

Structural characterization of *N*-glycans from the freshwater snail *Biomphalaria glabrata* cross-reacting with *Schistosoma mansoni* glycoconjugates

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The human parasitic trematode *Schistosoma mansoni* has a complex life cycle that includes the freshwater snail *Biomphalaria glabrata* as intermediate host. Within each stage, the parasite synthesizes a wide array of glycoconjugates, exhibiting, in part, unique carbohydrate structures. In addition, the parasite expresses definitive host-like sugar epitopes, such as Lewis X determinants, supporting the concept of carbohydrate-mediated molecular mimicry as an invasion and survival strategy. In the present study, we investigated whether common carbohydrate determinants occur also at the level of the intermediate host. To this end, a structural characterization of hemolymph glycoprotein-*N*-glycans of *B. glabrata* was performed. *N*-glycans were released from tryptic glycopeptides and labeled with 2-aminopyridine. Sugar chains serologically cross-reacting with *S. mansoni* glycoconjugates were isolated by immunoaffinity chromatography using a polyclonal antiserum directed against schistosomal egg antigens and fractionated by *Aleuria aurantia* lectin affinity chromatography and high-performance liquid chromatography. Obtained glycans were analyzed by different mass spectrometric techniques as well as by monosaccharide constituent and linkage analysis. The results revealed a highly heterogeneous oligosaccharide pattern. Cross-reacting species represented about 5% of the total glycans and exhibited a terminal Fuc(α 1-3)GalNAc unit, a (1-2)-linked xylosyl residue, or both types of structural motifs. In conclusion, our study demonstrates the presence of common carbohydrate epitopes also at the level of *S. mansoni* and its intermediate host.

Keywords: *Biomphalaria glabrata*/mass spectrometry/
mollusc glycoproteins/*N*-glycans/*Schistosoma*

Introduction

Schistosomiasis is one of the most important parasitic diseases with 200 million people infected worldwide, which is caused by digenetic trematodes of the genus *Schistosoma*. Schistosomes have a complex life cycle including freshwater

snails as intermediate hosts (van Dam and Deelder 1996). In the case of *Schistosoma mansoni*, adult worm pairs live in human mesenteric vessels. About one-third of the eggs produced by female worms reach the lumen of the intestine to be excreted with feces. Miracidia hatch from the eggs in freshwater reservoirs and infect specific snail hosts, such as *Biomphalaria glabrata*. Following asexual replication in the snail, large numbers of cercariae are released from sporocysts, which in turn penetrate the skin of their definitive host and transform into schistosomula. After development into adult worms and sexual maturation, the parasites start egg-production in the mesenteric blood veins again.

During its life cycle, the parasite generates a large array of carbohydrates representing, in part, unique, often highly fucosylated schistosome-specific glycans which have been shown to be major targets of the host's immune system (Cummings and Nyame 1999; Hokke and Deelder 2001; Khoo 2001; Wuhler and Geyer 2006). Furthermore, it has become increasingly evident that schistosomal glycoconjugates modulate the immune response of the host and play an important role in evasion mechanisms (Thomas and Harn 2004; Dunne and Cooke 2005; van Die and Cummings 2006). In addition to parasite-specific glycan motifs, schistosomes express also mammalian-type carbohydrate structures. Therefore, the question arises as to whether the worm might utilize sugar-based molecular mimicry (Damian 1989) as an additional survival strategy.

In previous studies, we have analyzed the carbohydrate structures of *S. mansoni* glycosphingolipids occurring in different life-cycle stages (Wuhler et al. 2000, 2002; Kantelhardt et al. 2002). The results revealed a stage-specific expression of glycosphingolipids decorated with definitive host-like Lewis X determinants in the cercarial stage, in agreement with the concept of molecular mimicry. In contrast, egg-stage glycolipids were shown to exhibit different terminal carbohydrate epitopes including, in particular, a Fuc(α 1-3)GalNAc-motif together with highly fucosylated carbohydrate moieties carrying oligofucosyl chains linked to terminal GalNAc as well as internal GlcNAc residues (Khoo et al. 1997a; Wuhler et al. 2002). Similar structural elements have also been observed in *O*-glycans of the cercarial glycocalyx of *S. mansoni* (Khoo et al. 1995; Huang et al. 2001) as well as in *N*-glycans of egg glycoproteins (Khoo et al. 1997b). As a characteristic feature, egg *N*-glycan core structures were found to carry optionally (β 1-2)-linked xylose at the central β -mannosyl residue and/or an additional (α 1-3)-fucose at their innermost GlcNAc.

Although it is already known since 20 years that *S. mansoni* shares protective carbohydrate epitopes with the freshwater snail *B. glabrata* (Dissous et al. 1986), the

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structural basis for this serological cross-reactivity has not yet been defined. In order to identify the carbohydrate motifs being relevant in this context, we have now initiated a detailed analysis of the N-glycans obtained from hemolymph glycoproteins of this snail. To this end, glycans were released from tryptic glycopeptides by peptide N-glycosidase F (PNGase F). Cross-reacting oligosaccharides were isolated by immunoaffinity chromatography using immobilized antibodies raised against soluble egg antigens (SEAs) from *S. mansoni*. Resulting glycans were fractionated by *Aleuria aurantia* lectin (AAL) affinity chromatography and normal-phase high-performance liquid chromatography (HPLC) and analyzed mainly by mass spectrometry (MS). The results revealed a great variety of cross-reacting carbohydrate species exhibiting core-linked xylose and/or terminal Fuc(α 1-3)GalNAc motifs. Hence, our results clearly manifest the existence and define the structures of two shared carbohydrate epitopes also at the level of *B. glabrata*, the intermediate host of *S. mansoni*.

Results

Serological cross-reactivity of *B. glabrata* hemolymph glycoproteins with *S. mansoni* glycoconjugates

B. glabrata hemolymph glycoproteins were tested for serological cross-reactivity with *S. mansoni* glycoconjugates using two types of antibodies: a monoclonal antibody, mAb M2D3H, specifically recognizing terminal Fuc(α 1-3)GalNAc carbohydrate units (Kantelhardt et al. 2002) and polyclonal antibodies which have been produced by immunizing rabbits with SEAs of *S. mansoni*. After western blotting, a number of polypeptide bands were found to be stained in both cases (Figure 1A). Serological recognition was, however, more pronounced when polyclonal anti-SEA antibodies were employed, although similar amounts of total protein have been applied in each case. Hence, it may be concluded that the immunoreactivity observed in the case of anti-SEA is mediated by more than one cross-reacting epitope, which could be corroborated by enzyme-linked immunosorbent assay (ELISA). Using the same antibodies and tryptic hemolymph (glyco)peptides before and after chemical defucosylation by hydrogen fluoride (HF) treatment (Haslam et al. 2000), maximum recognition was achieved, when untreated (glyco)peptides and anti-SEA were employed (Figure 1B). HF treatment led to a reduction in immunoreactivity by roughly 40%, demonstrating that fucosyl residues play an important, but not exclusive, role in antigen binding in this case. In agreement with data obtained by western blotting, mAb M2D3H exhibited a much lower immunoreactivity (representing only 8% of anti-SEA reactivity), which was reduced by 75% after defucosylation. From these data, it can be concluded that *B. glabrata* hemolymph glycoproteins carry both fucosylated and fucose-independent cross-reacting antigenic determinants.

Isolation of *B. glabrata* hemolymph N-glycans

B. glabrata hemolymph proteins were digested with trypsin, and resultant (glyco)peptides were treated with PNGase F. Released N-glycans were fluorescently labeled by reductive amination with 2-aminopyridine (PA) and analyzed by

matrix-assisted laser desorption/ionization (MALDI)-MS (Figure 2A). The spectrum revealed a highly heterogeneous pattern of different glycans comprising more than 100 different compositional species (Table I). Subsequent treatment of residual glycopeptides with peptide N-glycosidase A (PNGase A) resulted in an additional release of minute amounts of glycans exhibiting similar m/z values after fluorescent labeling and MALDI-MS analysis. The question remains open, as to whether PNGase A-released glycans represent different, PNGase F-resistant structural isomers or merely reflect an incomplete liberation of the present sugar chains by PNGase F. As MALDI-MS of the PA-labeled oligosaccharides recovered after hydrazinolysis of total hemolymph proteins displayed the same panel of glycan signals as carbohydrates obtained by PNGase F treatment (data not shown), the pool of PNGase F-released glycans was used in this study throughout.

Monosaccharide constituent analysis of the total PA-glycan fraction verified the presence of mannose (Man), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc), and xylose (Xyl) in addition to two methylated sugar derivatives, which could be identified as 3-O-methyl-mannose (3-O-MeMan) and 3-O-methyl-galactose (3-O-MeGal) by GC-MS analysis of the corresponding alditol acetates (Figure 3A and Supplementary Figure 1). Man, Gal, GlcNAc, GalNAc, Fuc, Xyl, 3-O-MeMan, and 3-O-MeGal occurred in molar ratios approaching 2.5:1.1:0.3:0.1:0.3:0.1:0.5:0.1, respectively. Subsequent linkage analyses revealed the presence of a great variety of partially methylated alditol acetate derivatives, reflecting the vast structural heterogeneity of this glycan fraction (Table II). In addition, obtained linkage data displayed a number of characteristic features including (1) the presence of 2,3,6-trisubstituted Man, allowing the assignment of the linkage position of xylose to C-2 of the central core mannose, (2) the occurrence of terminal 3-O-MeMan and 3-O-MeGal as well as 2-substituted and 6-substituted 3-O-MeMan, (3) the presence of internal 2-substituted fucosyl residues (Figure 3B), and (4) the occurrence of 3-substituted GalNAc, 3,4-disubstituted GlcNAc, and 4-substituted GlcNAc as well as trace amounts of terminal GlcNAc and 3-substituted GlcNAc residues (Table II). From these results, it may be, therefore, concluded that the obtained oligosaccharide mixture comprised, in part, species with core-linked xylose and/or fucosylated GalNAc(1-4)GlcNAc units in agreement with the observed mAb M2D3H immunoreactivity. The occurrence of terminal 3-O-MeMan in *B. glabrata* glycoprotein-N-glycans has already been reported by Marxen et al. (2003).

Isolation of N-glycans sharing carbohydrate epitopes with *S. mansoni* glycoconjugates

In order to enrich cross-reacting glycan species, total PA-oligosaccharides were subjected to preparative immunoaffinity chromatography employing immobilized polyclonal anti-SEA antibodies. Unbound and bound oligosaccharide fractions, representing about 95% and 5% of total PA-glycans, respectively, were individually analyzed by MALDI-MS (Figure 2B and C). The results revealed that oligosaccharides comprising a xylose residue were almost completely retained in the bound fraction, whereas unbound glycans, including the

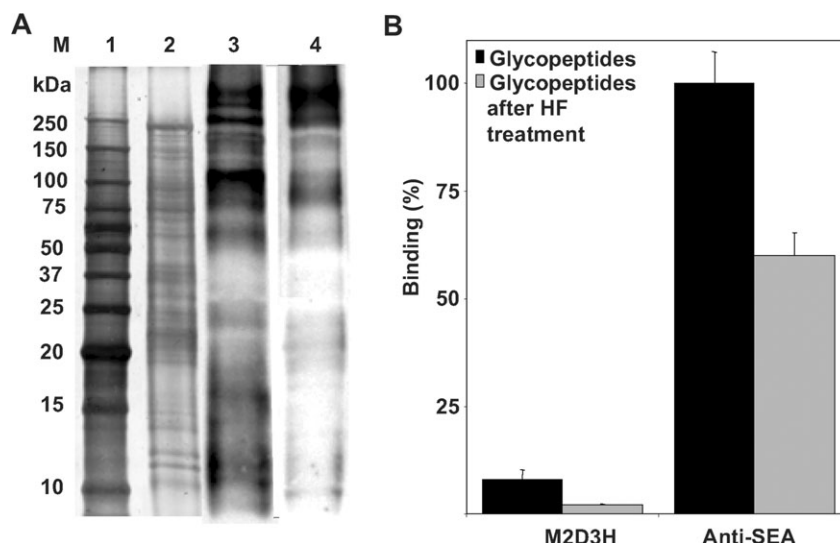


Fig. 1. Serological characterization of *B. glabrata* hemolymph glycoproteins and glycopeptides. (A) Analysis of total hemolymph proteins. Lane 1, molecular weight standards; lane 2, total proteins visualized by silver staining; lanes 3 and 4, western blot detection of cross-reacting glycoproteins using anti-SEA and mAb M2D3H, respectively. Ten micrograms of total protein was used in lanes 2–4. (B) ELISA of tryptic hemolymph (glyco)peptides. Peptides were applied either before or after HF treatment and assayed using mAb M2D3H and anti-SEA. Results are typical representatives of three independent experiments performed in triplicate. Binding registered in the case of anti-SEA was set to 100%.

most abundant compositional species present, represented mainly species with sugar compositions of Hex₃₋₉HexNAc₂, MeHex₁₋₃Hex₁₋₈HexNAc₂, and MeHex₀₋₂Hex₁₋₆HexNAc₂Fuc₁ in addition to minor compounds with compositions of MeHex₀₋₂Hex₂₋₆HexNAc₃₋₇Fuc₀₋₁ (Table I). Fragment ion analysis of monofucosylated, unbound species by tandem mass spectrometry (MS/MS) after laser-induced dissociation (LID) revealed that fucose was linked to the innermost, PA-labeled *N*-acetylhexosamine (HexNAc) residue throughout in these cases (data not shown). As monosaccharide constituent and linkage analyses confirmed again the presence of terminal as well as internal 3-*O*-MeMan derivatives (Table II), it may be concluded that these monosaccharide entities are not recognized by anti-SEA antibodies. Intriguingly, two major xylosylated species (MeHex₂Hex₁HexNAc₂Xyl₁ and MeHex₂Hex₁HexNAc₂Xyl₁Fuc₁) did not bind to the immunoaffinity column possibly due to sterical hindrance provided by the presence of two 3-*O*-methyl-hexose residues.

In contrast, glycans being retained by immunoaffinity chromatography displayed an entirely different pattern of compositional species covering a wide range of molecular masses, many of which have not been detected in the total glycan pool due to their low abundance (cf. Figure 2 and Table I). In particular, glycans with molecular masses between 1500 and 2500 Da were found to be enriched by this step (Supplementary Figure 2). As a striking feature, most of these oligosaccharides were characterized by the presence of xylose as well as more than two HexNAc and/or fucosyl residues. On the basis of their predicted monosaccharide compositions (Table I), cross-reacting glycans can be grouped into different classes: (1) xylose-containing, truncated species with or without core-fucose (MeHex₀₋₂Hex₁₋₃HexNAc₂Xyl₁Fuc₀₋₁), (2) xylose-containing, elongated, in part, mono-fucosylated structures with more than two HexNAc residues (MeHex₀₋₂Hex₂₋₅HexNAc₃₋₇Xyl₁Fuc₀₋₁), (3) xylose-containing, elongated structures with more than

two HexNAc and more than one fucose residues (MeHex₀₋₂Hex₂₋₅HexNAc₄₋₇Xyl₁Fuc₂₋₃), and (4) cross-reacting glycans devoid of xylose (MeHex₀₋₂Hex₁HexNAc₄₋₇Fuc₁₋₃). These results clearly demonstrate that, apart from the exceptions mentioned above, the presence of core-linked xylose is sufficient for cross-reactivity with anti-SEA. In contrast, cross-reacting species lacking xylose comprised preponderantly two fucose and, at least, four HexNAc residues. In agreement with these data, determination of monosaccharide composition (data not shown) and linkage analyses (Table II) revealed enrichment in the amounts of fucose, xylose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine in comparison with the unbound glycans.

