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Hexose Rearrangements upon Fragmentation of *N*-Glycopeptides and Reductively Aminated *N*-Glycans

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Tandem mass spectrometry of glycans and glycoconjugates in protonated form is known to result in rearrangement reactions leading to internal residue loss. Here we studied the occurrence of hexose rearrangements in tandem mass spectrometry of *N*-glycopeptides and reductively aminated *N*-glycans by MALDI-TOF/TOF-MS/MS and ESI-ion trap-MS/MS. Fragmentation of proton adducts of oligomannosidic *N*-glycans of ribonuclease B that were labeled with 2-aminobenzamide and 2-aminobenzoic acid resulted in transfer of one to five hexose residues to the fluorescently tagged innermost *N*-acetylglucosamine. Glycopeptides from various biological sources with oligomannosidic glycans were likewise shown to undergo hexose rearrangement reactions, resulting in chitobiose cleavage products that have acquired one or two hexose moieties. Tryptic immunoglobulin G Fc-glycopeptides with biantennary *N*-glycans likewise showed hexose rearrangements resulting in hexose transfer to the peptide moiety retaining the innermost *N*-acetylglucosamine. Thus, as a general phenomenon, tandem mass spectrometry of reductively aminated glycans as well as glycopeptides may result in hexose rearrangements. This characteristic of glycopeptide MS/MS has to be considered when developing tools for *de novo* glycopeptide structural analysis.

Mass spectrometry is a key method for the glycosylation analysis of biological samples. Several approaches are often combined to analyze protein glycosylation at the levels of intact glycoproteins, released glycans, or glycopeptides.[1] Glycans may be characterized in detail after enzymatic release by combining high-resolution separation techniques with tandem mass spectrometry.[2−7] Linkage information is provided by cross-ring cleavages, which are particularly prominent in high-energy collision-induced dissociation of sodium-adducts of native or permethylated glycans.[2] Moreover, negative-ion mode fragmentation of deprotonated, native species generally provides very detailed structural information.[8−10] (Tandem) mass spectrometry of tryptic glycopeptides is increasingly gaining popularity as this technique is part of proteomics work-flows aiming at protein identification, quantification, and characterization of various post-translational modifications.[11]

A significant portion of tandem mass spectrometric approaches in structural analysis of glycoconjugates deals with fragmentation of protonated species. These ions are easily generated from (tryptic) glycopeptides as well as reductively aminated glycans. Protonated glycans and glycoconjugates are typically less stable than the corresponding sodium adducts; therefore, dissociation may be induced after mild collisional activation. Fragmentation is characterized by the cleavage of (multiple) glycosidic bonds, resulting in B-type and Y-type ions.[11]

In addition to glycosidic-bond cleavages, rearrangements resulting in internal residue loss have been described in the collisionally induced dissociation (CID) of protonated oligosaccharides and glycoconjugates. These rearrangements have been reported to result in the loss of internal monosaccharide[12−16] or oligosaccharide units[14,17−19] which have been named "false" sugar sequence ions.[17] Next to proton adducts, ammonium adducts have been reported to result in such rearrangement products.[17] Recently, the MS/MS analysis of oligosaccharides derivatized by reductive amination with benzylamine and *N*,*N*-dimethylation of the secondary amine group revealed the migration of fucose to

