

Structural Characterization of Glycans on Omega-1, a Major *Schistosoma mansoni* Egg Glycoprotein That Drives Th2 Responses

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Received January 27, 2010

Soluble egg antigens (SEA) of the human parasite *Schistosoma mansoni* are among the strongest natural stimuli of Th2 responses. Omega-1, a major glycoprotein in SEA, initiates these characteristic Th2 responses through conditioning of dendritic cells (DCs). In view of the reported immunomodulatory potential of SEA glycans, we have investigated omega-1 glycosylation, using an approach combining mass spectrometric techniques and enzyme treatments at the glycopeptide level. We demonstrate that omega-1 has two fully occupied N-glycosylation sites, each mainly carrying core-difucosylated diantennary glycans with one or more Lewis X motifs in the antennae. Using a specific approach of nanoscale LC–MS(/MS) and MALDI-TOF(/TOF) MS in combination with exoglycosidase treatments of tryptic glycopeptides, we were able to provide a detailed, site-specific glycosylation analysis of a single, native *S. mansoni* glycoprotein. The obtained knowledge of the glycans present on omega-1 contributes to a full understanding of the mode of action of this immunomodulatory glycoprotein.

Keywords: *Schistosoma mansoni* • glycosylation • omega-1 • Lewis X

Introduction

Schistosomiasis, a disease caused by infection with parasitic flatworms, is a major public health burden in many developing countries in the tropics and subtropics. Schistosome larvae released from infested water snails penetrate through the skin of the host, and the developing schistosomes migrate toward the mesenteric venules of the bowel (*Schistosoma mansoni* and *S. japonicum*) or the venous plexus of the bladder (*S. hematobium*), where adult female and male worms pair and produce hundreds of eggs per day. Instead of being excreted with the feces or urine to continue the schistosome life cycle, many of the eggs get trapped inside organs where they induce granulomatous immune reactions, the main cause of morbidity associated with schistosomiasis.¹

During *S. mansoni* infection, an initial T-helper 1 cell (Th1)-mediated response to the parasite shifts to a highly Th2-polarized immune response. This shift coincides with the start of egg production by the mature worms, suggesting that egg-derived molecules are capable of inducing the Th2-polarization.² Indeed, schistosome eggs,³ soluble egg antigens (SEA),^{4,5} and more specifically a subfraction of SEA formed by the excretory/secretory (ES) egg proteins⁶ are highly potent inducers of a Th2 immune response. Recently, the major single factor

from ES that is responsible for Th2-priming *in vitro* and *in vivo* has been identified as omega-1, a glycoprotein with RNase activity and a major constituent of ES.^{7,8} *In vitro*, omega-1 conditions human monocyte-derived dendritic cells (DCs) to drive Th2-polarization, while SEA depleted from omega-1 has lost this ability.^{6,9} Furthermore, when injected in IL-4 dual reporter mice, omega-1 is sufficient for Th2-induction *in vivo*. Although it has been suggested that the RNase activity of omega-1 may be involved in its capacity to drive Th2-polarization,⁹ total SEA requires intact glycosylation to be able to induce Th2-type responses including antigen-specific IgE production and induction of IL-4 and IL-10.⁵ Moreover, synthetic conjugates of the Lewis X element occurring in SEA have been shown to harbor Th2-promoting properties.^{10,11} These observations suggest that glycans or glycan motifs carried by SEA glycoproteins such as omega-1 play a role in the mechanisms by which they exert their Th2-inducing activity.

Schistosome egg glycosylation has been studied extensively, mainly by mass spectrometric analysis of released N- and O-glycans. A wide spectrum of complex and partly stage-specific structures has been found.^{12–15} Glycoprotein glycans from SEA comprise high mannose, complex-type and truncated N-glycans which are predominantly α 3/ α 6-difucosylated at the Asn-linked GlcNAc, potentially accompanied by a core-xylose.¹² The nonreducing N-glycan termini are for a large part composed of Gal β 1–4GlcNAc (LacNAc, LN), Gal β 1–4(Fuca1–3)GlcNAc (Lewis X) and (mono or difucosylated) GalNAc β 1–4GlcNAc (LacdiNAc, LDN) motifs.^{12,13} Egg glycoprotein O-glycans are mainly based on the mucin-type core 1 (Gal β 1–3GalNAc) and

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core 2 (Gal β 1–3(GlcNAc β 1–6)GalNAc) structures, but also a *S. mansoni*-specific Gal β 1–3(Gal β 1–6)-GalNAc core was found,^{12,14} while O-glycan termini were mainly composed of Lewis X, LN and (multifucosylated) LDN motifs. Recently, the glycans released from the ES fraction of *S. mansoni* eggs were analyzed, showing that these display N-glycan α 3/ α 6-core fucosylation and terminal LN, Lewis X and (fucosylated) LDN motifs as main features.¹⁵

Although it is currently debated exactly which proteins make up the ES fraction^{16–18} of *S. mansoni* eggs, it is clear that multiple N- and O-glycosylated proteins are present.¹⁶ The presence or absence of specific glycans may confer antigenic properties to individual proteins in ES and influence binding to C-type lectins on immune cells, such as dendritic cell-specific intercellular adhesion molecule-2-grabbing nonintegrin (DC-SIGN), mannose receptor (MR) or macrophage galactose-type lectin (MGL).^{19–22} Therefore, it would be appropriate to analyze the glycosylation of ES in a protein-specific fashion, instead of by releasing glycans from the protein mixture and thus losing crucial information about how individual proteins are glycosylated. To date, only in the case of IPSE/ α -1,²³ another major component of ES, has a protein-specific in-depth glycosylation analysis been carried out.²⁴

In view of the immunomodulatory properties of omega-1 and the lack of structural information about omega-1 glycans, we performed a detailed analysis of omega-1 glycosylation using nanoscale LC–MS(/MS), and MALDI-TOF(/TOF) MS measurements of tryptic glycopeptides in combination with exoglycosidase treatments. We show that omega-1 has two fully occupied N-glycosylation sites, which carry mainly core-difucosylated diantennary glycans with one or two terminal Lewis X motifs. These data provide a molecular basis for investigations on functional properties of omega-1 glycosylation and shed light on the structural details of schistosome egg glycan antigens.

Material and Methods

Purification of Omega-1. Omega-1 was purified from SEA as previously described.^{7,23,25} In short, SEA was prepared from *Schistosoma mansoni* eggs and fractionated by cation exchange chromatography. Omega-1, together with IPSE/ α -1 a constituent of the highly cationic egg fraction CEF6, was separated from IPSE/ α -1 by affinity chromatography using specific anti-IPSE/ α -1 monoclonal antibodies coupled to an NHS-HiTrap Sepharose column according to the manufacturer's instructions (GE Healthcare, Little Chalfont, UK).

α -Fucosidase Treatment for Western Blot Analysis. Antigens were dissolved in 10 μ L of 100 mM sodium phosphate buffer, pH 5.0 and treated with α -1-(3,4)-fucosidase from *Xanthomonas manihotis* (0.5 mU; Sigma, Zwijndrecht, The Netherlands) overnight at 37 °C.

To confirm removal of fucoses, omega-1 was reduced by incubation with 0.025 volumes of 200 mM dithiothreitol for 30 min at 60 °C, after which the antigen solution was mixed with 0.075 volumes of 200 mM iodoacetamide and incubated for 30 min in the dark at room temperature. Trypsin (Promega, Leiden, The Netherlands) was added to the sample at a 1:100 trypsin/antigen ratio and incubated overnight at 37 °C. After incubation, the digestion was quenched with 1 μ L of 5% trifluoroacetic acid. Tryptic digests were measured using MALDI-TOF-mass analysis in the positive ion reflectron mode (results not shown).

Western Blot Analysis. One μ g of untreated and fucosidase-treated omega-1 was subjected to SDS-PAGE under reducing conditions on a 12% gel using the Mini-Protein Cell system (Biorad, Veenendaal, The Netherlands). The proteins were transferred onto a nitrocellulose membrane in a Biorad Criterion Blotter system according to manufacturer's instructions. The protein blots were stained as described previously.²⁶ In short, blots were blocked with BSA and incubated with the primary anticarbohydrate mAb 128–4F9-A, which binds to Lewis X antennae. Blots were subsequently incubated with AP-labeled goat-antimouse IgG (Caltag; Invitrogen, Breda, The Netherlands) and stained with NBT/BCIP.

Sample Preparation for Mass Spectrometric Analysis. Ten micrograms of omega-1 were separated on a 12% polyacrylamide gel by SDS-PAGE electrophoresis under reducing conditions using the Mini-Protein Cell system (Biorad) and stained with Colloidal blue (Invitrogen, Groningen, The Netherlands) according to manufacturer's recommendations. The stained omega-1 band (31 kD) was excised and stored in 1% acetic acid at 4 °C. The gel piece containing omega-1 was reduced, alkylated and digested with 0.015 μ g trypsin (Promega), as previously described.²⁷ After digestion, peptides were extracted twice with 20 μ L of 0.1% TFA. Extracts were pooled and stored at –20 °C.

Exoglycosidase Treatment for Mass Spectrometric Analysis. Tryptic glycopeptides were treated with β -galactosidase from bovine testis (1 mU; Sigma, Zwijndrecht, The Netherlands) in 10 μ L 100 mM sodium phosphate buffer, pH 5.0, for 24 h at 37 °C. α -Fucosidase treatment was performed on tryptic glycopeptides with α 1-(3,4)-Fucosidase from *Xanthomonas manihotis* (0.5 mU; Sigma) in 100 mM sodium phosphate buffer, pH 5.0, for 24 h at 37 °C. Part of the α -fucosidase-treated glycopeptides was subsequently treated with β -galactosidase for 24 h at 37 °C.

MALDI-TOF(/TOF) MS. Total tryptic digest of omega-1, as well as glycopeptides after β -galactosidase and/or α -fucosidase treatment, were purified by Zip-Tip μ -C18 following the manufacturer's instructions (Millipore, Billerica, MA), eluted with 10 mg mL^{–1} DHB in 50% acetonitrile, 0.1% trifluoroacetic acid onto a MALDI target plate and air-dried. MALDI-TOF(/TOF) MS data were obtained using an Ultraflex II time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) equipped with a LIFT-MS/MS facility. Spectra were acquired in the positive ion reflectron mode. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by metastable decomposition of the precursor in the field-free drift region were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after the ion reflector passage. MS/MS spectra with fragment ions showing a high mass deviation were subjected to internal calibration using chitobiose cleavage products as calibrant signals.

Nano-HPLC–ESI-ion trap-MS(/MS). Total tryptic digest of omega-1, as well as glycopeptides after β -galactosidase treatment, were applied to a reverse-phase column (PepMap, 3 μ m, 75 μ m $\times$ 100 mm; Dionex /LC Packings, Amsterdam, The Netherlands) using an Ultimate 3000 nano-LC system (Dionex /LC Packings). The column was equilibrated at room temperature with eluent A (0.1% formic acid in water) at a flow rate of 200 nL min^{–1}. After injection of the sample, elution conditions were switched to 10% solvent B (95% acetonitrile, 0.1% formic acid),

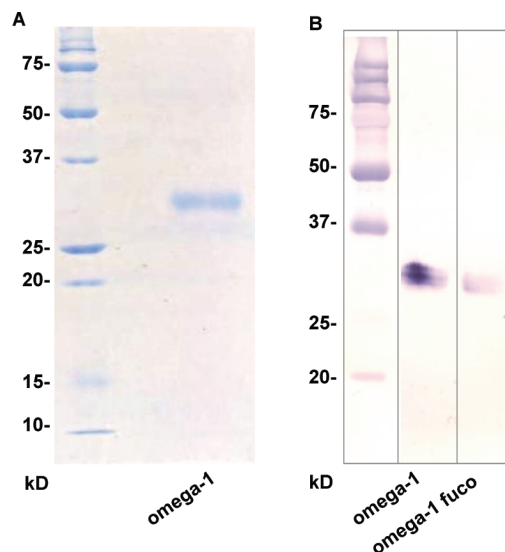


Figure 1. Visualization and characterization of omega-1. (A) Omega-1 was separated under reducing conditions by SDS-PAGE and stained with Colloidal blue. (B) For testing the presence of terminal Lewis X, omega-1 and omega-1 treated with α 1-3,4 fucosidase (Omega-1 fuco) were subjected to Western blot using a monoclonal antibody against Lewis X (128–4F9-A).

followed by a gradient to 60% B in 45 min and a subsequent isocratic elution of 10 min. The eluate was monitored by absorption at 215 nm.

