Structural elucidation of zwitterionic carbohydrates derived from glycosphingolipids of the porcine parasitic nematode *Ascaris suum*

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Carbohydrates substituted with phosphocholine (PC) and phosphoethanolamine (PE) were released from zwitterionic glycosphingolipids of the pig parasitic nematode *Ascaris suum* by treatment with endoglycoceramidase. Individual glycans were obtained by HPLC on porous graphitic carbon followed by high-pH anion-exchange chromatography. In addition to the known pentasaccharides Galα3GalNAcβ4[PC6]GlcNAcβ3Manβ4Glc and Galα3GalNAcβ4[PC6]GlcNAcβ3[PE6]Manβ4Glc, the corresponding tri- and tetra-saccharides, as well as components with elongated structures, could be identified by matrix-assisted laser-desorption ionization–time-of-flight MS, methylation analysis, ^{1}H- and ^{13}C-NMR spectroscopy, exoglycosidase cleavage and electrospray ionization ion-trap MS. The extended components comprised novel structural motifs such as di-substituted α-galactose carrying two β-linked galactosyl residues, which were found to bear, in part, further fucose, galactose, *N*-acetylgalactosamine and/or *N*-acetylglucosamine moieties. Furthermore, additional fucosylation of the PC-substituted *N*-acetylglucosamine and a non-terminal fucosyl motif were detected. In conclusion, this study contributes significant new information on the glycome of nematodes.

Key words: nanoelectrospray ionization-ion-trap MS (nano-ESI-IT MS^n), phosphocholine, phosphoethanolamine.

INTRODUCTION

Nematode infections, e.g. ascariasis, are characterized by low mortality but high morbidity, causing high economical costs, especially in developing countries. Although surface constituents, such as glycoconjugates, are known to be involved in early infection processes of nematodes, the knowledge of the structures and biological activities of parasitic helminth-derived glycolipids and glycoproteins is still fragmentary. The biosynthesis of glycoconjugates usually implies the action of various glycosyltransferases and glycosidases, thus leading to an heterogeneous mixture of glycans regarding their size and complexity [1]. During our studies on the structures of glycosphingolipids from parasitic and free-living nematodes [2–6] we identified a conserved structural motif consisting of the arthro-series carbohydrate backbone, substituted with phosphocholine (PC) and, in some cases, with phosphoethanolamine (PE). In *Ascaris suum* two major compounds were identified as Galα3GalNAcβ4[PC6]GlcNAcβ3Manβ4Glcβ-ceramide (component A) and Galα3GalNAcβ4[PC6]GlcNAcβ3[PE6]Manβ4Glcβ-ceramide (component C) [3]. Glycolipids of this type can therefore be regarded as a phylogenetic marker of nematodes.

The zwitterionic PC substituent of nematode glycoconjugates was found to play an important role in host–parasite interaction by interfering with key proliferative signal transduction pathways in B- and T-lymphocytes, thus leading to a reduced immune response towards parasitic infections (for review, see [7–9]). Furthermore, this epitope has been shown to induce pro-inflammatory and T-helper 2-type cytokines [3,10,11] and to promote dendritic cell maturation towards T-helper 2 cell development [12].

Most studies on carbohydrate structures of parasitic nematodes have been focused on immunogenic glycoconjugates and revealed novel structural motifs like tyvelose (3,6-dideoxy-D-arabino-hexose)-capped glycans in *Trichinella spiralis* [13], highly fucosylated chitobiose core structures in *Haemonchus contortus* [14], structures containing O-methylated monosaccharide substituents like in *Toxocara canis* and *T. cati* [15], or PC-substituted glycoprotein-N-glycans [16–18] and glycosphingolipids [2,3,5]. The first detailed structural analysis of *Caenorhabditis elegans* glycans revealed the presence of unusual glucose-containing O-linked mucin-type, short chondroitin-like as well as N-linked high-mannose-type oligosaccharides [19]. Some of these O-glycans were bound to the protein via GlcNAc and comprised O-methylated fucosyl residues. This finding is remarkable in so far as O-linked mucin-type glycans have been shown to play important roles in embryogenesis of *C. elegans* [20]. Furthermore, there is evidence for the presence of oligomannosidic N-glycans with glucosyl caps, complex-type glycans with pentasaccharide cores plus one additional GlcNAc residue, a range of core-fucosylated N-glycans containing three to five mannoses, up to four fucose residues and O-methylated sugars [21] as well as glycosaminoglycan-type structures in this organism [22,23]. The estimated theoretical complexity of glycosylation in nematodes is very high, since many different putative fucosyltransferases encompassing α1-2-, α1-3- and α1-6-fucosyltransferases, galactosyltransferases, *N*-acetylglucosaminyltransferases and xylosyltransferases have been predicted by similarity searches of the *C. elegans* genome [21]. The severe phenotypes observed in the case of *C. elegans* glycosylation mutants, however, have already emphasized the importance of correct glycosylation in embryogenesis, oocyte development and organ formation of nematodes [24–28].

We here describe the investigation of 14 oligosaccharide moieties derived from glycosphingolipids of the pig parasitic nematode *A. suum*, which represent the most complex glycolipid compounds found so far in nematodes.

Abbreviations used: dHex, deoxyhexose; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPAEC, high-pH anion-exchange chromatography; MALDI-TOF MS, matrix-assisted laser-desorption ionization–time-of-flight MS; nano-ESI-IT MS^n, nanoelectrospray ionization-ion-trap MS; PC, phosphocholine; PE, phosphoethanolamine; PGC, porous graphitic carbon; RCT, relayed coherence transfer; tGal, terminal galactose.

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EXPERIMENTAL

Isolation of zwitterionic oligosaccharides from *A. suum*

Fresh undamaged worms were collected at the local slaughterhouse. Washed worms were frozen at −80 °C, homogenized using a Waring blender and lyophilized. Isolation and purification of the zwitterionic glycosphingolipids was performed as described in [4]. Glycolipids (109 mg) were subsequently treated with endoglycoceramidase (EC 3.2.1.123) to remove the ceramide moieties as outlined previously [29].

Separation of zwitterionic oligosaccharides by two-dimensional HPLC

Released zwitterionic oligosaccharides (20 mg) were separated in the first dimension on a porous graphitic carbon (PGC) column (Hypercarb 5 μm, 100 mm × 4.6 mm; Hypersil, Runcorn, Cheshire, U.K.) at a flow rate of 1 ml/min at room temperature with water and acetonitrile as solvents. The pH of both solvents was adjusted to 8.0 with ammonia. After washing and equilibration with water, a linear gradient to 15 % acetonitrile in 50 min was applied. Elution of the oligosaccharides was monitored at 205 nm. In the second HPLC dimension, the fractions from the PGC column were applied to a CarboPak PA-100 column (Dionex, Sunnyvale, CA, U.S.A.) for high-pH anion-exchange chromatography (HPAEC). A linear gradient from 10 to 20 mM sodium acetate in 80 mM NaOH was raised in 35 min at room temperature and a flow rate of 1 ml/min using the instrumentation described elsewhere [30].

Desalting of oligosaccharides

Non-porous graphitized carbon solid-phase extraction cartridges (250 mg; ENVI-Carb; Supelco, Taufkirchen, Germany) were washed consecutively with 9 ml of water, 9 ml of water/acetonitrile (3:1, v/v) and 9 ml of water. The cartridges were equilibrated with 3 ml of 10 mM sodium acetate in 80 mM NaOH, and the oligosaccharide fractions were applied directly to the cartridges after HPAEC. Salt was removed with 9 ml of water, and the oligosaccarides were eluted from the cartridges with 6 ml of water/acetonitrile (3:1, v/v).

HF treatment

The PC and PE substituents were released by treatment with 48 % HF (Merck, Darmstadt, Germany) at 4 °C for 24 h as described in [31].

Matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI-TOF MS)

Mass spectra were recorded using a Vision 2000 instrument (Finnigan MAT, Bremen, Germany), equipped with an ultraviolet nitrogen laser ($\lambda = 337$ nm) in the positive-ion reflectron mode. The matrix consisted of 20 mg/ml 2,5-dihydroxybenzoic acid (Sigma, Deisenhofen, Germany), 1 mg/ml 5-methoxysalicylic acid (Aldrich, Steinheim, Germany) and 15 mg/ml α-L-fucose (Fluka, Buchs, Switzerland) dissolved in 0.1 % aqueous trifluoroacetic acid/acetonitrile (1:2, v/v) [32]. To 1 μl of matrix solution, 1 μl of oligosaccharide sample (30–50 pmol/μl) was added on a stainless-steel target and dried in a gentle stream of warm air. For on-target enzymic treatments 5 mg/ml 6-aza-2-thiothymine (Sigma) in double-distilled water was used as a matrix. For sequential exoglycosidase digestions, the preparation was redissolved in 1–2 μl of dialysed enzyme solution, incubated at 37 °C for 12–24 h [33] and dried at room temperature. For external calibration pseudomolecular ions $[M+Na]^+$ of dextran hydrolysate were used. Accumulated spectra of 10–50 individual records were registered, and monoisotopic masses are indicated throughout.