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(1) Geyer, H.; Geyer, R. *Biochim. Biophys. Acta* **2006**, *1764*, 1853–1869.
(2) Zaia, J. *Mass Spectrom. Rev.* **2004**, *23*, 161–227.
(3) Zaia, J. *Mass Spectrom. Rev.* **2009**, *28*, 254–272.
(4) Wuhrer, M.; de Boer, A. R.; Deelder, A. M. *Mass Spectrom. Rev.* **2009**, *28*, 192–206.
(5) Wuhrer, M.; Deelder, A. M.; Hokke, C. H. *J. Chromatogr., B* **2005**, *825*, 124–133.
(6) Mechref, Y.; Novotny, M. V. *Chem. Rev.* **2002**, *102*, 321–369.
(7) Mechref, Y.; Novotny, M. V. *Mass Spectrom. Rev.* **2009**, *28*, 207–222.
(8) Harvey, D. J. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 647–659.
(9) Harvey, D. J. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 631–646.
(10) Wuhrer, M.; Deelder, A. M. *Anal. Chem.* **2005**, *77*, 6954–6959.
(11) Wuhrer, M.; Catalina, M. I.; Deelder, A. M.; Hokke, C. H. *J. Chromatogr., B* **2007**, *849*, 115–128.
(12) Ma, Y. L.; Vedernikova, I.; Van den, Heuvel, H.; Claeys, M. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 136–144.
(13) Warrack, B. M.; Hail, M. E.; Triolo, A.; Animati, F.; Seraglia, R.; Traldi, P. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 710–715.
(14) Harvey, D. J.; Mattu, T. S.; Wormald, M. R.; Royle, L.; Dwek, R. A.; Rudd, P. M. *Anal. Chem.* **2002**, *74*, 734–740.
(15) Tadano-Aritomi, K.; Hikita, T.; Kubota, M.; Kasama, T.; Toma, K.; Hakomori, S. I.; Ishizuka, I. *J. Mass Spectrom.* **2003**, *38*, 715–722.
(16) Dal Piaz, F.; De Leo, M.; Braca, A.; De Simone, F.; De Tommasi, N. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 286–296.
(17) Ernst, B.; Müller, D. R.; Richter, W. J. *Int. J. Mass Spectrom.* **1997**, *160*, 283–290.
(18) Wuhrer, M.; Balog, C. I. A.; Catimel, B.; Jones, F. M.; Schramm, G.; Haas, H.; Doenhoff, M. J.; Dunne, D. W.; Deelder, A. M.; Hokke, C. H. *FEBS J.* **2006**, *273*, 2276–2292.
(19) Wuhrer, M.; Koeleman, C. A. M.; Hokke, C. H.; Deelder, A. M. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1747–1754.

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the tagged, innermost monosaccharide.[20] Notably, because of the fixation of the charge on the quarternary nitrogen, these derivatives are not supposed to have mobile protons[20] and the mechanism of these rearrangements is not clear.

Ions generated by internal residue loss, if mistakenly interpreted as conventional glycosidic bond cleavages, may lead to the false postulation of structural motifs and suggest the presence of a mixture of isomers. Because of the lack of basic rules for the occurrence of rearrangement products associated with internal residue loss, tandem mass spectrometry of proton adducts is of rather limited value for the *de novo* structural elucidation of glycoconjugates. Notably, fucosylated glycans and glycoconjugates have repeatedly been shown to undergo rearrangements reactions very efficiently,[11,14] but rearrangements involving other monosaccharide moieties have likewise been shown.[12,14,15,21]

Here, we describe for the first time hexose rearrangements in tandem mass spectrometry of proton adducts of *N*-glycopeptides and reductively aminated *N*-glycans. A profound knowledge of the occurrence of such rearrangement reactions would appear to be essential to establish reliable *de novo* sequencing algorithms for structural glycomics at the glycopeptide level.

EXPERIMENTAL SECTION

Materials and Reagents. Peptide *N*-glycosidase F (PNGase F) was purchased from Roche Diagnostics, Mannheim, Germany. Bovine ribonuclease B (RNase B), 2-aminobenzamide (2AB), 2-aminobenzoic acid (anthranilic acid, AA), sodium cyanoborohydride, formic acid, and ammonia (25% aqueous solution) were from Sigma-Aldrich (Zwijndrecht, The Netherlands). The HILIC HPLC column (Amide-80, 4.6 mm × 25 cm, particle size 5 μm) was from Tosoh Bioscience, Stuttgart, Germany. 2,5-Dihydroxybenzoic acid (DHB) was obtained from Bruker Daltonics (Bremen, Germany). The PepMap column (3 μm; 75 μm × 150 mm) and guard column (300 μm × 5 mm) were from Dionex, Amsterdam, The Netherlands. Platin coated fused-silica electrospray needles 360 μm o.d., 20 μm i.d. with 10 μm opening) were from New Objective (Cambridge, MA).