In order to verify that core-linked xylose is, indeed, sufficient for cross-reactivity with anti-SEA, a Man(α1-6)[Xyl(β1-2)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-3)]GlcNAc standard oligosaccharide from pineapple stem bromelain was similarly subjected to immunoaffinity chromatography before and after defucosylation by HF treatment. Starting glycans, unbound material obtained in the flow-through and oligosaccharides recovered in the immunoaffinity eluates were analyzed by MALDI-MS (Supplementary Figure 3). The results revealed binding of fucosylated species as well as glycans lacking fucose, thus demonstrating that polyclonal anti-SEA antibodies similarly recognize plant-derived oligosaccharides comprising solely a core-linked xylose residue.

Subfractionation of cross-reacting glycans by lectin affinity chromatography

In order to facilitate further structural characterization, obtained cross-reacting glycans were fractionated by affinity chromatography using immobilized AAL, which is known to interact with core-(α1-6)-fucosylated oligosaccharides and, although with lower affinity, with glycans comprising outer Gal(β1-4)[Fuc(α1-3)]GlcNAc units or Fuc(α1-3)GalNAc(β1-4)[Fuc(α1-3)]GlcNAc-motifs (Yamashita et al. 1985; Geyer

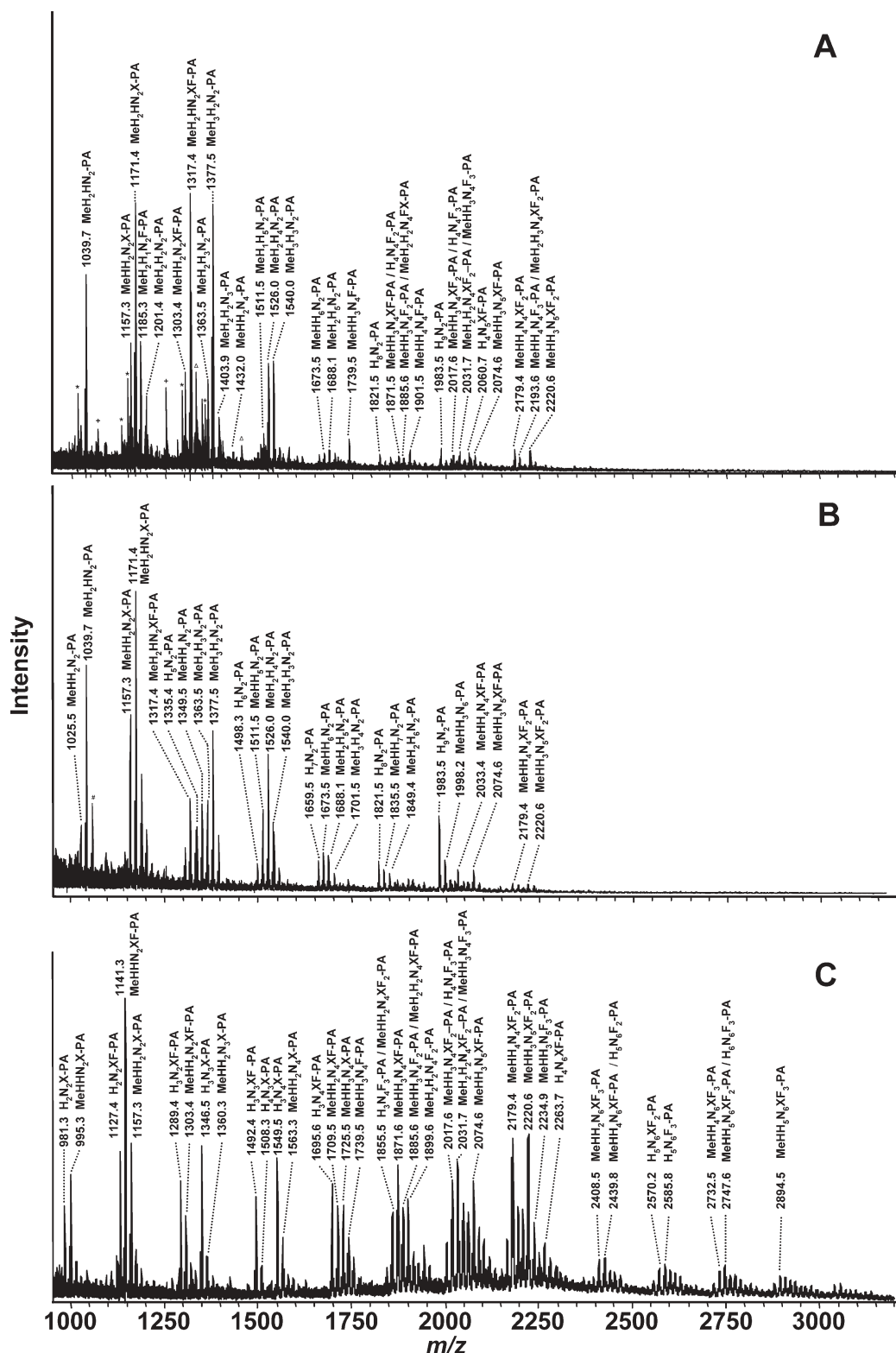


Fig. 2. MALDI-mass spectra of PA-labeled *B. glabrata* N-glycans. The oligosaccharides of total hemolymph proteins were released from tryptic glycopeptides by PNGase F-treatment, labeled with 2-aminopyridine, and screened by MALDI-MS either before or after immunoaffinity chromatography using immobilized anti-SEA antibodies. (A) Total N-glycan fraction, (B) unbound oligosaccharides, and (C) glycans recovered in the immunoaffinity eluate. Sodiated pseudomolecular ions $[M + Na]^+$ were registered with isotopic resolution. Differences in the relative intensities of unbound glycan signals observed in some cases [compare (A) and (B)] are assumed to originate from an inhomogeneous co-crystallization of oligosaccharide and matrix molecules. The presence of these compounds in the unbound (B) and their absence in the retained oligosaccharide fraction (C), however, are clearly significant. Monosaccharide compositions of major species are assigned. H, hexose; MeH, 3-*O*-methyl-hexose; N, *N*-acetylhexosamine; F, fucose; X, xylose. *, proton adducts $[M + H]^+$; +, glucose oligomers; Δ, noncarbohydrate contaminants; #, potassium adduct $[M + K]^+$.

Table I. Compilation of PA-labeled *N*-glycans obtained from *B. glabrata* hemolymph glycoproteins

Observed mass [M + Na] ⁺	Calculated mass [M + Na] ⁺	Proposed composition					Occurrence ^a	Recognized by anti-SEA	Diagnostic fragment ions ^b
		MeH	H	N	X	F			
981.3	981.4		2	2	1		— ^c	+	n.d.
995.3	995.4	1	1	2	1		— ^c	+	1, 3
1011.5	1011.4		3	2			— ^c	—	2, 9
1025.5	1025.4	1	2	2			— ^c	—	2, 3
1039.7	1039.7	2	1	2			++++	—	3
1127.4	1127.4		2	2	1	1	— ^c	+	1, 2, 6
1141.3	1141.4	1	1	2	1	1	— ^c	+	1, 3, 6 (317, 97)
1143.3	1143.4		3	2	1		— ^c	+	1, 2, 9
1157.3	1157.4	1	2	2	1		++++	+ / — ^d	1, 2, 3, 10, 16 (479, 245, 259)
1171.4	1171.5	2	1	2	1		++++ +	—	1, 2, 3, 6, 13
		1	2	2		1			
1173.8	1173.4		4	2			— ^c	—	n.d.
1185.3	1185.5	2	1	2		1	++++	—	3, 6
1187.4	1187.5	1	3	2			— ^c	—	n.d.
1201.4	1201.5	2	2	2			+++	—	3, 10
1289.4	1289.5		3	2	1	1	— ^c	+	1, 2, 6
1303.4	1303.5	1	2	2	1	1	++++	+ / — ^d	1, 2, 3, 6, 16 (479, 245, 259)
1317.4	1317.5	2	1	2	1	1	++++ +	—	1, 3, 6
1335.4	1335.5		5	2			— ^c	—	2, 9
1346.5	1346.5		3	3	1		— ^c	+	2, 9, 11
1349.5	1349.5	1	4	2			+++	—	2, 3, 9, 10
1360.3	1360.5	1	2	3	1		— ^c	+ / — ^d	n.d.
			3	3		1			
1363.5	1363.5	2	3	2			++++	—	2, 3, 10
1377.5	1377.5	3	2	2			++++ +	—	3, 10
1403.9	1404.6	2	2	3			++	—	n.d.
1432.0	1431.6	1	2	4			+	—	n.d.
1492.4	1492.6		3	3	1	1	— ^c	+	2, 6, 14
1498.3	1497.5		6	2			++	—	n.d.
1506.4	1506.6	1	2	3	1	1	— ^c	+	3, 6, 15
1508.3	1508.6		4	3	1		— ^c	+	n.d.
1511.5	1511.6	1	5	2			++	—	n.d.
1526.0	1525.6	2	4	2			++++	—	2, 3, 10
1540.0	1539.6	3	3	2			++++	—	2, 3, 10
1549.5	1549.6		3	4	1		— ^c	+	2, 5, 11
1563.3	1563.6	1	2	4	1		+	+	2, 3, 5, 10
1659.5	1659.6		7	2			+	—	2, 9
1673.5	1673.6	1	6	2			+	—	2, 3, 9
1688.1	1687.6	2	5	2			++	—	2, 3, 9, 10
1695.6	1695.6		3	4	1	1	— ^c	+	4, 5, 6, 7, 11
1701.5	1701.6	3	4	2			++	—	n.d.
1709.5	1709.7	1	2	4	1	1	— ^c	+	n.d.

Continued

Table I. Continued

Observed mass [M + Na] ⁺	Calculated mass [M + Na] ⁺	Proposed composition					Occurrence ^a	Recognized by anti-SEA	Diagnostic fragment ions ^b
		MeH	H	N	X	F			
1711.6	1711.6		4	4	1		— ^c	+	5, 11
1723.5	1723.7	1	2	4		2	— ^c	+	n.d.
1725.5	1725.7	1	3	4	1		+	+	n.d.
1739.5	1739.7	1	3	4		1	++	+	n.d.
1752.5	1752.6		3	5	1		— ^c	+	n.d.
1781.3	1780.7	1	2	5		1	— ^c	—	n.d.
1821.5	1821.6		8	2			+	—	9
1835.5	1835.6	1	7	2			(+)	—	n.d.
1841.5	1841.7		3	4	1	2	— ^c	+	n.d.
1849.4	1849.6	2	6	2			(+)	—	n.d.
1855.5	1855.7		3	4		3	— ^c	+	4, 5, 6, 7, 8, 10,
		1	2	4	1	2			12, 15
1857.6	1857.7		4	4	1	1	— ^c	+	6, 7, 11, 14
1869.3	1869.7	1	2	4		3	— ^c	+	n.d.
1871.6	1871.7	1	3	4	1	1	— ^c	+	n.d.
			4	4		2			
1885.6	1885.7	1	3	4		2	+	+	n.d.
		2	2	4	1	1			
1887.3	1887.7	1	4	4	1		— ^c	+	n.d.
1899.6	1899.7	2	2	4		2	— ^c	+	n.d.
1901.5	1901.7	1	4	4		1	++	—	n.d.
1912.5	1912.7	1	2	5	1	1	— ^c	+	n.d.
			3	5		2			
1915.8	1915.7	2	3	4		1	— ^c	+	n.d.
1926.5	1926.6	1	2	5		2	— ^c	+	n.d.
1940.6	1940.7	2	1	5		2	— ^c	+	n.d.
1955.5	1955.8		3	6	1		— ^c	+	n.d.
1983.5	1983.7		9	2			++	—	2, 9
1985.4	1985.8		4	6			— ^c	—	n.d.
1998.2	1997.7	1	8	2			+	—	2, 3
2001.4	2001.7	1	2	4	1	3	— ^c	+	n.d.
2003.5	2003.8		4	4	1	2	— ^c	+	n.d.
2012.2	2011.7	2	7	2			+	—	n.d.
2017.6	2017.8	1	3	4	1	2	+	+	4, 5, 6, 7, 8, 10,
			4	4		3			12, 15
2026.2	2026.7		3	7			+	—	n.d.
2031.7	2031.8	2	2	4	1	2	++	+	n.d.
		1	3	4		3			
2033.4	2033.8	1	4	4	1	1	+	+/- ^d	5, 10, 12
2045.2	2044.8		3	5	1	2	— ^c	+	n.d.
		2	2	4		3			
2047.6	2047.8	2	3	4	1	1	+	+	n.d.
		1	4	4		2			
2058.6	2058.8	1	2	5	1	2	— ^c	+	4, 5, 6, 7, 8, 12,
			3	5		3			13, 15

Continued

Table I. Continued

Observed mass [M + Na] ⁺	Calculated mass [M + Na] ⁺	Proposed composition					Occurrence ^a	Recognized by anti-SEA	Diagnostic fragment ions ^b
		MeH	H	N	X	F			
2060.7	2060.8		4	5	1	1	+	+	2, 6, 9
2072.4	2072.8	1	2	5		3	— ^c	+	n.d.
2074.6	2074.7	1	3	5	1	1	+	+ / — ^d	4, 5, 10, 12, 16 (858)
2088.7	2088.8	1	3	5		2	(+)	+	n.d.
		2	2	5	1	1			
2101.7	2101.8		3	6	1	1	(+)	+	n.d.
2117.7	2117.8		4	6	1		(+)	+	n.d.
2163.5	2163.8	1	3	4	1	3	— ^c	+	n.d.
2177.4	2177.8	2	2	4	1	3	— ^c	+	n.d.
2179.4	2179.8	1	4	4	1	2	++	+ / — ^d	4, 5, 6, 7, 12, 13, 15
2193.6	2193.8	1	4	4		3	+	+	n.d.
		2	3	4	1	2			
2204.6	2204.9	1	2	5	1	3	(+)	+	4, 5, 6, 7, 15
2220.6	2220.9	1	3	5	1	2	++	+ / — ^d	4, 5, 6, 7, 15, 16 (858, 624, 638)
2234.9	2234.9	1	3	5		3	(+)	+	n.d.
		2	2	5	1	2			
2238.9	2238.8		6	5	1		— ^c	—	n.d.
2247.6	2247.6		3	6	1	2	— ^c	+	n.d.
2263.7	2263.9		4	6	1	1	(+)	+	n.d.
2277.7	2277.9	1	3	6	1	1	(+)	+	n.d.
			4	6		2			
2293.6	2293.8	1	4	6	1		— ^c	+	n.d.
			5	6		1			
2393.6	2393.9		3	6	1	3	— ^c	+	n.d.
2408.5	2407.9	1	2	6	1	3	— ^c	+	n.d.
2425.7	2425.9		5	6	1	1	(+)	+	n.d.
2439.8	2439.9	1	4	6	1	1	— ^c	+	n.d.
			5	6		2			
2450.6	2450.9		3	7	1	2	— ^c	+	n.d.
		2	2	6		3			
2466.8	2466.9		4	7	1	1	— ^c	+	n.d.
2555.6	2556.0		4	6	1	3	— ^c	+	n.d.
2570.2	2570.0		5	6	1	2	— ^c	+	n.d.
2585.8	2586.0		5	6		3	— ^c	+	n.d.
		1	4	6	1	2			
2599.6	2600.0	2	3	6	1	2	— ^c	+	n.d.
		1	4	6		3			
2612.6	2613.0		4	7	1	2	— ^c	+	n.d.
2626.8	2627.0	1	3	7	1	2	— ^c	+	n.d.
			4	7		3			
2732.5	2732.0	1	4	6	1	3	— ^c	+	n.d.
2747.6	2748.0	1	5	6	1	2	— ^c	+	n.d.
			6	6		3			

Continued

Table I. Continued

Observed mass [M + Na] ⁺	Calculated mass [M + Na] ⁺	Proposed composition					Occurrence ^a	Recognized by anti-SEA	Diagnostic fragment ions ^b
		MeH	H	N	X	F			
2759.5	2759.1		4	7	1	3	— ^c	+	n.d.
2773.3	2773.1	1	3	7	1	3	— ^c	+	n.d.
2787.5	2787.1	2	2	7	1	3	— ^c	+	n.d.
2877.5	2876.1	1	5	8		1	— ^c	+	n.d.
2894.5	2894.1	1	5	6	1	3	— ^c	+	n.d.
2908.1	2908.5	2	4	6	1	3	— ^c	+	n.d.
2921.5	2921.1		5	7	1	3	— ^c	+	n.d.
2935.5	2935.1	1	4	7	1	3	— ^c	+	n.d.