Off-line nanoelectrospray ionization-ion-trap MS (nano-ESI-IT MS^n)

ESI-IT MS^n experiments were performed with either an LCQ quadropole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, U.S.A.) or an Esquire 3000 (Bruker Daltonics GmbH, Bremen, Germany), both equipped with a nano-ESI off-line source. Oligosaccharide solutions (3–5 μl) containing approx. 10–25 pmol/μl in methanol/water (1:1, v/v) were loaded into laboratory-made gold-coated glass capillaries and electrosprayed at 700–1000 V. For the LCQ instrument the metal-transfer capillary was heated to 200 °C. The capillary and tube lens were held at 48 V. Positive- and negative-ion spectra were obtained by averaging 10 scans, each scan consisting of three microscans. For consecutive MS^n experiments, the relative collision energy in the trap varied between 30 and 70 %, corresponding to the LCQ 1.0 software definitions, depending on the chemical nature of the precursor ions. For the Esquire 3000 instrument the glass capillary was set to a potential of 700–1000 V and the dry gas (4 l/min) was heated to 80 °C. Positive- and negative-ion spectra were obtained by averaging 10 scans. The fragmentation amplitude for MS^n experiments was set between 1.2 and 0.4, referring to the Esquire 3000 software settings (esquireControl version 6.08).

Carbohydrate constituent analysis

Oligosaccharides were hydrolysed with 4 M trifluoroacetic acid (Merck) for 4 h at 100 °C under an argon atmosphere. For the analysis of peracetylated alditol acetates the samples were dried in a stream of nitrogen at 40 °C and the monosaccharides were reduced and peracetylated as described elsewhere [34]. For GLC analysis fused-silica capillary columns (RTX 200 MS, 0.25 mm inner diameter, 30 m; Restek, Bad Soden, Germany; or ZB-5, 0.25 mm inner diameter, 60 m; Phenomenex, Aschaffenburg, Germany) were used. For both columns the temperature was increased from 130 °C, at 4 °C/min, to 290 °C.

Alternatively, monosaccharides were monitored by HPLC and fluorescence analysis as their anthranilic acid derivatives [35].

Methylation analysis

Oligosaccharides were permethylated [36] without, before or after HF treatment. After hydrolysis, reduction and peracetylation, the partially methylated alditol acetates obtained were analysed by capillary GLC/MS using the instrumentation and microtechniques described elsewhere [37].

Exoglycosidase treatment

Prior to digestion the enzymes were dialysed for 2–3 h on a floating nitrocellulose membrane (catalogue no. VSWP02500; Millipore, Bedford, MA, U.S.A.) against 25 mM ammonium acetate buffer adjusted to the recommended pH, i.e. pH 6.0 for α-galactosidase from green coffee beans (EC 3.2.1.22; Boehringer Mannheim, Mannheim, Germany) and pH 4.5 for β-*N*-acetylglucosaminidase from bovine kidney (EC 3.2.1.52; Boehringer Mannheim). β-Galactosidase from jack beans (EC 3.2.1.23; Glyko, Bicester, Oxon, U.K.) was directly diluted with 25 mM ammonium acetate buffer (pH 4.0). Treatments with α-fucosidase II (EC 3.2.1.51; Glyko) were performed according to the

manufacturer's instructions. Free oligosaccharides (700 pmol) with or without HF pretreatment were lyophilized and subsequently dissolved in 20 μl of the appropriate buffer solution. About 2–3 μl (10–100 m-units) of the dialysed enzyme were added and the sample solution was incubated at 37 °C for 12–24 h. To remove the volatile buffer, samples were lyophilized twice. In the case of low amounts of material, the exoglycosidase treatment was performed directly on the MALDI target, employing the method described previously [33].

Smith degradation

Smith degradation was performed according to Khoo et al. [38]. Desalting was achieved on graphitic carbon cartridges (50 mg; Hypersil). The cartridges were washed and equilibrated consecutively with 3 ml of water, 3 ml of water/acetonitrile (3:1, v/v) and 3 ml of water before adding the sample. Salts were removed with 1.5 ml of water and oligosaccharides eluted with 1.0 ml of water/acetonitrile (3:1, v/v).

NMR spectroscopy

For deuterium-exchange, samples were dissolved in ^2H_2O (99.96% ^2H; Aldrich), lyophilized three times and filtered (Ultrafree-MC, 0.1 μm low-binding Durapore; Millipore, Eschborn, Germany). ^1H-NMR spectra were recorded at 600 MHz on an Avance DRX 600 Bruker spectrometer (Bruker, Rheinstetten, Germany). The gradient-selected COSY, relayed coherence transfer (RCT)-1 COSY, RCT-2 COSY, TOCSY (mixing time of 100 ms) and NOESY (mixing time of 300 ms) experiments were performed at 300 K in ^2H_2O (99.996% ^2H; Cambridge Isotope Laboratories, Andover, MA, U.S.A.) using standard Bruker software (XWINNMR 2.6).

RESULTS

Isolation of zwitterionic oligosaccharides from *A. suum* glycosphingolipids

Glycosphingolipids were separated into a neutral and an acidic fraction by anion-exchange column chromatography. The neutral species were further subfractionated into neutral and neutral zwitterionic compounds by silica gel column chromatography. Resulting zwitterionic glycosphingolipids were treated with endoglycoceramidase to release their oligosaccharide moieties, which were fractionated on a PGC column (Figure 1a). Twenty fractions were collected and tested for homogeneity by MALDI-TOF MS. Heterogeneous fractions were subjected to HPAEC, as shown for fraction 13 (Figure 1b). Compounds were named in accordance with their elution properties in PGC chromatography and HPAEC, respectively (e.g. compound 13/3 was obtained in fraction 13 in PGC chromatography and subfraction 3 after HPAEC). All fractions were again tested for homogeneity by MALDI-TOF MS and quantified by carbohydrate constituent analysis.

Structural characterization of zwitterionic oligosaccharides

Carbohydrate constituent analysis of the individual oligosaccharide fractions revealed the presence of Glc, Man, GlcNAc, GalNAc, Gal and Fuc as neutral sugar components. Corresponding MALDI-TOF MS spectra were characterized by the presence of two pseudomolecular ions $[M+Na]^+$ and $[(M-86)+2Na]^+$, due to the presence of zwitterionic PC, whereas nano-ESI-IT MS^n spectra showed a mass difference of either 59 Da (loss of trimethylamine) or 124 Da (subsequent elimination of the resulting phosphotriester) as described previously [29]. To

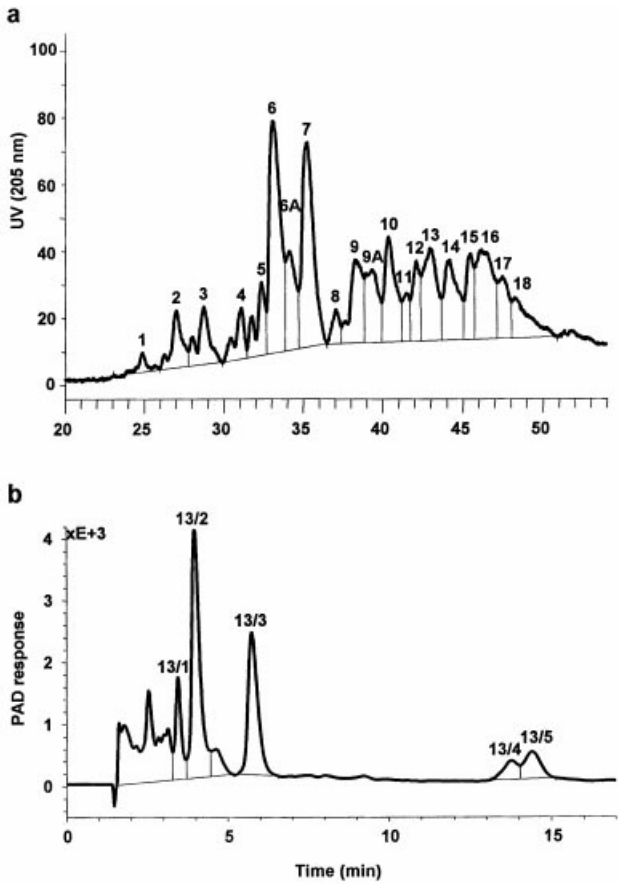

Figure 1 Fractionation of glycosphingolipid-derived zwitterionic oligosaccharides from *A. suum*

(**a**) Elution profile of oligosaccharides using a PGC column. (**b**) Subfractionation of fraction 13 by HPAEC. Individual oligosaccharide fractions were collected and numbered as indicated. PAD, pulsed amperometric detector.

Table 1 MALDI-TOF MS data of zwitterionic oligosaccharides

Oligosaccharides were analysed in the positive-ion reflectron mode. Due to the PC substituent, monoisotopic masses corresponded to pseudomolecular ions $[M+Na]^+$ or $[(M-86)+2Na]^+$, whereas the components 4/4 and 4/5, which contained an additional PE substituent, gave pseudomolecular ions $[M-H+2Na]^+$ and $[(M-86)-H+3Na]^+$, which are marked by * and †, respectively. Hex, hexose; HexNAc, *N*-acetylhexosamine; dHex, deoxyhexose.

