 4 °C. The supernatant was discarded after centrifugation, with the retained pellet dissolved in chloroform : methanol : water (10 : 10 : 1, by volume) and dialysed against distilled water for 3 days at 4 °C. After rotary evaporation, the preparation was dissolved in chloroform : methanol : water (30 : 60 : 8, by volume) and applied to a DEAE-Sephadex column (A-25, acetate-form, 30×100 mm; Pharmacia, Germany). Neutral (**N**-/**Nz**-)glyco(sphingo)lipids were eluted with 500 ml chloroform : methanol : water (30 : 60 : 8, by volume) and acidic (**A**-)glyco(sphingo)lipids with 500 ml chloroform : methanol : 0.8 M aqueous sodium acetate (30 : 60 : 8, by volume). Neutral (**N**-) and neutral zwitterionic (**Nz**-)glyco(sphingo)lipids were separated on a silica gel_{60} column (70–250 mesh, 30×150 mm; Merck, Germany); equilibrated with chloroform : methanol (9 : 1, by volume). **N**-Glycosphingolipids were eluted with 2000 ml chloroform : methanol : water (65 : 25 : 4, by volume) and **Nz**-glyco(sphingo)lipids with 900 ml chloroform : methanol : water (10 : 70 : 20, by volume). To remove saponifiable and non-saponifiable phospholipids, **N**-glycosphingolipids were peracetylated in pyridine : acetic anhydride (2 : 1, by volume) for 18 h at room temperature. Dried, peracetylated **N**-glycosphingolipids were dissolved in 1,2-dichloroethane : n-hexane (4 : 1, by volume) and applied to a Florisil column (30×150 mm, 60–100 mesh; Merck) equilibrated with 1,2-dichloroethane : n-hexane (4 : 1, by volume). The column was washed with 400 ml each of 1,2-dichloroethane : n-hexane (4 : 1 and 1 : 1, by volume) and 1,2-dichloroethane. Peracetylated glycosphingolipids were eluted with 800 ml 1,2-dichloroethane : acetone (1 : 1, by volume), evaporated to dryness and desiccated overnight over P_2O_5. **N**-Glycosphingolipids were deacetylated with methanolic sodium methylate (1%, by weight) for 3 h at room temperature and desalted by dialysis. To remove lipid contaminants and free fatty acids, **N**-glycosphingolipids were applied to a silica gel_{60} column (30×50 mm; Merck) equilibrated with chloroform : methanol (98 : 2, by volume). The column was washed with 600 ml chloroform : methanol (98 : 2, by volume), three times with 300 ml chloroform : methanol (95:5, by volume), 300 ml acetone : methanol (90 : 10, by volume), 300 ml chloroform : methanol (80 : 20, by volume) and 300 ml chloroform : methanol : water (10 : 70 : 20, by volume). The fractions were evaporated to dryness, desalted on reverse-phase cartridges (Chromabond $C18_{ec}$, Macherey & Nagel, Germany) and monitored for **N**-glycosphingolipids by HPTLC. Corresponding fractions were pooled. For isolation of individual **N**-glycosphingolipid components, HPLC fractionation on a porous silica gel column (Iatrobeads 6RS-8010, 10 μm, 4.6×500 mm; Macherey & Nagel) was performed. Glycosphingolipids were eluted with a linear gradient from 100% eluant A (2-propanol : n-hexane : water 55 : 44 : 1, by volume) to 100% eluant B (2-propanol : n-hexane : water 55 : 35 : 10, by volume) in 30 min at a flow rate of 2 ml min^{-1}; 2 ml fractions were collected and monitored for glycosphingolipids by HPTLC. Corresponding fractions were pooled and rotary evaporated to dryness.

HPTLC

For HPTLC separation, HPTLC-silica gel_{60} plates from Merck were used. **N**-Glycosphingolipids were dissolved at 2 μg μl^{-1} in chloroform : methanol : water (60 : 35 : 8, by volume). For reproducibility, HPTLC was performed according to Nores *et al.* [6] using a motor-driven propeller in the

lid of the chromatographic tank for better saturation of the atmosphere. During the period of saturation, the plates were suspended in the tank for 15 min before lowering into the running solvent. For 1-dimensional HPTLC, chloroform : methanol : water (65 : 25 : 4, by volume) was used as the running solvent. Glycosphingolipids were visualized, after drying of the HPTLC-plates, by spraying with orcinol : sulfuric acid.

Treatment with exoglycosidases

N-Glycosphingolipids (100–200 pmol) were treated either with α-D-galactosidase (EC 3.2.1.22) from coffee beans (Boehringer, *Germany*), *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52) from jack beans (Sigma, Germany) or β-mannosidase (EC 3.2.1.25) from *Helix pomatia* (Oxford Glycosystems, UK). For cleavage, the dried **N**-glycosphingolipids were taken up in 50 µl 0.05 M sodium citrate, pH 4.0, with 0.1% (by weight) sodium taurodeoxycholate and incubated at 37 °C for 24 h with 0.83 nkat α-galactosidase, 2.78 nkat β-*N*-acetylhexosamindase and 0.42 nkat β-mannosidase, respectively. After addition of 200 µl water, resultant glycosphingolipids were extracted three times with 200 µl n-butanol and analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and HPTLC.

Methylation analysis

N-Glycosphingolipids (1–10 nmol) were permethylated and hydrolysed [7]. Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analysed by capillary GLC/MS using the instrumentation and microtechniques described elsewhere [8].

Preparation of lyso-glycosphingolipids

To study the heterogeneity of the sphingoid-base moiety, the amide linkage of dried glycosphingolipids (100–200 pmol) was hydrolysed by incubation with 1 M methanolic KOH for 16 h at 100 °C. After neutralization with acetic acid, the lyso-glycosphingolipids were desalted on reverse-phase cartridges and analysed by MALDI-TOF-MS.