The LC column was coupled to an Esquire HCT-Ultra ESI-ion trap-MS (Bruker-Daltonics, Bremen, Germany) equipped with an online nanospray source operating in the positive-ion mode. For electrospray (1100–1250 V), electropolished, stainless steel LC–MS emitters (150 μ m OD, 30 μ m ID) from Proxeon A/S (Odense, Denmark) were used. The solvent was evaporated at 175 °C employing a nitrogen stream of 7 L min^{−1}. Ions from m/z 500 to m/z 1800 were registered in the MS mode. When operated in the auto MS/MS mode, registering ions from m/z 140 to 2200, each MS scan was followed by the acquisition of MS/MS spectra of up to three of the most abundant ions in the MS spectrum.

Results

Initial Analysis of Omega-1 Glycosylation. Omega-1 is produced in the subshell area of *S. mansoni* eggs where also IPSE/alpha-1 is produced.^{8,24} To investigate whether omega-1, like IPSE/alpha-1, carries Lewis X motifs,²⁴ purified omega-1 (Figure 1A) was subjected to Western blot analysis. The anti-Lewis X mAb 128–4F9-A binds to omega-1 (Figure 1B), demonstrating the presence of Lewis X. Treatment of omega-1 with α -fucosidase to remove terminal α 1-3,4-linked fucoses (omega-1 fuco, Figure 1B) strongly reduces binding of the antibody, confirming the presence of the fucosylated Lewis X motif on omega-1.

For subsequent in depth glycosylation analysis of omega-1, the purified glycoprotein was subjected to SDS-PAGE and the gel piece containing omega-1 was cut, reduced, alkylated and treated with trypsin to generate (glyco)peptides. Extracted (glyco)peptides were subjected to nano-RP-LC–MS analysis. Omega-1 has two consensus glycosylation sites at residues N71 (tryptic peptide Q69-R77) and N176 (tryptic peptide E172-R183).⁸ In the LC chromatogram, based on the observation of the glycosylation marker ions at m/z 366 (LN antenna) and m/z

512 (Lewis X antenna), three glycopeptide regions were registered at time windows 7.4–9.3, 14.3–16.9 and 19.3–20.9 min (data not shown). The first and second glycopeptide cluster are indistinguishable in terms of glycan heterogeneity and both arise from peptide Q69-R77. Evidently, the glutamine at the amino terminus of the second cluster had undergone cyclization, forming a pyroglutamate, leading to a mass shift of $m/z - 5.3$ [$M + 3H$]³⁺ compared to the glycopeptide cluster with native N-terminal glutamine and a shift in elution time due to an increase in hydrophobicity upon cyclization. The nano-LC–mass spectrum belonging to the first cluster (site N71) is shown in Figure 2A. The nano-LC–mass spectrum of tryptic glycopeptides containing site N176 which eluted at 19.3–20.9 min is shown in Figure 2B.

The glycans of both glycosylation sites are composed of variable numbers of hexoses (H), *N*-acetylhexosamines (N) and deoxyhexoses (F), as reflected by corresponding mass differences (162, 203, and 146 Da, respectively) between the registered glycopeptides. For glycopeptides containing site N71 (Figure 2A), the assumption of a Q69-R77 peptide moiety decorated with an N-glycan leads for the major glycopeptide species ([$M + 3H$]³⁺ at m/z 1080.5) to the deduced glycan composition H₅N₄F₄. This composition may represent an N-glycan with two Lewis X antennae and core-difucosylation, which has also been found on IPSE/alpha-1.^{15,24} Other major signals include N-glycans composed of H₅N₄ and H₄N₅ substituted with two to six fucoses, suggesting a high abundance of multifucosylated LN and, to a lesser extent, LDN antennae. Tryptic glycopeptides covering site N176 (Figure 2B) were found to contain glycans which were similar in composition to those of site N71. However, ratios of glycan variants were significantly different for the two sites (Figure 2). In general, glycopeptides of site N176 showed a lower degree of fucosylation than those of site N71. Nonglycosylated forms of the peptides Q69-R77 and E172-R183 were not detected by LC–MS, indicating that in both peptides the N-glycosylation sites are fully occupied (data not shown).

N-Glycosylation Analysis of Glycopeptide E172-R183. To confirm the tentative assignment of the glycopeptide clusters presented in Figure 2, the tryptic glycopeptides were subjected to enzymatic treatment with β -galactosidase and/or α -fucosidase and analyzed by MALDI-TOF MS (Figures 3 and 4). Selected untreated and α -fucosidase treated glycopeptides were in addition subjected to fragmentation using MALDI-TOF/TOF MS to further substantiate the structural assignments. A set of illustrative glycopeptide fragmentation spectra is shown in Figures 5–7 and Supplementary Figures 1 and 2 (Supporting Information). For the other glycopeptides, the fragmentation data are summarized in Table 1 (untreated) and Supplementary Table 1 (α -fucosidase treated, Supporting Information).

First, we confirmed that the glycopeptides assigned to site N176 (Figure 2B, 3A, and 4A) comprised the peptide moiety E172-R183: for the glycopeptide at m/z 3594.7 (Figure 3A), a characteristic MALDI-TOF/TOF MS pattern of fragment ions was observed (Figure 5), including m/z 1370.2 (protonated peptide-NH₃), m/z 1470.2 (^{0,2}X-ring fragmentation of the innermost *N*-acetylglucosamine), m/z 1736.3 (protonated peptide with *N*-acetylglucosamine and one fucose) and m/z 1882.2 (protonated peptide with *N*-acetylglucosamine and two fucoses). This indicates a peptide mass of 1387.3 Da [$M + H$]⁺ and a glycan moiety of H₅N₄F₄. The deduced peptide mass of 1387.3 Da fits the theoretical mass of the tryptic peptide E172-R183 of 1388.6 Da [$M + H$]⁺. In addition, peptide fragments in

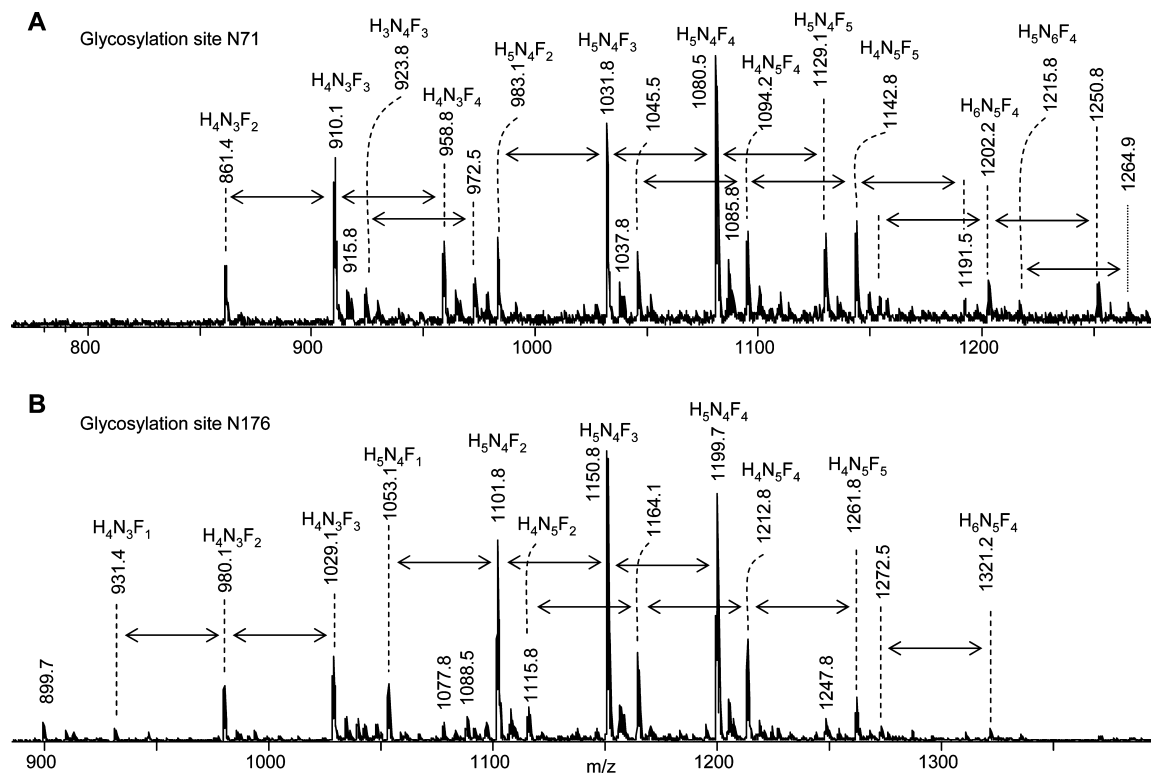


Figure 2. Nano-LC–MS of glycopeptides from a tryptic digest of omega-1, covering glycosylation sites (A) N71 and (B) N176. Signals are triple positively charged and labeled with monoisotopic masses. Compositions of the glycan moieties are given in terms of hexose (H), *N*-acetylhexosamine (N) and fucose (F). Differences in fucose content are indicated by double-headed arrows.

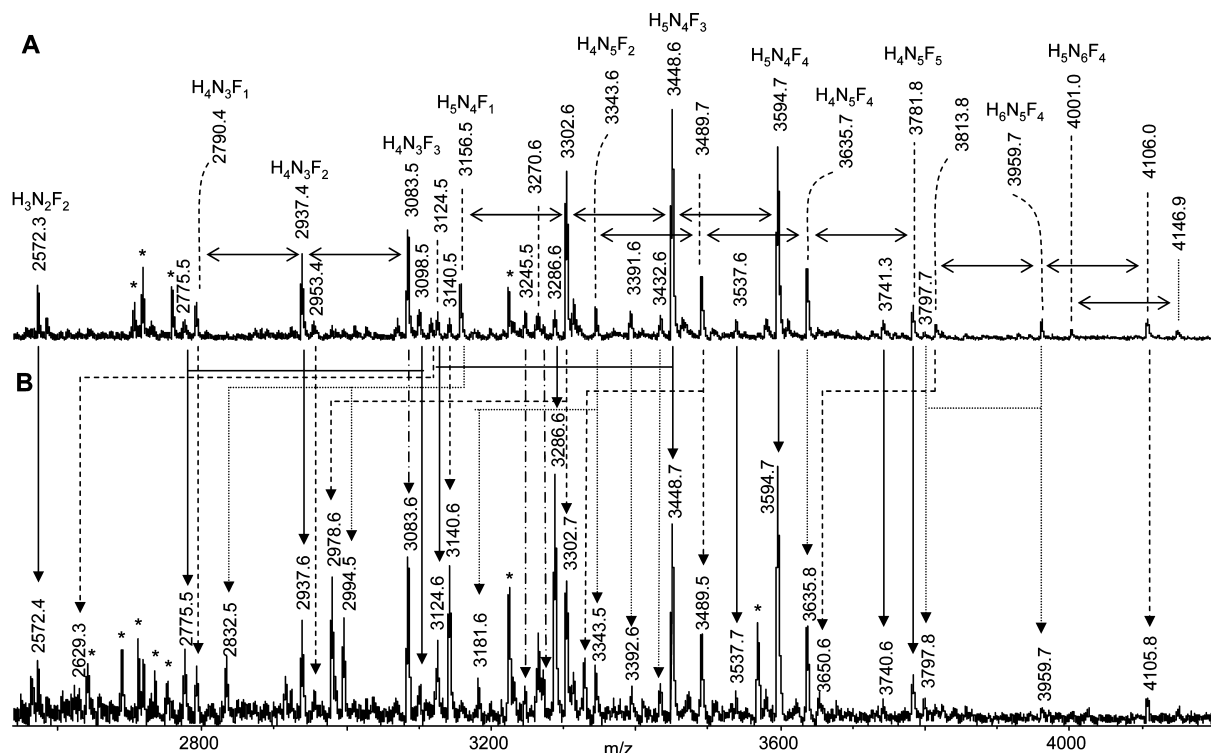


Figure 3. β -Galactosidase treatment of omega-1 glycopeptides. Omega-1 was subjected to reduction and alkylation, digested with trypsin and the resulting (glyco)peptides of glycosylation site N176 were analyzed by MALDI-TOF MS (A) before and (B) after treatment with β -galactosidase. Signals are singly positively charged and labeled with monoisotopic masses. Composition of the glycan moieties are given in terms of hexose (H), *N*-acetylhexosamine (N) and fucose (F). Differences in fucose content are indicated by double-headed arrows. Nonglycopeptide signals are marked with asterisks (*).

the low mass region provided a GSANCIR sequence tag that further confirms the assignment. For the other fragmented

glycopeptides belonging to the peptide moiety E172-R183 containing glycosylation site N176, similar signal patterns were