^a(+) to +++++; 0–1000; 1001–1500; 1501–2500; 2501–5000; 5001–10 000; 10 001–15 000 area counts, respectively. Values given are mean values of three independently averaged mass spectra.

^bNumber key for the diagnostic fragment ions (measured as [M + H]⁺ in the LID mode): 1, Xyl (*m/z* 133); 2, Hex (*m/z* 163); 3, MeHex (*m/z* 177); 4, FucHexNAc (*m/z* 350); 5, HexNAc₂ (*m/z* 407); 6, HexNAcFuc-PA (*m/z* 446); 7, HexNAc₂Fuc (*m/z* 553); 8, HexNAc₂Fuc₂ (*m/z* 699); 9, Hex₃HexNAc₂-PA (*m/z* 989); 10, MeHexHex₂HexNAc₂-PA (*m/z* 1003); 11, Hex₃HexNAc₂X-PA (*m/z* 1121); 12, MeHexHex₂HexNAc₂X-PA (*m/z* 1135); 13, MeHexHex₂HexNAc₂F-PA (*m/z* 1149); 14, Hex₃HexNAc₂XF-PA (*m/z* 1267); 15, MeHexHex₂HexNAc₂XF-PA (*m/z* 1281); 16, D-ion and ring-fragments measured as [M + Na]⁺ (*m/z* D-ion, ^{0,4}A, ^{3,5}A) in the CID mode. n.d., not done.

^cSpecies only detectable after immunoaffinity chromatography, lectin (AAL) affinity enrichment and/or HPLC subfractionation.

^d+/-, detected in flow-through as well as eluate.

et al. 2005). MALDI-MS analyses of the unbound and retained carbohydrate fractions, each of which amounting roughly half of total cross-reacting glycans, demonstrated an enrichment of fucosylated species in the AAL-bound fraction, whereas the flow-through contained preponderantly nonfucosylated species with compositions of MeHex₀₋₁Hex₁₋₃HexNAc₂Xyl₁ or MeHex₀₋₂Hex₂₋₄HexNAc₃₋₆Xyl₁ (Figure 4 and Table I). In addition, minor amounts of fucosylated species with or without xylose (MeHex₀₋₂Hex₃₋₅HexNAc₄₋₇Xyl₀₋₁Fuc₁₋₂) were obtained in the flow-through of the AAL column. Because the same compositional species were also recovered in the AAL-bound fractions, these glycans might exhibit only weak binding affinities to this lectin. Each carbohydrate fraction comprised a highly heterogeneous mixture of different compositional species, which might still represent isomeric and/or isobaric variants.

In order to further reduce oligosaccharide heterogeneity, AAL-bound and flow-through fractions were separated by normal-phase HPLC (Figure 4C and D), resulting in a large number of individual peak fractions, each of which still comprised up to 10 different compositional species (see insets in Figure 4C and D). In agreement with previous studies (Geyer et al. 2005), the glycans obtained within one peak still covered a wide molecular mass range, demonstrating that clear-cut size-fractionation of these highly heterogeneous mixtures was not possible. Therefore, subsequent structural analyses had to be performed on oligosaccharide mixtures, thus ruling out, for example, linkage analyses of individual glycan species.

Structural assignment of cross-reacting carbohydrate determinants

Owing to the large structural heterogeneity of the glycans under study, characterization of serologically cross-reacting carbohydrate epitopes has been restricted primarily to distinct

structural prototypes carrying exclusively a core-linked xylose, a fucosylated immunoreactive determinant, or both epitopes. Glycans were mainly characterized by MALDI-MS and MALDI-MS/MS(LID). In some cases, MALDI-MS/MS after collision-induced dissociation (CID) and electrospray ionization (ESI)-ion trap (IT)-MS/MS experiments have been additionally performed.

Glycans with compositions of Hex₂HexNAc₂Xyl₁ and MeHex₁Hex₁HexNAc₂Xyl₁ (*m/z* 981.3 and 995.3 in Table I) as well as core-fucosylated variants thereof (*m/z* 1127.4 and 1141.3) were the smallest cross-reacting carbohydrate species. The majority of glycans, however, was further elongated by additional hexose (Hex) and/or methyl-hexose residues (Table I). As shown for the glycan with the composition of MeHex₁Hex₂HexNAc₂Xyl₁Fuc₁ (*m/z* 1303.4), representing a weakly cross-reacting compound, the MALDI-MS/MS(LID) spectrum (Figure 5A) clearly demonstrated the presence of core-linked fucose (fragment Y₁) as well as terminal methyl-hexose (Y_{3α}), hexose (Y_{3β}), and xylose residues (Y_{3γ}). The linkage positions of the outer hexose and methyl-hexose residues were assigned by MALDI-MS/MS(CID). The spectrum revealed the formation of a so-called “D-ion” B₂/Y_{3α} (*m/z* 478.9) comprising two hexoses and one xylose, the corresponding B₂/Y_{3α}/Y_{3γ} ion (*m/z* 346.9) as well as the diagnostically relevant ring fragment ions ^{3,5}A₂ (*m/z* 258.9) and ^{0,4}A₂ (*m/z* 245.0). In accordance with the literature data (Harvey et al. 1997; Harvey 1999; Mechref et al. 2003), the obtained results thus verify the presence of a (1-3)-linked methyl-hexose and a (1-6)-linked hexose in addition to xylose at the central mannose residue of the core (Figure 5B). From the signal intensities of the D-ion and the corresponding ion lacking xylose, it is evident that the intense signal, generated by preferential loss of the substituent at the 3-position, still contains Xyl. Because xylosylation of the 4-position is ruled out by the observed ^{3,5}A₂ fragment at

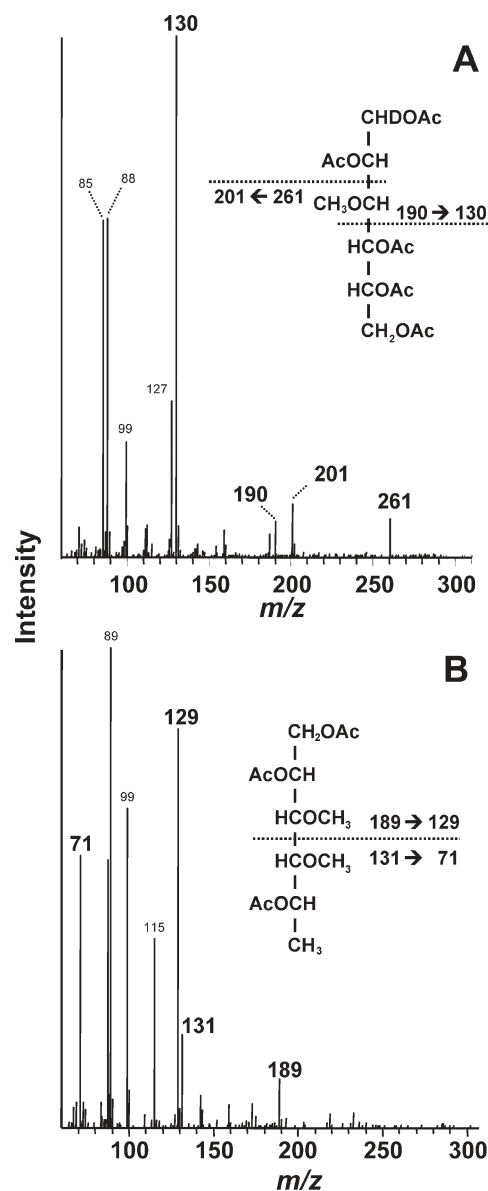


Fig. 3. Electron impact mass spectra of partially methylated alditol acetates. (A) Spectrum of 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-mannitol obtained after acid hydrolysis of native PA-glycans, sodium borodeuteride reduction, and peracetylation. (B) Spectrum of 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-fucitol obtained after permethylation of total PA-glycans, acid hydrolysis, sodium borohydride reduction, and peracetylation. Alditol acetate derivatives were analyzed by GC–MS in the electron impact mode. Characteristic fragment ions are assigned.

m/z 259, the xylose residue could be assigned to the 2-position of the branching mannose in agreement with the linkage data. The deduced assignment of outer hexose and methyl-hexose residues was further confirmed by ESI-IT-MS³ of the corresponding B₃ fragment ion at m/z 858.2 (Supplementary Figure 4). Similar to MALDI-MS/MS(CID), the ESI-IT-MS³ spectrum revealed the presence of a D-ion B₂/Y_{3α} (m/z 479.3), the corresponding B₂/Y_{3α}/Y_{3γ} ion (m/z 347.1) as well as the fragment ions ^{3,5}A₂ (m/z 259.2) and ^{0,4}A₂ (m/z 245.0), which is in agreement with the data published by Morelle et al. (2005) on the ESI-IT-MS/MS fragmentation of oligosaccharides derivatized with 2-aminobenzamide.

Both core-xylosylated Hex₃HexNAc₂Xyl₁Fuc₀₋₁ (m/z 1143.3 and 1289.4) and MeHex₁Hex₂HexNAc₂Xyl₁Fuc₀₋₁ (m/z 1157.4 and 1303.4) glycan variants were found to be further elongated by additional HexNAc residues. As shown for species with the composition of Hex₃HexNAc₂Xyl₁ as an example, cross-reacting glycans of this type exhibited, in part, one or two additional HexNAc residues (Figure 6). In the latter case, MALDI-MS/MS(LID) unambiguously demonstrated a terminal Hex (Y_{3β}) and a terminal HexNAc unit (Y_{5α}). The presence of a nonreducing HexNAc–HexNAc disaccharide is verified by a corresponding B_{2α} fragment ion (Figure 6C). In agreement with linkage data demonstrating the presence of small amounts of both terminal GalNAc and GlcNAc in the cross-reacting glycan fraction (Table II), the identified HexNAc–HexNAc unit could represent a laccinac (GalNAc(β1-4)GlcNAc) or a chitobiose (GlcNAc(β1-4)GlcNAc) moiety.

Cross-reacting glycans with a composition of Hex₃HexNAc₄Xyl₁Fuc₁ (m/z 1695.6; Table I) were only partially retained by the AAL affinity column. Bound and unbound species were individually analyzed by MALDI-MS/MS(LID). The results revealed that bound sugar chains were mostly core-fucosylated and xylosylated, comprising a terminal HexNAc₂ unit as evidenced by the diagnostically relevant fragment ions at m/z 445.9 (Y₁) and 406.9 (B_{2α}) (Figure 7A). In contrast, unbound species represented preponderantly isomeric structures carrying fucose at the terminal, nonreducing HexNAc in agreement with fragment ions observed at m/z 300.1 (Y₁), 350.1 (B_{2α}), and 553.1 (B_{3α}) as well as the almost entire absence of a signal at m/z 446 (Figure 7B). In conjunction with linkage data (Table II) and the observed mAb M2D3H immunoreactivity, this structural entity is assumed to represent a Fuc(α1-3)GalNAc motif. In both cases, the assignment of the overall structure was corroborated by the additional fragment ions observed. The weak signals at m/z 350.1 and 553.1 or m/z 1470.6, marked by asterisks in Figure 7A or B, respectively, may suggest a slight cross-contamination by the complementary isomer. The intense signal at m/z 445.9 (Figure 7A) and its almost complete lack (Figure 7B), however, clearly demonstrate that fractionation of isomeric fucosylated structures can be achieved with high efficiency by AAL affinity chromatography.

The last prototypic representatives of cross-reacting glycans to be discussed were obtained as a mixture of isobaric glycans with compositions of Hex₃HexNAc₄Fuc₃ and MeHex₁Hex₂HexNAc₄Xyl₁Fuc₂ (m/z 1855.5; Table I), which could be neither separated by AAL affinity chromatography nor by subsequent HPLC. MALDI-MS/MS(LID) clearly revealed fragment ions indicative for both types of structures (Figure 8). The presence of compound MeHex₁Hex₂HexNAc₄Xyl₁Fuc₂ is verified by the lack of a signal at m/z 1631 (release of terminal HexNAc), a Y_{4α} fragment at m/z 1281.3 (loss of HexNAc₂Fuc₁) as well as the fragments Y_{4α}/Y_{3γ}/Y_{1δ} and Y_{3α}/Y_{3γ}/Y_{1δ} or Y_{4α}/Y_{3β}/Y_{1δ} at m/z 1003.2 and 841.2 or m/z 959.0 which differ from fragments Y_{4α}/Y_{1δ} and Y_{3α}/Y_{1δ} by the mass increment of one xylose or methyl-hexose, respectively. By the same line of reasoning, the presence of compound Hex₃HexNAc₄Fuc₃ is evidenced by fragment B_{3α} (HexNAc₂Fuc₂) at m/z 698.8. Intriguingly, similar cross-reacting glycan species with three

Table II. Linkage analysis of PA-glycans derived from *B. glabrata* hemolymph glycoproteins^a

Linkage	Total glycan fraction	Anti-SEA unbound glycans	Glycans bound by anti-SEA
Xyl(1-	+	(+)	+
Fuc(1-	+	(+)	+
-2)Fuc(1-	+	(+)	+
Man(1-	++	++	++
3- <i>O</i> -MeMan(1- ^b	+	+	+
-2)Man(1-	++	++	++
-2)3- <i>O</i> -MeMan(1- ^b	+	+	(+)
-6)Man(1-	(+)	nd	nd
-6)3- <i>O</i> -MeMan(1- ^b	(+)	(+)	(+)
-2,4)Man(1-	+	nd	(+)
-2,6)Man(1-	(+)	nd	(+)
-3,6)Man(1-	++	++	++
-2,3,6)Man(1- ^c	++	+	++
Gal(1-	+	+	++
3- <i>O</i> -MeGal(1- ^b	+	+	nd
-3,6)Gal(1-	(+)	nd	(+)
GalNAc(1-	nd	nd	(+)
-3)GalNAc(1-	+	(+)	+
GlcNAc(1-	(+)	nd	(+)
-3)GlcNAc(1-	(+)	nd	nd
-4)GlcNAc(1-	++	++	++
-3,4)GlcNAc(1-	+	+	+

^aTotal PA-oligosaccharides and glycan fractions obtained after immunoaffinity chromatography using immobilized anti-SEA antibodies were permethylated and hydrolyzed. The partially methylated alditol acetates obtained after reduction and peracetylation were analyzed by capillary GC-MS. Relative amounts are given as follows: ++, major component; +, minor compound; (+), trace; nd, not detected.