MALDI-TOF-MS

MALDI-TOF-MS data were obtained using a Vision 2000 instrument (Finnigan MAT, Germany), operating in the positive-ion reflectron mode. Ions were formed by a pulsed ultraviolet laser beam (nitrogen laser, $\lambda = 337$ nm). The matrix used was 2,5-dihydroxybenzoic acid (Sigma), 10 g l^{-1}, in 0.1% aqueous trifluoroacetic acid : acetonitrile (1 : 2, by volume). **N**-Glycosphingolipids were dissolved in chloroform : methanol : water (10 : 10 : 3, by volume). To 1 µl of matrix solution, 1 µl of the glycosphingolipid sample (approximately 5 pmol μl^{-1}) was added on to the stainless steel target. After drying in a cold air stream, a further 1 µl of matrix solution was added. In the case of lyso-glycosphingolipids, 1 µl of 10 mM LiCl in methanol was added to improve sensitivity. The droplet was allowed to dry in a cold air stream before introduction into the mass spectrometer. External mass calibration was provided by the $[M + H]^+$ ions of angiotensin and 2,5-dihydroxybenzoic acid (1296.7 and 155.0 Da; Sigma).

Liquid secondary-ion mass spectrometry (LSIMS)

LSIMS was carried out with a MAT 900 mass spectrometer (Finnigan MAT, Germany) equipped with a caesium gun, which was operated at an emission current of 2–3 µA. Mass spectra were recorded at an acceleration potential of 5 kV with a resolution of approximately 3000, and were acquired using a DEC 2100 data system. Spectra of native or permethylated oligosaccharide alditols were recorded in the negative-ion or positive-ion mode using triethanolamine or 3-nitrobenzyl alcohol (Aldrich, Germany) as matrix, respectively.

Fatty acid analysis

For fatty acid analysis, **N**-glycosphingolipids (1–10 nmol) were hydrolysed according to Gaver and Sweeley [9] with 200 µl of aqueous and methanolic HCl (1 M HCl, 10 M H_2O) at 100 °C for 16 h. The resultant fatty acid methyl esters were extracted three times with 500 µl n-hexane and analysed by capillary GLC/MS using the instrumentation described previously [8]. For the separation of fatty acid species a fused-silica capillary column (DB1, 0.25 mm ID, 60 m; ICT, Germany) was used. The column temperature was increased from 80 °C, at 7 °C per min, to a final temperature of 320 °C and held isothermally for 10 min. Spectra were recorded either after chemical ionization (CI-MS) with ammonia or electron-impact ionization at an electron energy of 2.4033×10^{-17} J or 1.1215×10^{-17} J, respectively. For determination of the absolute configuration at C2 of the hydroxy fatty acids they were converted to the corresponding (*R*)-phenylethylamides and trifluoroacetylated [10]. For the separation of the diastereomers a fused-silica capillary column (Optima 1, 0.25 mm ID, 10 m, Macherey & Nagel, Germany) was used. The column temperature was increased from 100 °C, at 6 °C per min, to a final temperature of 320 °C and held isothermally for 10 min. For detection an electron-capture detector was used. For identification a 2-(*R*,*S*)-hydroxy tetracosanoic acid standard was used.

Sphingoid-base analysis

For the structural analysis of sphingoid-bases, intact glycosphingolipids (1–10 nmol) were hydrolysed according to Kadowaki [11]. Fatty acids were extracted with n-hexane after acidification. Sphingoid-bases were then oxidized with 0.2 M methanolic periodic acid for 2 h at room temperature in the dark. The resulting aldehydes were oxidized at room temperature overnight in a mixture of 0.2 ml 1-butanol,

0.6 ml 0.02 M Na_2CO_3, 0.27 ml aqueous sodium metaperiodate/potassium permanganate (5 mg $NaIO_4$, 0.4 mg $KMnO_4$; Merck) and 0.17 ml H_2O. Under these conditions the aldehydes and the double bond of sphingosine-base derived aldehydes were oxidized yielding the corresponding free fatty acids [12]. Sphingosine, sphinganine and phytosphingosine bases led to fatty acids with alkyl-chains lacking four, two and three carbon atoms, respectively. Fatty acids were extracted, after addition of 1 ml 0.1 M aqueous HCl, three times with 1 ml n-hexane. After drying under a stream of nitrogen the fatty acids were either methylated with diazomethane [13] or derivatized as their picolinyl esters [14]. For the latter derivatization, following formation of the fatty acid imidazolide in 20 µl *N*,*N*′-carbonyldiimidazole in methylene chloride at room temperature for 5 min, the picolinyl ester was generated on heating the sample at 37 °C for 15 min in the presence of 5 µl of 3-pyridylmethanol; the solvent was then removed in a stream of dry nitrogen. Structural analysis was the same as described for the fatty acids.

^{1}H-NMR

A deuterium-exchanged sample of CMH from *A. suum* (300 µg) was dissolved in dimethyl sulphoxide-d_6 (99.9% D, Aldrich, Germany) containing 2% D_2O (100% D, Aldrich). Spectra were recorded at 360 MHz on a Bruker AM-360 spectrometer operated at 330 K. Homonuclear ^{1}H connectivities were determined by COSY experiments using standard Bruker software. Chemical signals were referenced to tetramethylsilane as an internal standard.