Component	Carbohydrate constituent composition	$[M+Na]^+$	$[(M-86)+2\ Na]^+$
1/2	$Hex_2HexNAc_2dHex_1PC$	1082.9	1020.1
3/2	$Hex_3HexNAc_2dHex_1PC$	1243.7	1181.9
3/3	$Hex_2HexNAc_1PC$	732.3	670.8
3/3A	$Hex_4HexNAc_2dHex_1PC$	1406.0	1343.2
4/2	$Hex_2HexNAc_2PC$	936.9	874.1
4/4	$Hex_2HexNAc_2PCPE$	1080.9*	1018.3†
4/5	$Hex_2HexNAc_1PCPE$	877.6*	815.9†
8/4	$Hex_4HexNAc_2PC$	1260.5	1197.5
8/5	$Hex_4HexNAc_2PC$	1259.8	1197.0
9	$Hex_5HexNAc_2PC$	1422.3	1360.3
13/2	$Hex_5HexNAc_3dHex_1PC$	1771.1	1708.7
13/3	$Hex_6HexNAc_2dHex_1PC$	1730.8	1666.8
13/4	$Hex_5HexNAc_3PC$	1625.9	1562.9
17	$Hex_5HexNAc_2dHex_1PC$	1568.8	1505.6

Table 2 Carbohydrate constituent analyses of zwitterionic oligosaccharides

In most cases, the sensitivity of detection in GLC/MS analysis was lower for *N*-acetylhexosamines than for hexosyl residues. The GlcNAc residue of the arthro-series core is not detectable due to PC substitution. +, substoichiometric amount.

	Component peak ratios															
Monosaccharide	1/2	3/2	3/3	3/3A	4/2	4/4	4/4*	4/5	4/5*	8/4	8/5	9	13/2	13/3	13/4	17
Gal	–	0.95	+	1.75	+	–	–	–	–	2.35	2.40	2.55	2.95	4.25	3.35	2.95
Man†	1.00	1.00	1.00	1.00	1.00	–	1.00	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glc	1.65	1.10	1.05	0.90	1.10	1.00†	1.45	1.00†	1.00	1.20	1.20	1.25	1.15	1.30	1.35	1.20
GalNAc	0.85	0.85	+	0.85	0.70	0.75	0.70	–	–	1.15	1.15	0.65	1.55	0.85	1.05	1.10
GlcNAc	+	–	–	+	–	–	0.45	–	0.45	–	–	–	–	–	0.80	–
Fuc	1.00	0.85	+	0.90	+	–	–	–	–	–	–	–	1.00	1.00	–	0.70

* After HF treatment.
† Set to 1.00.

Table 3 Data obtained after methylation analyses of zwitterionic oligosaccharides

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 based on 2,4,6-ManOH = 1.0. As a general feature, *N*-acetylhexosamine and terminal galactose derivatives were characterized by a lower response. PC-substituted GlcNAc derivatives are not registered. Derivatives occurring in substoichiometric amounts (+) resulted from minor contaminants.

	Component peak ratios														
Linkage	1/2	3/2	3/3	3/3A	4/2	4/4*	4/5*	8/4	8/5	9	13/2	13/3	13/4	17	Partially methylated alditol acetates
Gal(1-	–	0.80	+	1.00	–	–	–	0.75	0.65	1.25	0.40	1.15	0.60	0.85	2,3,4,6-GalOH
-2)Gal(1-	–	–	–	–	–	–	–	–	–	–	–	–	–	0.85	3,4,6-GalOH
-3)Gal(1-	–	–	–	–	–	–	–	–	1.20	–	–	–	–	–	2,4,6-GalOH
-6)Gal(1-	–	–	–	–	–	–	–	1.40	–	–	–	–	1.05	–	2,3,4-GalOH
-3,6)Gal(1-	–	–	–	–	–	–	–	–	–	0.90	0.85	0.70	1.15	0.85	2,4-GalOH
-2,3)Gal(1-	–	–	–	–	–	–	–	–	–	–	0.85	0.90	+	–	4,6-GalOH
-3)Man(1-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	2,4,6-ManOH
-4)Glc(1-	1.05	1.00	1.05	1.25	1.20	1.05	1.65	1.25	1.20	1.15	1.10	1.00	1.15	1.00	2,3,6-GlcOH
GalNAc(1-	0.20	+	–	–	0.15	0.40	–	–	–	–	0.20	–	–	–	3,4,6-GalN(Me)AcOH
-3)GalNAc(1-	–	0.55	–	0.25	–	–	–	0.25	0.30	0.40	0.40	0.40	0.20	0.35	4,6-GalN(Me)AcOH
GlcNAc(1-	–	–	–	–	–	–	0.50	–	–	–	–	–	0.20	–	3,4,6-GlcN(Me)AcOH
-4)GlcNAc(1-	–	–	–	–	–	0.40	–	–	–	–	–	–	–	–	3,6-GlcN(Me)AcOH
Fuc(1-	0.25	0.70	–	–	–	–	–	–	–	–	0.25	0.40	–	0.55	2,3,4-FucOH
-2)Fuc(1-	–	–	–	0.40	–	–	–	–	–	–	–	–	–	–	3,4-FucOH

* After HF treatment.

localize the position of the PC substituent, methylation analysis was performed in conjunction with HF treatment either before or after permethylation. Glycans which had been treated with HF before permethylation exhibited a 4-substituted GlcNAc, whereas glycans treated with HF after permethylation displayed a 4,6-disubstituted GlcNAc (results not shown), demonstrating that the PC substituent was always located at C-6 of the GlcNAc residue present in the arthro-core, in agreement with previous findings for components A and C [3]. Without HF treatment, the substituted GlcNAc residue was not registered in methylation analysis.

Structural analysis of zwitterionic oligosaccharides

The structures of individual zwitterionic glycans were elucidated by different techniques: MALDI-TOF MS (Table 1), carbohydrate constituent analysis (Table 2), methylation analysis (Table 3), sequential exoglycosidase digestions (Table 4), nano-ESI-IT MS^n and, for the major compounds, NMR spectroscopy (Table 5). Interpretation of ESI-IT MS^n spectra was performed as described previously [29] using the nomenclature proposed by Domon and Costello [39]. For final structural assignments, a biosynthetic route based on the arthro-series core structure was assumed, leading to three different types of oligosaccharide structure, which are summarized in Figure 2.

Tri- and tetrasaccharides containing PC and PE substituents

The smallest zwitterionic oligosaccharide structure found in this study was a trisaccharide in fraction 3/3. Carbohydrate constituent analysis showed Man and Glc to occur in equal amounts. In methylation analysis 4-substituted Glc and 3-substituted Man were found in agreement with a PC-substituted arthro-series core structure, PC-GlcNAcβ3Manβ4Glc. Exoglycosidase treatment was not successful due to the steric hindrance by the PC residue. MALDI-TOF MS analysis and carbohydrate constituent analysis of fraction 4/2 indicated a tetrasaccharide with nearly equal amounts of Man, Glc and GalNAc in addition to PC-substituted GlcNAc. Methylation analysis revealed a 4-substituted Glc, a 3-substituted Man and a terminal GalNAc. Treatment with β-*N*-acetylhexosaminidase from bovine kidney after removal of PC by HF treatment resulted in the loss of two *N*-acetylhexosamine

Table 4 MALDI-TOF MS data obtained after exoglycosidase treatments of zwitterionic oligosaccharide components

Digestions were performed in solution or directly on the stainless-steel MALDI target. Oligosaccharides were analysed in the positive-ion reflectron mode. Monoisotopic masses are given throughout, reflecting pseudomolecular ions $[M+Na]^+$. Fucosylated structures lost about 50% of Fuc during HF treatment. n.c., not cleaved.

Enzyme	1/2*	3/2*	3/3A	4/2*	8/4*	8/5*	9	13/2*	13/3*	13/4*	17
None	771.3†	933.0†	1342.7‡	771.4	1095.4	1095.6	1422.6/1360.6‡	1606.8/1460.7†	1565.6/1419.4†	1459.7	1568.8/1506.8‡
α-Galactosidase	–	771.1	1180.4	–	–	n.c.	n.c.	–	1403.7/1257.6†	–	n.c.
β-N-Acetylhexosaminidase	365.2	–	–	364.8	–	–	–	1403.5/1257.4†	–	1256.3	–
α-Fucosidase II	–	–	–	–	–	–	–	–	–	–	1423.1/1360.7‡
β-Galactosidase	–	–	n.c.	–	933.1	933.2	1098.6/1036.6‡	933.2†	933.3†	932.5	1035.9‡
α-Galactosidase	–	–	–	–	771.0	771.2	–	770.8†	771.3†	–	873.3‡

* After HF treatment.
† $[M\text{-Fuc}+Na]^+$.
‡ $[(M\text{-}86)+2Na]^+$.