^b3-*O*-methyl-hexoses were identified by constituent analysis employing sodium borodeuteride reduction. Linkage positions were assigned using trideuteromethyl iodide for permethylation.

^cSubstitution pattern was verified by permethylation and sodium borodeuteride reduction.

fucose residues have also been found in the hemocyanin of the keyhole limpet *Megathura crenulata* (Geyer et al. 2005).

Allocation of the fucosylated laciNAc unit within the molecule was performed by MALDI-MS/MS(CID). As shown for compound MeHex₁Hex₃HexNAc₅Xyl₁Fuc₂ (*m/z* 2220.6) as an example, the obtained fragment ion spectrum of the [M + Na]⁺ ion (Figure 9, inset) revealed the respective D-ion B₄/Y_{3α} (*m/z* 857.8) with a composition of MeHex₁Hex₂HexNAc₁Xyl₁, the corresponding B₄/Y_{3α}/Y_{3γ} ion (*m/z* 725.9) lacking xylose (MeHex₁Hex₂HexNAc₁) as well as the diagnostically relevant ring fragment ions ^{3,5}A₅ (*m/z* 637.9) and ^{0,4}A₅ (*m/z* 624.0). This result demonstrates that the central mannose carried at its C6 position a trisaccharide unit comprising one methyl-hexose, one hexose, and one *N*-acetylhexosamine. In agreement with previous data on glycans derived from keyhole limpet hemocyanin (KLH) (Geyer et al. 2005), it can be, therefore, concluded that the fucosylated laciNAc unit has been attached to the 3-linked mannose of the pentasaccharide core. MALDI-MS/MS(LID) of the protonated pseudomolecular ion [M + H]⁺ (*m/z* 2198.8) revealed that the present methyl-hexose is directly linked to the central mannose and is itself further substituted by a Hex₁HexNAc₁ disaccharide unit (see, for example, fragment ions Y_{4α}Y_{4β} and Y_{3α}Y_{4β}

in Figure 9). The presence of terminal HexNAc is evidenced by the Y_{5α} fragment ion. Consequently, the fucosyl residue linked to the laciNAc unit had to be assigned to the subterminal HexNAc, which is corroborated by the observation that this fucose moiety could not be released by α-fucosidase from bovine kidney (data not shown) in accordance with previous findings (Kantelhardt et al. 2002; Geyer et al. 2005). Hence, recognition of this glycan by anti-SEA antibodies is obviously mediated by the core-linked xylose residue and is not due to the presence of a Fuc(α1-3)GalNAc unit.

On the basis of the results obtained by MS and linkage analysis, structures of the discussed prototypic cross-reacting glycans are proposed in agreement with recent data on KLH (Kurokawa et al. 2002; Wuhrer et al. 2004; Geyer et al. 2005) and common literature data (Figure 10).

In addition to the prototypic, short-chained cross-reacting glycans described in detail above, a large number of compositional species has been found, mostly in trace amounts, which can be considered as structural variants due to the presence of additional hexose, methyl-hexose, *N*-acetylhexosamine, xylose, and/or fucose units, thus leading to a highly heterogeneous mixture of isomeric or isobaric oligosaccharides. Therefore, MALDI-MS/MS(LID) spectra of

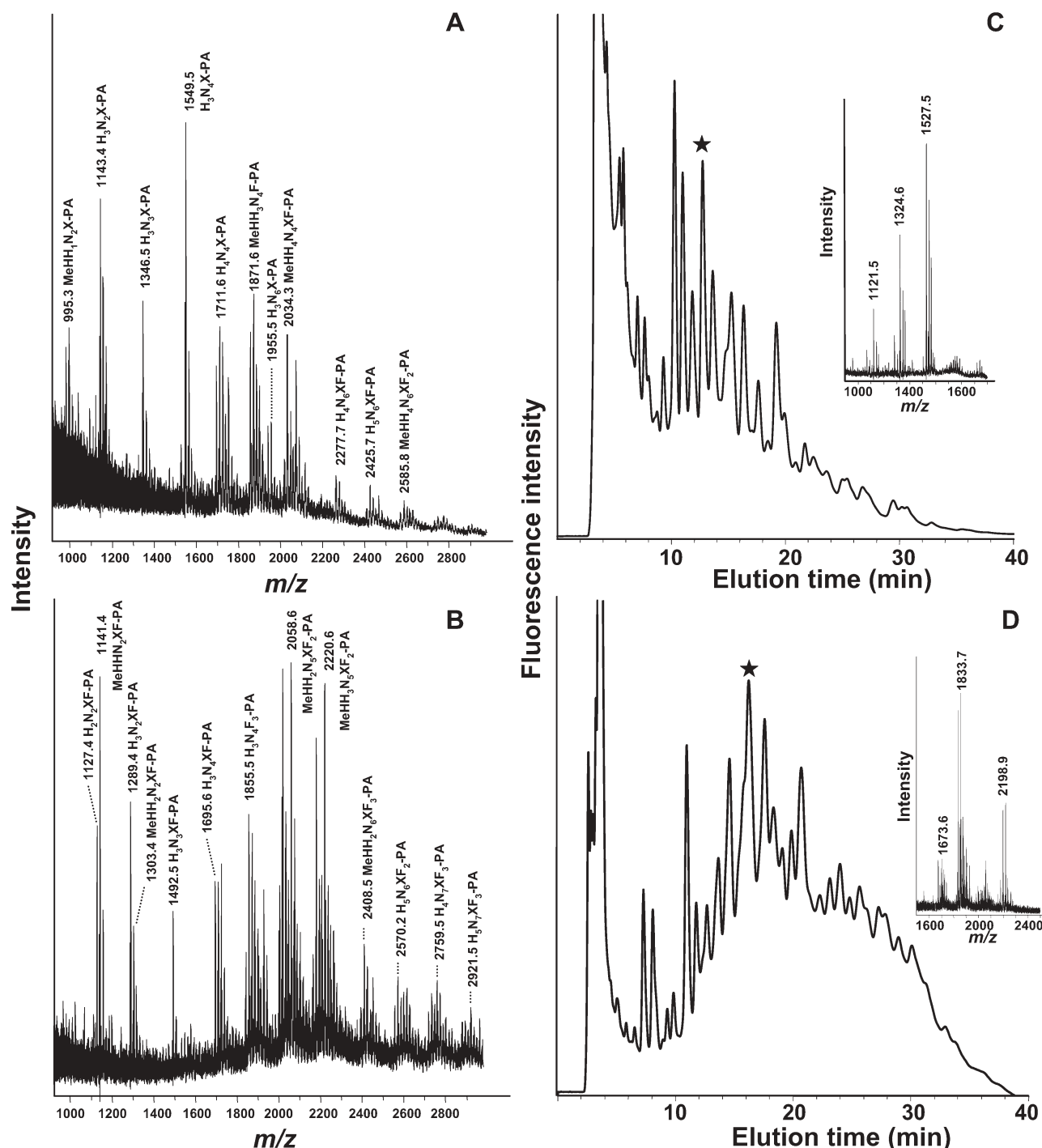


Fig. 4. Fractionation of cross-reacting *B. glabrata* N-glycans. PA-glycans recovered in the immunoaffinity eluate were fractionated by AAL affinity chromatography and subfractionated by HPLC using a TSK-amide column. (A) and (B) MALDI-mass spectra of glycans obtained in the flow-through and in the bound fraction eluted with 1 mM Fuc, respectively; (C) HPLC subfractionation of unbound species; and (D) HPLC separation of bound glycans. Insets in (C) and (D), MALDI-mass spectra recorded in the case of individual HPLC peak fractions marked by asterisks. Sodiated pseudomolecular ions $[M + Na]^+$ were registered in (A) and (B) with isotopic resolution, whereas protonated pseudomolecular ions $[M + H]^+$ were recorded in the insets in (C) and (D). Monosaccharide compositions of major species are assigned. H, hexose; MeH, 3-O-methyl-hexose; N, N-acetylhexosamine; F, fucose; X, xylose.

the respective glycan species were particularly inspected for the occurrence of diagnostically relevant fragment ions, indicating the presence of cross-reacting carbohydrate determinants (Table 1). In all cases, obtained results revealed the presence of xylose and/or monofucosylated and difucosylated lacdiNAc

units in agreement with the structures shown in Figure 10. It has to be pointed out, however, that this approach does not exclude the presence of further carbohydrate determinants also cross-reacting with anti-SEA, which applies, in particular, to the potential occurrence of glycans carrying Schistosoma-like

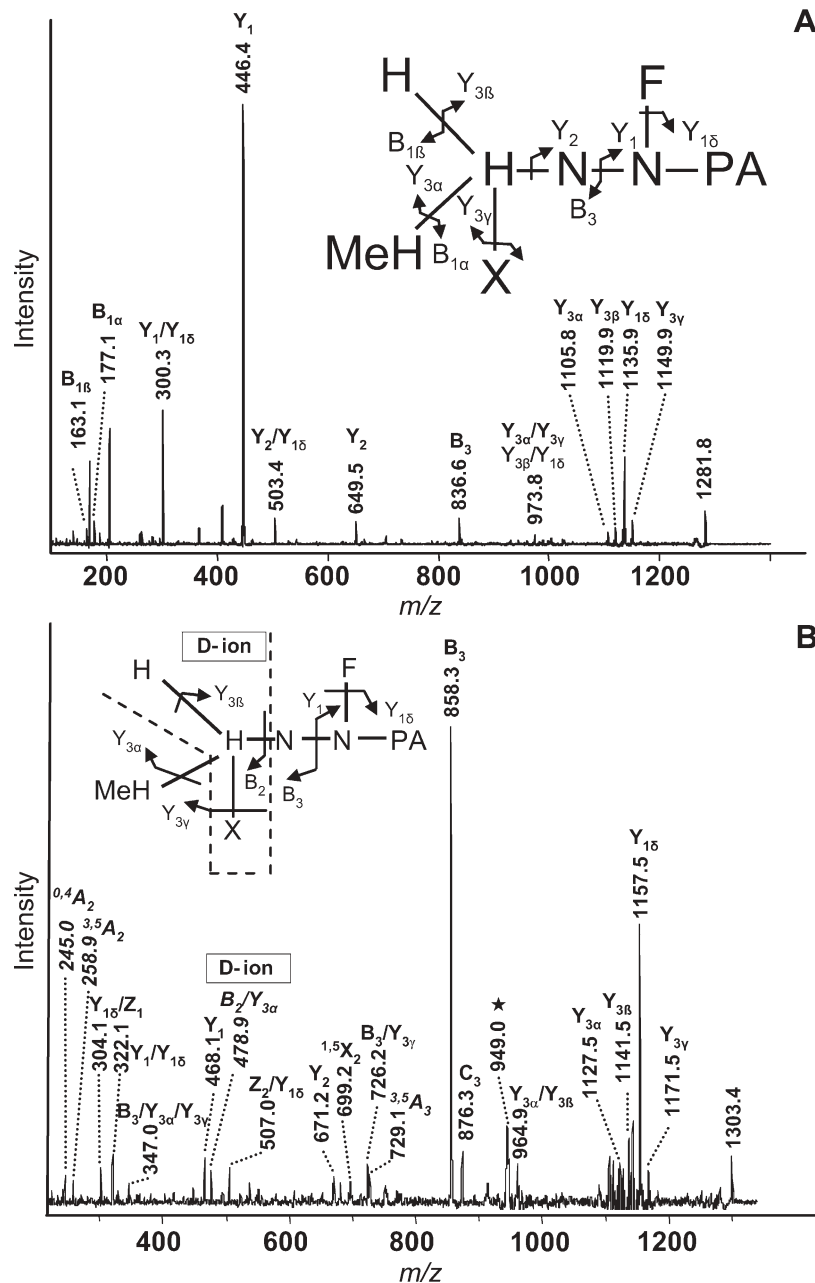


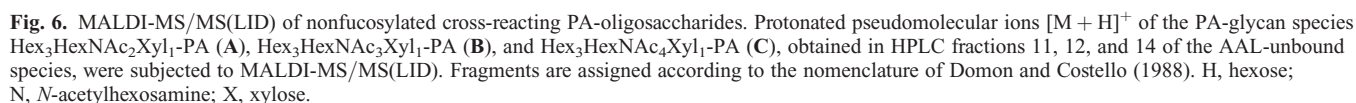
Fig. 5. Mass spectrometric analysis of cross-reacting PA-oligosaccharides carrying a methyl-hexose unit. (A) Protonated pseudomolecular ions $[M + H]^+$ of the PA-glycan species MeHex₁Hex₂HexNAc₂Xyl₁Fuc₁-PA, obtained in HPLC fraction 21 of AAL-bound glycans, were analyzed by MALDI-MS/MS(LID). (B) MALDI-MS/MS(CID) analysis of the corresponding sodiated pseudomolecular ions $[M + Na]^+$ (m/z 1303.4). Fragments are assigned using the nomenclature of Domon and Costello (1988). The signal marked by asterisk reflects a fragment produced from Y_{3α} by cleavage of the linkage between C-2 and C-3 of the pyridylaminated GlcNAc (−178.1 Da). H, hexose; MeH, methyl-hexose; N, N-acetylhexosamine; F, fucose; X, xylose.

Fuc(α1-2)Fuc-motifs. This assumption is especially corroborated by the detection of increased amounts of 2-substituted fucosyl residues by linkage analysis of cross-reacting hemolymph glycoprotein glycans (cf. Figure 3B and Table II). Moreover, MALDI-MS revealed a clear enrichment of oligosaccharide species with two or three fucoses in the immunoaffinity eluate preponderantly in the higher mass region (Figure 2C and Supplementary Figure 2). Although such glycans may represent good candidates for species with oligofucosyl sidechains, they could not be analyzed in detail due to their low abundance and the vast heterogeneity of

the respective glycan fractions even after lectin affinity chromatography and HPLC subfractionation (Figure 4).

Discussion

On the basis of the previous indications (Dissous et al. 1986), the focus of this study is to investigate whether *S. mansoni* and its intermediate host, *B. glabrata*, share common carbohydrate epitopes, which might provide a basis for carbohydrate-mediated molecular mimicry. In a first approach, *B. glabrata* hemolymph has been used as a source of



glycoproteins from this snail. Glycans were enzymatically released, fractionated according to cross-reactivity by immunoaffinity chromatography using immobilized anti-soluble egg antigen (anti-SEA) antibodies, and subfractionated by AAL affinity chromatography and normal-phase HPLC. Resultant glycan fractions still comprised, in part, highly heterogeneous mixtures. Hence, structural assignments were preponderantly made by MS. As it was not possible to isolate individual oligosaccharide species by this approach, linkage data have only been obtained from glycans mixtures.