Results

Isolation and purification of neutral-fraction glycosphingolipids

Glycosphingolipids were extracted with chloroform : methanol : water, chloroform : methanol : 0.8 M aqueous sodium acetate and 2-propanol : n-hexane : water, respectively. Contaminant triglycerides were removed from the raw extract with an acetone-wash. Glycosphingolipids were separated into a neutral and acidic fraction by anion-exchange column chromatography. The neutral fraction was further subdivided into a neutral and a neutral zwitterionic fraction by silica gel column chromatography. The **N**-glycosphingolipid fraction was further purified by a peracetylation/Florisil chromatography/deacetylation-procedure (Figure 1). Preparative HPLC of this fraction yielded four individual glycosphingolipid components (Figure 2). These

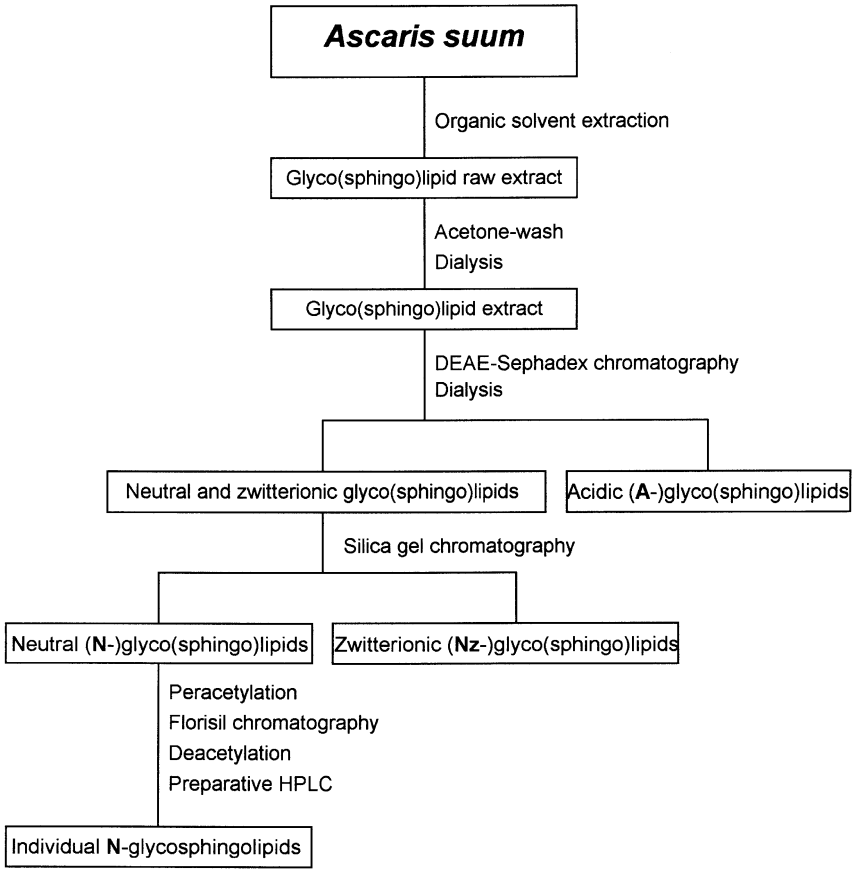

Figure 1. Schematic protocol for the extraction, isolation and fractionation of neutral (**N**-)glycosphingolipids of *A. suum.*

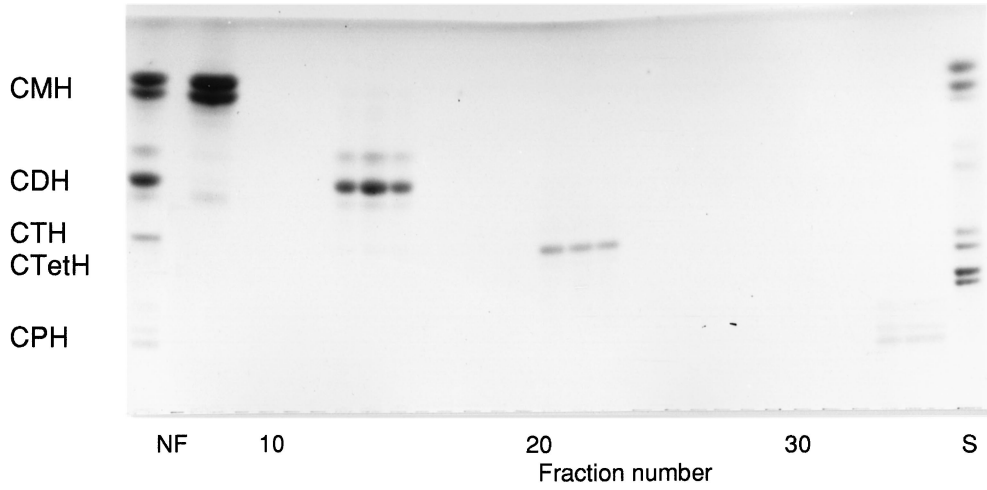

Figure 2. HPLC separation of individual neutral glycosphingolipids of *A. suum*. **N**-glycosphingolipids were fractionated on a HPLC column (500 × 4.6 mm; Iatrobeads 6RS-8010) at room temperature and a flow rate of 2 ml min^{-1}. Fractions of 2 ml were collected. Solvents A and B were 2-propanol : n-hexane : water (55 : 44 : 1 and 55 : 30 : 10, by volume, respectively). For fractionation, solvent B was increased linearly from 0% to 100% in 30 min. Aliquots of the fractions were spotted on HPTLC plates, developed with chloroform : methanol : water (65 : 25 : 4 by volume) and sprayed with orcinol : sulphuric acid reagent. NF, total neutral glycosphingolipids of *A. suum*; **S**: standard glycosphingolipids.

components were designated – according to their HPTLC migration properties – as ceramide monohexoside (CMH), ceramide dihexoside (CDH), ceramide trihexoside (CTH) and ceramide pentahexoside (CPH), respectively. A glycosphingolipid corresponding to a ceramide tetrahexoside (CTetH) could not be detected.

Carbohydrate structure analysis of individual glycosphingolipids

Individual glycosphingolipids were studied by methylation analysis (see Table 1), LSIMS (data not shown) and positive-ion MALDI-TOF-MS (Figure 3 and Table 2). Monosaccharide sequencing and determination of the anomeric configurations of corresponding glycosidic linkages were achieved by cleavage with the corresponding exoglycosidases (Table 2). Cleavage was confirmed by HPTLC and by MALDI-TOF-MS.