Glycan Release and Labeling. *N*-Glycans were released from RNase B (50 mg) by PNGase F as described previously.[22] Briefly, protein was dissolved in PBS containing SDS (1%) and 2-mercaptoethanol (0.5%) and incubated for 10 min at 100 °C. Chaps was added to a final concentration of 1%, followed by overnight PNGase F treatment at 37 °C. Released glycans were sequentially purified by using RP- and graphitized carbon cartridges.[22] Glycans were labeled with the fluorescent compounds 2AB and AA by reductive amination with sodium cyanoborohydride.[23] The labeled glycans were purified by HILIC HPLC with fluorescence detection (λ_{ex}–λ_{em} 360–425 nm). Eluent A consisted of 50 mmol/L ammonium formiate (pH 4.4), and eluent B consisted of eluent A/acetonitrile 20:80. A linear gradient from 100% to 40% eluent B was applied at a flow rate of 1 mL/min. Peak-fractions were analyzed by MALDI-TOF-MS, and fractions containing isomers were pooled.

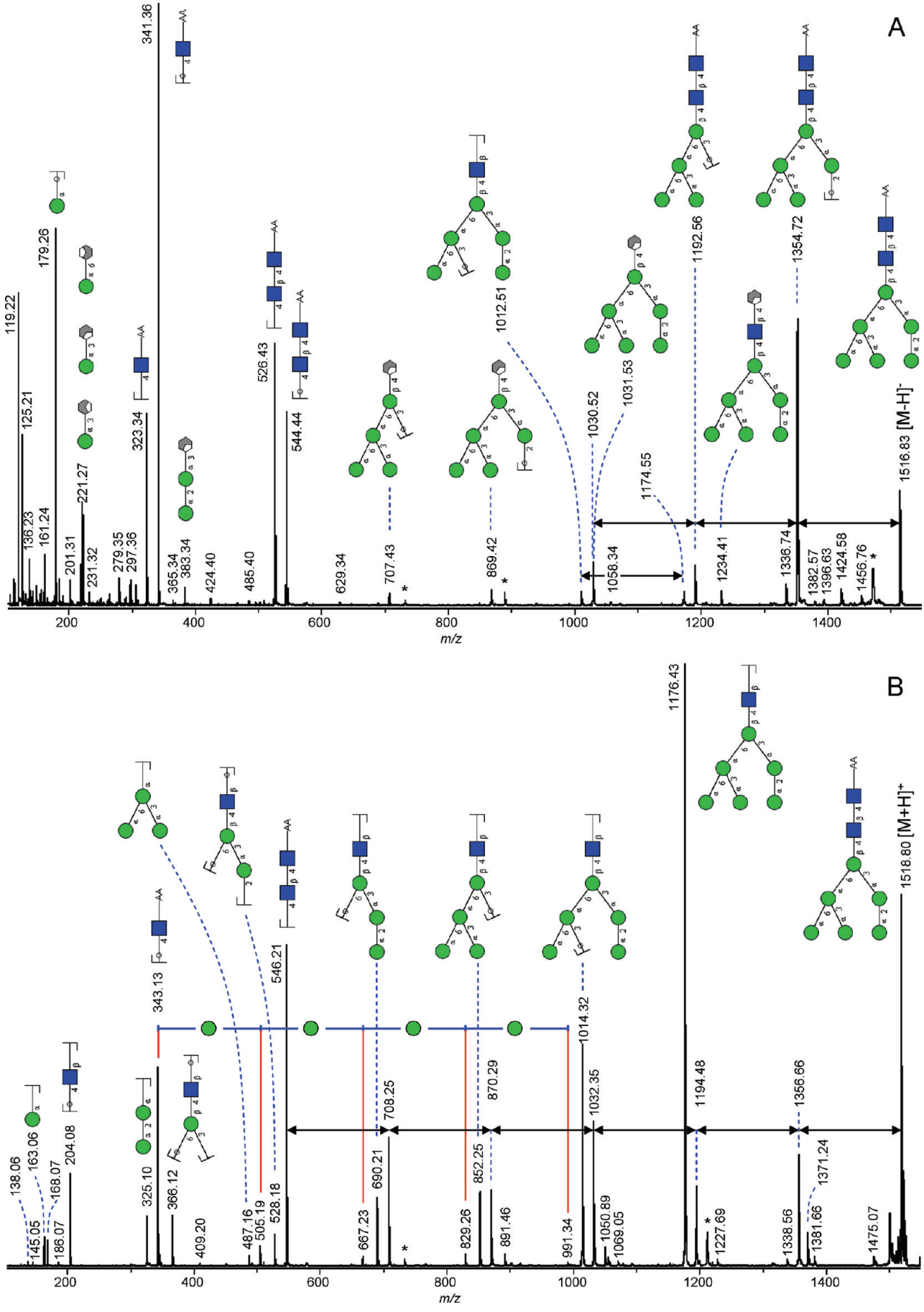
MALDI-TOF(/TOF)-MS(/MS). Samples were spotted with DHB (10 mg/mL in 30% acetonitrile) matrix and analyzed in the positive and/or negative (reflectron) mode by MALDI-TOF(/TOF)-MS(/MS) on a Ultraflex II mass spectrometer (Bruker Daltonics) containing a Smartbeam Nd:YAG laser (266 nm). Fragment analysis was achieved by laser-induced decomposition using the LIFT-TOF/TOF-MS/MS facility. Between 2 000 and 10 000 tandem mass spectra were acquired from different sites on the sample spot and were accumulated to obtain a representative fragment spectrum. Fragment ions were assigned using GlycoWorkbench.[24]