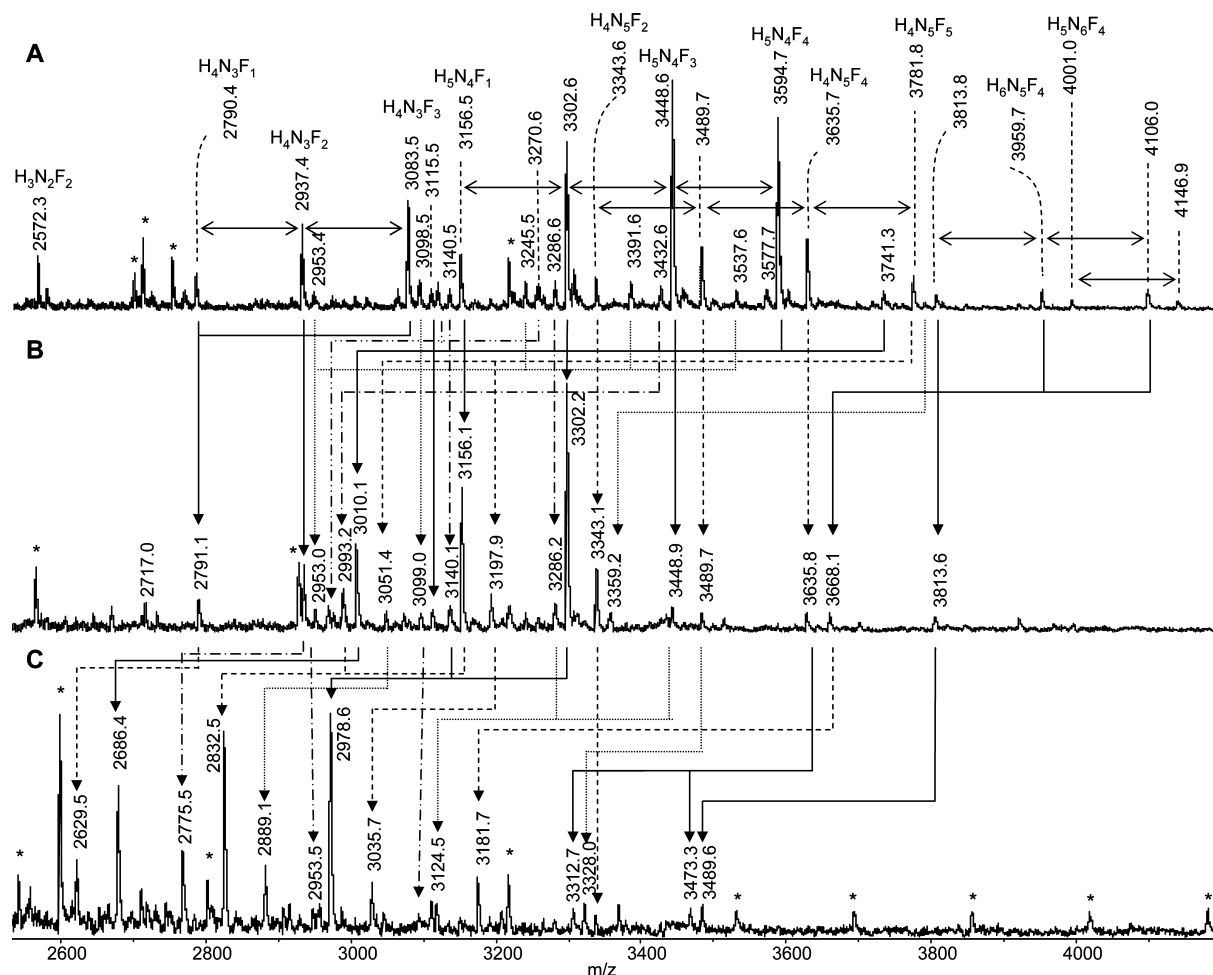


Figure 4. α -Fucosidase and β -galactosidase treatment of omega-1 glycopeptides. Omega-1 was subjected to reduction and alkylation, digested with trypsin and the resulting (glyco)peptides of glycosylation site N176 were analyzed by MALDI-TOF MS (A) before treatment, (B) after treatment with α -fucosidase only or (C) in combination with β -galactosidase. Signals are singly positively charged and labeled with monoisotopic masses. Composition of the glycan moieties are given in terms of hexose (H), *N*-acetylhexosamine (N) and fucose (F). Differences in fucose content are indicated by double-headed arrows. Nonglycopeptide signals are marked with asterisks (*).

observed (Figures 6 and 7, Supplementary Figures 1 and 2 and Table 1, Supporting Information).

The combined data from enzymatic treatments and MALDI-TOF/TOF MS analysis showed that $H_5N_4F_4$ on N176 is an N-glycan constructed of a trimannosyl core with two fucoses on the innermost *N*-acetylglucosamine and two fucosylated LN antennae. Based on the reactivity of omega-1 with an anti-Lewis X antibody (Figure 1B) and previously published data on the glycosylation of ES proteins including IPSE/alpha-1,^{15,24} the fucosylated LN antennae were interpreted as Lewis X motifs. Both enzyme treatments (Figures 3 and 4) and fragmentation data (Figure 5) are consistent with this interpretation. The galactose β 1–4 linkage in a Lewis X trisaccharide is not cleavable by β -galactosidase because of the 3-substitution of GlcNAc by fucose. In line with the assumption that the $H_5N_4F_4$ species contains two Lewis X antennae, β -galactosidase treatment did not lead to the disappearance of the signal at m/z 3594.7 and at the same time no increased signal at m/z 3432.6 was observed (Figures 3A and B). On the other hand, when the glycopeptides were treated with α -fucosidase from *Xanthomonas manihotis*, the fucoses in the Lewis X motif were removed (loss of signals at m/z 3594.7 ($H_5N_4F_4$) and m/z 3448.6 ($H_5N_4F_3$); Figure 4A and B), yielding a set of signals at m/z 3302.7, 3156.1, and 3010.1 from $H_5N_4F_2$, $H_5N_4F_1$ and H_5N_4

glycoforms, respectively (Figure 4B). Treatment with β -galactosidase subsequent to this fucosidase treatment leads to a complete removal of the antenna galactoses from each of the $H_5N_4F_2$, $H_5N_4F_1$ and H_5N_4 species (shift of two times 162 Da; e.g. the signal at m/z 3302.2 in Figure 4B shifts to m/z 2978.6 in Figure 4C). The resistance of the $H_5N_4F_4$ glycopeptide to β -galactosidase treatment and the gained susceptibility after α -fucosidase treatment further supports our proposal that the terminal fucosylated structures are Lewis X motifs. To corroborate this, a MALDI-TOF/TOF MS fragmentation spectrum of the E172-R183- $H_5N_4F_4$ glycopeptide was recorded (Figure 5) in which the losses of 511 and 673 Da (signals at m/z 3083.3 and 2920.7) from the parent ion are observed, corresponding to the loss of a Lewis X ($H_1N_1F_1$) and a Lewis X-Man fragment ($H_2N_1F_1$), respectively. In addition, a core cleavage pattern with an intense signal at m/z 1882.2 (peptide- N_1F_2) (Figure 5) is observed, indicating that two fucose residues in the $H_5N_4F_4$ species are linked to the core GlcNAc residue. The lower intensity signal at m/z 1736.3 (peptide- N_1F_1), is interpreted as the result of a chitobiose cleavage in combination with loss of a core fucose. This interpretation was further confirmed by MALDI-TOF/TOF MS of the E172-R183- $H_5N_4F_2$ glycopeptide species which is formed after the α -fucosidase treatment (Supplementary Figure 1, Supporting Information). In this

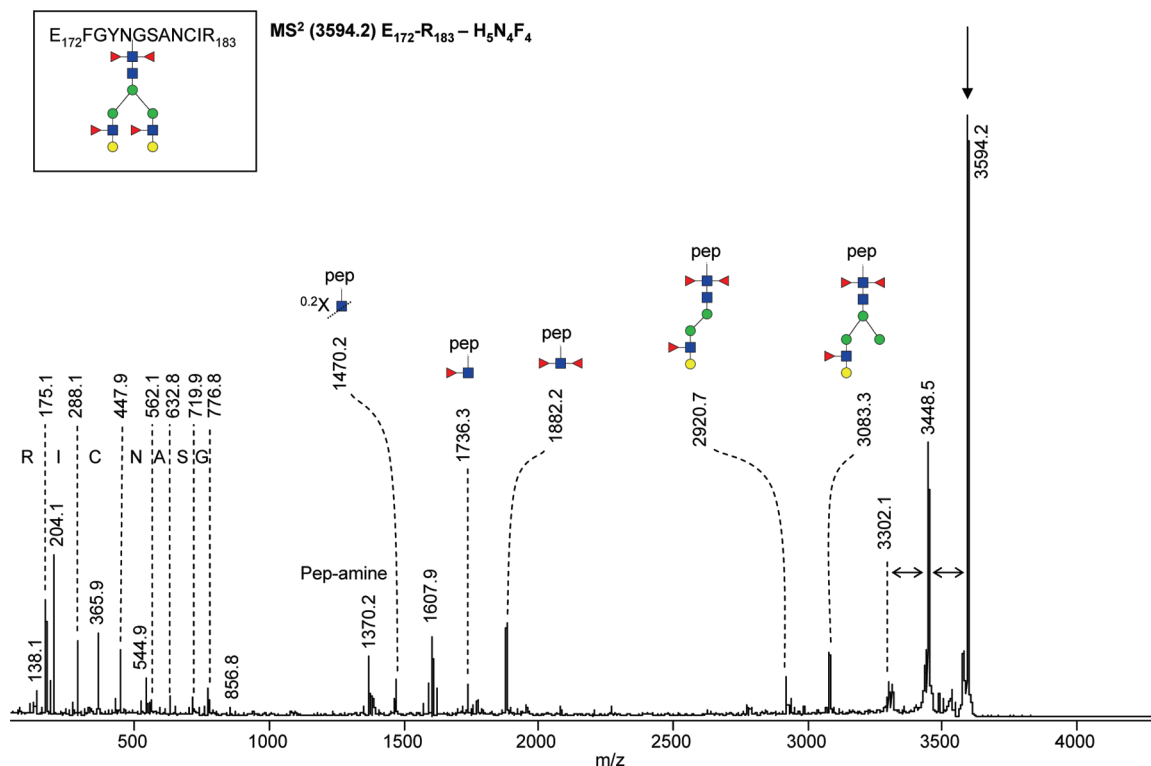


Figure 5. MS/MS of peptide E172-R183 carrying $H_5N_4F_4$ N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF MS of a tryptic digest of omega-1. The single-headed arrow indicates the precursor ion. Differences in fucose content are indicated by double-headed arrows. Red triangle, fucose; yellow circle, galactose; blue square, N-acetylglucosamine; green circle, mannose.