Table 5 ^{1}H Chemical shifts of major and minor oligosaccharide fractions (600 MHz, 300 K)

	Chemical shift, δ (p.p.m.)						
Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
Component A							
α-Glc	5.08	3.43	3.72	3.53	3.78	3.62	3.67
β-Glc	4.25	3.14	3.53	3.54	3.43	3.58	3.72
β-Man	4.56	3.98	3.68	3.51	3.27	3.59	3.79
β-GlcNAc-6*P*	4.55	3.65	3.62	3.60	3.51	3.90	4.02
β-GalNAc	4.54	3.98	3.71	4.06	3.61		
α-Gal	4.99	3.68	3.62	3.85	3.69		
Choline	4.35‡	3.62‡	3.14§				
Fraction 9							
α-Glc	5.11	3.47	3.75	3.56	3.81	3.65	3.69
β-Glc	4.56	3.17	3.56	3.56	3.47	3.62	3.76
β-Man	4.59	4.02	3.71	3.54	3.31	3.63	3.82
β-GlcNAc-6*P*	4.59	3.68	3.66	3.64	3.54	3.94	4.01
β-GalNAc	4.59	4.01	3.78	4.08	3.64		
α-Gal	5.02	3.89	3.81	4.21	3.72	3.91	3.95
β-Gal	4.38	3.45	3.55	3.82			
β-Gal	4.47	3.77	3.56	3.81			
α-Gal*	5.02	3.71	3.65	3.88			
β-Gal*	4.38	3.45	3.55	3.82			
Choline	4.25‡	3.68‡	3.16§				
Fraction 17							
α-Glc	5.11	3.46	3.74	3.55	3.82	3.65	3.71
β-Glc	4.55	3.17	3.56	3.57	3.46	3.62	3.76
β-Man	4.60	4.02	3.72	3.52	3.31	3.63	3.82
β-GlcNAc-6*P*	4.59	3.69	3.66	3.65	3.55	3.93	4.05
β-GalNAc	4.59	4.01	3.75	4.09	3.65		
α-Gal	4.99	3.80	3.79	4.17			
β-Gal	4.38	3.45	3.55	3.82			
β-Gal	4.57	3.57	3.75	3.79	4.00		
α-Fuc	5.18	3.66	3.76	3.67	4.48	1.09	
α-Gal*	5.02	3.71	3.65	3.88			
β-Gal*	4.38	3.45	3.55	3.82			
α-Gal†	5.05	3.88	3.80	4.17	3.73	3.91	3.95
β-Gal†	4.38	3.45	3.55	3.82			
β-Gal†	4.47	3.48	3.56	3.81			
Choline	4.27‡	3.64‡	3.17§				

* First minor compound lacking the 3-linked Gal residue (structure 8/4).
† Second minor compound 9.
‡ Non-resolved multiplet.
§ Singlet $-N^+(CH_3)_3$ (integral 9 H).

(HexNAc) residues, confirming the core structure GalNAcβ4GlcNAcβ3Manβ4Glc. Tri- and tetrasaccharides with PC plus additional PE substituents were found in 4/5 and 4/4. MALDI-TOF MS analysis revealed signals $[(M\text{-}86)\text{-}H+3Na]^+$ and $[M\text{-}H+2Na]^+$, indicative of the PE substituent. Carbohydrate constituent analysis showed Glc for 4/5 and Glc and GalNAc for 4/4. After treatment with HF, Man and GlcNAc were additionally found in both fractions. To elucidate all linkage positions, methylation analyses were performed after HF treatment, revealing a terminal GlcNAc in addition to a 4-substituted Glc and 3-substituted Man in the case of the trisaccharide, whereas a 4-substituted GlcNAc and a terminal GalNAc were found for the tetrasaccharide. To confirm that the PE substituent is attached to the mannosyl residue, both structures were analysed by nano-ESI-IT MS^n. The MS^1 spectrum of the trisaccharide 4/5 indicated pseudomolecular ions $[M\text{-}H+2Na]^+$ at m/z 877.8 (spectrum not shown). MS^2 (877.8) showed a main signal resulting from the loss of trimethylamine (−59 Da), but also a small ion which is characteristic for the presence of PE (−43 Da; see Figure 3). Further fragmentation resulted in a loss of Glc at the reducing end, yielding a disaccharide with PC and PE. Fragmentation of this remaining unit led to three signals confirming the attachment of PE to mannose, namely a C_1 fragment (m/z 349.9), a B_1 fragment (m/z 331.9) and a Z_1 fragment (m/z 329.9). The linkage position of PE in the tetrasaccharide 4/4 was similarly determined.

Structures based on the component A core structure

Seven zwitterionic oligosaccharide structures were characterized in which the pentasaccharide core from component A with the PC substituent at C-6 of the GlcNAc had been elongated at the terminal galactose residue. Two fractions (9 and 17) were obtained in larger amounts, thus allowing NMR measurements. To this end, component A was first analysed as a reference substance for signal assignment [3]. In the gradient-selected COSY spectrum, two sugars were found to have the α- and four the β-configuration. The six spin systems belonging to these anomeric protons were assigned by RCT-1 COSY, RCT-2 COSY and TOCSY NMR experiments. The chemical shifts of the protons are given in Table 5. The Gal and GalNAc residues were assigned in the TOCSY spectrum due to the missing H-3–H-5 signals. The downfield shift of the anomeric galactosyl proton at 4.99 p.p.m. indicated an α-configuration, whereas the GalNAc (H-1 4.54 p.p.m.) is β-linked. The anomeric proton (H-1) at 4.56 p.p.m. could be assigned to the Man, due to the missing interactions in RCT-1 COSY, RCT-2 COSY and TOCSY spectra of the H-2 with the H-4 and H-5 protons. The two anomeric protons at 5.08 (indicative of an α-configuration) and 4.25 p.p.m. (β-configuration) could be assigned to the glucosyl residue at the reducing end of the oligosaccharide, due to the strong coupling

Proposed structures	Fraction	Amount [μg]
PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc	Component A	
PC6 PE6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc	Component C	
PC6 GlcNAcβ3Manβ4Glc	3/3	28
PC6 PE6 GlcNAcβ3Manβ4Glc	4/5	6
PC6 GalNAcβ4GlcNAcβ3Manβ4Glc	4/2	59
PC6 PE6 GalNAcβ4GlcNAcβ3Manβ4Glc	4/4	2
Galβ6 PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc	8/4	9
PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc Galβ3	8/5	19
Galβ6 PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc Galβ3	9	280
Galβ6 PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc Galβ3 Fucα2	17	540
GlcNAcβ6Galβ6 PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc Galβ3	13/4	1
Galβ6 PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc Galα3Galβ3 Fucα2	13/3	24
Galβ6 PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc GalNAcβ3Galβ3 Fucα2	13/2	38
PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc Fucα3	3/2	46
PC6 GalNAcβ4GlcNAcβ3Manβ4Glc Fucα3	1/2	9
PC6 Galα3GalNAcβ4GlcNAcβ3Manβ4Glc Gal2Fucα3	3/3A	7

Figure 2 Structures of glycosphingolipid-derived zwitterionic oligosaccharides found in *A. suum*

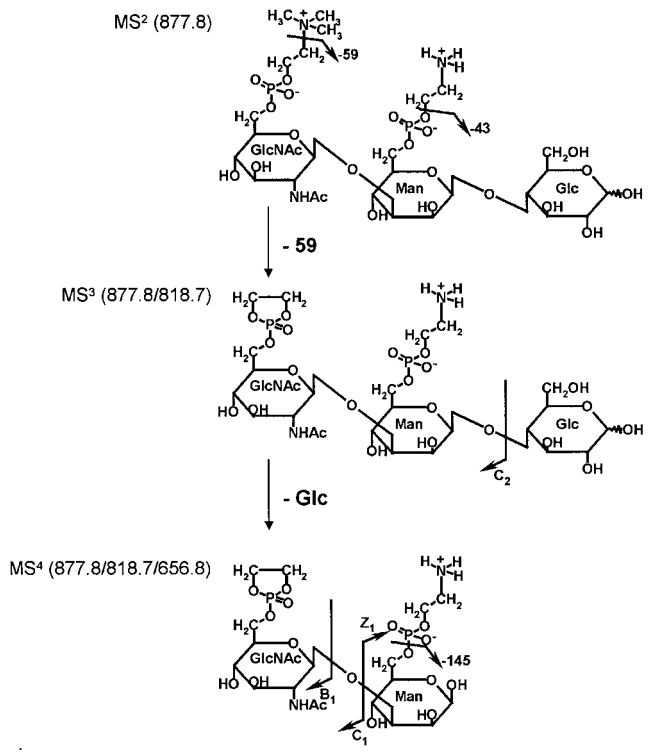

Figure 3 Consecutive fragmentation of pseudomolecular ions $[M-H+2Na]^+$ of fraction 4/5 by nano-ESI-IT MS^n in the positive-ion mode

The precursor ions selected for consecutive MS^n experiments are given in parentheses.

constants ($J > 9$ Hz) indicative of Glc. The sequential assignment of the sugar residues in the oligosaccharide was done by NOESY experiments. Both Glc residues are at the reducing end because of the unique H-1–H-2 intra-residual cross-peaks. Both H-4 (α, 3.53 p.p.m.; β, 3.54 p.p.m.) protons showed a nuclear Overhauser effect cross-peak with the H-1 proton (4.56 p.p.m.) of Man, thus indicating a 1-4 linkage between Glc and Man. A cross-peak between the H-3 proton (3.68 p.p.m.) of the Man and the H-1 proton (4.55 p.p.m.) of the GlcNAc residue showed a 1-3 linkage, whereas the GlcNAc residue is substituted at C-4 by the GalNAc residue, as found by the GlcNAc(H-4)-GalNAc(H-1) cross-peaks (3.60 p.p.m./4.54 p.p.m.). Finally the H-3 (3.71 p.p.m.) and H-4 (4.06 p.p.m.) protons of the GalNAc residue showed cross-peaks with the anomeric proton (4.99 p.p.m.) of the α-Gal residue, displaying the 1-3 linkage of these residues. In the distortionless enhancement by polarization transfer ('DEPT')-135 spectrum, the negative C-6 signal of GlcNAc (66.8 p.p.m.) showed a diagnostic $^3J_{C\text{-}6,P}$ coupling of ≈ 4 Hz, indicating substitution of C-6 by a phosphate residue. This interpretation was further supported by a $^1H,^{31}P$-heteronuclear multiple quantum correlation experiment whereby two cross-peaks with H-1a,b of choline (4.35 and 0 p.p.m.) and H-6b (4.02 and 0 p.p.m.) could be observed, whereas that of H-6a (3.90 and 0 p.p.m.) was lacking (results not shown).