The results revealed that about 5% of the total glycans exhibited serological cross-reactivity with anti-SEA antibodies. In particular, most of the major carbohydrate species did not bind to the immunoaffinity matrix. Exceptions were glycans with compositions of 3-*O*-MeMan₁Man₂GlcNAc₂Xyl₁Fuc₀₋₁, which were found in both the flow-through and the retained fractions. The reason for this partial fractionation has not been further investigated, but may reside in a substantial sterical hindrance of antibody binding by the present 3-*O*-MeMan, thus leading to a partial release of these sugar chains already during washing the immunoaffinity column. Detailed analysis of these bound glycans revealed that each one comprised a (1-3)-linked methyl-mannose residue. All elongated cross-reacting glycans investigated in this study, however, expressed mannose in this position (see, for example, Figures 6–8). Hence, it may be speculated that the presence of a methyl-hexose at C3 of the central mannose of the pentasaccharide core prevented further elongation of this antenna. This assumption is in agreement with data from van Kuik et al. (1986, 1987) on *N*-glycans from hemocyanin of *Lymnaea stagnalis*, which demonstrated that (α1-3)-linked 3-*O*-MeMan units of the pentasaccharide core are obviously not elongated in this snail. Consequently, glycans of this type might have been accumulated in *B. glabrata* for similar reasons. Intriguingly, corresponding species carrying two methyl-hexose units did not bind to the immunoaffinity matrix possibly due to sterical hindrance. Likewise, glycans containing methyl-hexose residues, but lacking xylose and fucose, were not bound, indicating that 3-*O*-methylated hexose is not recognized by anti-SEA antibodies. This holds also true for the major MeHex₂Hex₁HexNAc₂-glycan ([M + Na]⁺ *m/z* 1039.7), which has been described to occur as a main *N*-glycan in *B. glabrata* dermatopontin (Marxen et al. 2003).

Analysis of *B. glabrata* hemolymph glycans was largely impeded by their vast structural heterogeneity, thus preventing an isolation of pure, homogeneous oligosaccharide fractions, although in some cases (see, for example, Figure 7) separation of different oligosaccharide isomers could be achieved. Therefore, structural assignments have been restricted to a limited set of prototypic structures. The results obtained closely resemble the data obtained for KLH glycans similarly cross-reacting with *S. mansoni* glycoconjugates (Geyer et al. 2005). KLH cross-reacting species comprised primarily a difucosylated lacdiNAc-entity, i.e. Fuc(α1-3)GalNAc(β1-4)[Fuc(α1-3)]GlcNAc unit, whereas glycans carrying a core-linked xylose have only been detected in smaller quantities. In contrast, core-linked xylose represented a major cross-reacting carbohydrate epitope in *B. glabrata* *N*-glycans. Furthermore, *B. glabrata* glycoproteins contained also monofucosylated lacdiNAc units, i.e. Fuc(α1-3)

GalNAc(β1-4)GlcNAc. Similar to *B. glabrata* glycans, KLH expressed a wide variety of different isomeric structural variants, which were found to be multiply decorated by additional galactosyl residues (Geyer et al. 2005). As composition analyses revealed an enrichment of Gal during immunoaffinity chromatography of *B. glabrata* glycans, this type of *N*-glycan variation might also occur in *B. glabrata* hemolymph glycoproteins.

N-glycans comprising (β1-2)-linked xylose have also been found in hemocyanins of *Helix pomatia* (van Kuik et al. 1985; Lommerse et al. 1997) and *L. stagnalis* (van Kuik et al. 1986, 1987) as major carbohydrate constituents of these molecules. Intriguingly, both hemocyanins contain also 3-*O*-methyl-hexose derivatives and, at least in part, lacdiNAc antennae. Oligosaccharides from *L. stagnalis* resemble, in particular, *B. glabrata* glycans as they similarly contain 3-*O*-Me-Gal and/or 3-*O*-Me-Man units, the latter of which were assigned to C-3 or C-6 of the central β-linked mannose. Hence, the major *N*-glycan of *L. stagnalis* hemocyanin with a composition of 3-*O*-MeMan₂Man₁GlcNAc₂Xyl₁ (van Kuik et al. 1986) can be assumed to be identical with the PA-glycan with *m/z* 1171.4 ([M + Na]⁺) obtained in this study (Figure 2 and Table I).

Throughout its life cycle, *S. mansoni* expresses a great variety of, in part, highly immunogenic, fucosylated carbohydrate motifs including Gal(β1-4)[Fuc(α1-3)]GlcNAc (Lewis X), Fuc(α1-3)GalNAc(β1-4)GlcNAc, GalNAc(β1-4)[Fuc(α1-3)]GlcNAc, Fuc(α1-3)GalNAc(β1-4)[Fuc(α1-3)]GlcNAc, and GalNAc(β1-4)[Fuc(α1-2)Fuc(α1-3)]GlcNAc (Hokke and Deelder 2001; Nyame et al. 2004). Using monoclonal antibodies with defined carbohydrate specificities (van Remoortere et al. 2000), it could be demonstrated that these epitopes occur with high abundance on egg-derived glycoproteins (Robijn et al. 2005) and SEAs circulating in the serum of infected animals (Nourel Din et al. 1994). Therefore, it may be assumed that rabbit hyperimmune sera against SEAs from *S. mansoni* contain also antibodies recognizing glycans with oligofucosyl, i.e. Fuc(α1-2)Fuc(α1-3) side chains. This is insofar of relevance as *B. glabrata* hemolymph glycoproteins similarly express glycans with 2-substituted fucose moieties, which were enriched in the immunoaffinity eluate. Although potentially oligofucosylated glycans, representing mostly larger carbohydrate species, have not been analyzed in detail in this study, it appears to be highly likely that such oligosaccharides also occur in *B. glabrata*. As our data provided also evidence for the presence of GalNAc(β1-4)[Fuc(α1-3)]GlcNAc and Fuc(α1-3)GalNAc(β1-4)[Fuc(α1-3)]GlcNAc structural motifs, *S. mansoni* appears to share further fucosylated carbohydrate epitopes with its intermediate host in addition to core-linked xylose and Fuc(α1-3)GalNAc(β1-4)GlcNAc units.

In addition, many of the high molecular weight *B. glabrata* hemolymph glycans carry up to eight HexNAc units, which might reflect the occurrence of chito-oligomeric antennae as found in *N*-glycans of the snake venom glycoprotein batroxobin (Lochnit and Geyer 1995), *N*-glycans of filarial nematodes (Haslam et al. 1999), or, in fucosylated form, in glycosphingolipids of *S. mansoni* eggs (Khoo et al. 1997a; Wuhler et al. 2002). Alternatively, the latter oligosaccharide species might comprise GalNAc(β1-4)GlcNAc (lacdiNAc) repeating units, as described for adult *S. mansoni*

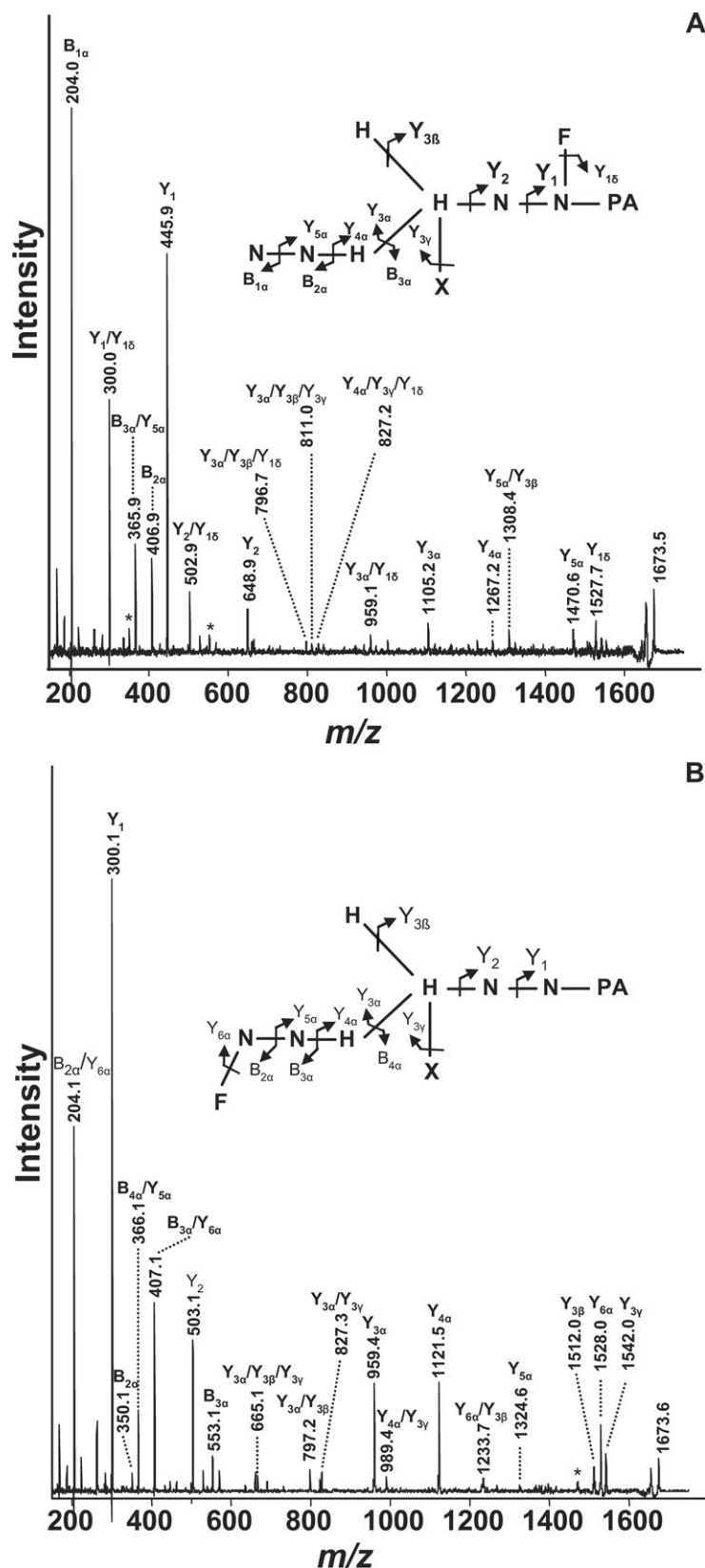


Fig. 7. MALDI-MS/MS(LID) of cross-reacting PA-glycan isomers. Protonated pseudomolecular ions $[M + H]^+$ of isomeric PA-glycan species with a composition of $\text{Hex}_3\text{HexNAc}_4\text{Xyl}_1\text{Fuc}_1\text{-PA}$, obtained in HPLC fraction 29 of the AAL-bound fraction (A) or in HPLC-fraction 16 of the AAL-unbound species (B), were subjected to MALDI-MS/MS(LID). The weak signals at m/z 350.1 and 553.1 or m/z 1470.6 marked by asterisks in (A) or (B), respectively, suggest a slight cross-contamination by the complementary isomer. Fragments are assigned according to the nomenclature of Domon and Costello (1988). H, hexose; N, *N*-acetylhexosamine; F, fucose; X, xylose.

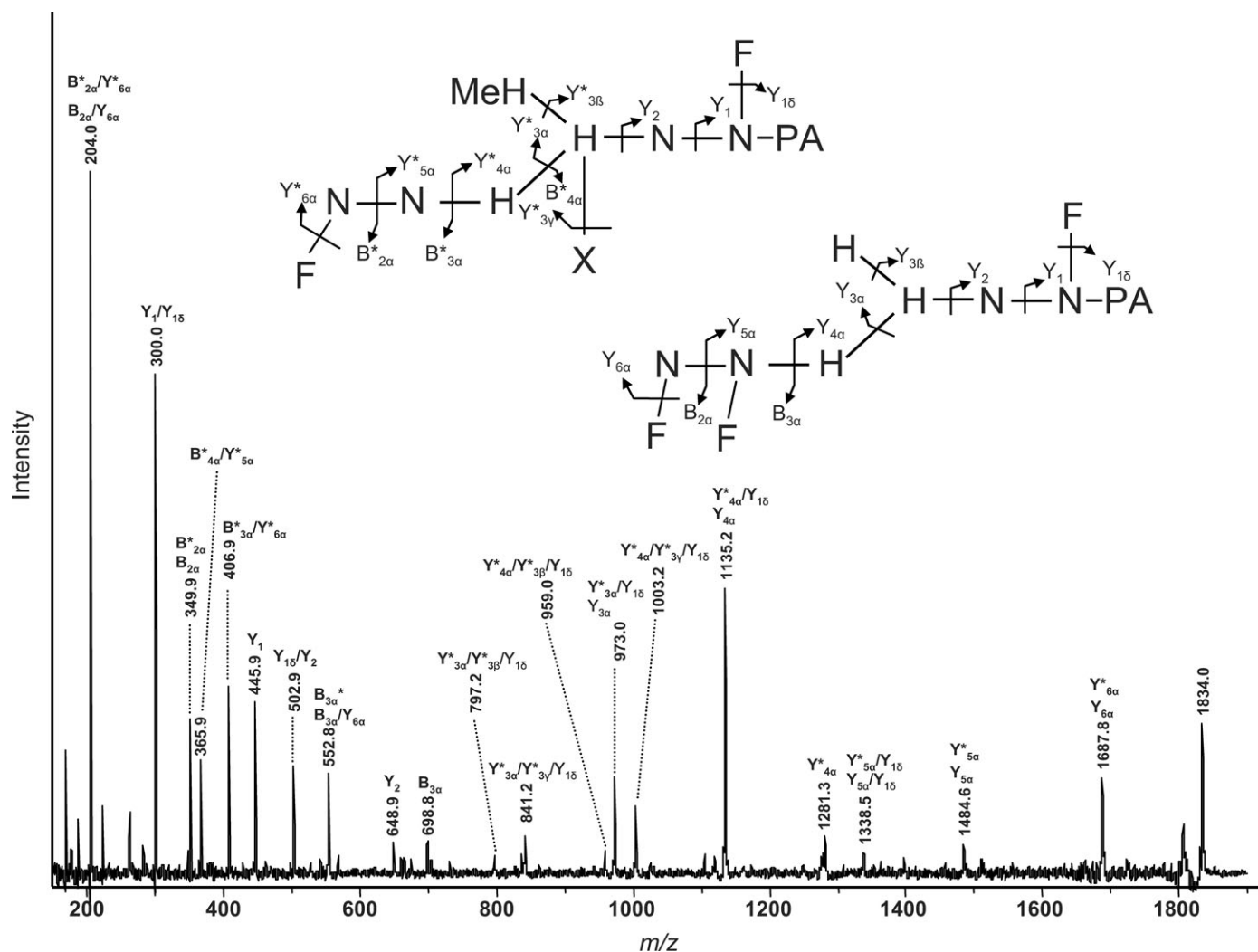


Fig. 8. MALDI-MS/MS(LID) of an isobaric PA-glycan mixture. Protonated pseudomolecular ions $[M + H]^+$ of isobaric PA-oligosaccharide species with compositions of Hex₃HexNAc₄Fuc₃ and MeHex₁Hex₂HexNAc₄Xyl₁Fuc₂ obtained in HPLC fraction 33 of the AAL-bound glycans were subjected to MALDI-MS/MS(LID). Fragments are assigned according to the nomenclature of Domon and Costello (1998). H, hexose; N, *N*-acetylhexosamine; F, fucose; X, xylose.

glycoprotein-*N*-glycans (Wuhrer et al. 2006). Because these species represented mostly minor compounds and were only detectable after enrichment by immunoaffinity chromatography, they could not be analyzed in detail either.