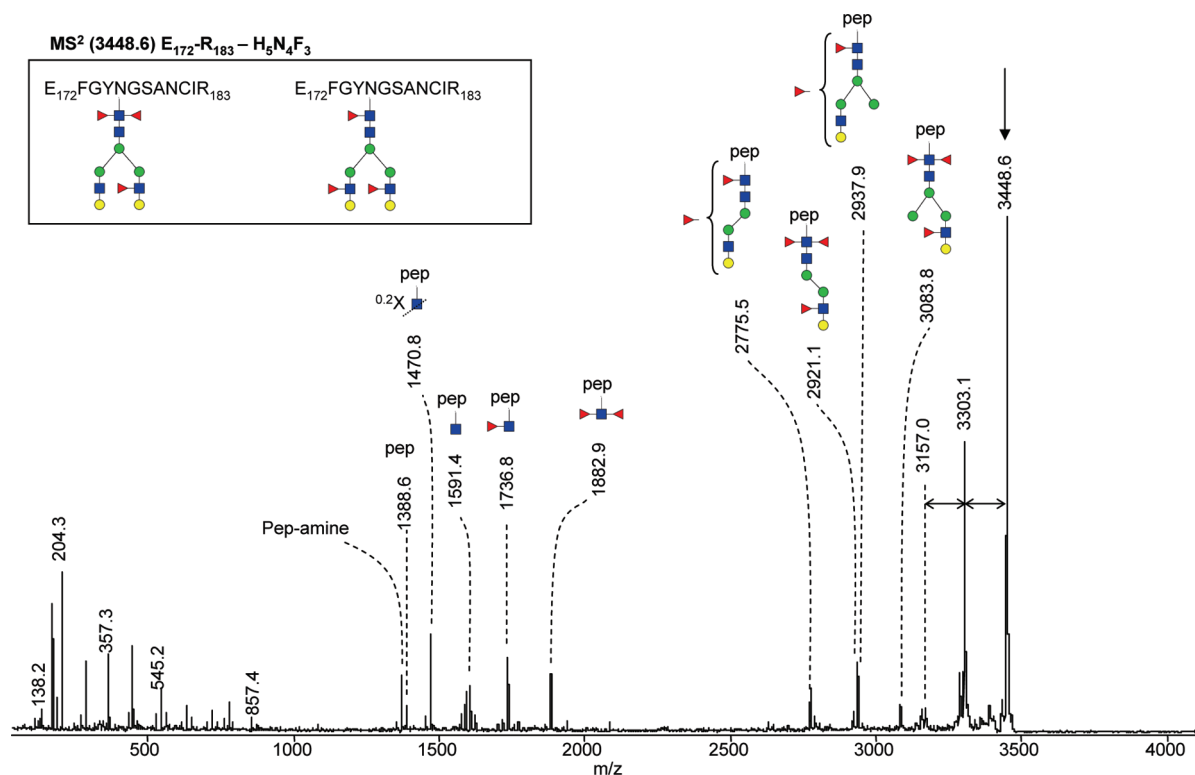


Figure 6. MS/MS of peptide E172-R183 carrying $H_5N_4F_3$ N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF MS of a tryptic digest of omega-1. The single-headed arrow indicates the parent ion. Differences in fucose content are indicated by double-headed arrows. Red triangle, fucose; yellow circle, galactose; blue square, N-acetylglucosamine; green circle, mannose.

spectrum, signals derived from antenna fragmentations are observed at m/z 2937.1 and 2774.9 (loss of H_1N_1 and H_2N_1 , respectively), but no loss of $H_1N_1F_1$ is observed, indicating that

all antenna fucoses had been removed. In addition, the core fragmentation pattern including an intense signal at m/z 1882.4 (peptide- N_1F_2) accompanied by a minor signal at m/z 1736.4

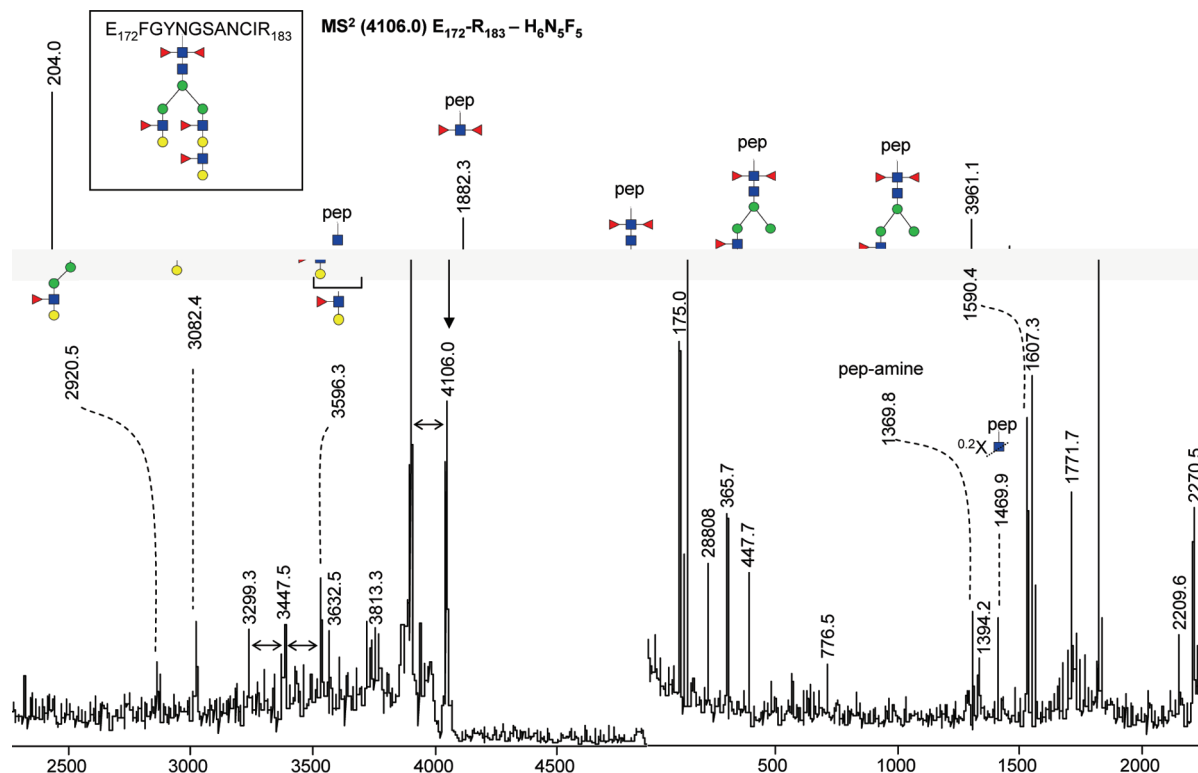


Figure 7. MS/MS of peptide E172-R183 carrying $H_6N_5F_5$ N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF MS of a tryptic digest of omega-1. The single-headed arrow indicates the parent ion. Differences in fucose content are indicated by double-headed arrows. Red triangle, fucose; yellow circle, galactose; blue square, *N*-acetylglucosamine; green circle, mannose.

(loss of one fucose from peptide- N_1F_2), a pattern highly similar to that present in Figure 5, indicates that two core fucoses remain.

A second abundant signal in the MALDI-TOF MS spectrum in Figures 3A/4A is that of glycopeptide E172-R183- $H_5N_4F_3$. Our data indicate that the $H_5N_4F_3$ species carry two LN antennae and comprise two differentially fucosylated isomers. A fraction of the E172-R183- $H_5N_4F_3$ glycopeptides is resistant to β -galactosidase treatment (part of the signal at m/z 3448.6 in Figure 3A remains in Figure 3B), which, as previously explained for $H_5N_4F_4$, indicates the presence of two Lewis X antennae. The other fraction of the glycopeptides exhibited the loss of one hexose after β -galactosidase treatment (loss of 162 Da, resulting in a signal at m/z 3286.6 in Figure 3B), which suggests the presence of one nonfucosylated and one fucosylated LN. Based on these results, we suggest that $H_5N_4F_3$ on site N176 consists of two isomeric glycans, one containing two Lewis X antennae and a monofucosylated core, while the other carries one Lewis X and one LN antenna and two core fucoses. The assumed presence of Lewis X and LN motifs on the isomers was verified using MALDI-TOF/TOF MS (Figure 6). The fragmentation spectrum reveals the loss of 511 and 673 Da (signals at m/z 2937.9 and 2775.5) from the parent ion, corresponding to the loss of a Lewis X ($H_1N_1F_1$) and a Lewis X-Man ($H_2N_1F_1$) moiety, respectively. Furthermore, the observed losses of 365 and 527 Da (signals at m/z 3083.8 and 2921.1) indicate the presence of LN (H_1N_1) and LN-Man (H_2N_1) antennae, respectively, in one of the isomers. In addition, a core cleavage pattern is observed (Figure 6) which clearly differs from the pattern characteristic of core-difucosylation (Figure 5 and Supplementary Figure 1, Supporting Information). In Figure 6, the signal at m/z 1736.8 (pep+ N_1F_1) is more intense than the signal at m/z 1882.9

(pep+ N_1F_2). By comparison with the core cleavage patterns in Figure 5 and Supplementary Figure 1 (Supporting Information), the pattern in Figure 6 can only partially be explained as resulting from an isomer with difucosylated core (left structure in Figure 6). The fragment ion at m/z 1736.8 (pep+ N_1F_1) is in addition formed by chitobiose cleavage of the second isomer having a monofucosylated core (right structure in Figure 6).

The composition of the $H_4N_5F_4$ variant on glycosylation site N176 indicates the presence of an N-glycan with two fucoses at the Asn-linked GlcNAc, combined with one Lewis X and one fucosylated LDN antenna, LDN being a common structural element in helminth glycans.²⁸ The $H_4N_5F_4$ fragmentation data (Supplementary Figure 2, Supporting Information) are in line with this interpretation. Signals derived from the loss of N_2F_1 (m/z 3081.8) and $H_1N_1F_1$ (m/z 3124.7) from the precursor ion are observed, as well as the characteristic core fragmentation pattern indicative of a difucosylated core GlcNAc. The mass spectrometric data do not allow differentiation between $Fuc\alpha 1-3GalNAc\beta 1-4GlcNAc(F-LDN)$ and $GalNAc\beta 1-4(Fuc\alpha 1-3)GlcNAc$ (LDN-F) structures. However, using synthetic F-LDN and LDN-F glycoconjugates,²⁹ the α -fucosidase from *Xanthomonas manihotis* was shown to cleave only the fucose $\alpha 1-3$ linked to the GlcNAc in LDN-F, but not the $\alpha 1-3$ linkage between the fucose and GalNAc in F-LDN (data not shown). α -Fucosidase treatment of omega-1 glycopeptides (Figure 4B) results in removal of antenna fucoses from $H_4N_5F_4$, as the signal at m/z 3635.7 in Figure 4A almost completely shifts to signals at m/z 3051.4, 3197.1 and 3343.1, representing glycans with one LN and one LDN motif and zero, one or two core fucoses, respectively (Supplementary Table 1, Supporting Information). Similar shifts were observed for other glycans that carry fucosylated LDN based on their monosaccharide composition