LC–MS(/MS). Reverse phase-nanoLC–ion trap (IT)-MS/MS of 2AB-labeled glycans was performed on a PepMap column using an Ultimate 3000 nanoLC system (Dionex) equipped with a guard column. The system was equilibrated with eluent A (0.4% acetonitrile, 0.1% formic acid in water) at a flow rate of 300 nL/min. After injection of the sample, a linear gradient to 25% eluent B (H_2O/acetonitrile 5:95, v/v, containing 0.1% formic acid) in 15 min was applied, followed by a gradient to 70% eluent B in another 10 min and a final wash with 70% B for 5 min. The nanoLC system was directly coupled to an Esquire High Capacity Trap (HCTultra) ESI-IT-MS (Bruker) equipped with an online nanospray source operating in the positive-ion mode at 900–1200 V. The solvent was evaporated at 170 °C with a nitrogen stream of 6 L/min. Ions from m/z 400–2000 were registered. Automatic fragment ion analysis was enabled, resulting in MS/MS spectra of the most abundant peaks. Fragment ions were assigned using GlycoWorkbench.[24]

RESULTS

N-Glycans of bovine ribonuclease B (RNase B) were released using PNGase F, labeled with 2-aminobenzoic acid (AA) and 2-aminobenzamide (2AB), purified by HILIC with fluorescence detection, and analyzed by MALDI-TOF/TOF-MS/MS. Fragmentation of the deprotonated, AA-labeled hexamannosidic (Man6GlcNAc2-AA) *N*-glycan in negative-ion mode is shown in Figure 1A. The tandem mass spectrum is characterized by cleavages of glycosidic bonds as well as some cross-ring cleavages, which are in full compliance with the structure of the RNase B Man6 *N*-glycan as described in the literature.[25,26] Analysis of the HPLC-purified Man6GlcNAc2-AA by MALDI-TOF-MS in positive-ion mode resulted in the registration of sodium adducts ($[M + Na]^+$ at m/z 1378), proton adducts ($[M + H]^+$ at m/z 1356), and potassium adducts ($[M + K]^+$, at m/z 1394) at relative intensities of approximately 60:20:20, respectively. Proton adducts of the RNase B Man6 in AA-labeled form (Figure 1B) and 2AB-labeled form (Figure 1C) were analyzed by MALDI-TOF/TOF-MS/MS. The fragmentation of the $[M + H]^+$ species did predominantly result in cleavages of glycosidic bonds, and no cross-ring cleavages were observed. The most intense fragment ions at m/z 1176 arose from chitobiose cleavage (Figure 1B,C). Moreover, a combination of chitobiose cleavage with loss of mannoses accounted for a series of ions at m/z 1014, m/z 852, m/z 690,