Positive-ion MALDI-TOF-MS of CMH revealed pseudomolecular ions ($[M + Na]^+$) at *m/z* 837, 839 and 855, which corresponded to a composition of Hex-ceramide with heterogeneity in the ceramide moiety. Methylation analysis revealed the presence of terminal glucose. The ^{1}H NMR spectrum displayed characteristic signal splitting of all glucose protons due to the presence of different sphingoid bases (data not shown). The intensity ratios of the splitted signals were found to be in the range of the proportions identified by MALDI-TOF-MS for sphingosine, sphinganine and phytosphingosine (approximately 1 : 1 : 1). Such splitting for sugar proton signals are characteristic for heterogeneous ceramides and has also been observed in other glycosphingolipids expressing different chemical species in the sphingoid base and fatty acid moieties, respectively [15]. The chemical shift and coupling constant of the anomeric proton H-1 (δ 4.13 ppm, $J_{1,2}$ 7.8 Hz) clearly revealed a β-linked glucose. Other signals of the unsubstituted glucose moiety were assigned based on a 2D COSY experiment being in excellent agreement with data from literature [16].

MALDI-TOF-MS of CDH led to pseudomolecular ions at *m/z* 998 and 1000, which was consistent with a composition of Hex_2-ceramide. Methylation analysis demonstrated the presence of terminal mannose and a 4-substituted glucose. Treatment with β-mannosidase from jack beans released the terminal mannose residue, as evidenced by MALDI-TOF-MS, of the cleaved glycosphingolipid yielding pseudomolecular ions at *m/z* 837 and 839.

CTH revealed pseudomolecular ions at *m/z* 1201 and 1203, which was consistent with a composition of $HexNAcHex_2$-ceramide. Subsequently, methylation analysis demonstrated the presence of a terminal *N*-acetylglucosamine, 3-substituted mannose and 4-substituted glucose. The terminal *N*-acetylglucosamine residue was removed by treatment with β-*N*-acetylhexosaminidase from jack beans generating pseudomolecular ions at *m/z* 998 and 1000. On subsequent β-mannosidase-treatment, the resulting glycosphingolipid was degraded to CMH with pseudomolecular ions at *m/z* 837 and 839. These results led to the conclusion that the CTH-oligosaccharide chain sequence belonged to the so-called arthro-series of insect glycosphingolipids [17].

The compound CPH, which was present in only low amounts, revealed a poorly resolved pseudomolecular ion at *m/z* 1567. The composition, $HexNAc_2Hex_3$-ceramide, was

Table 1. Methylation analysis of **N**-glycosphingolipids[a]

Linkage	***N**-Glycosphingolipid compound*					*Alditol acetate*
	CMH	*CDH*	*CTH*	*CPH*	*CPH[G]*[b]	
Glc(1-	1.0	–	–	–	–	2,3,4,6-GlcOH
-4)Glc(1-	–	1.0	1.0	1.0	1.0	2,3,6–GlcOH
Man(1-	–	0.5	–	–	–	2,3,4,6-ManOH
-3)Man(1-	–	–	0.9	0.8	0.8	2,4,6-ManOH
GlcNAc(1-	–	–	0.4	–	–	3,4,6-GlcN(Me)AcOH
-4)GlcNAc(1-	–	–	–	0.7	0.8	3,6-GlcN(Me)AcOH
GalNAc(1-	–	–	–	–	0.5	3,4,6-GalN(Me)AcOH
-3)GalNAc(1-	–	–	–	0.6	–	4,6–GalN(Me)AcOH
Gal(1-	–	–	–	0.4	–	2,3,4,6-GalOH

[a]*A. suum* **N**-glycosphingolipids were permethylated and hydrolysed. The partially methylated sugar derivatives obtained after reduction and peracetylation were analysed by capillary GLC/MS. Results are expressed as peak ratios of the alditol acetates found. The low yields of terminal monosaccharides are due to their higher volatility, *ie* a higher level of methylation. 2,3,4,6-GlcOH, 2,3,4,6-tetra-*O*-methylglucitol; 3,4,6-GlcN(Me)AcOH, 2-deoxy-2-(*N*-methyl)acetamido-3,4,6-tri-*O*-methylglucitol, etc.
[b]obtained from CPH by treatment with *α*-galactosidase.