Oligosaccharides composed of component A with additional Gal residues, at either C-3 or C-6 of α-Gal, were found in fractions 8/4 and 8/5, respectively. Carbohydrate constituent analysis indicated the presence of two Gal, one Man, one Glc and one GalNAc in addition to PC-substituted GlcNAc. Digestion of HF-treated glycans with β-galactosidase from jack beans released one galactose, and consecutive treatment with α-galactosidase from green coffee beans released a second Gal residue (Table 4). Both structures differed by a 3-substituted

Gal present in 8/5 and a 6-substituted Gal in 8/4, as found by methylation analysis. The structure of 8/5 was additionally confirmed by nano-ESI-IT MS^n (results not shown).

Methylation analysis of fraction 9 glycans demonstrated the presence of one 3,6-di-substituted and two terminal Gal (tGal) residues, both of which could be released by β-galactosidase treatment. Nano-ESI-IT MS^1 revealed pseudomolecular ions $[M+Na]^+$ at m/z 1422.6 and $[M+2\ Na]^{2+}$ at m/z 723.3 (results not shown). MS^2 (1422.6) displayed the liberation of trimethylamine (-59 Da) and MS^3 (1422.6/1363.4) the subsequent loss of 124 Da, due to the PC substituent. Additionally, Y_3, Y_4, B_3 and B_4 fragments were observed at m/z 674.4, 877.4, 712.4 and 897.5, respectively (Figure 4a). MS^4 (1422.6/1363.4/674.4) of Y_3 yielded the fragments B_1 at m/z 332.1, B_2 at m/z 494.3, C_2 at m/z 512.3 and Y_2 at m/z 365.4, verifying a monosaccharide sequence HexNAc(PC)-Hex-Hex (where Hex is hexose) at the reducing end (Figure 4b). MS^4 (1422.6/1363.4/712.4) of the B_3 resulted in a C_2-fragment at m/z 527.2, a B_2 fragment at m/z 509.2, Y_{2x} fragments at m/z 550.3, lacking one terminal galactose and $B_{1x/2}$ fragments at m/z 347.3 (Figure 4c). The absence of Y-type fragments with two hexosyl mass increments confirmed the di-substituted galactose. MS^5 (1422.6/1363.4/712.4/347.3) of the $B_{1x/2}$ fragment resulted in a C_1 fragment at 203.4 and a B_1 or Y_1 fragment at m/z 184.8. A-type fragmentation was observed in the case of $B_{1\beta/2}$, containing a double bond between C-1 and C-2, which yielded fragment ions $^{2,4}A_2$ at m/z 287.3, $^{0,3}A_2$ at m/z 275.3 and $^{0,4}A_2$ at m/z 245.4 (Figure 4d). The $B_{1\alpha/2}$ ion did not generate ring fragments, due to the 3-substitution of the subterminal Gal [29].

Compared with the ^{1}H-NMR spectra of component A two additional anomeric protons at 4.38 and 4.47 p.p.m. were observed in fraction 9 (see Table 5). The chemical shifts, as well as the strong coupling constants $J_{1,2}$, confirmed their β-configuration. The missing H-3–H-5 interaction in the TOCSY spectrum revealed these residues as Gal. The anomeric proton of β-Gal (4.47 p.p.m.) showed a cross-peak to the H-3 proton of the α-Gal in NOESY experiments, indicating the 1-3 linkage of this residue to the α-Gal.

In the ^{1}H one-dimensional spectrum a minor signal of an anomeric proton at 5.02 p.p.m. occurred, showing cross-peaks with H-2, H-3 and H-4 in the TOCSY spectrum, but not with H-5, indicating a further α-Gal. In the NOESY spectrum a cross-peak at 5.02/3.82 p.p.m. was observed, corresponding to H-4 of the 1-6-linked β-Gal. Furthermore, the intensity of the 6-linked terminal Gal was found to be slightly more prominent than the 3-linked Gal. Therefore it may be concluded that in ≈ 20–$30\,\%$ of the molecules the 3-linked galactose is missing, thus reflecting a contamination by fraction 8/4.

Carbohydrate constituent analysis and MALDI-TOF MS of fraction 17 indicated the presence of an additional fucosyl residue compared with compound 9. Methylation analysis revealed one terminal Gal, one 2-substituted Gal and a terminal Fuc, indicating that one of the terminal galactosyl residues of compound 9 is substituted at C-2 by a fucosyl residue. Due to steric hindrance the terminal galactose could not be removed enzymically. After liberation of the fucose by α-fucosidase II, however, two Gal could be released by treatment with β-galactosidase from jack beans (Table 4). Nano-ESI-IT MS^1 revealed pseudomolecular ions $[M+Na]^+$ at m/z 1568.7 and $[M+2\ Na]^{2+}$ at m/z 796.4. MS^2 (1568.7) resulted in the loss of trimethylamine (-59 Da), whereas chain fragmentation was observed in the MS^3 (1568.7/1509.5) spectrum yielding $Y_{6\alpha}$ (m/z 1363.4), Y_4 (m/z 877.3), Y_3 (m/z 674.2), B_5 (m/z 1043.4), B_4 (m/z 858.3) and $B_{1\alpha/4}$ (m/z 712.4) ions (Figure 5a). The absence of a Y_{5x} fragment is characteristic of the branching at this position. The exact position of Fuc could not be elucidated, due to its lability under the MS conditions used. The MS^3 spectrum, however, demonstrated Fuc to be located at one of the Gal residues at the non-reducing end.

In the NMR spectra of structure 17, two minor compounds (9 and 8/4) were also identified. For the major species, all protons of those residues between the reducing end and the β-GalNAc showed nearly identical chemical shifts to component 9. Additional peaks of the anomeric protons were found at 5.18, 4.99, 4.57 and 4.38 p.p.m. The peaks at 4.99, 4.57 and 4.38 p.p.m. were assigned to α-Gal, the 1-3 linked β-Gal and the 1-6 linked β-Gal, respectively. The Fuc, with its anomeric proton in the α-configuration (5.18 p.p.m.), was easily identified by its methyl group (1.09 p.p.m.). A cross-peak between H-2 of the 3-linked β-Gal in the NOESY experiment and H-1 of the Fuc indicated the latter to be linked in 2-position of this β-Gal residue.

Fraction 13/4 glycans represented compound 9 elongated by a GlcNAc residue, as indicated by carbohydrate constituent analysis and MALDI-TOF MS. Methylation analysis revealed one of the two outer galactosyl residues to be substituted at the C-6 position. Following HF treatment, β-N-acetylhexosaminidase from bovine kidney released this residue. Subsequently the two galactosyl residues could be cleaved with β-galactosidase from jack beans. Nano-ESI-IT MS^1 measurements revealed only pseudomolecular ions $[M+2Na]^{2+}$ at m/z 824.8 (results not shown). Loss of trimethylamine in MS^2 (824.8) resulted again in doubly charged pseudomolecular ions at m/z 795.3. MS^4 (824.8/795.3/915.7) fragmentation of the B_4 ion, obtained in MS^3, yielded, in addition to B_3 or Y_3, fragments at m/z 712.6, a C_3 ion at m/z 730.7 and a $Y_{2\beta}$ fragment at m/z 753.6, resulting from the loss of the tGal residue (Figure 6). This $Y_{2\beta}$ ion lost in MS^5 (824.8/795.3/915.7/753.6) one HexNAc residue, thus resulting in a B_3 or Y_3 fragment at m/z 550.8. In addition, a C_3 fragment at m/z 568.6 was formed. The concomitant $^{0,2}A_3$ ring fragment at m/z 508.9 gave a first indication that the GlcNAcGal branch is located at C-6 of the α-linked Gal. MS^6 of the B_3 precursor ion at m/z 550.8 yielded the ring fragments $^{2,4}A_3$ at m/z 490.7 and $^{0,3}A_3$ at m/z 478.8 of the α-Gal residue, thus confirming the above localization.

Two further species were found to elongate compound 17 by either a Gal (13/3) or a GalNAc (13/2) residue, as revealed by carbohydrate constituent analysis. Methylation analysis showed for both structures a 2,3-di-substituted Gal, in addition to two tGal residues for 13/3 and one tGal and one terminal *N*-acetylgalactosamine for 13/2. Assuming the same biosynthetic route for both compounds (13/2 and 13/3), the two additional monosaccharides should be located at the fucosylated Gal residue. Smith degradation and subsequent methylation analysis confirmed this assumption. Former glucosyl-, fucosyl-, terminal *N*-acetylgalactosaminyl and terminal galactosyl residues could no longer be detected. Instead, only 3-substituted Man, 3-substituted GalNAc and, in addition, a 3-substituted as well as a tGal were observed in both cases. α-Galactosidase from green coffee beans cleaved the additional Gal residue in 13/3, whereas the terminal GalNAc in 13/2 was sensitive towards treatment with β-N-acetylhexosaminidase from bovine kidney.