In the definitive host, sugar entities of glycoconjugates present in SEA seem to play a major role in immune modulation and are implicated in the induction of T helper 2 immune responses by these antigens (Hokke and Deelder 2001; Pearce and MacDonald 2002; Thomas and Harn 2004; Dunne and Cooke 2005). Likewise, it has been postulated that excretory–secretory products of schistosomes could affect the snail’s cellular defense system through inhibiting hemocyte motility as well as decreasing hemocyte phagocytic activity and production of cytotoxic superoxide (El-Ansary 2003). Further evasion strategies appear to imply molecular mimicry as discussed, for example, on the basis of the high structural homology of tropomyosins from *S. mansoni* and *B. glabrata* (Dissous et al. 1990). Our present study revealed that uninfected *B. glabrata* and *S. mansoni* share, at least, two carbohydrate determinants: core-linked

xylose and/or a terminally fucosylated lactiNAc unit expressing the Fuc(α1-3)GalNAc-motif recognized by mAb M2D3H. As miracidia have been also shown to express corresponding fucosylated carbohydrate motifs at their surface (Köster and Strand 1994), the detection of cross-reacting glycans in their snail host supports the hypothesis that molecular mimicry may also exist at the level of sugar epitopes. It would be, therefore, interesting to investigate the regiospecific expression of the identified cross-reacting carbohydrate epitopes within the snail. Possibly, these structures occur preponderantly in tissue that acts as the main interface of the parasite and the host. Corresponding immunohistochemical studies are in progress.

Materials and methods

Materials

For the structural analyses of cross-reacting *N*-glycans from *B. glabrata* hemolymph glycoproteins, 120 mL snail blood

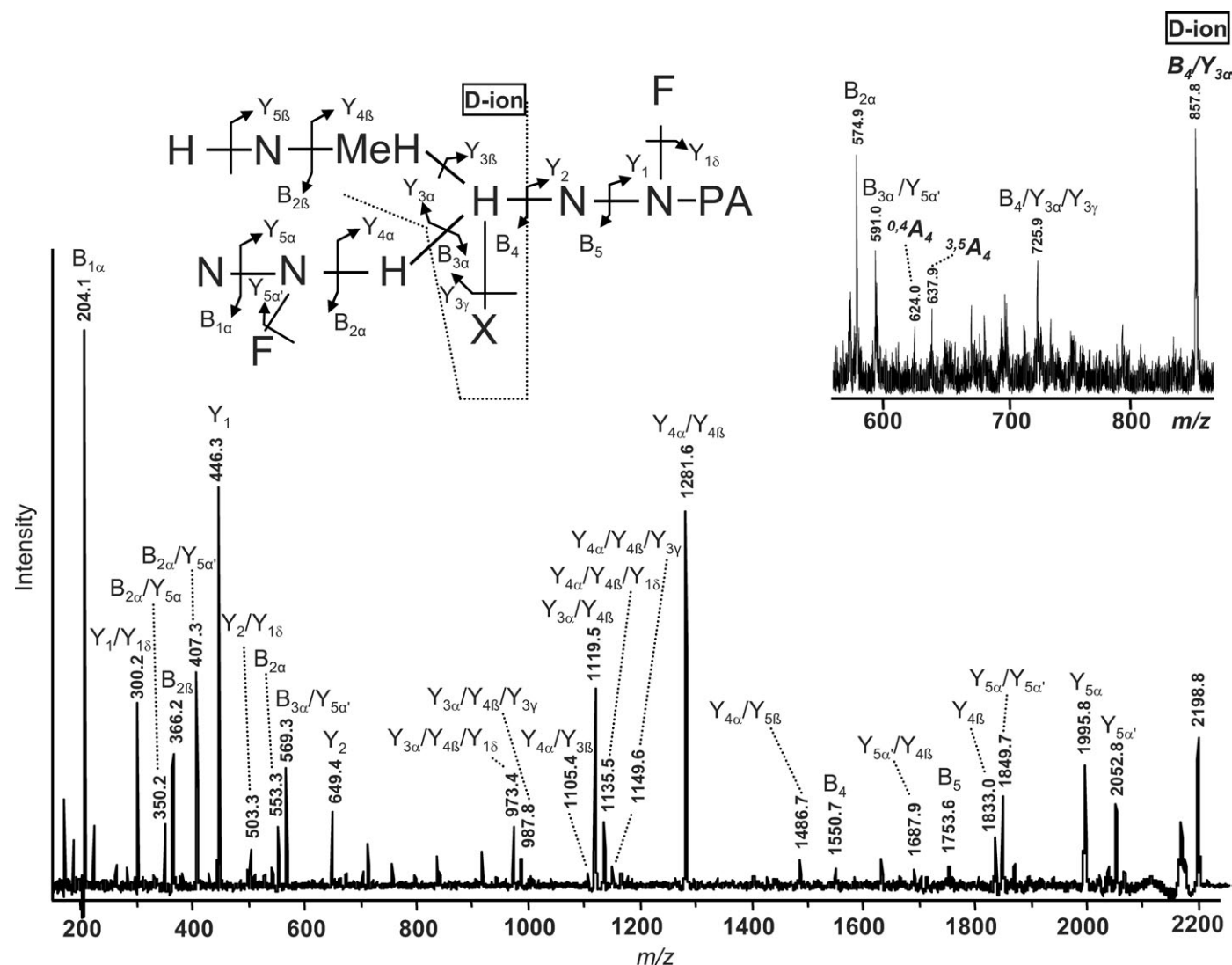


Fig. 9. Allocation of fucosylated laciNac units. Protonated pseudomolecular ions $[M + H]^+$ of compound MeHex₁Hex₃HexNAc₅Xyl₁Fuc₂ (m/z 2198.8) were analyzed by MALDI-MS/MS(LID). Inset: sodiated pseudomolecular ions $[M + Na]^+$ (m/z 2220.6) were subjected to MALDI-MS/MS(CID). Only the diagnostically relevant portion of the CID mass spectrum is displayed. Fragment ions are assigned according to the nomenclature of Domon and Costello (1998). H, hexose; MeH, 3-*O*-methyl-hexose; N, *N*-acetylhexosamine; F, fucose; X, xylose.

from an *S. mansoni* susceptible strain was used. The hemolymph was collected by cardiac puncture of the snails, shortly centrifuged after sampling, and stored at -20°C . The supernatant from M2D3H hybridoma cell culture (Bickle and Andrews 1988), producing a monoclonal antibody (mAb M2D3H) recognizing the Fuc(α 1-3)GalNAc-epitope (Kantelhardt et al. 2002), was used 10-fold concentrated for ELISA and western blot analysis. Rabbit hyperimmune serum raised against SEAs from *S. mansoni* [anti-SEA (Hamilton et al. 1999)] was used for immunoaffinity chromatography as well as for ELISA and western blot analyses. PNGase F from *Flavobacterium meningosepticum* (PNGase F; EC 3.5.1.52) and PNGase A from almond (PNGase A; EC 3.5.1.52) were obtained from Roche Diagnostics (Mannheim, Germany). The Man(α 1-6)[Xyl(β 1-2)]Man(β 1-4)GlcNAc(β 1-4)[Fuc(α 1-3)]GlcNAc (MUXF³) standard oligosaccharide from pineapple stem bromelain was kindly provided by

E. Staudacher, Department of Chemistry, BOKU-University of Natural Resources and Applied Sciences, Vienna, Austria.

Tryptic digestion of hemolymph proteins

Obtained hemolymph was centrifuged for 15 min at 4000 rpm (1780g) to remove hemocytes and cell debris and dialyzed for 48 h against 25 mM (NH₄)HCO₃ changing the buffer every 6 h. After dialysis, the amount of protein was determined using the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany), and an aliquot from the hemolymph was set apart for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and stored at -20°C . The volume of the remaining sample was reduced to about 50% by controlled freeze-drying. L-1-Tosylamido-2-phenylethyl-chloromethylketone–treated trypsin (Sigma, Taufkirchen, Germany; EC 3.4.21.4) was activated by incubation in 100 μL 1 mM HCl and added to the hemolymph sample

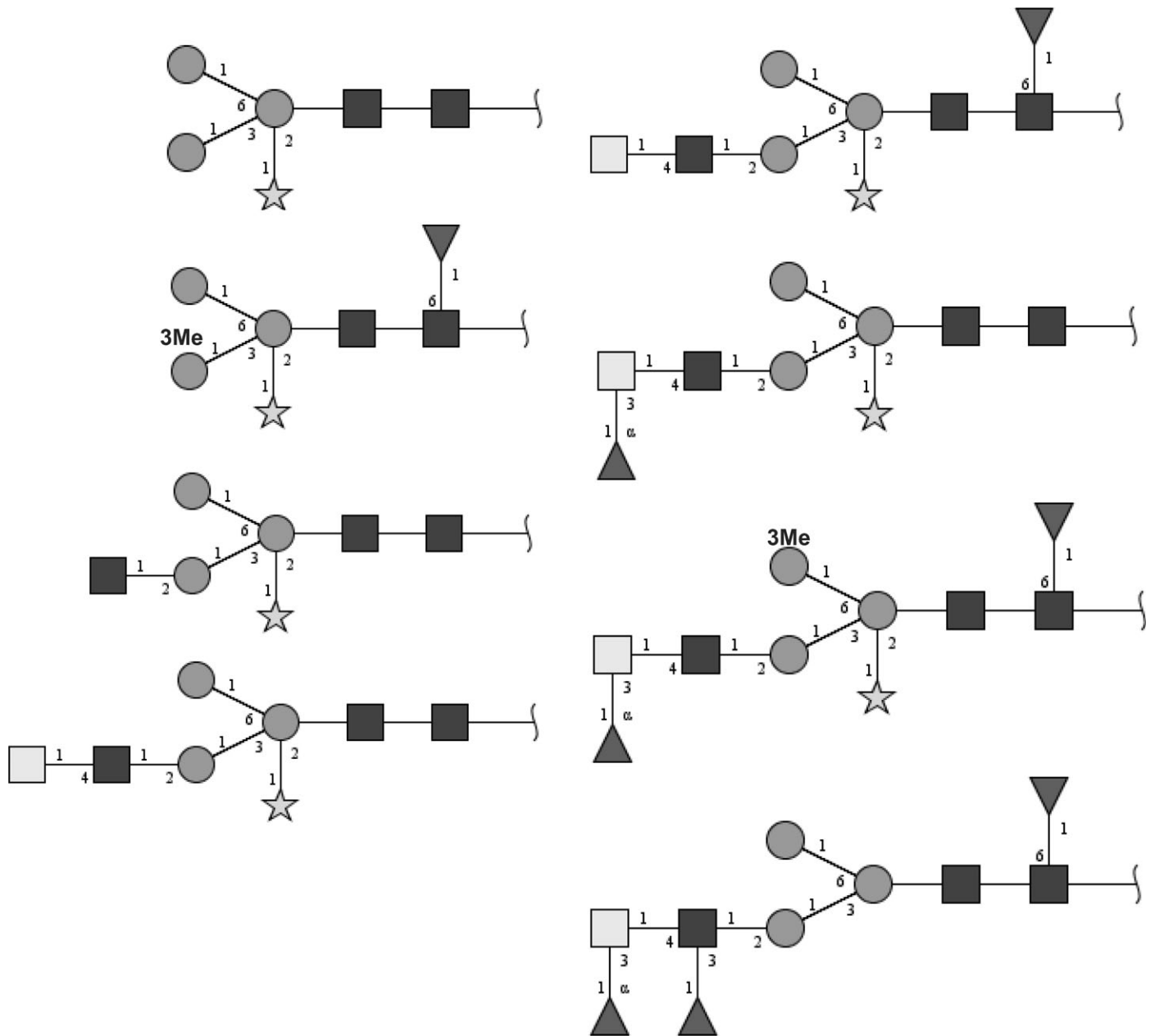


Fig. 10. Prototypes of *B. glabrata* hemolymph glycoprotein glycans cross-reacting with *S. mansoni* glycoconjugates. Key symbols used for constituent monosaccharides: ●, Man; 3Me, 3-O-methylated; □, GalNAc; ■, GlcNAc; ▲, Fuc; ☆, Xyl

adjusting a ratio of protein versus trypsin of 40:1 (by weight). Following incubation overnight at 37 °C under shaking, trypsin was inactivated by boiling (100 °C, 10 min) and tryptic (glyco)peptides were freeze-dried.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

One-dimensional SDS–PAGE was performed according to Laemmli (1970), using a Mini-PROTEAN 3 electrophoresis system (Bio-Rad). Gels (9 × 6 × 0.75 cm) were prepared containing 8.5% acrylamide. An aliquot of the hemolymph was first desalted using a nucleic acid purification column (Amersham, Uppsala, Sweden), according to manufacturer's instructions. The desalted hemolymph sample was freeze-dried,

dissolved in sample buffer (62.5 mM Tris–HCl, pH 6.8, 8.7% glycerol, 2% SDS, 1 mM EDTA, 0.00625% bromophenol blue) containing mercaptoethanol, and boiled for 5 min. In each lane, 10 µg of protein was applied. After electrophoresis, the gels were either stained with silver or electroblotted to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford).

Western blot analysis

Following electroblotting, the PVDF membrane was blocked by incubation in Roti-Block (Roth, Karlsruhe, Germany), diluted 1:10 (by vol.) with water, overnight at 4 °C. Monoclonal M2D3H-antibodies and anti-SEA were diluted

1:4 and 1:100 000 with 10-fold diluted Roti-Block, respectively. PVDF membranes were incubated for 2 h at room temperature. The blot stripes were washed 3 times for 10 min with 10-fold diluted Roti-Block. Porcine anti-rabbit or rabbit-anti-mouse immunoglobulins conjugated with horseradish peroxidase (DAKO, Hamburg, Germany), used as secondary antibodies, were diluted 1:1000 with 10-fold diluted Roti-Block. After incubation for 2 h, the PVDF membranes were washed 5 times for 10 min with 10-fold diluted Roti-Block and once with Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.5, 100 mM NaCl). Antibody staining was visualized by chemoluminescence (Pierce, Rockford, IL) and documented using 9 × 13 cm Kodak X-Omat AR Film (Sigma).