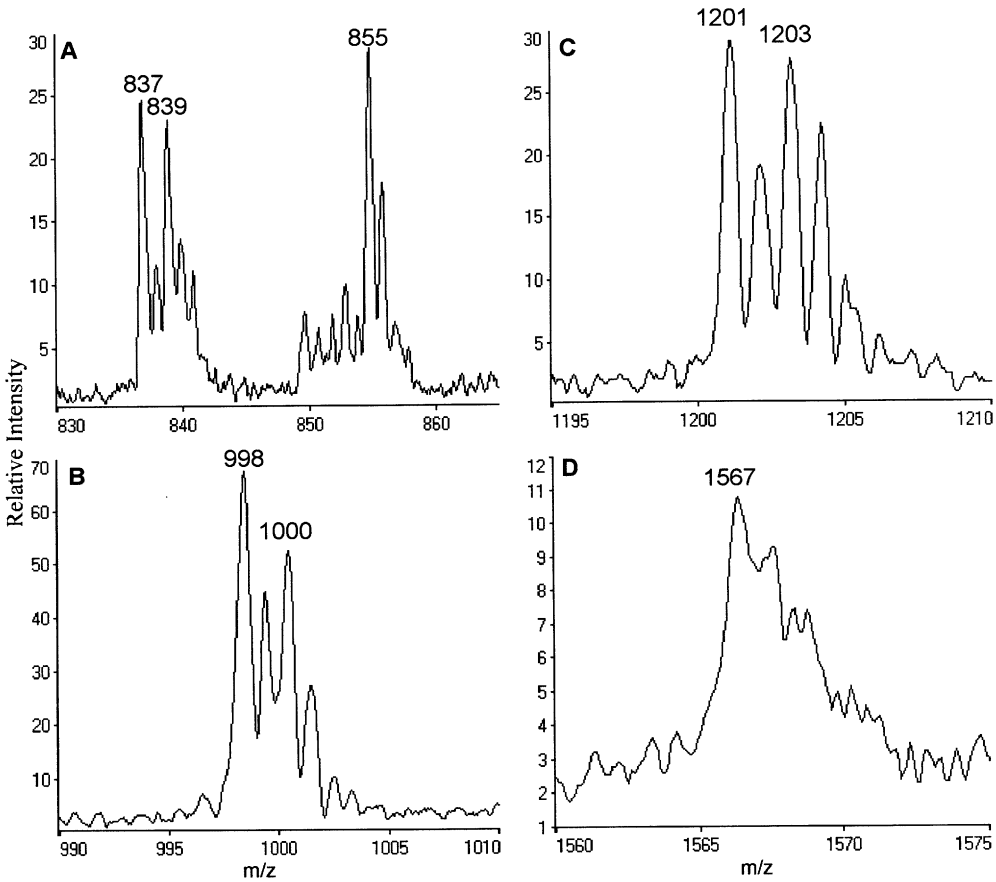

Figure 3. Analysis of **N**-glycosphingolipids by MALDI-TOF-MS. (A) CMH, (B) CDH, (C) CTH and (D) CPH were analysed in the positive-ion reflectron mode using 2,5-dihydroxybenzoic acid as matrix. Pseudomolecular ions ([M + Na^+]) are given in accurate mass values rounded to the nearest mass unit.

Table 2. Sequential cleavage of **N**-glycosphingolipids with exoglycosidases[a]

Enzyme used	*Pseudomolecular ions ([M + Na]$^+$) of fragments obtained from*			
	CMH	*CDH*	*CTH*	*CPH*
None	837, 839, 855	998, 1000	1201, 1203	1567
α-Galactosidase from coffee beans				1405, 1407
β-N-Acetylhexosaminidase from jack beans			998, 1000	998, 1000
β-Mannosidase from *H. pomatia*		837, 839	837, 839	837, 839

[a]*A. suum* **N**-glycosphingolipids were cleaved with the enzymes indicated and the products were analysed by MALDI-TOF-MS in the positive-ion reflectron mode using 2,5-dihydroxybenzoic acid as matrix. Pseudomolecular ions ([M + Na]$^+$) are given in accurate mass values rounded to the nearest mass unit.

proved by methylation analysis demonstrating the presence of a terminal galactose, 3-substituted *N*-acetylgalactosamine, 4-substituted *N*-acetylglucosamine, 3-substituted mannose and 4-substituted glucose. Treatment with α-galactosidase from coffee beans led to pseudomolecular ions at *m/z* 1405 and 1407. Sequential digestion with *β-N*-acetylhexosaminidase from jack beans and *β*-mannosidase liberated two *N*-acetylhexosaminyl and one mannosyl residues, as evidenced by the pseudomolecular ions at *m/z* 998 and 1000 and *m/z* 837 and 839. Cleavage of the terminal galactose with α-galactosidase (CPH [G]); Table 1) and determination of the resultant terminal monosaccharide as *N*-acetylgalactosamine confirmed the sequence of the two *N*-acetylhexosaminyl residues, as for arthro-series glycosphingolipids [17, 18].

Ceramide analysis

For fatty acid analysis, **N**-glycosphingolipids were hydrolysed, the resultant fatty acid methyl esters extracted with n-hexane and analysed without further purification by GLC/MS (Figure 4A). Fatty acid methyl esters were identified, after chemical ionization with ammonia, as their pseudomolecular ions ([M + NH_4]$^+$). The fatty acid distribution for the **N**-glycosphingolipids is summarized in Table 3. Hydroxy-tetracosanoic acid comprised nearly 90% of the fatty acids present, whilst tetracosanoic acid with about 4% was the only non-hydroxy fatty acid present. Odd-numbered fatty acids were represented by hydroxy-tricosanoic and -pentacosanoic acid with 2.5% and 1.8%, respectively. To localize the hydroxyl group, GLC/MS spectra of the fatty acid methyl esters were recorded after electron-impact ionization. The mass spectrum of hydroxy-cerebronic acid is shown in Fig. 4B. Two abundant ions at *m/z* 398 and 339 were identified as the molecular ion ([M]$^+$) and ([M-59]$^+$; loss of the carbomethoxy radical $CH_3OC{=}O^{\bullet}$). Less abundant ions at *m/z* 380 ([M-H_2O]$^+$), 366 ([M-methanol]$^+$) and 320 ([M-{60 + 18}]$^+$) were of minor structural significance. The ion at *m/z* 90, resulting from the McLafferty rearrangement and 2,3-bond cleavage,

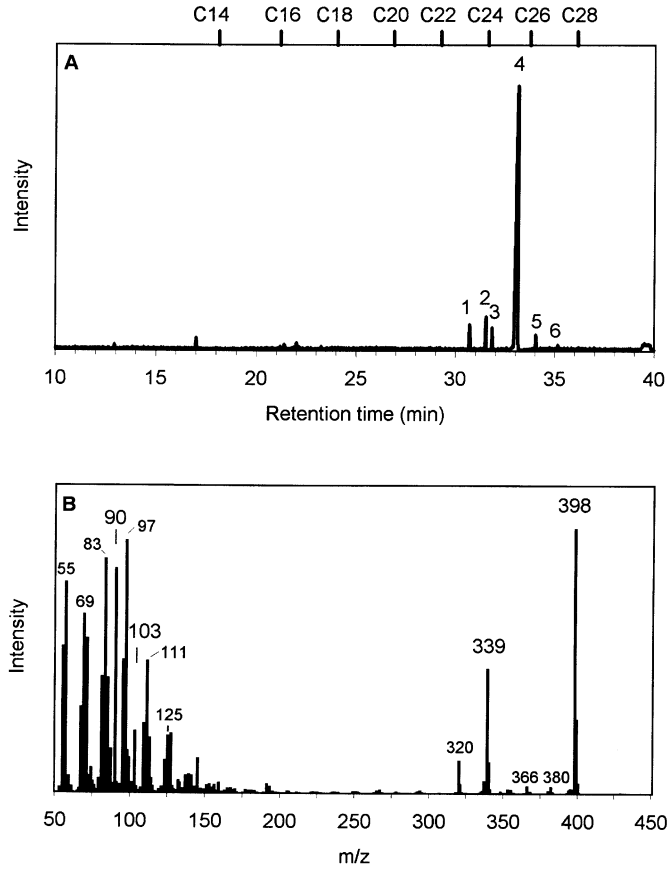

Figure 4. GLC/MS analysis of fatty acid methyl esters of **N**-glycosphingolipids of *A. suum*. Fatty acids were liberated from glycosphingolipids by acid hydrolysis as their fatty acid methyl esters and used for GLC/MS analysis without further purification. (A) Total ion chromatogram of fatty acid methyl esters after chemical ionization with ammonia; (B) Electron-impact mass spectrum of peak 4. Relevant diagnostic ions are marked by larger numbering. C14–C28, retention times of corresponding saturated fatty acid methyl esters.