Table 1. Omega-1 Tryptic Glycopeptides

Glycan composition ^a	Glycopeptide signal (m/z) ^b	Relative peak height (%) ^c	Fragment ions ^d	Proposed structures ^e
Peptide E ₁₇₂ FGYNGSANCIR ₁₈₃ H ₂ NF ₂	2572.3 (β-gal; 2572.4; 0 Hex) 858.1 (M+3H) ⁺ ; 1286.4 (M+2H) ²⁺	3	1591.4 (pep ⁺ N ₁) 1882.9 (pep ⁺ N ₁ F ₁) ESI-MS/MS: 366.1 (H ₂ N); 528.2 (H ₂ N) ₂ 690.3 (H ₂ N) ₃	Trimannosyl core, ¹⁴ F
H ₂ NF ₂	2775.5 (β-gal; 2777.5; 0 Hex)	1		HexNAc antenna, ¹⁴ F
H ₂ NF ₂	2790.4 (β-gal; 2791.4; 0 Hex, and 2629.3; -1 Hex)	1	1390.6 (pep ⁺ N ₁) 1736.4 (pep ⁺ N ₁ F ₁) 2279.4 (pep ⁺ N ₁ H ₂) H ₂ NF ₂	Trimannosyl core, ¹⁴ F
H ₂ NF ₂	2937.4 (β-gal; 2937.6; 0 Hex, and 2775.5; 1 Hex); 979.8 (M+3H) ²⁺	6	1590.9 (pep ⁺ N ₁) 1737.9 (pep ⁺ N ₁ F ₁ ; 1.5) 1882.9 (pep ⁺ N ₁ F ₂ ; 1.5) 2265.0 (pep ⁺ H ₂ NF ₂) 2426.5 (pep ⁺ H ₂ NF ₂) 2572.3 (pep ⁺ H ₂ NF ₂) ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 868.8 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: 1 Lewis X, monofucosylated core; ant: 1 LN, ¹⁴ F
H ₂ NF ₂	2953.4 (α-fuc; 2953.0; 0 Fuc)	1		1 LN, mono-fucosylated core
H ₂ NF ₂	3083.5 (β-gal; 3083.6; 0 Hex); 1028.5 (M+3H) ²⁺	7	1591.4 (pep ⁺ N ₁) 1737.2 (pep ⁺ N ₁ F ₁ ; 0.33) 1883.3 (pep ⁺ N ₁ F ₂ ; 1.0) 2410.4 (pep ⁺ H ₂ NF ₂) 2426.5 (pep ⁺ H ₂ NF ₂) 2572.3 (pep ⁺ H ₂ NF ₂) ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 941.9 (pep ⁺ N1F2; [M+2H] ²⁺)	1 Lewis X and ¹⁴ F
H ₂ NF ₂	3098.5 (β-gal; 3099.5; 0 Hex, and 2977.6; -1 Hex)	2	1590.4 (pep ⁺ N ₁) 1735.9 (pep ⁺ N ₁ F ₁ ; 0.5) 1881.8 (pep ⁺ N ₁ F ₂ ; 1) 2570.7 (pep ⁺ H ₂ NF ₂) 2425.3 (pep ⁺ H ₂ NF ₂) H ₂ NF ₂	Mixture: 1 LN with core and/or antenna fucosylation
H ₂ NF ₂	3115.5 (α-fuc; 3115.1; 0 Fuc)	1		1 LN, mono-fucosylated core
H ₂ NF ₂	3128.5 (α-fuc; 2978.5; -1 Fuc)	2		1 fucosylated LDN, ¹⁴ F
H ₂ NF ₂	3146.5 (α-fuc; 3146.1; 0 Fuc)	1		1 LN and 1 HexNAc, ¹⁴ F
H ₂ NF ₂	3156.5 (β-gal; 2994.5; -1 Hex, and 2832.5; -1 Hex)	4	1591.4 (pep ⁺ N ₁) 1737.2 (pep ⁺ N ₁ F ₁) 2483.4 (pep ⁺ H ₂ N) 2629.1 (pep ⁺ H ₂ NF ₂) 2664.0 (pep ⁺ H ₂ N) 2792.0 (pep ⁺ H ₂ NF ₂) ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 868.8 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: biantennary LN with one fucose at the core or antenna
H ₂ NF ₂	3245.5 (β-gal; 3245.5; 0 Hex)	2	1881.4 (pep ⁺ N ₁ F ₂)	1 Lewis X, ¹⁴ F
H ₂ NF ₂	3270.6 (β-gal; 3270.5; 0 Hex; α-fuc; 2978.5)	1		¹⁴ F
H ₂ NF ₂	3286.6 (β-gal; 3286.6; 0 Hex, and 3126.6; -1 Hex)	2	1592.0 (pep ⁺ N ₁) 1737.9 (pep ⁺ N ₁ F ₁ ; 1.0) 1883.3 (pep ⁺ N ₁ F ₂ ; 1.0) 2630.2 (pep ⁺ H ₂ NF ₂) 2773.7 (pep ⁺ H ₂ NF ₂) 2919.9 (pep ⁺ H ₂ NF ₂) H ₂ NF ₂	1 Lewis X or fucosylated HexNAc, ¹⁴ F
H ₂ NF ₂	3302.6 (β-gal; 3302.7; 0 Hex, 3146.6; -1 Hex, and 2978.6; -2 Hex); 1101.5 (M+3H) ²⁺	12	1590.3 (pep ⁺ N ₁) 1736.3 (pep ⁺ N ₁ F ₁ ; 1.0) 1882.4 (pep ⁺ N ₁ F ₂ ; 1.0) 2628.7 (pep ⁺ H ₂ NF ₂) 2775.1 (pep ⁺ H ₂ NF ₂) 2789.7 (pep ⁺ H ₂ NF ₂) 2937.1 (pep ⁺ H ₂ NF ₂) ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 941.9 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: biantennary LN with core and/or antenna fucosylation
H ₂ NF ₂	3343.6 (β-gal; 3343.5; 0 Hex, and 3181.6; -1 Hex)	2	1590.3 (pep ⁺ N ₁) 1736.4 (pep ⁺ N ₁ F ₁ ; 1.0) 1882.4 (pep ⁺ N ₁ F ₂ ; 1.0) 2628.7 (pep ⁺ H ₂ NF ₂) 2775.1 (pep ⁺ H ₂ NF ₂) 2789.7 (pep ⁺ H ₂ NF ₂) 2937.1 (pep ⁺ H ₂ NF ₂) ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 941.9 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: biantennary (1 LN, 1 LDN) with core and/or antenna fucosylation
H ₂ NF ₂	3391.6 (β-gal; 3391.6; 0 Hex)	2	1591.3 (pep ⁺ N ₁) 2880.4 (pep ⁺ H ₂ NF ₂) 3080.4 (pep ⁺ H ₂ NF ₂) H ₂ NF ₂	1 Lewis X and 1 fucosylated mannose, ¹⁴ F
H ₂ NF ₂	3432.6 (β-gal; 3432.6; 0 Hex)	1		1 Lewis X and 1 fucosylated HexNAc, ¹⁴ F
H ₂ NF ₂	3448.6 (β-gal; 3448.7; 0 Hex, 3286.6; -1 Hex); 1180.2 (M+3H) ²⁺	16	1591.4 (pep ⁺ N ₁) 1736.8 (pep ⁺ N ₁ F ₁ ; 1.3) 1883.0 (pep ⁺ N ₁ F ₂ ; 1.0) 2629.4 (pep ⁺ H ₂ NF ₂) 2775.4 (pep ⁺ H ₂ NF ₂) 2789.9 (pep ⁺ H ₂ NF ₂) 2921.1 (pep ⁺ H ₂ NF ₂) 2937.4 (pep ⁺ H ₂ NF ₂) 3083.8 (pep ⁺ H ₂ NF ₂) ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 941.9 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: biantennary LN with core and antenna fucosylation
H ₂ NF ₂	3489.7 (β-gal; 3489.5; 0 Hex, and 3327.7; -1 Hex); 1163.8 (M+3H) ²⁺	4	1591.2 (pep ⁺ N ₁) 1736.7 (pep ⁺ N ₁ F ₁ ; 0.33) 1883.3 (pep ⁺ N ₁ F ₂ ; 1.0) 2774.8 (pep ⁺ H ₂ NF ₂) 2818.9 (pep ⁺ H ₂ NF ₂) 2937.0 (pep ⁺ H ₂ NF ₂) 2976.9 (pep ⁺ H ₂ NF ₂) 3080.4 (pep ⁺ H ₂ NF ₂) 3124.1 (pep ⁺ H ₂ NF ₂) ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 941.9 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: biantennary (1 LN, 1 LDN) with one antenna fucose, ¹⁴ F
H ₂ NF ₂	3537.6 (β-gal; 3537.7; 0 Hex)	1		Lewis X, ¹⁴ F
H ₂ NF ₂	3594.7 (β-gal; 3594.7; 0 Hex); 1198.9 (M+3H) ²⁺	14	1590.9 (pep ⁺ N ₁) 1736.3 (pep ⁺ N ₁ F ₁ ; 0.33) 1882.1 (pep ⁺ N ₁ F ₂ ; 1.0) 2774.2 (pep ⁺ H ₂ NF ₂) 2920.5 (pep ⁺ H ₂ NF ₂) 2936.5 (pep ⁺ H ₂ NF ₂) 3083.2 (pep ⁺ H ₂ NF ₂) ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 941.9 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	2 Lewis X, ¹⁴ F
H ₂ NF ₂	3635.7 (β-gal; 3635.8; 0 Hex); 1212.5 (M+3H) ²⁺	5	1590.4 (pep ⁺ N ₁) 1736.7 (pep ⁺ N ₁ F ₁ ; 0.33) 1882.3 (pep ⁺ N ₁ F ₂ ; 1.0) 2774.2 (pep ⁺ H ₂ NF ₂) 2920.8 (pep ⁺ H ₂ NF ₂) 2936.5 (pep ⁺ H ₂ NF ₂) 3081.4 (pep ⁺ H ₂ NF ₂) 3124.7 (pep ⁺ H ₂ NF ₂) ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 941.9 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	1 Lewis X, 1 fucosylated LDN and ¹⁴ F
H ₂ NF ₂	3741.0 (β-gal; 3740.6; 0 Hex)	1	1590.4 (pep ⁺ N ₁) 1737.9 (pep ⁺ N ₁ F ₁ ; 0.12) 1883.4 (pep ⁺ N ₁ F ₂ ; 1.0) 2937.5 (pep ⁺ H ₂ NF ₂) 3082.4 (pep ⁺ H ₂ NF ₂) H ₂ NF ₂	1 Lewis X and 1 pseudo LewisY, ¹⁴ F
H ₂ NF ₂	3781.8 (β-gal; 3781.6; 0 Hex)	2		1 Lewis X and 1 difucosylated LDN, ¹⁴ F
H ₂ NF ₂	3797.7 (β-gal; 3799.8; 0 Hex; α-fuc; 3359.2; -2 Fuc)	1		¹⁴ F
H ₂ NF ₂	3813.8 (β-gal; 3810.6; -1 Hex)	1		Mixture: biantennary (1 LN, 1 di-LDN) with core and antenna fucosylation
H ₂ NF ₂	3959.7 (β-gal; 3959.7; 0 Hex, and 3797.7; -1 Hex)	1	1592.4 (pep ⁺ N ₁) 1737.5 (pep ⁺ N ₁ F ₁ ; 0.33) 1883.8 (pep ⁺ N ₁ F ₂ ; 1.0) 2776.4 (pep ⁺ H ₂ NF ₂) 2940.2 (pep ⁺ H ₂ NF ₂) 3083.2 (pep ⁺ H ₂ NF ₂) 3302.1 (pep ⁺ H ₂ NF ₂) 3450.7 (pep ⁺ H ₂ NF ₂) 3596.0 (pep ⁺ H ₂ NF ₂) H ₂ NF ₂	One of several possible isomers is shown
H ₂ NF ₂	4001.0	1	1592.7 (pep ⁺ N ₁) 1737.3 (pep ⁺ N ₁ F ₁ ; 0.25) 1884.7 (pep ⁺ N ₁ F ₂ ; 1.0) 2939.9 (pep ⁺ H ₂ NF ₂) 3302.5 (pep ⁺ H ₂ NF ₂) 3448.9 (pep ⁺ H ₂ NF ₂) 3593.5 (pep ⁺ H ₂ NF ₂) H ₂ NF ₂	1 LN and LN4LDN or 1 LDN and di-LN, with core and antenna fucosylation
H ₂ NF ₂	4106.0 (β-gal; 4105.8; 0 Hex)	1	1590.4 (pep ⁺ N ₁) 1882.3 (pep ⁺ N ₁ F ₂) 2920.5 (pep ⁺ H ₂ NF ₂) 3082.4 (pep ⁺ H ₂ NF ₂) 3399.5 (pep ⁺ H ₂ NF ₂) 3447.5 (pep ⁺ H ₂ NF ₂) 3596.3 (pep ⁺ H ₂ NF ₂) H ₂ NF ₂	One of several possible isomers is shown
H ₂ NF ₂	4146.9	1	1591.4 (pep ⁺ N ₁) 1737.5 (pep ⁺ N ₁ F ₁ ; 0.1) 1883.9 (pep ⁺ N ₁ F ₂ ; 1.0) 3083.8 (pep ⁺ H ₂ NF ₂) 3303.6 (pep ⁺ H ₂ NF ₂) 3447.8 (pep ⁺ H ₂ NF ₂) 3594.7 (pep ⁺ H ₂ NF ₂) H ₂ NF ₂	Mixture: 1 LN and LN4LDN or 1 LDN and di-LN, with core and antenna fucosylation
Peptide Q ₆₀ PCNC ₇₇ (GLN-R)				One of several possible isomers is shown
H ₂ NF ₂	861.4 (M+3H) ²⁺ (β-gal; 861.4; 0 Hex, 807.7; -1 Hex)	4	ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 528.2 (H ₂ N) 618.2 (pep ⁺ N ₁ ; [M+2H] ²⁺) 691.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺) 764.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: 1 LN with core and/or antenna fucosylation
H ₂ NF ₂	910.1 (M+3H) ²⁺ (β-gal; 910.1; 0 Hex)	10	ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 691.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺) 764.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	1 Lewis X, ¹⁴ F
H ₂ NF ₂	915.8 (M+3H) ²⁺ (β-gal; 915.8; 0 Hex, 807.7; -1 Hex)	2		Mixture: 1 LN with core and/or antenna fucosylation
H ₂ NF ₂	923.7 (M+3H) ²⁺ (β-gal; 923.7; 0 Hex)	2		
H ₂ NF ₂	929.8 (M+3H) ²⁺	1		
H ₂ NF ₂	938.8 (M+3H) ²⁺ (β-gal; 938.8; 0 Hex)	5	ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 691.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺) 764.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Lewis X, ¹⁴ F
H ₂ NF ₂	964.4 (M+3H) ²⁺ (β-gal; 964.4; 0 Hex)	2		1 Lewis X, ¹⁴ F
H ₂ NF ₂	972.5 (M+3H) ²⁺ (β-gal; 972.5; 0 Hex)	3		¹⁴ F
H ₂ NF ₂	977.8 (M+3H) ²⁺ (β-gal; 977.8; 0 Hex)	1		1 Lewis X and 1 HexNAc, ¹⁴ F
H ₂ NF ₂	983.1 (M+3H) ²⁺ (β-gal; 983.1; 0 Hex, 929.8; -1 Hex, 875.1; -2 Hex)	5	ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 691.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺) 764.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: biantennary LN with core and/or antenna fucosylation
H ₂ NF ₂	991.4 (M+3H) ²⁺ (β-gal; 991.5; 0 Hex)	1		1.0% 2.0% 2.0%
H ₂ NF ₂	1031.8 (M+3H) ²⁺ (β-gal; 1031.8; 0 Hex, 977.8; -1 Hex)	12	ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 764.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: biantennary LN with core and antenna fucosylation
H ₂ NF ₂	1045.5 (M+3H) ²⁺ (β-gal; 1045.5; 0 Hex, 991.5; -1 Hex)	5	ESI-MS/MS: 366.1 (H ₂ N); 407.2 (N ₂); 512.2 (H ₂ NF ₂); 553.2 (N ₂) 764.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: Lewis X or fucosylated LDN, ¹⁴ F
H ₂ NF ₂	1080.5 (M+3H) ²⁺ (β-gal; 1080.5; 0 Hex, 1026.4; -1 Hex)	16	ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 638.2 (H ₂ NF ₂) 691.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺) 764.4 (pep ⁺ N ₁ F ₂ ; [M+2H] ²⁺)	Mixture: 2 Lewis X, ¹⁴ F or 1 LN with two fucoses, ¹⁴ F
H ₂ NF ₂	1086.2 (M+3H) ²⁺ (β-gal; 1086.2; 0 Hex)	4	ESI-MS/MS: 366.1 (H ₂ N); 512.2 (H ₂ NF ₂) 553.2 (N ₂)	Lewis X, ¹⁴ F
H ₂ NF ₂	1094.2 (M+3H) ²⁺ (β-gal; 1094.2; 0 Hex)	5	ESI-MS/MS: 366.1 (H ₂ N); 407.2 (N ₂); 512.2 (H ₂ NF ₂) 553.2 (N ₂)	1 Lewis X, 1 fucosylated LDN and ¹⁴ F
H ₂ NF ₂	1099.8 (M+3H) ²⁺ (β-gal; 1099.8; 0 Hex)	2		14.0% 2.0%
H ₂ NF ₂	1129.1 (M+3H) ²⁺ (β-gal; 1129.1; 0 Hex			