(20) Broberg, A. *Carbohydr. Res.* **2007**, *342*, 1462–1469.
(21) Hsu, F. F.; Turk, J. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 536–546.
(22) Wuhrer, M.; Koeleman, C. A. M.; Deelder, A. M.; Hokke, C. H. *FEBS J.* **2006**, *273*, 347–361.
(23) Bigge, J. C.; Patel, T. P.; Bruce, J. A.; Goulding, P. N.; Charles, S. M.; Parekh, R. B. *Anal. Biochem.* **1995**, *230*, 229–238.
(24) Ceroni, A.; Maass, K.; Geyer, H.; Geyer, R.; Dell, A.; Haslam, S. M. *J. Proteome Res.* **2008**, *7*, 1650–1659.
(25) Fu, D.; Chen, L.; O'Neill, R. A. *Carbohydr. Res.* **2009**, *261*, 173–186.
(26) Zhuang, Z.; Starkey, J. A.; Mechref, Y.; Novotny, M. V.; Jacobson, S. C. *Anal. Chem.* **2007**, *79*, 7170–7175.


A
119.22
125.21
136.23
161.24
179.26
201.31
221.27
231.32
279.35
297.36
323.34
341.36
365.34
383.34
424.40
485.40
526.43
544.44
629.34
707.43
869.42
1012.51
1030.52
1031.53
1056.34
1174.55
1192.56
1234.41
1336.74
1354.72
1382.57
1396.63
1424.58
1456.76
1516.83 [M-H]-
m/z
B
138.06
145.05
163.06
168.07
186.07
204.08
325.10
343.13
366.12
409.20
487.16
505.19
528.18
546.21
667.23
690.21
708.25
829.26
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991.34
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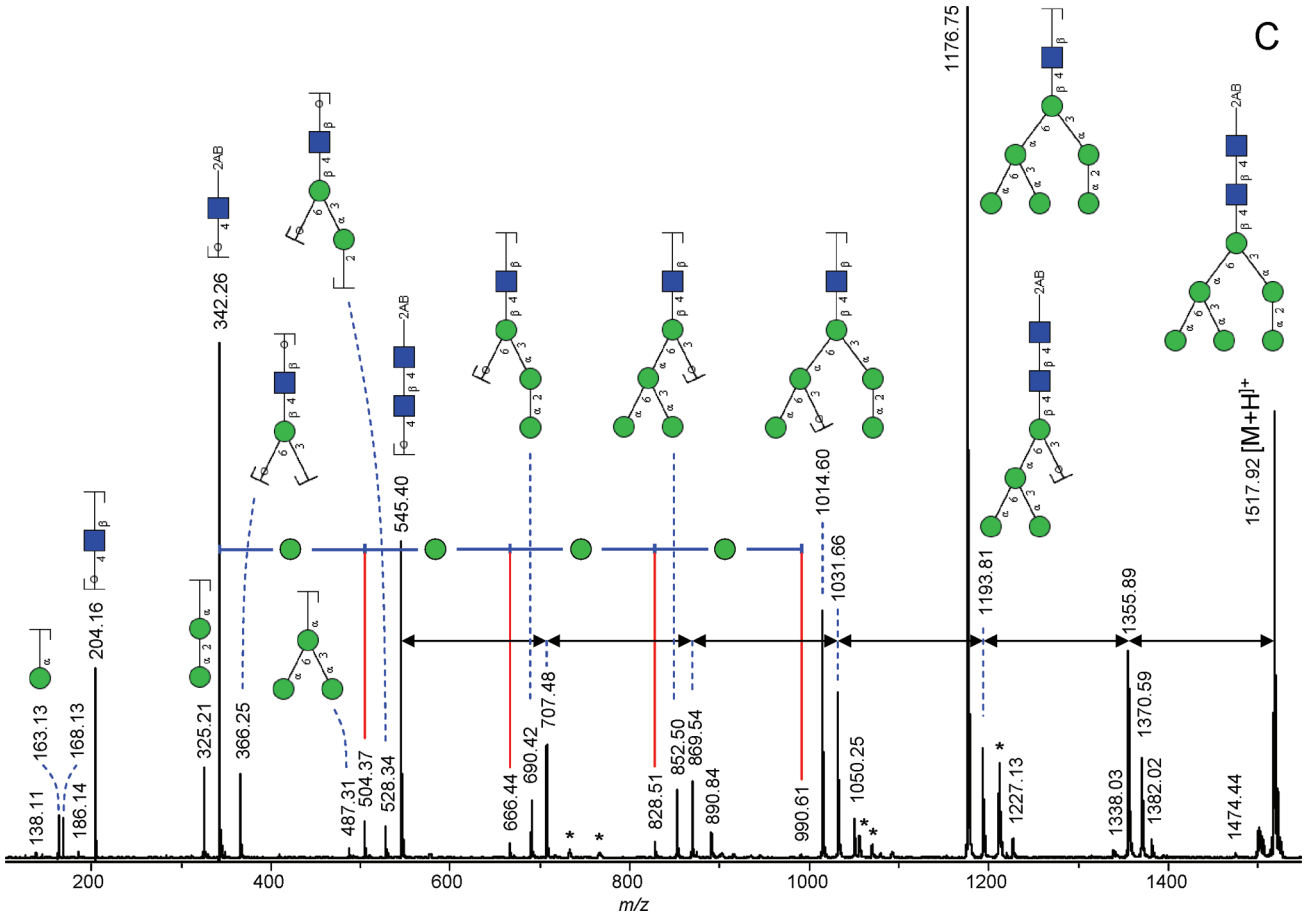