clearly demonstrated that the corresponding fatty acid is a 2-hydroxy derivative [19]. The carbomethoxy series-ions shifted to *m/z* 103 were less abundant, as expected for a 2-hydroxy fatty acid. Ions at *m/z* 125, 111, 97, 83, 69 and 55

Table 3. Fatty acid composition of *A. suum* **N**-glycosphingolipids[a]

Peak number	*Fatty acid*	*Pseudomolecular ion* $[M + NH_4]^+$	*Relative amounts*
1	C22h : 0	388	2.72
2	C23h : 0	402	2.50
3	C24 : 0	400	3.95
4	C24h : 0	416	88.15
5	C25h : 0	430	1.78
6	C26h : 0	444	0.90

[a]Fatty acid methyl esters were analysed by capillary GLC/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 per cent. C24 : 0, saturated fatty acid with 24 carbon atoms; C22h : 0, saturated hydroxy fatty acid with 22 carbon atoms, etc. For C24h : 0, the hydroxyl group was assigned to C2.

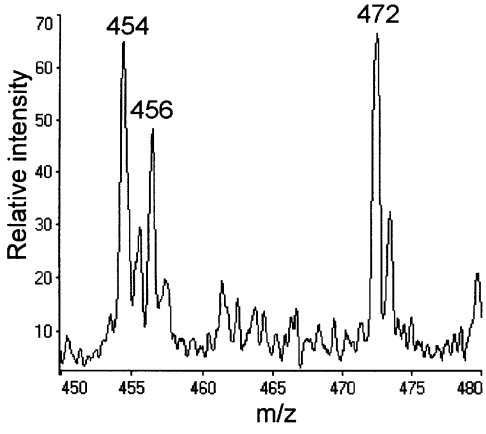

Figure 5. Analysis of lyso-CMH by MALDI-TOF-MS in the positive-ion reflectron mode using 2,5-dihydroxybenzoic acid as matrix. Lyso-CMH was prepared by incubation of CMH with 1 M methanolic KOH for 16 h at 100 °C. Pseudomolecular ions ($[M + Li]^+$) are given in accurate mass values rounded to the nearest mass unit.

belong to the C_nH_{2n-1} series ($n = 9$–4). The absolute configuration at C2 was found to be (*R*) (data not shown).

To study the heterogeneity of the sphingoid-base moiety, the fatty acids were released from the glycosphingolipids by strong alkaline treatment. The resulting lyso-glycosphingolipids were analysed by MALDI-TOF-MS. The pseudomolecular ions ($[M + Li]^+$) of lyso-CMH at *m/z* 454, 456 and 472 indicated the presence of C17-sphingosine, -sphinganine and -phytosphingosine bases (Figure 5), whereas CDH, CTH and CPH comprised solely C17-sphingosine and -sphinganine (data not shown). The detection of only fatty acids and sphingosine(s) in the ceramide moiety defined the **N**-glycolipids extracted from *A. suum* as **N**-glycosphingolipids. To chemically determine the structure of the sphingoid-base(s), **N**-glycosphingolipids were hydrolysed and the sphingoid-bases were oxidized with periodic acid and periodate/permanganate in order to generate the corresponding free fatty acids. After derivatization as their methyl esters with diazomethane or picolinyl esters with 3-pyridylmethanol, the fatty acids were analysed by GLC/MS with chemical ionization with ammonia and electron-impact ionization. C13 : 0, C14 : 0 and C15 : 0 fatty acids were detected (Figure 6A) and identified as