Structures containing a fucosylated core

Fraction 3/2 comprised the fucosylated component A. Methylation analysis revealed a 4-substituted Glc, 3-substituted Man, 3-substituted GalNAc and a tGal. Localization of the fucosyl residue was done by nano-ESI-IT MS^n. MS^1 showed pseudomolecular ions $[M+Na]^+$ at m/z 1244.7 and $[M+2Na]^{2+}$ at m/z 634.3. The loss of trimethylamine was observed in MS^2 of

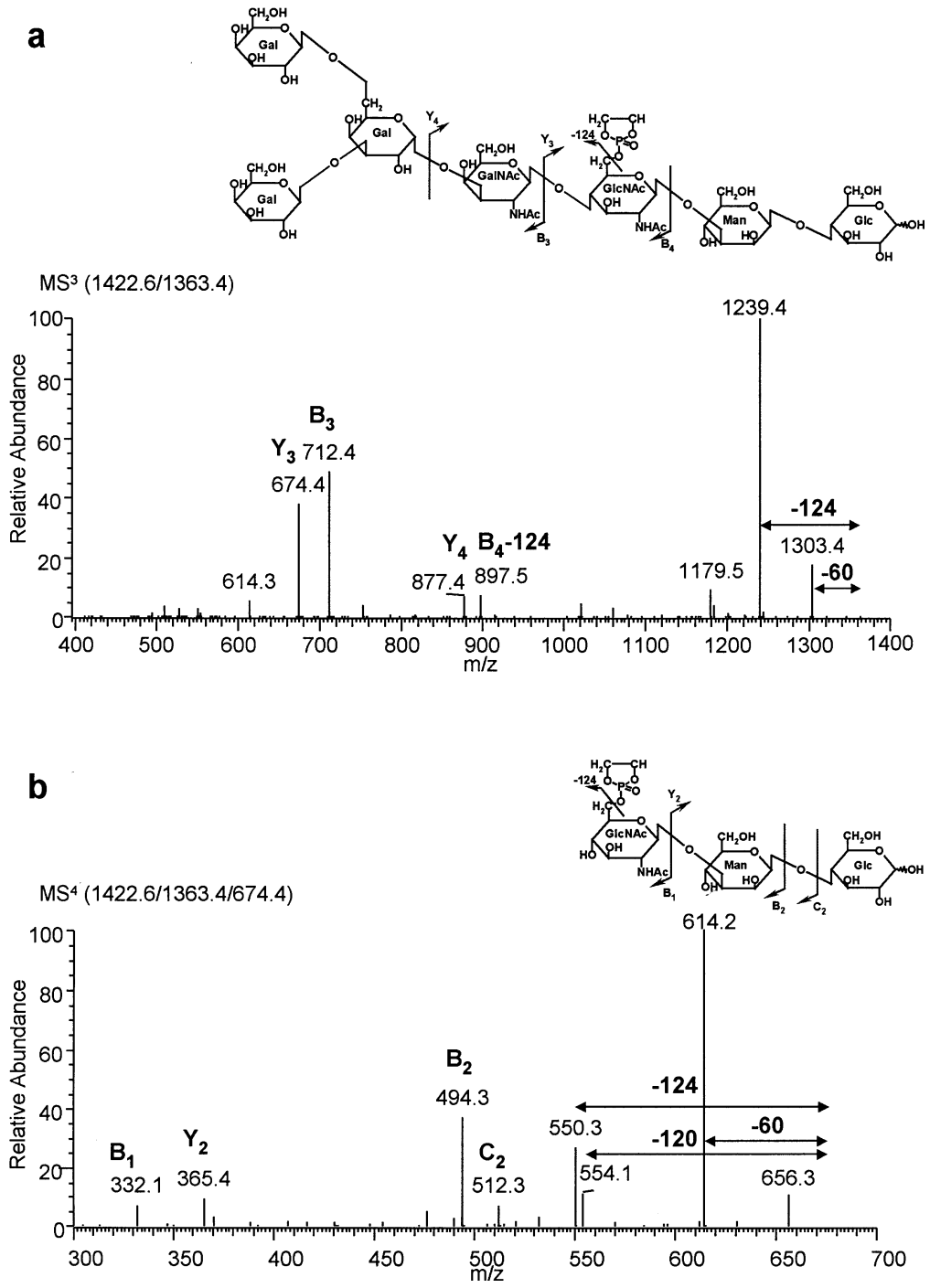

Figure 4 **For legend, see facing page**

m/z 1244.7. The MS^3 (1244.7/1185.5) spectrum was dominated by the loss of the Fuc residue evidenced by a $Y_{3\beta}$ fragment at m/z 1039.4, but $Y_{4\alpha}$ (1024.5), $Y_{3\alpha}$ (820.4) and B_4 ions (1005.4) could also be detected (Figure 5b). Fragmentation of the $Y_{3\alpha}$ precursor ion yielded again a Y_3 fragment at m/z 674.3 in addition to a B_3 fragment at m/z 640.3, which was subjected to further fragmentation. In MS^5 (1244.7/1185.5/820.4/640.3) a Y_2 signal at m/z 494.3, resulting from the loss of the fucosyl residue, and a B_2 fragment at m/z 478.3 formed by the loss of the Man residue were found (Figure 5c). The remaining molecule at m/z 478.3 is composed of one GlcNAc, one Fuc and the cyclic phosphotriester derived from the PC substituent. Thus the Fuc residue could be assigned to C-3 of the central GlcNAc residue. The smallest structure of this type was found in 1/2. Methylation analysis revealed a terminal Fuc, a terminal *N*-acetylgalactosamine, a 3-substituted Man and a 4-substituted Glc. Localization of the fucosyl residue was performed by nano-ESI-IT MS^n similar to 3/2 (results not shown).

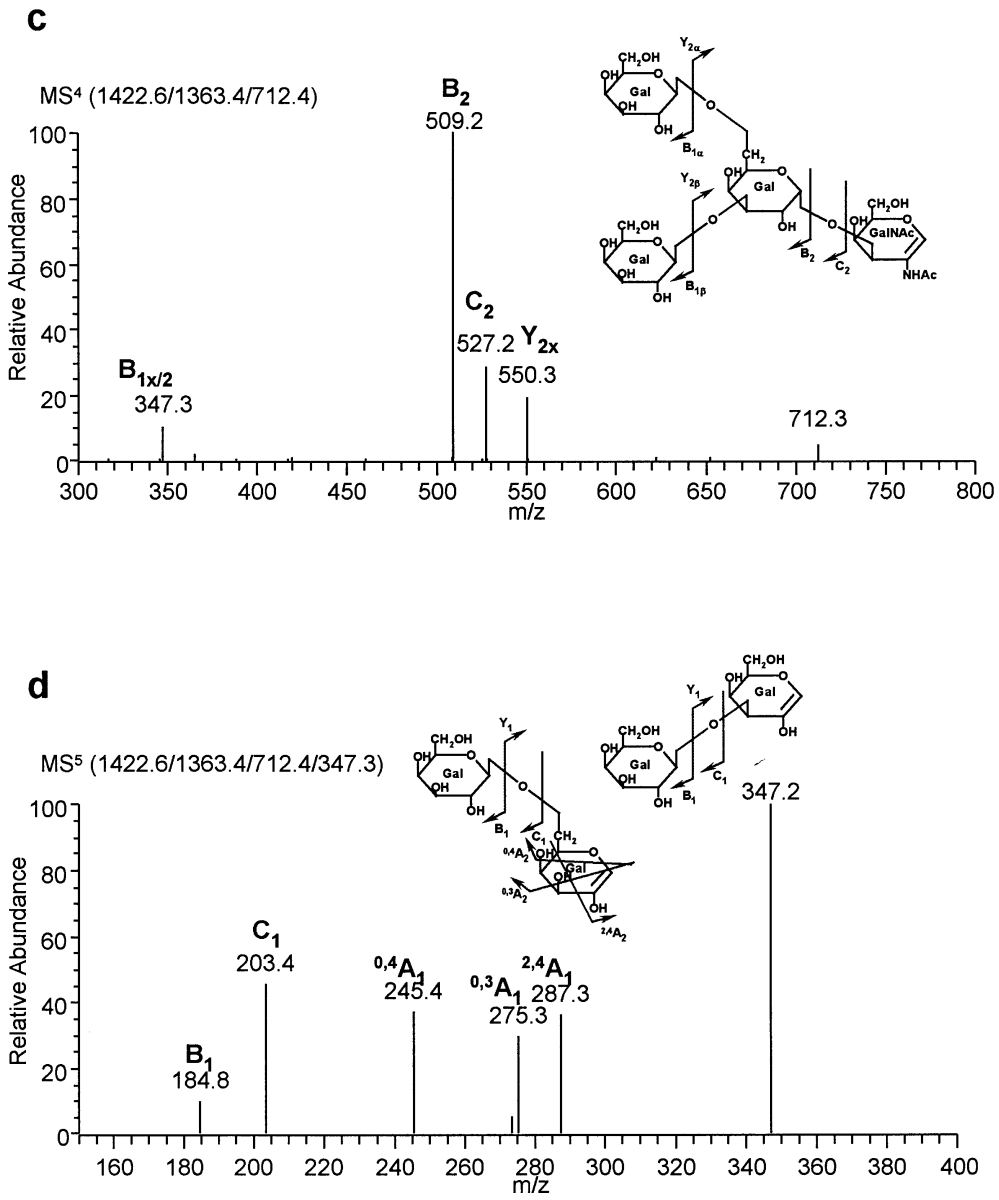

Figure 4 Positive-ion mode nano-ESI-IT MS^n spectra from $[M+Na]^+$ of fraction 9

Ions formed by loss of 60 or 120 Da are due to ring fragmentation of the reducing Glc. The precursor ions selected for consecutive MS^n experiments are given in parentheses.