Enzyme-linked immunosorbent assay

Tryptic hemolymph (glyco)peptides were assayed by ELISA with/without defucosylation by HF treatment (see below). The (glyco)peptides were adsorbed (200 ng per well in 100 μ L 0.1 M Na₂CO₃, pH 9.6; 2 h at 37 °C) to microtiter plates (Maxisorb; Nunc, Wiesbaden, Germany). Plates were washed twice with TBS and blocked for 1 h with 0.5% bovine serum albumin (BSA) in TBS. Primary antibodies (mAb M2D3H and anti-SEA, diluted 1:100 and 1:5000, respectively) were added in TTBS-10 (TBS 1:10 diluted, 0.05% Tween-20) containing 0.25% BSA (100 μ L per well) and incubated for 1 h at 37 °C. After multiple washes with TTBS-10, alkaline phosphatase-conjugated goat anti-mouse Ig (Dako Diagnostics; diluted 1:1000) or goat anti-rabbit Ig (Sigma; diluted 1:1000) in TTBS-10 containing 0.25% BSA was applied. Plates were washed with TTBS-10 and incubated with 0.1% *p*-nitrophenyl phosphate (Biomol, Hamburg, Germany) in 100 mM glycine buffer, pH 10.4, containing 1 mM ZnCl₂ and 1 mM MgCl₂ at 37 °C. After 30 min, the reaction was stopped by addition of 100 μ L of 50 mM aqueous EDTA solution per well and absorption was determined at 405 nm.

Release of N-glycans

Freeze-dried tryptic (glyco)peptides (1200 mg) were dissolved in 5 mL 20 mM sodium phosphate buffer, pH 7.2. N-glycans were released by exhaustive treatment with PNGaseF (750 U; Roche) at 37 °C overnight under shaking. After addition of the same amounts of enzyme, incubation was repeated once. The resulting mixture of products was applied to a reverse-phase cartridge (C18ec; Macherey und Nagel, Düren, Germany). Released oligosaccharides were recovered in the flow-through and collected. For desalting, glycans were applied to a porous graphitic-carbon cartridge (Supelclean ENVI-Carb, Supelco, Bellefonte, PA). The cartridges were washed with water and oligosaccharides were eluted with 25% (v/v) aqueous acetonitrile. In parallel, tryptic glycopeptides were also subjected to analytical scale hydrazinolysis (Kuraya and Hase 1992; Hase 1994). Residual glycopeptides obtained after digestion with PNGase F were further treated with PNGase A (10 m-units; Roche) in 50 mM ammonium acetate buffer, pH 5.0, for 16 h at 37 °C. Released glycans were purified using a reverse-phase cartridge, as described above.

Labeling of N-glycans

Released N-glycans were fluorescently labeled with sublimation-purified 2-aminopyridine (PA; Merck, Darmstadt, Germany) according to Kuraya and Hase (1992). Excess 2-aminopyridine was removed by gel filtration using a TSK-gel Toyopearl HW-40F column (1.6 × 80 cm; TosoHaas, Philadelphia, PA). A 10 mM ammonium acetate buffer, pH 6.0, was used as running solvent. Column flow was adjusted to 12 mL per hour and monitored by fluorescence detection at 320/400 nm. Fractions (6 mL) were collected and PA-labeled oligosaccharides containing fractions were pooled.

Immunoaffinity chromatography of PA-labeled N-glycans

Rabbit IgG was isolated from anti-SEA by affinity chromatography using a DEAE Affi-Gel Blue (Bio-Rad) column (30 × 3 cm), as described by the manufacturer. In brief, anti-SEA (20 mL) was dialyzed for 48 h against running buffer (20 mM Tris-HCl, pH 8.0, containing 25 mM NaCl and 0.02% sodium azide) before loading it onto the column. Chromatography was performed at 4 °C in running buffer at a flow rate of 12 mL per hour and fractions of 6 mL were collected. Elution of proteins was monitored by absorbance at 280 nm. Rabbit IgG obtained in the flow-through of the column was pooled, concentrated by vacuum dialysis, and checked for purity by SDS-PAGE. The column was regenerated by applying two column volumes of 2 M guanidine-hydrochloride solution in running buffer and five column volumes of washing buffer [0.1 M acetic acid, 1.4 M NaCl, 40% (by vol.) isopropanol, pH 3]. For re-equilibration, the column was finally washed with 12 column volumes of running buffer.

The pooled IgG-fraction (66 mg) was dialyzed against phosphate-buffered saline (PBS; 0.1 M NaH₂PO₄, 150 mM NaCl, pH 7.2) and coupled to N-hydroxysuccinimide-activated Sepharose 4B fast flow (Amersham) according to the manufacturer's protocol. Resultant anti-SEA-Sepharose (7 mL) was packed into a column (20 × 0.5 cm) and washed with TBS (5 column volumes). PA-oligosaccharides were applied in 500 μ L TBS, followed by 1 h incubation at room temperature. After washing the column with 50 mL of TBS, bound PA-oligosaccharides were eluted with 50 mL of 100 mM triethylamine, pH 11.5, containing 150 mM NaCl. Unbound glycans were re-loaded onto the column 5 times. Obtained flow-through and eluate fractions were collected, freeze-dried, and applied separately to a 25-mg porous graphitic-carbon cartridge (Thermoquest, Kleinostheim, Germany) for desalting. The cartridge was washed with 10 mL of water and PA-oligosaccharides were eluted with 5 mL of 25% (by vol.) aqueous acetonitrile. Samples were dried in a speed-vac concentrator.

Lectin affinity chromatography

Serologically cross-reacting PA-glycans were subfractionated by AAL affinity chromatography as described elsewhere (Geyer et al. 2005). Unbound glycans and oligosaccharides eluted with 1 mM fucose in PBS were collected and desalted as described above.

HPLC fractionation of PA-glycans

PA-glycans were fractionated at 40 °C by HPLC using a TSK-Amide-80 column (4 × 250 mm; Tosoh Bioscience, Montgomeryville, PA) at a flow rate of 1 mL/min and monitored by fluorescence detection at 320/400 nm (Hase 1994). The column was equilibrated with 3% (by vol.) aqueous triethylamine/acetic acid, pH 7.3 in acetonitrile (35:65, by vol.). After sample injection, elution was performed applying a gradient of 0–100 % of 3% aqueous triethylamine/acetic acid, pH 7.3 in acetonitrile (50:50) within 50 min. Fractions of 1 mL (AAL-unbound carbohydrates) or 0.5 mL (AAL-bound glycans) were collected.

Mass spectrometry

MALDI-MS was performed on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a LIFT-MS/MS facility as described recently (Geyer et al. 2005). The instrument was operated in the positive-ion reflectron mode using 6-aza-2-thiothymine (Sigma) as matrix. Fragment ion analyses by MS/MS after LID or high-energy CID were performed as detailed earlier (Wuhrer et al. 2004; Geyer et al. 2005). External mass calibration was performed by utilizing $[M + H]^+$ ions of PA-isomaltosyl oligosaccharides.

For nano-LC-ESI-IT-MS, N-glycans were separated on a nano-scale Amide-80 column (5 µm, 80 Å; 75 µm × 150 mm; Tosoh, Montgomeryville, PA) as outlined previously (Geyer et al. 2005). The system was directly coupled with Esquire 3000 ESI-IT-MS (Bruker) equipped with an online nano-spray source operating in the positive-ion mode. For electrospray (900–1200 V), capillaries (360 µm OD, 20 µm ID with 10 µm opening) from New Objective (Cambridge, MA) were used. The solvent was evaporated at 180 °C with a nitrogen stream of 6 L/min. Ions from m/z 100 to m/z 3000 were registered.

Interpretation of MS spectra was assisted by using “Glyco-Peakfinder”. Structure plots in the notation of the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>) were generated with the visual editor of “Glycoworkbench”. Both software applications are developed and available as part of the EUROCarbDB project (<http://www.eurocarbodb.org/applications/ms-tools>).

Monosaccharide composition and linkage analysis

For constituent analyses, samples were hydrolyzed in 250 µL 4 M aqueous trifluoroacetic acid (100 °C, 4 h). After removing the trifluoroacetic acid by vacuum centrifugation, released monosaccharides were analyzed as alditol acetates by capillary GC or GC–MS using flame-ionization or electron impact detection, respectively. Monosaccharide constituents, including 3-*O*-MeMan and 3-*O*-MeGal, were identified by co-chromatography with the respective (partially methylated) alditol acetate standards (Supplementary Figure 1) as detailed elsewhere (Geyer et al. 1982). For linkage analyses, PA-oligosaccharides were permethylated using either methyl iodide (CH₃I) or trideuteromethyl iodide (C²H₃I) and hydrolyzed. Partially methylated alditol acetates, obtained after sodium borohydride or sodium borodeuteride reduction and peracetylation, were analyzed by capillary GC–MS in the chemical ionization as well as by electron impact

ionization mode using the instrumentation and microtechniques described elsewhere (Geyer et al. 1983; Geyer and Geyer 1994).

HF treatment

Aliquots of tryptic glycopeptides were chemically defucosylated (Haslam et al. 2000) by treatment with 48% HF solution (16 h at 4 °C) in the dark. HF was removed by a stream of nitrogen. Residual samples were taken up twice with 100 µL methanol and dried again in order to ensure complete HF removal.

Supplementary material

Supplementary data are available at *Glycobiology* online (<http://glycob.oxfordjournals.org/>).

Acknowledgments

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Conflict of interest statement

None declared.

Abbreviations

AAL, *Aleuria aurantia* lectin; BSA, bovine serum albumin; CID, collision-induced dissociation; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexNAc, *N*-acetylhexosamine; HF, hydrogen fluoride; HPLC, high-performance liquid chromatography; IT, ion trap; KLH, keyhole limpet hemocyanin; lacdiNAc, GalNAc(β1-4)GlcNAc; LID, laser-induced dissociation; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption/ionization; Man, mannose; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PA, pyridylamine (2-aminopyridine); PBS, phosphate-buffered saline; PNGase A, peptide *N*-glycosidase A; PNGase F, peptide *N*-glycosidase F; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEA, soluble egg antigen; TBS, Tris-buffered saline; Xyl, xylose.

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SUPPLEMENTAL DATA

Figure S1. Monosaccharide constituent analysis of total PA-glycans obtained from *B. glabrata* hemolymph glycoproteins. Alditol acetates and mono-*O*-methylated alditol acetates obtained after hydrolysis, sodium borohydride reduction and peracetylation were analyzed by capillary GC/MS and identified by co-chromatography with authentic alditol acetate standards. The linkage positions of the methyl groups present in 3-*O*-methyl-mannose and 3-*O*-methyl-galactose were verified by electron impact mass spectrometry after reduction with sodium borodeuteride (see Figure 3A). Registered glucose derivatives (marked by asterisk) are referred to glucose-containing contaminants.

Figure S2. MALDI-MS of cross-reacting PA-glycans obtained by immunoaffinity chromatography. The given mass spectrum displays enlarged mass range details (m/z 1500-2550) of the spectrum shown in Figure 2C. Sodiated pseudomolecular ions $[M + Na]^+$ were registered with isotopic resolution. Respective monosaccharide compositions are assigned in Table I.

Figure S3. Immunoaffinity chromatography of a plant-derived standard oligosaccharide. $\text{Man}(\alpha 1-6)[\text{Xyl}(\beta 1-2)]\text{Man}(\beta 1-4)\text{GlcNAc}(\beta 1-4)[\text{Fuc}(\alpha 1-3)]\text{GlcNAc}$ (MUXF³) from pineapple stem bromelain was subjected before (A-C) and after HF treatment (D-F) to immunoaffinity chromatography using immobilized anti-SEA antibodies. Starting material (A,D), unbound material obtained in the flow-through of the column (B,E) and glycans recovered in the immunoaffinity eluate (C,F) were analyzed by MALDI-MS employing similar aliquots of flow-through and bound fractions. Sodium and potassium adducts ($[M + Na]^+$ and $[M + K]^+$) were registered with isotopic resolution. The remaining signals at m/z 1049.9 and 1065.9 in

(D) and (F) are due to an incomplete removal of (α 1-3)-linked fucose. The absence of the defucosylated compound (MUX) at m/z 903.7 and 919.7 in the flow-through (E) and its presence in the immunoaffinity eluate (F) unambiguously demonstrate that xylose attached in (β 1-2)-linkage to the central core mannose is sufficient for recognition by anti-SEA antibodies. The observation that fucosylated glycans were also obtained in the flow-through (B) might indicate a weaker binding due to sterical hindrance by the (α 1-3)-linked fucosyl residue or overloading of the immunoaffinity column. Key symbols used for constituent monosaccharides: $\bigcirc$ = Man, $\blacksquare$ = GlcNAc, $\triangle$ = Fuc, $\star$ = Xyl.

Figure S4. Analysis of cross-reacting MeHex₁Hex₂HexNAc₂Xyl₁Fuc₁-PA species by normal phase-nano-LC-ESI-IT-MS/MS. (A), Assignment of fragments obtained upon cleavage of glycosidic bonds. (B), Postulated ring fragments obtained in the MS³ spectrum of the B₃ ion. (C), MS³ spectrum of the B₃ ion (m/z 858.0) obtained from the sodiated pseudomolecular ion [M + Na]⁺ (m/z 1303.8) by an additional ion isolation/fragmentation cycle. In spectra recorded at other time intervals a weak signal at m/z 275.2 (^{0,3}A₂) has been also detected. Fragments are assigned using the nomenclature of Domon and Costello (Domon and Costello, 1988). It has to be noted, however, that some signals may also originate from other fragmentation pathways which are not indicated for the sake of clarity. H, hexose; MeH, methyl-hexose; N, *N*-acetylhexosamine; F, fucose; X, xylose.