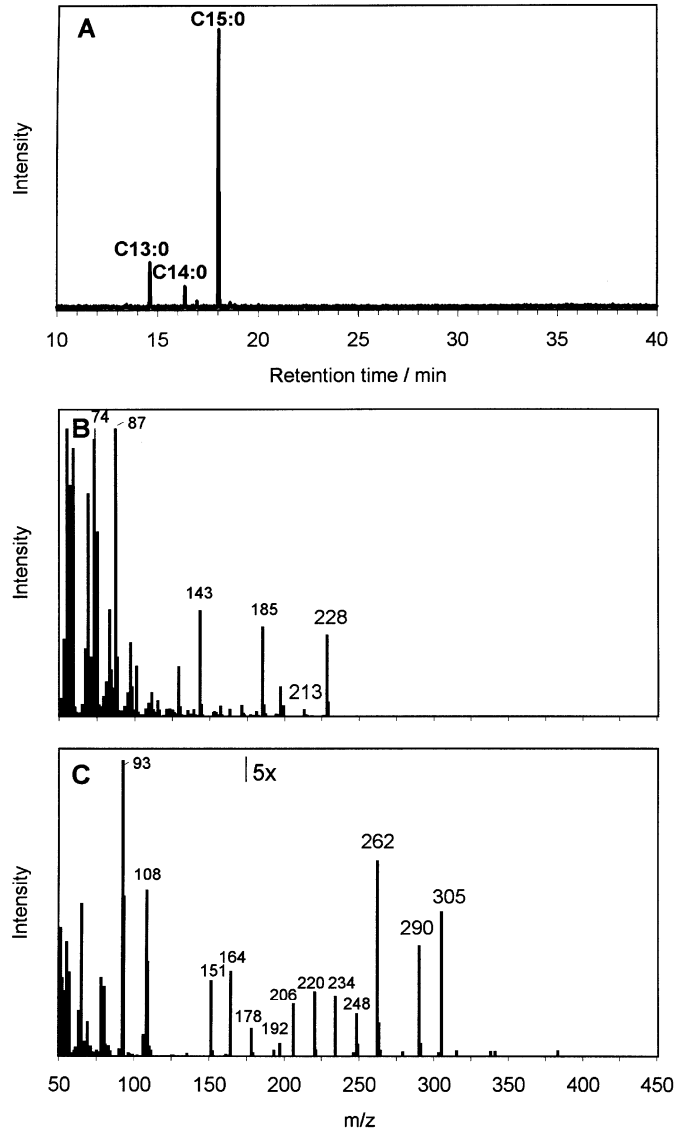

Figure 6. GLC/MS analysis of fatty acid methyl esters and picolinyl esters of *A. suum* **N**-glycosphingolipids derived from oxidized sphingoid bases. CMH was subjected to acid hydrolysis. After extraction of the free fatty acids, sphingoid bases were oxidized with periodic acid and periodate/permanganate. Resulting fatty acid compounds were extracted with hexane. After esterification with diazomethane or 3-pyridylmethanol, the fatty acid derivatives were analysed by GLC/MS without further purification. (A) Total ion chromatogram of fatty acid methyl esters after chemical ionization with ammonia; (B) electron-impact mass spectrum of C13 : 0, (C) electron-impact mass spectrum of the corresponding C13 : 0 fatty acid picolinyl ester. Relevant diagnostic ions are marked by larger numbering.

corresponding to the iso-branched structures of C17 sphingosine, phytosphingosine and sphinganine. In contrast to the determination of the three sphingoid-bases in approximately equimolar proportions by physical analysis (^{1}H NMR, MALDI-TOF-MS; Figure 3A), this was not reflected in their yields following chemical analysis (methodology as above; Figure 6A). The mass spectrum of the C13:0 fatty acid methyl ester given in Figure 6B showed a molecular ion ($[M]^+$) at *m/z* 228. The less abundant ion at *m/z* 213 ($[M\text{-}15]^+$) indicated an iso-branched fatty acid whereas more abundant ions at *m/z* 185, 143 and 87 (carbomethoxy series) and *m/z* 74 (McLafferty rearrangement) were of less structural significance. In contrast, the mass spectrum of the C13:0 fatty acid picolinyl ester showed a series of fragment ions resulting from cleavage after each carbon atom (Figure 6C). The molecular ion at *m/z* 305 ($[M]^+$) was accompanied by fragment ions at *m/z* 290, 262, 248, 234, 220, 206, 192, 178 and 164. Ions at *m/z* 151, 108 and 93 result from McLafferty rearrangement or fragmentations within the picolinyl ester unit and are, therefore, of no diagnostic value. The missing fragment ion at *m/z* 276 clearly indicated the presence of an iso-branched C13:0 fatty acid. These results led to the conclusion of a 2-amino-15-methyl-4-hexadecene-1,3-diol as the characteristic sphingosine base and the corresponding C17 iso-branched sphinganine and phytosphingosine bases for *A. suum* **N**-glycosphingolipids. The absolute (*S*/*R*) configurations at the carbon atoms have not been established.

Discussion

That various glycoconjugates, in particular, those of the cuticular surface, are of importance to the parasitic mode of life of nematodes can be inferred from the stage-dependent changes in the expression of cuticular carbohydrates [20], such as, in the pig parasitic nematode, *A. suum* [21]. Glycoconjugates have been implicated in various aspects of the host-parasite interaction, including, chemoreception [22] and penetration [23]. However, structural analysis of the putative glycoproteins, proteoglycans and glycosphingolipids involved have only been performed on the immunodominant O-glycans of *Toxocara* spp., excretory-secretory glycoprotein antigens [24], and in addition, the monoglucosylceramide species from the free-living nematode, *Caenorhabditis elegans* [25]. The specific intention of this investigation was to determine the structural characteristics of the neutral glycosphingolipids derived from adults of the prototypic, parasitic nematode, *A. suum.*

The structures of the four detected neutral fraction glycosphingolipids from *A. suum* (phylum Nematoda: order Ascaridida) have been established on the basis of: methylation analysis; exoglycosidase cleavage; GLC/MS; LSIMS; and, in particular, MALDI-TOF-MS. From the data obtained, the chemical structures for the **N**-glycosphingolipid components could be proposed (Figure 7). The most outstanding features of these **N**-glycosphingolipids were: homology in the carbohydrate chain with the arthro-series glycosphingolipids of insects (*C. vicina* [5, 17, 18] and *Lucilia caesar* [26]); a ceramide moiety dominated by the 2-(*R*)-hydroxy C24:0 fatty acid, cerebronic acid, and the *iso*-branched sphingosine-base d17:1 and the sphinganine-base d17:0 in approximately equal proportions. The phytosphingosine-base t17:0 was only identified in CMH.

MALDI-TOF-MS was found to be an excellent analytical tool for glycosphingolipid analysis [27]. The detection of glycosphingolipids at the low picomole level without derivatization allowed control of all purification and analytical steps. The usage of MALDI-TOF-MS in reflectron mode with a resolution of 2000–3000 in most cases permitted isotopic resolution, the detection of microheterogeneity, for example, in the ceramide portion, and a clear discrimination between saturated and unsaturated sphingoid-bases or fatty acids. Exoglycosidase experiments could be examined in an easy and less time consuming way requiring little sample clean-up. As MALDI-TOF-MS spectra of glycosphingolipids rarely showed fragmentation, the spectra were easy to interpret but omitted structural information concerning the carbohydrate moiety. Post-source-decay experiments may overcome this disadvantage, but are in general more complex [28]. GLC/MS analysis of fatty acid methyl esters after electron-impact ionization allowed structural elucidation of fatty acid modifications, such as, hydroxylation. Using this method, 2-hydroxy and 3-hydroxy fatty acids can be clearly distinguished by their McLafferty rearrangement ions at *m/z* 90 or *m/z* 74, respectively. Because of the lack of a wide range of standards and the low