Figure 1. MALDI-TOF/TOF-MS/MS analysis of a reductively aminated hexamannosidic *N*-glycan. The Man6GlcNAc2 *N*-glycan from RNase B was labeled with 2-aminobenzoic acid (AA) and analyzed by MALDI-TOF/TOF-MS/MS in negative ion mode ((A) $[M - H]^-$) and positive-ion mode ((B) $[M + H]^+$). Man6GlcNAc2 from RNase B was labeled with 2AB and analyzed by MALDI-TOF/TOF-MS/MS in positive-ion mode ((C) $[M + H]^+$). Schemes often represent one of several possible assignments. Blue square, GlcNAc; green circle, mannose; double-headed arrow, hexose; *, poorly resolved metastable ion. Hexagons indicate A-type cross-ring cleavages, with the white segment corresponding to the carbon atoms retained on the A-type ion.[24]

m/z 528, *m/z* 366, and *m/z* 204. All the intense fragment ions included part of the chitobiose core, which clearly showed a preferred association of the proton with this part of the molecule. Ions of low intensity at *m/z* 163, *m/z* 325, and *m/z* 487 were oxonium ions and indicated that protons did also associate with oligomannosidic motifs of the molecule. Strikingly, the fragment ion of the AA-labeled glycan at *m/z* 343 (Figure 1B) arising from chitobiose cleavage (Y_1-ion) was accompanied by a series of related ions with added hexoses at *m/z* 505 (1 hexose), *m/z* 667 (2 hexoses), *m/z* 829 (3 hexoses), and *m/z* 991 (4 hexoses). A similar series of hexose additions was observed for the chitobiose cleavage (*m/z* 342) product of Man6GlcNAc2-2AB resulting in signals at *m/z* 504, *m/z* 666, *m/z* 828, and *m/z* 990 with one to four hexoses added (Figure 1C). Notably, these Y_1-ions "decorated" with hexoses were only observed in MALDI-TOF/TOF-MS/MS of proton adducts, and no corresponding hexose additions to the chitobiose cleavage products had been observed in negative-ion mode MALDI-TOF/TOF-MS/MS (Figure 1A) and on fragmentation of sodium adducts (not shown). Moreover, these fragment ions did not correspond to known structural motifs on RNase B glycans and were, therefore, interpreted as rearrangement products. In order to confirm the composition of these rearrangement products, the ion observed at *m/z* 828 (Figure 1C) was subjected to further investigation. Fragmentation of the 2AB-labeled *N*-glycan was achieved by in-source decay, and the product ion at *m/z* 828 was selected and further fragmented by MALDI-TOF/TOF-MS/MS (Figure 2). The resulting fragment-ion spectrum clearly identified this species as the chitobiose cleavage product carrying three hexose moieties.