Fraction 3/3A was found to carry an additional Gal residue, as revealed by carbohydrate constituent analysis and MALDI-TOF MS. Methylation analysis revealed a monosubstituted Fuc residue and two tGal. The electron impact spectrum of the partially methylated fucitol acetate indicated 2-substitution (results not shown). Nano-ESI-IT MS^1 displayed pseudomolecular ions $[M+Na]^+$ at m/z 1406.0 and $[M+2\,Na]^{2+}$ at m/z 714.5. After the loss of trimethylamine (-59 Da) in MS^2, the MS^3 (1406.0/1346.9) spectrum did not reveal an immediate loss of fucose, but starting from the $Y_{3\alpha}$ fragment at m/z 981.9 the loss of one Hex residue (m/z 819.6) and, subsequently, one Fuc residue (m/z 673.9) were observed (Figure 5d). Fragmentation of the $Y_{3\alpha}$ in MS^4 (1406.0/1346.9/981.9) similarly led to the loss of a Hex residue, resulting in a signal at m/z 819.8, and subsequent release of fucose, yielding a signal at m/z 673.9. Localization of the Fuc residue at GlcNAc was corroborated by MS^4 (1406.0/1346.9/639.8) of $B_{2\alpha/3}$, showing again the characteristic fragment at m/z 478 (compare with B_2 in Figure 5c). The anomeric configuration of the Gal residue linked to Fuc could not be elucidated, due to its resistance to exoglycosidase treatments.

Minor zwitterionic oligosaccharide structures

In addition to the oligosaccharide structures described above, further compounds occuring in minor amounts were detected by MALDI-TOF MS which, however, could not be analysed in detail due to quantitative limitations. The monosaccharide compositions deduced from the MALDI-TOF MS analyses, however, revealed a wide range of $Hex_{2-10}HexNAc_{2-6}dHex_{1-3}PC$

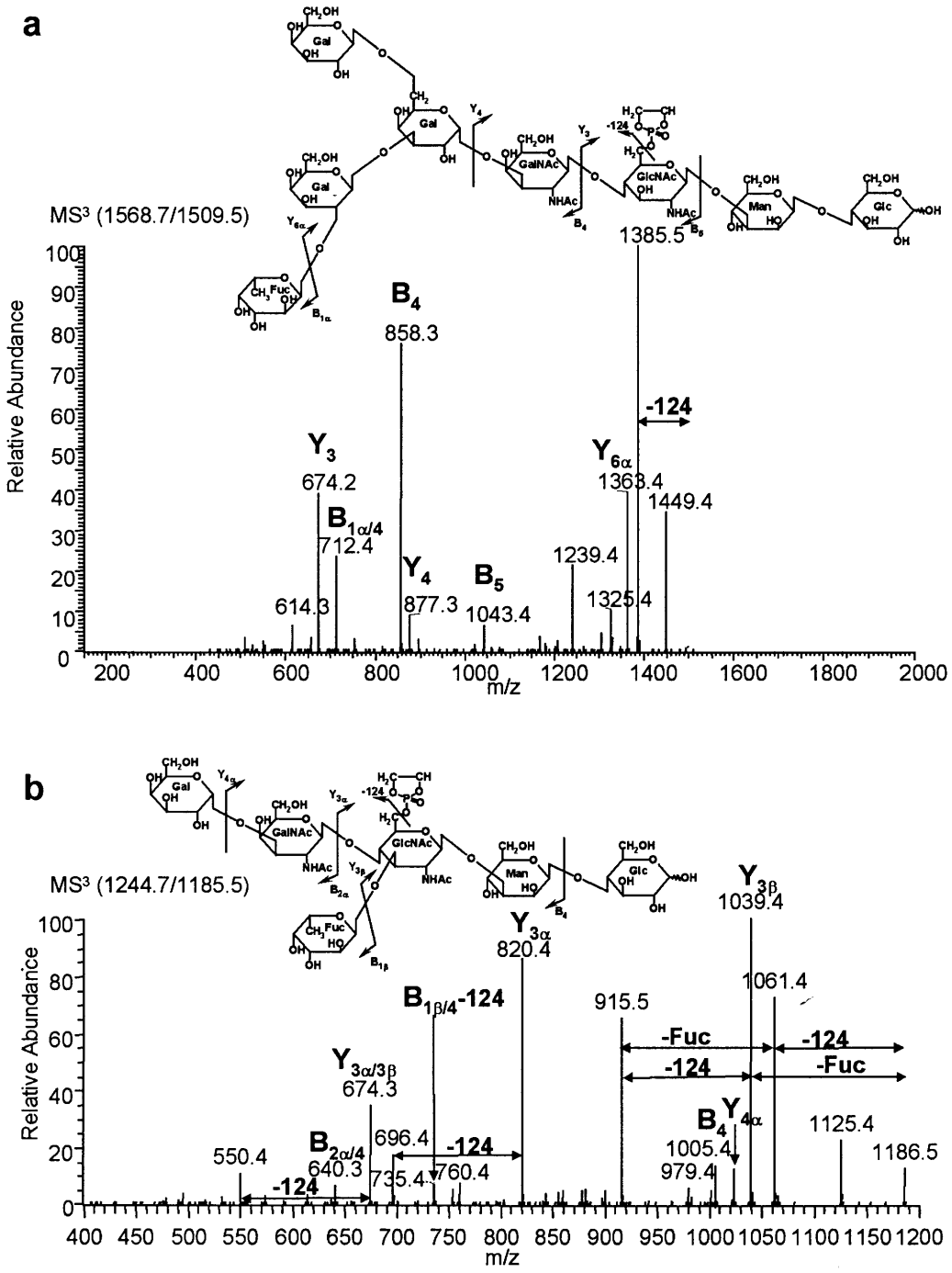

Figure 5 For legend, see facing page

(where dHex is deoxyhexose) structures, thus underlining the high glycosylation potential and variability of glycolipids in this nematode.

DISCUSSION

PC may be considered a major post-translational modification of parasitic helminth antigens, since this antigenic determinant has been detected in nematodes [40–43], in trematodes, including *Schistosoma mansoni* [44] and in the cestode *Bothriocephalus scorpii* [45]. In fact, the frequency of serological cross-reactivity between cestodes, trematodes and, in particular, nematodes [46] may be due to the broad distribution of PC-bearing molecules. The (macro)molecular location of the PC moiety is in most cases unknown, but at least in the case of filarial nematodes [16,17] and *T. spiralis* [18], it has been shown to be attached to the protein backbone via N-linked glycans.

Zwitterionic glycosphingolipids have been structurally characterized from various members of the invertebrate phyla, including the identification of the monosaccharide-amphoteric moiety:

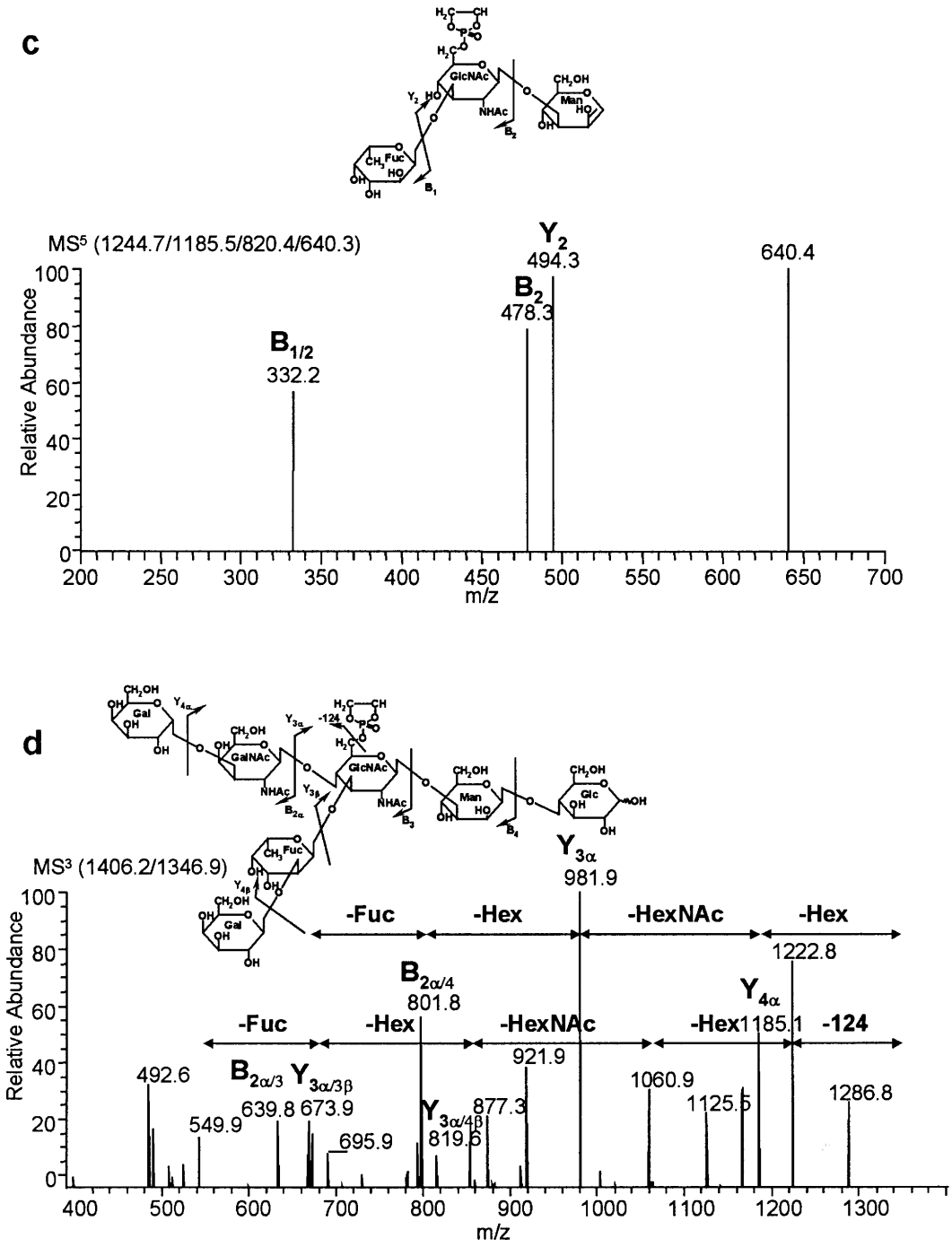

Figure 5 Nano-ESI-IT MS^n spectra for localization of fucose

The precursor ions selected for consecutive MS^n experiments are given in parentheses. (**a**) MS^3 spectrum of 17. (**b**) MS^3 of 3/2 showing the terminal fucose. (**c**) MS^5 of 3/2 showing the localization of the fucose. (**d**) MS^3 spectrum of 3/3A showing the substituted fucose.

in Annelida as Gal-PC, in Arthropoda (Crustacea) as Glc-PE, in Arthropoda (Insecta) as GlcNAc-PE, in Mollusca (freshwater Bivalvia) as Man-PE, in Mollusca (marine Gastropoda) as Gal-phosphonoethanolamine, and in Nematoda as GlcNAc-PC and Man-PE ([2,3,5,31] and references therein).

Structural elucidation of the two major zwitterionic glycosphingolipids (components A and C) of the porcine parasitic nematode *A. suum* has shown their common pentasaccharide core to belong to the arthro-carbohydrate series [3]. Besides the corresponding trisaccharides and tetrasaccharides carrying either PC or PC plus PE substituents, we could now elucidate structures with elongated pentasaccharide cores. These components comprised novel structural motifs such as a disubstituted α-Gal carrying two β-linked Gal residues, which may be further substituted by fucose and/or additional Gal, GalNAc or GlcNAc residues. The structural unit with three galactosyl residues

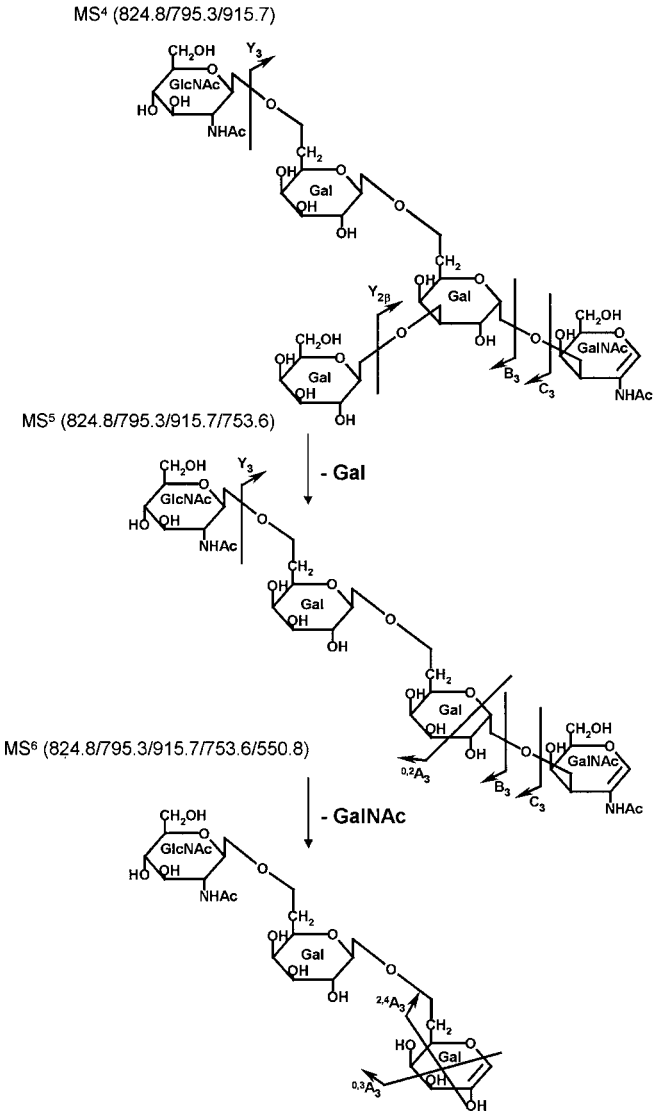

Figure 6 Nano-ESI-IT MS^n positive-ion mode fragmentation pattern from $[M + Na]^+$ of fraction 13/4

The precursor ions selected for consecutive MS^n experiments are given in parentheses.

resembles to some extent the mucin-type O-glycans of *C. elegans*, comprising a GalNAc substituted by two β-Gal residues [19], where 2-fucosylation of the 3-linked β-Gal has also been observed. In contrast with *C. elegans* O- and N-glycans [19,21], however, no evidence for the presence of methylated or negatively charged monosaccharides could be obtained.

In addition to the stepwise elongation of the arthro-series pentasaccharide at its terminal α-galactosyl residue, we identified a second biosynthetic pathway starting with a fucosylation of the PC-substituted GlcNAc, thus generating a completely substituted monosaccharide unit. It could be further demonstrated that this fucose may, in part, bear an additional Gal residue. Although internal fucosyl residues are a characteristic feature of *S. mansoni* egg glycosphingolipids [47,48] as well as O-glycans from the cercarial glycocalyx [38], where they are part of oligofucosyl side chains, this is the first report on the occurrence of internal fucosyl residues in glycoconjugates from nematodes. Hence the structures found reflect the action of novel glycosyltransferases, which demonstrates the enormous glycosylation potential of these organisms.

In this study, nano-ESI-IT MS^n contributed valuable information on the respective oligosaccharide structures and the localization of non-carbohydrate substituents present. The spectra not only provided monosaccharide sequence data, but also revealed, in part, linkage positions. In contrast with previous investigations, in which ring fragmentation had only been observed in the case of C-type fragments [29], we found additional ring fragments of B-type ions, which supplied further structural information. A second observation of diagnostic value was the frequent occurrence of C-type fragments at branching positions in positive-ion mode spectra. Together with the dominating B-type ion species, this pair of ions with an 18 Da mass difference might be a useful marker to identify branch-points in carbohydrate structures.

PC-substituted glycolipids with arthro-series trisaccharide, tetrasaccharide or pentasaccharide units have been also found in the filarial nematode *Onchocerca volvulus* [2] and in *C. elegans* [5], demonstrating that these compounds represent highly conserved glycoconjugate markers of nematodes. Except for the structural analogue of compound 8/5, however, which has been also detected in *C. elegans* [5], more complex glycosphingolipids have not been obtained from these organisms, possibly due to the smaller amounts of available starting material.

The biological function of these complex glycolipids remains unclear. It is, however, interesting to note that the zwitterionic glycosphingolipids of *A. suum* stimulated rather than suppressed human peripheral blood mononuclear cell production of the cytokines tumour necrosis factor α, interleukin-1 and interleukin-6 in a concentration range similar to lipopolysaccharide [3]. The mechanism by which the *A. suum*-derived zwitterionic glycosphingolipids induce cytokine production is still unknown. It may be postulated that they act in a direct way by replacing intracellular lipid second messengers, such as ceramide, via not yet identified PC-specific receptors. Furthermore, the carbohydrate structures of these glycolipids might play a role in the development of nematodes. The finding that the three *sqv* (*sqv*-3, -7 and -8) mutants of *C. elegans*, lacking a galactosyltransferase, glucuronyltransferase or a nucleotide sugar transporter, showed that severe phenotypes clearly emphasize the importance of correct glycosylation in embryogenesis, oocyte development and organ formation [24–28]. Likewise, the *gly*-13 mutation, affecting GlcNAc-transferase I, is associated with lethality at an early larval stage [49], underlining again the functional significance of glycoconjugates during embryogenesis of *C. elegans* [50]. Knowledge of the exact glycan structures will allow the identification of further glycosyltransferases and the investigation of putative functions of the respective carbohydrate structures by selectively silencing the genes by RNA-interference technology or knockout mutations.

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