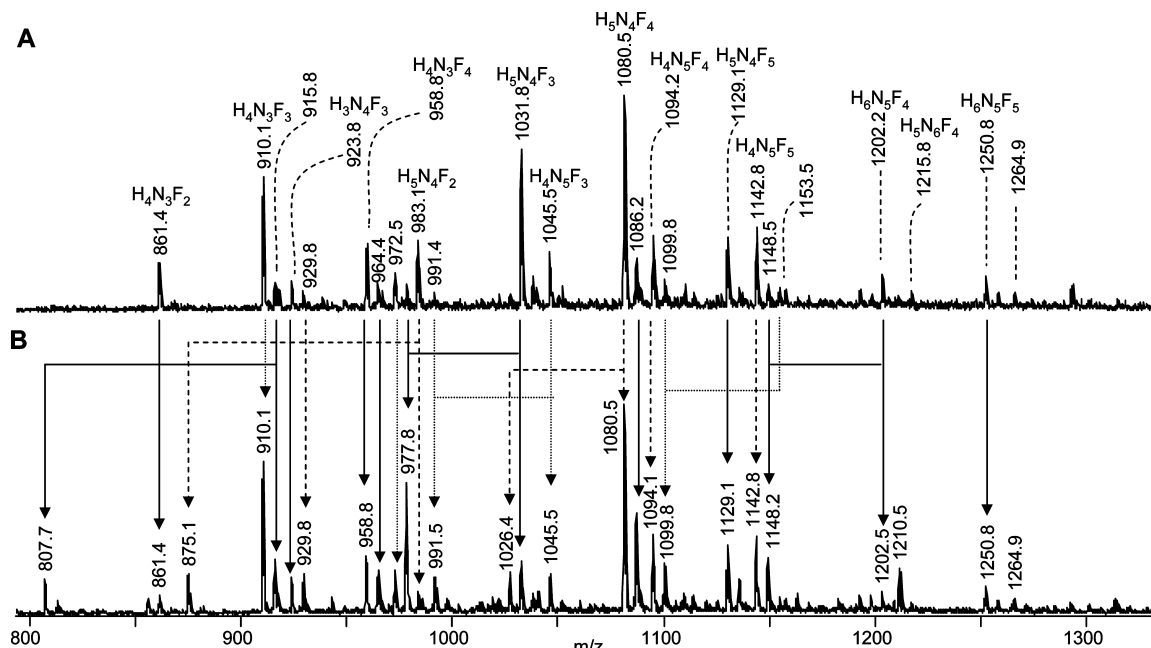


Figure 8. β -Galactosidase treatment of omega-1 glycopeptides. Omega-1 was subjected to reduction and alkylation, digested with trypsin and the resulting (glyco)peptides of glycosylation site N71 were analyzed by nano-LC-MS (A) before and (B) after treatment with β -galactosidase. Signals are triple positively charged and labeled with monoisotopic masses. Composition of the glycan moieties are given in terms of hexose (H), N-acetylhexosamine (N) and fucose (F).

(Figure 4B). Therefore, we propose that some glycoforms of omega-1 carry LDN-F motifs, but F-LDN motifs do not occur.

Another representative example of a fragmentation is given by the MALDI-TOF/TOF mass spectrum of E172-R183- $H_6N_5F_5$ which carries one Lewis X and one dimeric Lewis X antenna accompanied by two fucoses at the core (Figure 7). The presence of the dimeric Lewis X motif was demonstrated by a high signal at m/z 3082.4 after fragmentation, indicative of the loss of two interlinked Lewis X elements. The results of the successive treatment of α -fucosidase and β -galactosidase support this interpretation. α -Fucosidase treatment of $H_6N_5F_5$ glycopeptides results in the loss of up to three antenna fucoses (signal at m/z 4106.0 in Figure 4A shifts to m/z 3813.6 and 3668.1 in Figure 4B). After subsequent treatment with β -galactosidase, the signals at m/z 3668.1 and 3813.6 (Figure 4B) shift to m/z 3343.1 and 3489.6 respectively (Figure 4C), indicative of the loss of two galactoses. This observation is in line with the occurrence of one nonsusceptible internal galactose in the proposed structure of $H_6N_5F_5$. In addition to the aforementioned signals, a signal at m/z 3181.7 appears, which might be interpreted as the loss of three galactoses from the signal at m/z 3668.1. However, we believe that the latter signal is not derived from fucosidase/galactosidase treatment of $H_6N_5F_5$, but arises from $H_4N_5F_2$ (m/z 3343.1 in Figure 4B), which represents a core difucosylated glycopeptide with one LN and one LDN antenna. Upon β -galactosidase treatment, the $H_4N_5F_2$ glycoform loses one galactose residue from the LN antenna, thereby giving rise to a signal at m/z 3181.7 in Figure 4C, derived from the similar intense signal at m/z 3343.1 in Figure 4B.

In a similar manner, combination of fragmentation analysis by MALDI-TOF/TOF MS and α -fucosidase/ β -galactosidase treatment led to structure proposals for most other glycoforms observed for the E172-R183 peptide. The data are summarized in Table 1 and Supplementary Table 1 (Supporting Information).

N-Glycosylation Analysis of Glycopeptide Q69-R77. Unlike from the E172-R183 glycopeptides, signals from peptide Q69-R77 containing the glycosylation site N71 could not be detected by MALDI-TOF MS. The most abundant Q69-R77 glycopeptide, carrying a $H_5N_4F_4$ glycan (Figure 2A), would correspond to a $[M + H]^+$ ion of a theoretical mass of 3095.4 Da, but this was not observed in the spectrum in Figure 3A, nor were any other signals detected that would correspond to glycopeptides from Q69-R77. Therefore, with the specific aim of further characterizing the glycosylation of the Q69-R77 glycopeptides, untreated and β -galactosidase-treated glycopeptides from omega-1 were also measured by nano-LC-MS (Figure 8) and LC-MS/MS (Figure 9 and Table 1). Registered masses, fragmentation data as well as deduced glycan compositions for all the detected glycopeptide species are given in Table 1.

For the most abundant glycopeptide of Q69-R77 carrying the N-glycan $H_5N_4F_4$, the main structure is composed of a trimannosyl core with two fucoses attached to the innermost N-acetylglucosamine and two Lewis X antennae (left structure in Figure 9), using similar reasoning as for the $H_5N_4F_4$ glycan on site N176. However, $\sim 15\%$ of the $H_5N_4F_4$ glycans on site N71 contain a nonfucosylated LN antenna, as demonstrated by the removal of one hexose after β -galactosidase treatment (m/z 1080.5 $[M + H]^{3+}$ in Figure 8A shifts to m/z 1026.4 in Figure 8B). This indicates that two fucoses are located at the other LN branch. To verify this, the $H_5N_4F_4$ glycopeptide was fragmented using nano-LC-MS/MS (Figure 9). Losses of 365 and 657 Da (signals at m/z 1437.6 and 1291.6) were observed from the parent ion, which corresponds to the loss of LN (H_1N_1) and difucosylated LN ($H_1N_1F_2$), respectively. Notably, fragmentation data of protonated glycans and glycopeptides may be compromised due to the occurrence of fucose rearrangements, possibly contributing to the generation of the above-described fragments. This phenomenon is commonly seen in the analysis of N-glycans by MALDI as LC-MS fragmentation.³⁰ However,

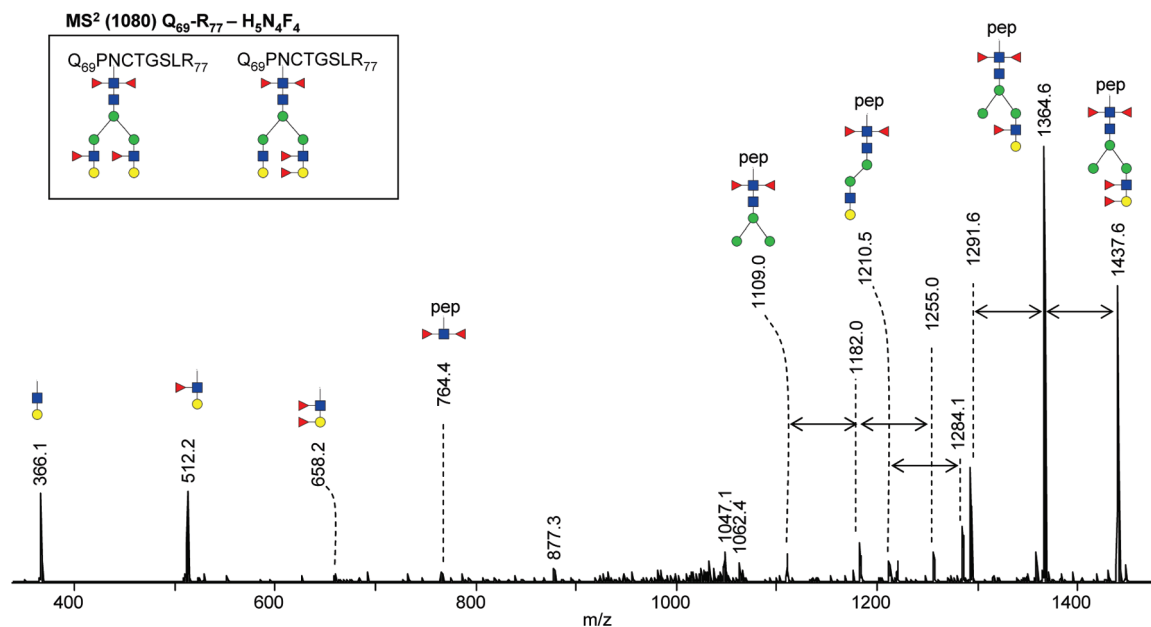


Figure 9. MS/MS of peptide Q69-R77 carrying $H_5N_4F_4$ N-glycans. The MS/MS spectrum was acquired by nano-LC–MS/MS of a tryptic digest of omega-1. Differences in fucose content are indicated by double-headed arrows. Red triangle, fucose; yellow circle, galactose; blue square, N-acetylglucosamine; green circle, mannose.

based on the results from the β -galactosidase treatment, it was clearly demonstrated that a small part of $H_5N_4F_4$ glycans on site N71 contain one LN and one difucosylated LN antenna, a glycan structure which was not observed for $H_5N_4F_4$ on site N176. In analogy with this, we propose for glycopeptide Q69-R77 containing an N-glycan with composition $H_4N_5F_5$ a similar structural assignment as for $H_4N_5F_4$, being one isomer with a difucosylated core in combination with one monofucosylated LDN and one difucosylated LN antenna, and another with a difucosylated core and one Lewis X and one difucosylated LDN antenna (Table 1).