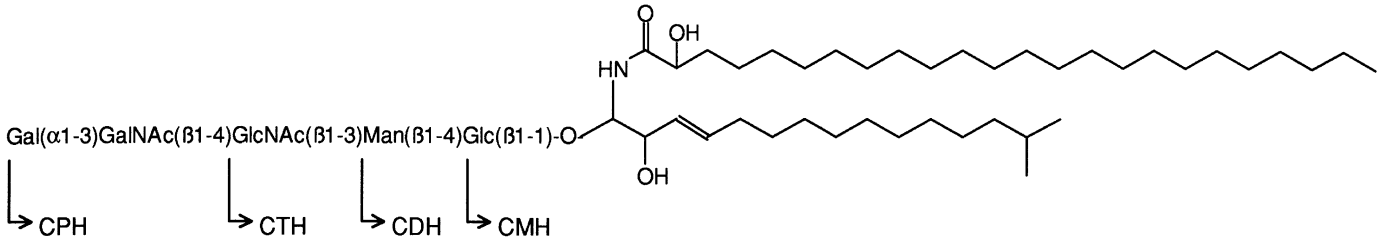

Figure 7. Summarized structures of the analysed **N**-glycosphingolipids from *A. suum.*

sensitivity of common analytical methods for sphingoid-bases, we applied the periodic acid and periodate/permanganate (Rudloff-)oxidation to generate fatty acids from the corresponding sphingoid-bases. Following derivatization to their picolinyl esters, the structural analysis of the alkyl-chain with modifications far from the carboxyl group was possible. Compared with fatty acid methyl esters, the simple electron-impact spectra of these picolinyl esters allowed precise localization of double bonds and branching [29–31] at high sensitivity.

It is evident from the established structures that the four **N**-glycosphingolipid components belong to a biosynthetic pathway proceeding by the stepwise addition of the respective monosaccharide. This generalization is emphasized in the summarized structural formulae of the four **N**-glycosphingolipid components (Figure 7; with the *iso*-branched d17 : 1 as the common sphingoid-base, whilst CTetH was not detected during the analysis) from *A. suum*. The occurrence of *iso*-branched sphingoid-bases in nature, when present, is either in trace amounts or of restricted distribution, such as, in certain atypical bacteria and protozoa [32, 33]. The only glycosphingolipid(s) to demonstrate close homology with the ceramide moiety characteristic of *A. suum* were the monoglucosylceramides derived from the free-living nematode, *C. elegans* [25]. In comparison, the *A. suum* ceramide moiety exhibited increased microheterogeneity in the sphingoid-base and reduced microheterogeneity amongst the 2-hydroxy fatty acids. Between more distantly related members of the Protostomia, the composition of glycosphingolipids that exhibited the closest, structural homology to the **N**-glycosphingolipid components of *A. suum* were those derived from the millipede, *Parafontaria laminata armigera* ([34]; phylum Arthropoda: class Chilopoda). The carbohydrate chain could be interpreted as belonging to the arthro-series or to a modified, *ie* fucosylated, arthro-series. The ceramide moiety indicated increased microheterogeneity in the sphingoid-base (non-branched and branched species of d17 : 0, d17 : 1 and d18 : 1) and fatty acids (non-hydroxylated, 2- and 3-hydroxylated fatty acids of C22 : 0, C23 : 0 and C24 : 0).

In an attempt to establish potential, phylogenetic relationships within the Protostomia, according to the composition of neutral glycosphingolipids, it was evident that the carbohydrate moieties of **N**-glycosphingolipids of *A. suum* were not structurally related to those of the Cestoda [15, 35–37] and Annelida [38], or to the Trematoda [39, 40]. The frequency of ceramide-derived, medium- and long-chained, hydroxylated fatty acids amongst free-living and parasitic nematodes, cestodes and trematodes (*ie* helminths), however, may indicate a common, functional property for these entities in the plasma membrane and/or helminth surface. This tentative conclusion, as to the phylogenetic relatedness of the Nematoda and Arthropoda, based on glycosphingolipid composition is supported by studies on the molecular phylogeny of the Invertebrata, whereby the arthropods have not been found to be closely related to the annelids and represent an early divergence from other metameric lineages [41, 42].

The frequent occurrence of hydroxylated fatty acid-containing ceramide moieties in the neutral glycosphingolipids of non-parasitic and parasitic helminths could indicate a particular, evolutionary advantage in the synthesis of hydroxyceramides by these organisms. In fact, an increased hydroxyl group content of sphingolipid-ceramide moieties has been observed in biological membranes subjected to high 'stress' environments. In addition, evidence could be provided that the stability and impermeability of model membranes is a function of the glycosphingolipid-derived hydroxyl group network of lateral hydrogen bonds at the boundary between the hydrocarbon matrix and polar head groups [43]. As ceramides are known to be second messengers [44], these 'hydrophilic' ceramides could play an important role in both host-parasite interaction and parasite survival. The preferential incorporation of hydroxy-fatty acids into ceramides during ceramide biosynthesis or hydroxyceramide into glycosphingolipids could be envisaged to occur either by hydroxy fatty acid-specific sphinganine *N*-acyltransferases or hydroxyceramide-specific glycosyltransferases, respectively [45]. Therefore, both enzymes could be good candidates for blocking glycosphingolipid biosynthesis in regard to the development of anthelmintic drugs.

Acknowledgements

We thank Peter Kaese, Werner Mink and Siegfried Kühnhardt for methylation, GLC/MS and LSIMS analyses. This study was supported by the German Research Council (SFB 272 and Graduiertenkolleg 'Molecular Biology and Pharmacology'). This paper is in partial fulfilment of the requirements of G.L. for the degree of Dr. rer. nat. at Giessen University.

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Received 22 August 1996, revised 15 October 1996