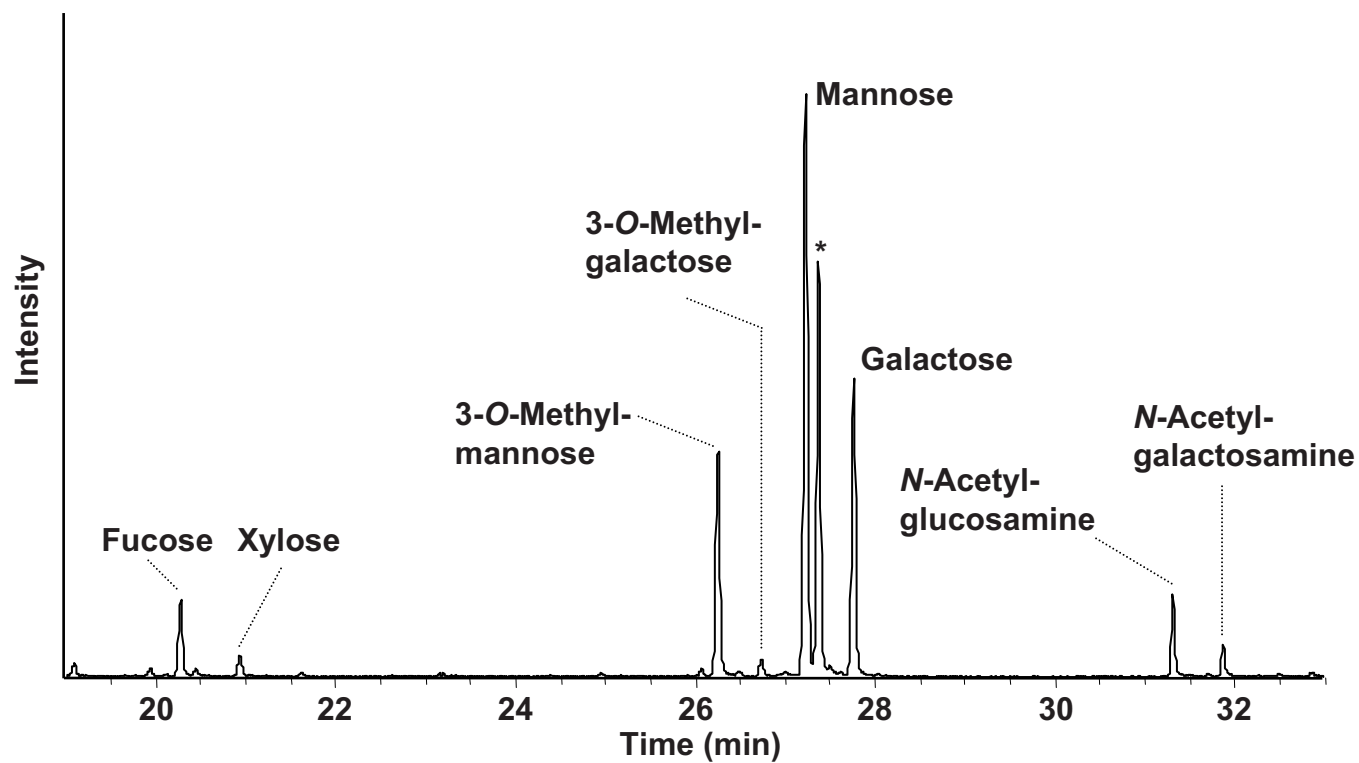


Figure S1

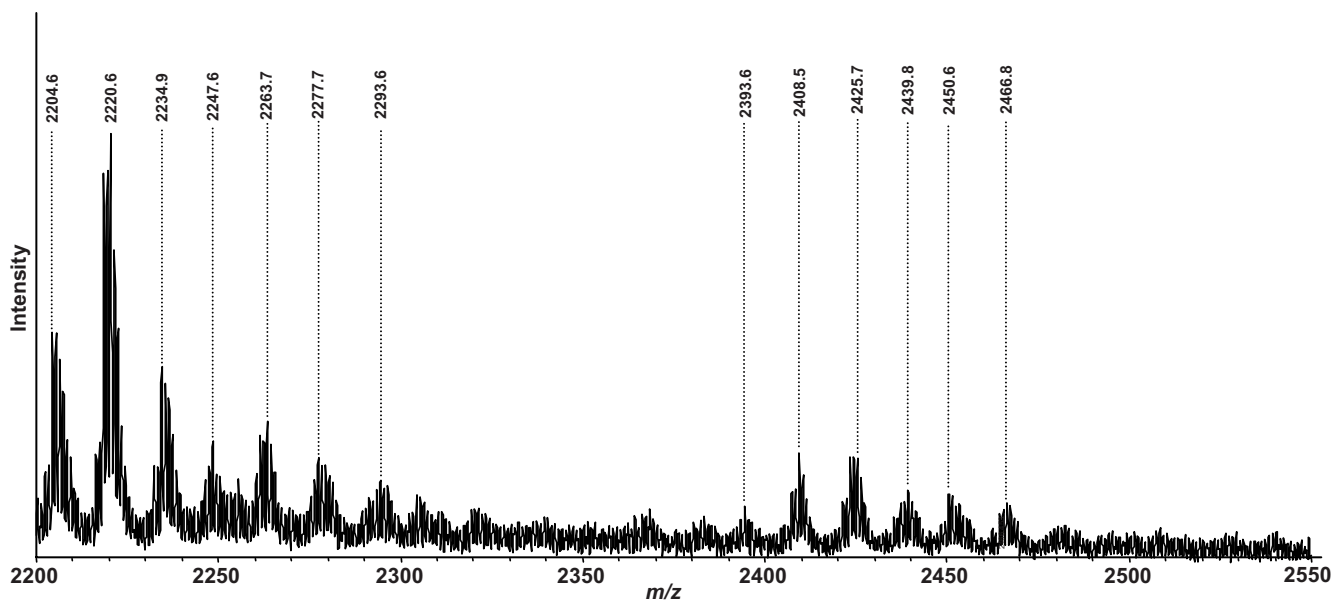
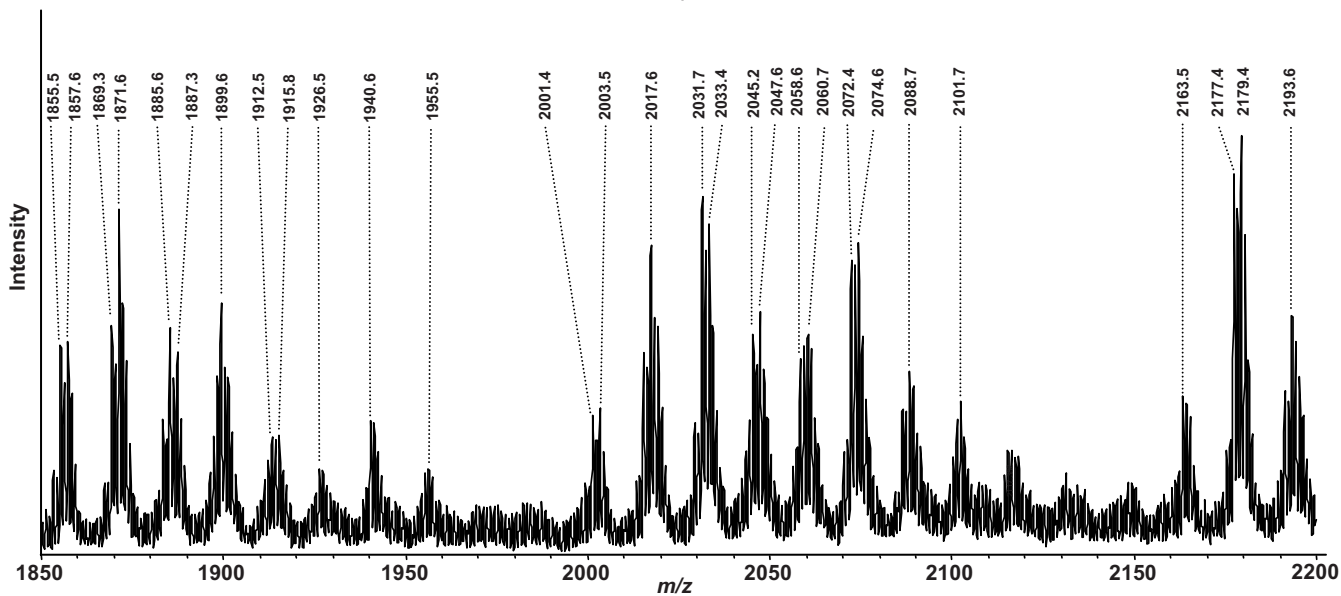
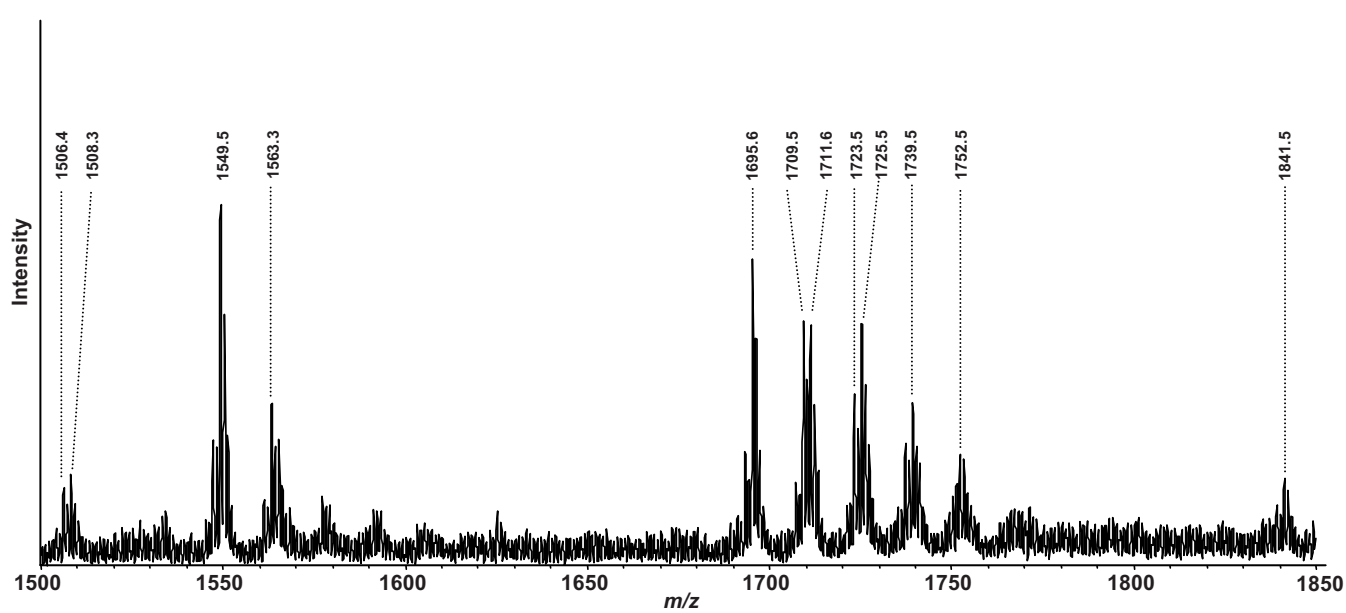


Figure S2

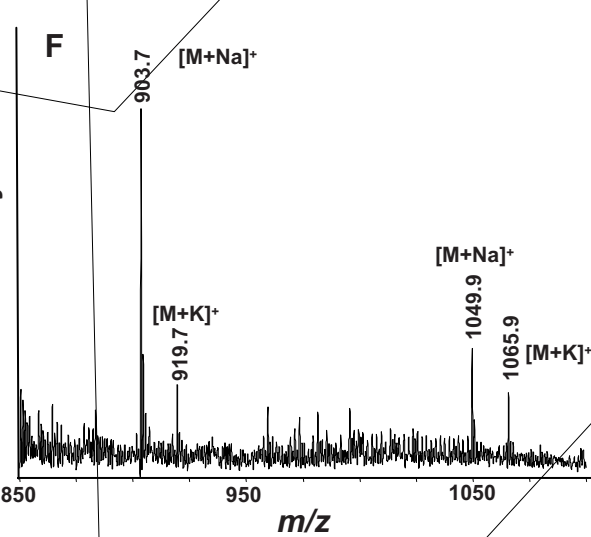
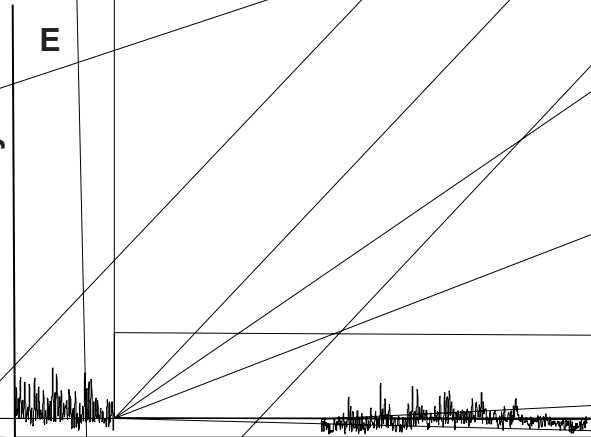
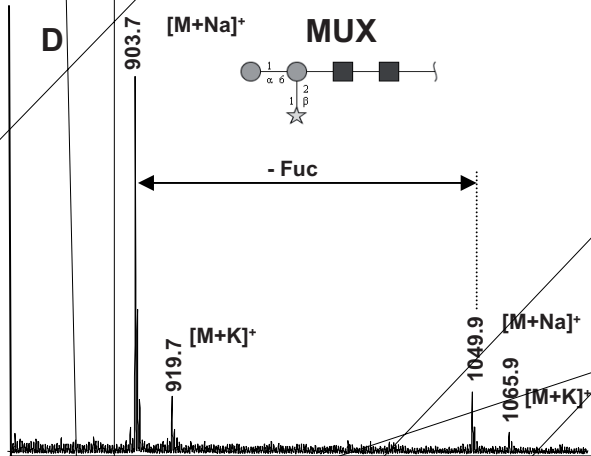
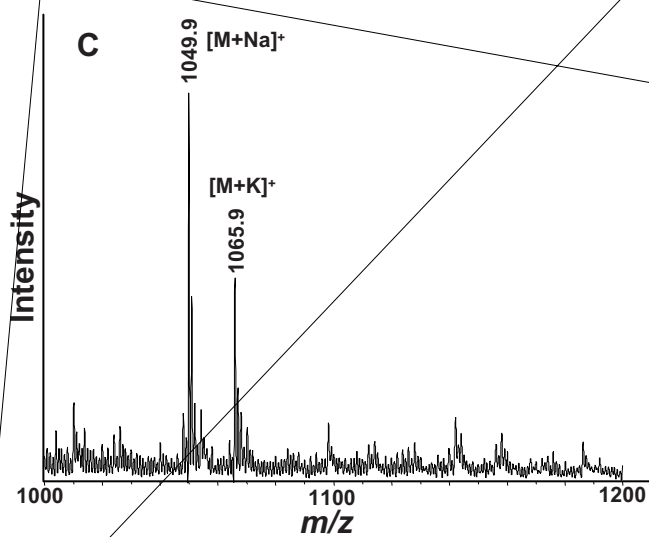
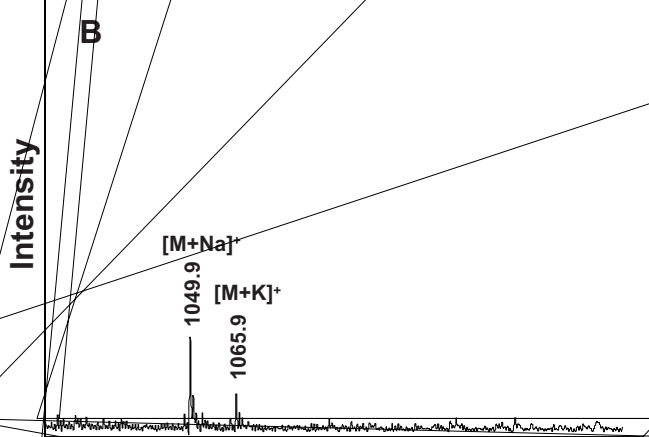
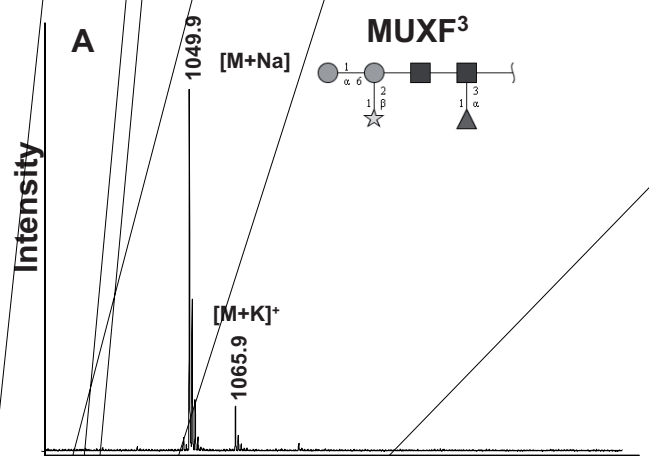


Figure S3