For most of the other N-glycans at site N71, similar information was obtained, as summarized in Table 1. Most of the glycan structures at site N71 were identical to the structures of glycosylation site N176, however in some cases with differences in fucosylation as described above. The main differences between the sites are in the relative abundance of glycan structures, with site N71 containing a higher amount of multifucosylated structures compared to site N176.

General Overview of Omega-1 Glycosylation. An overview of the most abundant glycosylation forms of omega-1, combining the data from glycosylation sites N71 and N176, is given in Table 2. Notably, the abundance of individual glycan structures was calculated using signal intensities derived from LC–MS data for site N71 (Figure 8A) and MALDI-TOF MS data for site N176 (Figure 3A), which might have implications for the comparability of the two sites. On the other hand, when comparing the LC–MS derived data (Figure 2A) and MALDI derived data (Figure 3A) from site N176, the ratio of the high intensity signals are very comparable, although the low abundant species show some deviations.

The majority of the glycan structures on the two sites exhibit two fucoses on the innermost N-acetylglucosamine and one or two Lewis X antennae. In view of the documented immunogenic properties of Lewis X and core fucosylation,^{10,31} we estimated the abundance of these structures on omega-1 using the most abundant glycans as markers (Table 2). One omega-1

glycan carries an average of 1.3 Lewis X motifs, meaning that on average 2.6 Lewis X units are present on the omega-1 glycoprotein. To estimate the occurrence of core fucosylation, the data from the α -fucosidase treatment of site N176 were used. As evident from the mass spectra in Figure 4B/C, recorded after the removal of antenna fucoses, the vast majority of N176 glycans carry one or two core fucoses. This conclusion was verified by MALDI-TOF/TOF MS for the high abundant glycans (Table 2). We calculated that 60% of the glycans at N176 carry two fucoses at the core, 30% carry one core fucose and 10% have no core fucosylation. For site N71, when focusing on the twelve most abundant glycan structures of site N71 (Table 2), we calculated that 74% of these glycans carry two core fucoses, 10% carry one core fucose and 16% have no core fucosylation. As already reported for the egg and ES glycoprotein mixture,^{12,15} the observed difucosylated core structures are expected to be comprised of $\alpha 1-3, \alpha 1-6$ -linked fucoses. It remains unclear whether the fucose at the monofucosylated core is $\alpha 1-3$ or $\alpha 1-6$ linked.

To conclude, the analysis of tryptic glycopeptides by MALDI-TOF/TOF MS and nano-LC–MS/MS, in combination with β -galactosidase and/or α -fucosidase treatment reveal that the majority of N-glycans of omega-1 express two fucoses on the innermost N-acetylglucosamine and contain one or two terminal Lewis X motifs. Less abundant antenna structures include LN and difucosylated LN, non, mono- and difucosylated LDN and (fucosylated) LN and LDN repeats.

Discussion

Omega-1 is a single glycoprotein in *S. mansoni* soluble egg antigens (SEA) that harbors the potent Th2-inducing properties characteristic for this complex antigen mixture.^{6,9} Glycans from schistosome egg glycoproteins are ligands for multiple C-type lectins present on immune cells,^{20,32} and these glycans appear to be involved in immunomodulatory activities attributed to SEA.^{5,10} Prompted by these observations and the lack of

Table 2. Schematic Representation of the Most Abundant N-Glycans Detected on Omega-1

Glycans ^a	relative abundance on site N176 (%) ^b	relative abundance on site N71 (%) ^b	relative abundance on Omega-1 (%) ^b
	14	14	14
	7	10	9
	8	8	8
	8	4	6
	5	5	5
	4	2	3
	1	5	3
	4	2	3
	4	1	2.5
	2	3	2.5
	2	3	2.5
	2	3	2.5

^a Red triangle, fucose; yellow circle, galactose; yellow square, N-acetylgalactosamine; blue square, N-acetylglucosamine; green circle, mannose. ^b Numbers are based on glycopeptide signal intensities in MALDI-TOF MS (site N176) or LC-MS (site N71), in combination with signal intensities from fragmentation and enzyme data for isomeric structures.

structural information about omega-1 glycans, we performed a site-specific analysis of the glycosylation pattern of omega-1.

This study reveals that omega-1 has two fully occupied N-glycosylation sites. Both sites display similar glycan heterogeneity, but with different relative abundances of the individual glycans present (Figure 2; Table 2). All glycans on omega-1 are of a diantennary complex type, and most display an α 3 and/or α 6 fucosylation of the innermost N-acetylglucosamine (Tables 1 and 2). Lewis X was found to be the major antenna motif, but also other terminal glycan elements were found to be expressed on omega-1, including LDN-F, tandem repeats of Lewis X, and difucosylated LN and LDN. In addition, we searched the LC-MS-derived data for O-glycosylated peptides, focusing on O-glycosylation marker oxonium ions at m/z 204 (HexNAc antenna), m/z 350 (fucosylated HexNAc antenna), m/z 407 (LDN antenna), m/z 496 (difucosylated HexNAc antenna), m/z 512 (Lewis X antenna) and m/z 699 (difucosylated LDN antenna). Using these marker ions, we could not find any indications for O-linked glycosylation on omega-1.

The occurrence of these types of N-glycan elements in a schistosome egg glycoprotein is in line with data generated by

nonprotein-specific analysis of the N-glycans of *S. mansoni* egg glycoproteins in SEA¹² as well as in ES,¹⁵ the latter being a mixture of excretory/secretory glycoproteins of which omega-1 is a major component.^{16,17} Simultaneous expression of LDN- and LN-based antenna elements has been reported in the egg glycoprotein mixtures as well as in the protein-specific glycosylation analysis of IPSE/alpha-1.²⁴ Based on the LN and LDN backbones, both Lewis X and LDN-F antigens are present on omega-1, most likely due to action of a Lewis X-type fucosyltransferase (FucT) in *S. mansoni* eggs that is also capable of transferring fucose to the GlcNAc residue in LDN in addition to that in LN.³³ We also detected difucosylated LN and LDN variants on omega-1. The F-LDN-F element has been previously described in schistosome glycoproteins and glycolipids.^{12,15,26} In contrast, the difucosylated LN element has not been detected before in schistosome eggs or ES. This element likely represents the pseudo-Lewis Y motif (Fuc α 1-3Gal β 1-4[Fuc α 1-3]GlcNAc) as has previously been described in a major fraction of *S. mansoni* cercarial glycolipids.³⁴ Hypothetically, a difucosylated LN motif also may contain the Fuc α 1-2Fuc α 1-3 sequence. This disaccharide sequence is abundantly present on schistosome egg O-glycans,¹⁵ glycolipids²⁶ and free excretory oligosaccharides,³⁵ but only in the context of chito-oligomeric or LDN backbones. We did not find indications for the occurrence of the Fuc α 1-2Fuc element on omega-1. With respect to the biosynthesis of the different fucosylated elements present in omega-1, some may be formed by the action of the same FucT (e.g., Lewis X and LDN-F³³), as has also been suggested for the formation of the Fuc α 1-3GalNAc linkage in F-LDN-F and the Fuc α 1-3Gal in pseudo-Lewis Y.^{34,36} Still, at least 4 different FucTs appear to be active at the site of production of omega-1, and taking into account the occurrence of the other fucosylated motifs in *S. mansoni* eggs and other life stages, it is not surprising that at least 20 FucT genes are present in the *S. mansoni* genome,³⁷ some of which display highly regulated expression levels by transcriptome analysis.³⁸ It is unclear at present what the precise acceptor and linkage specificities of these FucTs are, as current annotation based on sequence homology is not sufficient to classify these FucTs.

With respect to the overall set of N-glycans found on ES glycoproteins,¹⁵ truncated and high-mannose type glycans as well as core xylosylation observed in a minor set of ES glycoproteins were not found for omega-1. On the other hand, the glycosylation of IPSE/alpha-1, the only other *S. mansoni* egg glycoprotein of which specific glycosylation data is available to date, appears to be highly homologous to omega-1.²⁴ IPSE/alpha-1 is produced in the subshell area of the schistosome egg, at the same location as omega-1, which suggests that these two proteins, both major constituents of *S. mansoni* ES, are subjected to the same glycosylation machinery. According to Mathieson et al., at least four other major glycoproteins are present in *S. mansoni* egg ES, including thioredoxin and three microexon-gene (MEG) proteins,¹⁸ and it would be interesting to see if these proteins carry the O-glycans as well as the subset of N-glycans found in ES, but not present on omega-1 and IPSE/alpha-1. In this respect, it must be noted that proteomic analysis of ES by another group led to the identification of 188 proteins¹⁶ instead of only a few.¹⁸ This discrepancy may be explained by the different methods used for the preparation of ES. Furthermore, while Cass et al. analyzed the whole ES mixture,¹⁶ Mathieson and Wilson separated ES proteins on a 2-DE gel and merely investigated the spots visible after Sypro Ruby staining, possibly missing low-abundant proteins.¹⁸

However, in spite of the large differences between the protein contents of ES fractions, in both cases omega-1 and IPSE/alpha-1 were identified as the most abundant ES proteins, underlining also the relative abundance of the glycans they carry in the overall ES glycome.

The finding that omega-1 contains the Lewis X motif is of importance. In the context of a LNFPIII conjugate, Lewis X has previously been shown to induce Th2 responses in a mouse model following intranasal immunization.¹⁰ Furthermore, it can stimulate murine DCs *in vitro* toward a type 2 DC capable of inducing the development of Th2 cells from naive mouse-derived CD4+ T cells.¹¹ Based on these data, it would be tempting to attribute the recently reported Th2-polarizing properties of omega-1 to its Lewis X structures.^{6,9} However, IPSE/alpha-1, which carries virtually identical Lewis X-containing glycans to omega-1, is not able to induce the strong DC-mediated type 2 responses observed for omega-1 in the same assay,^{6,24} indicating that in this case also properties provided by the underlying protein backbone play a role. Interestingly, Steinfeldt et al. recently demonstrated in an *in vitro* bystander polarization assay that upon treatment with diethyl pyrocarbonate, a chemical which irreversibly modifies histidine residues, omega-1 is no longer able to condition DCs for Th2-induction.⁹ Clearly, the possible contributions of the glycan and protein parts to the immunological properties of omega-1 need to be further investigated. In this respect, it is interesting to note that fucosylated glycan structures abundantly present on omega-1, are recognized by C-type lectin immune receptors such as DC-SIGN and MR,^{19,39} which have been reported to mediate internalization of SEA constituents by DCs.²⁰

The detailed examination of the site-specific glycosylation of omega-1 was made possible by an approach based on MALDI-TOF(/TOF) MS and nano-LC-MS(/MS) analysis. Importantly, by combining the MS analyses with specific exoglycosidase treatments, we could validate structural assignments of important glycan elements, exclude potential false interpretations of the MS/MS spectra due to frequently occurring fucose rearrangements,³⁰ and thus provide a solid basis for the identification and characterization of the glycan structures. This methodology, based on gel-derived tryptic glycopeptides, is also easily applicable to many other glycoproteins as it does not require extensive or complicated sample workup.

Acknowledgment. We thank Isabel Catalina for performing initial experiments and Carolien Koeleman for expert technical assistance. This work was supported by the Dutch Organization for Scientific Research (NWO), Grant No CW ECHO 700.55.013.

Supporting Information Available: Supplementary Figures 1 and 2 and Table 1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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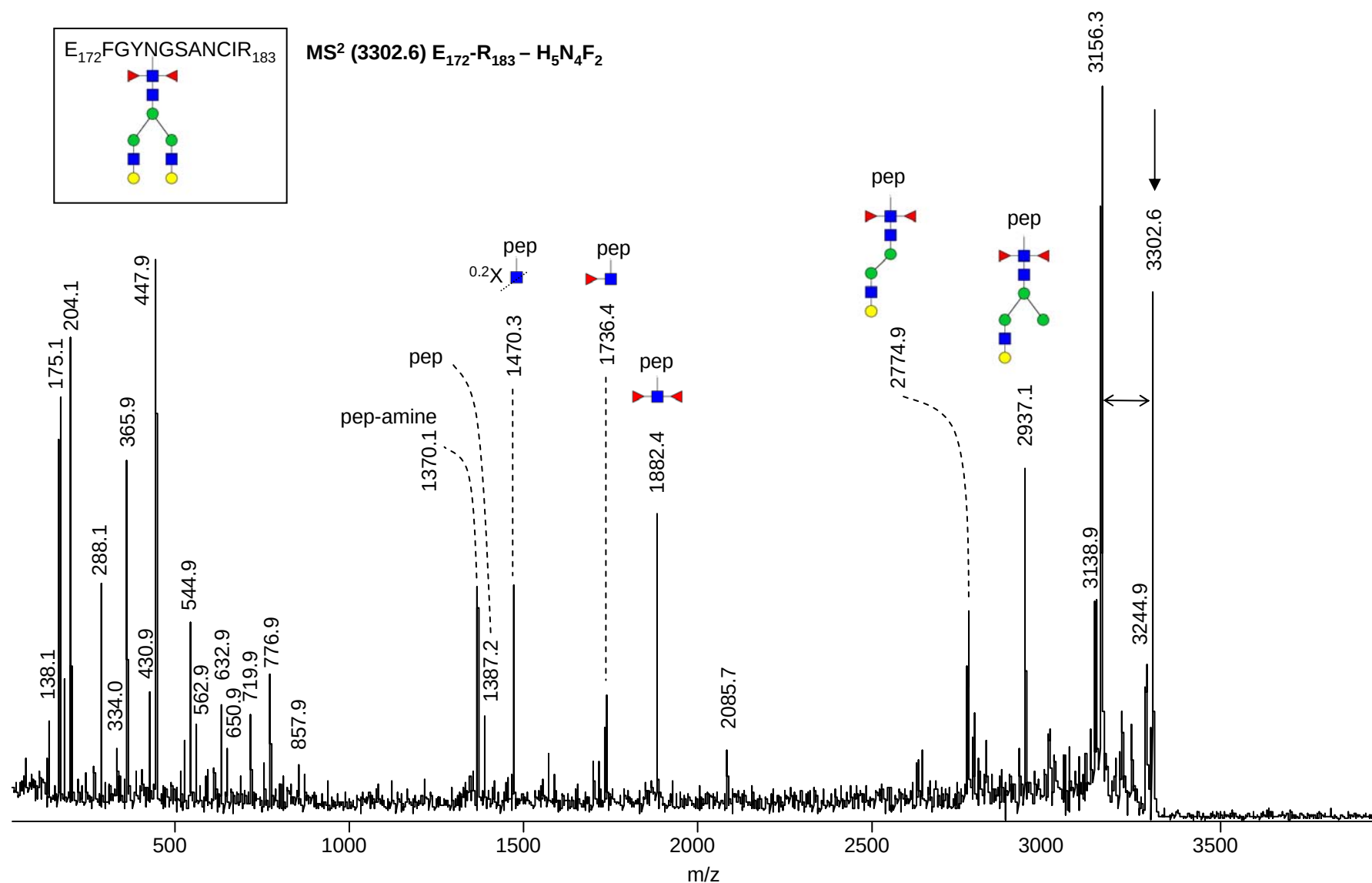
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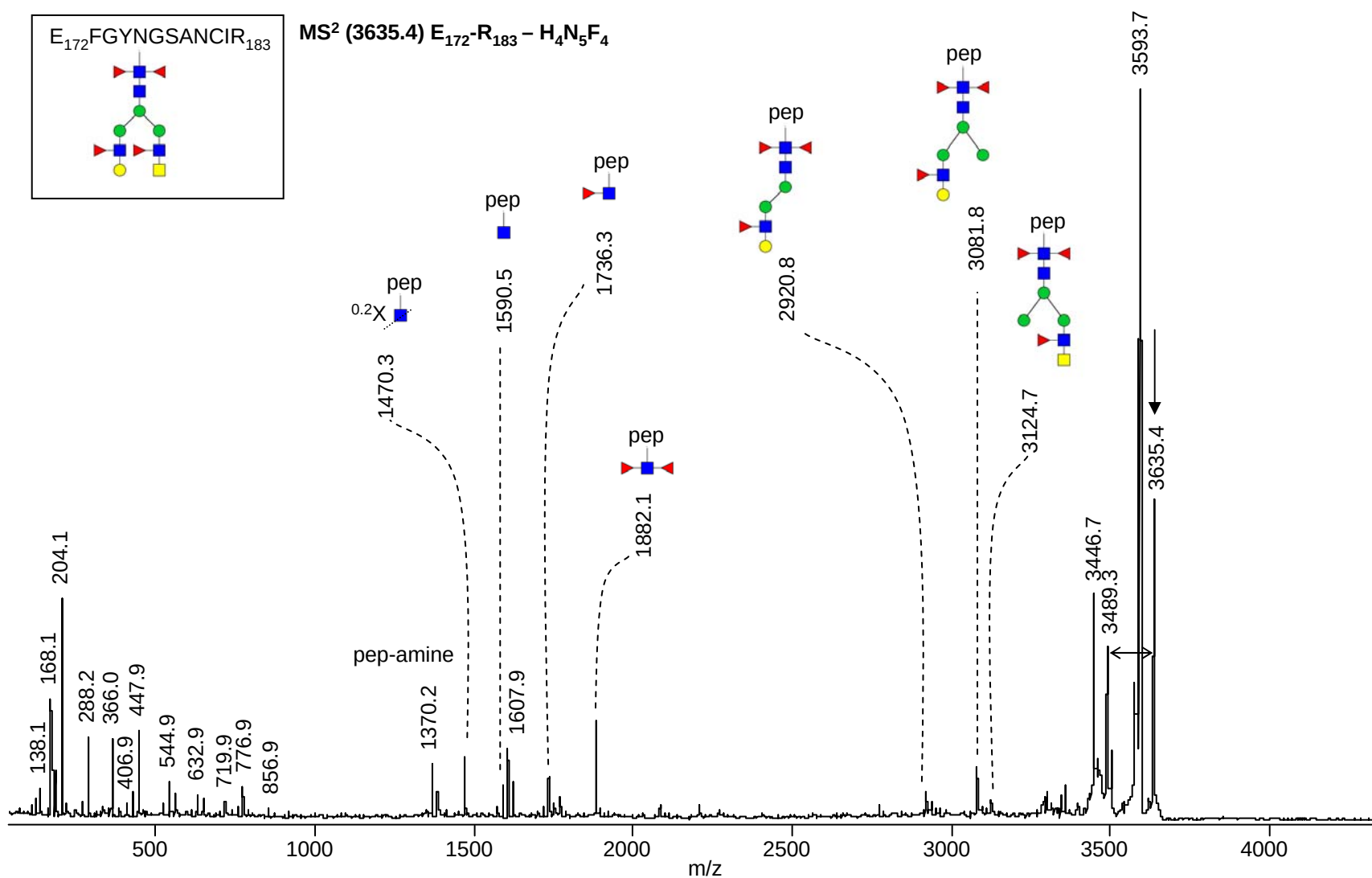
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Supporting Figure 1



Supporting Fig. 1. MS/MS of peptide E172–R183 carrying H5N4F2 N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF-MS of a tryptic digest of omega-1 treated with α -fucosidase. The single-headed arrow indicates the parent ion. Differences in fucose content are indicated by double-headed arrows. Red triangle, fucose; yellow circle, galactose; blue square, N-acetylglucosamine; green circle, mannose.

Supporting Figure 2



Supporting Fig. 2. MS/MS of peptide E172-R183 carrying H4N5F4 N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF-MS of a tryptic digest of omega-1. The single-headed arrow indicates the parent ion. Differences in fucose content are indicated by double-headed arrows. Red triangle, fucose; yellow circle, galactose; blue square, N-acetylglucosamine; yellow square, N-acetylgalactosamine; green circle, mannose.

Supporting Table 1. Omega-1 glycopeptide species detected after trypsin digestion and α -fucosidase treatment.

Glycan composition ^a	Glycopeptide signal (m/z) ^b	Relative peak height (%) ^c	Fragment ions ^d	Proposed structures ^e
H ₄ N ₃ F ₁	2791.1 (β -gal: 2629.5; -1 Hex)	3		1 LN, 1 core fucose
H ₄ N ₃ F ₂	2937.4 (β -gal: 2775.5; -1 Hex)	7	1736.5 (pep ¹ +N ₁ F ₁ ; 0.5) 1882.6 (pep ¹ +N ₁ F ₂ ; 1.0) 2572.2 (pep ¹ +H ₃ N ₂ F ₂) - H ₁ N ₁	1 LN, ^{3,6} F 
H ₅ N ₃ F ₁	2953.4 (β -gal: 2953.6; -0 Hex)	2		Mono-fucosylated core
H ₅ N ₄	3010.5 (β -gal: 2686.4; -2 Hex)	10	1590.7 (pep ¹ +N ₁) 2483.2 (pep ¹ +H ₃ N ₃) - H ₂ N ₁ 2645.4 (pep ¹ +H ₄ N ₃) - H ₁ N ₁	2 LN, no core fucosylation 
H ₄ N ₅	3051.4 (β -gal: 2889.1; -1 Hex)	2		1 LN, 1 LDN, no core fucosylation 
H ₅ N ₃ F ₂	3099.0 (β -gal: 3099.1; -0 Hex)	1		1 LN, ^{3,6} F
H ₆ N ₃ F ₁	3115.1	2		Mono-fucosylated core
H ₄ N ₄ F ₂	3140.1 (β -gal: 2978.6; -1 Hex)	3		1LN and 1 HexNAc, ^{3,6} F
H ₅ N ₄ F ₁	3156.6 (β -gal: 2832.5; -2 Hex)	17	1590.6 (pep ¹ +N ₁) 1736.6 (pep ¹ +N ₁ F ₁) 3629.3 (pep ¹ +H ₃ N ₃ F ₁) - H ₂ N ₁ 2791.4 (pep ¹ +H ₄ N ₃ F ₁) - H ₁ N ₁	2 LN, mono-fucosylated core 
H ₄ N ₅ F ₁	3197.9 (β -gal: 3035.7; -1 Hex)	4	1736.7 (pep ¹ +N ₁ F ₁) 2628.4 (pep ¹ +H ₃ N ₃ F ₁) - H ₁ N ₂ 2790.7 (pep ¹ +H ₄ N ₅ F ₁) - N ₂ 2833.4 (pep ¹ +H ₃ N ₄ F ₁) - H ₁ N ₁	1 LN, 1 LDN, monofucosylated core 
H ₄ N ₄ F ₃	3286.2 (β -gal: 3124.5; -1 Hex)	3		
H ₅ N ₄ F ₂	3302.6 (β -gal: 2978.6; -2 Hex)	30	1736.4 (pep ¹ +N ₁ F ₁ ; 0.4) 1882.4 (pep ¹ +N ₁ F ₂ ; 1.0) 2774.9 (pep ¹ +H ₃ N ₃ F ₂) - H ₂ N ₁ 2937.1 (pep ¹ +H ₄ N ₃ F ₂) - H ₁ N ₁	2 LN, ^{3,6} F 
H ₄ N ₅ F ₂	3343.1 (β -gal: 3181.7; -1 Hex)	7	1590.8 (pep ¹ +N ₁) 1736.9 (pep ¹ +N ₁ F ₁ ; 0.33) 1882.7 (pep ¹ +N ₁ F ₂ ; 1.0) 2936.3 (pep ¹ +H ₄ N ₃ F ₂) - N ₂ 2979.2 (pep ¹ +H ₃ N ₄ F ₂) - H ₁ N ₁	1 LN, 1 LDN, ^{3,6} F 
H ₅ N ₄ F ₃	3448.9 (β -gal: 3125.5; -2 Hex)	3		
H ₄ N ₅ F ₃	3489.7 (β -gal: 3327.9; -1 Hex)	1		1 LN, 1 fucosylated LDN, ^{3,6} F 
H ₄ N ₅ F ₄	3635.8 (β -gal: 3473.3; -1 Hex, and 3312.7; -2 Hex)	2		^{3,6} F
H ₆ N ₅ F ₂	3668.1 (β -gal: 3343.1; -2 Hex)	2		1 LN, 1 di-LN, ^{3,6} F
H ₆ N ₅ F ₃	3813.6 (β -gal: 3489.6; -2 Hex)	1		^{3,6} F

^aGlycan compositions are given in terms of hexose (H), *N*-acetylhexosamine (N) and fucose (F). ^bMonoisotopic masses of glycopeptide precursors are given throughout. Glycopeptides are singly positively charged, as registered by MALDI-TOF-MS. For many glycopeptides, masses after β -galactosidase (β -gal) treatment are listed and the loss of *n* hexose is concluded (- *n* hex). ^cRelative peak height is expressed as a percentage of cumulative peak height. ^dFragment ions were determined by MALDI-TOF/TOF-MS. Pep¹ corresponds to peptide E₁₇₂FGYNGSANCIR₁₈₃ containing glycosylation site N176. ^eProposed glycan structures are deduced from glycopeptide MS after enzyme treatments and fragmentation data. Red triangle, fucose; yellow circle, galactose; yellow square, *N*-acetylgalactosamine; blue square, *N*-acetylglucosamine; green circle, mannose.