AA-labeled and 2AB-labeled RNase B N-glycans with 5, 7, 8, and 9 mannose residues were similarly analyzed by negative-ion mode ($[M - H]^-$) and positive-ion mode ($[M + H]^+$) MALDI-TOF/TOF-MS/MS (mass spectra not shown). Consistently, mannose additions to the chitobiose cleavage products were observed upon fragmentation of the protonated species, while all negative mode fragment ions were in accordance with the known RNase B *N*-glycans structures and the rules established for negative mode tandem mass spectrometry of glycans.[8−10] In order to visualize the efficacy of the rearrangement events, the intensities of the mannose-containing chitobiose cleavage products were normalized to the intensity of the related ion lacking the mannose (Y_1-ion at *m/z* 343 and *m/z* 342 for AA-labeled and 2AB-labeled glycans, arising from chitobiose cleavage) and are displayed in histograms (Figure 3B,C). The rearrangement ion comprising one mannose had an intensity of between 6% and 11%

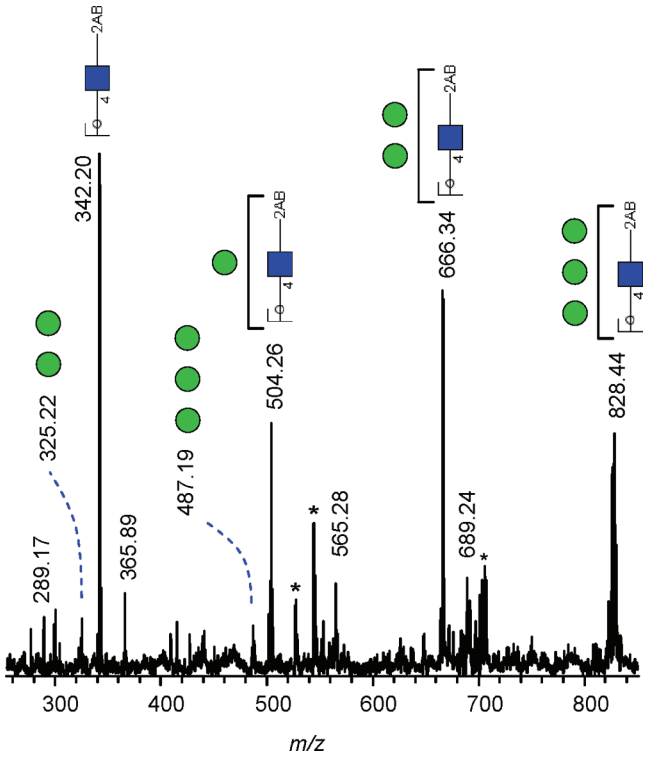

Figure 2. MALDI-TOF/TOF-MS/MS analysis of a hexose rearrangement product. The fragment ion at *m/z* 828, which was observed in MALDI-TOF/TOF-MS (postsource decay; Figure 1C) was selected as an in-source decay ion for further fragment analysis (postsource decay) by MALDI-TOF/TOF-MS/MS. Annotation was performed similar to Figure 1.

of the intensity of the Y_1-ion (Figure 3B,C). This ion had the highest intensity of all mannose rearrangement products in all 10 fragment ion spectra of protonated oligomannosidic *N*-glycans. In general terms, the more mannoses had to be transferred during rearrangement, the lower the relative intensity of the product. Strikingly, while the intensities of the rearrangement products with two and three mannoses were still rather high for Man5 and Man6 *N*-glycans, rearrangement products with four or more mannoses were of much lower intensity (Figure 3B,C). For Man7 and Man8, a similarly sharp drop in intensities was observed: rearrangement products with up to four mannoses were of high intensity, while those with five and more mannoses were hardly detectable. For Man9, in contrast, up to five mannoses were rather efficiently transferred to fluorescently labeled innermost GlcNAc, while the transfer of six or more mannoses was hardly observed. When comparing the mannose transfer patterns (Figure 3B,C) with the major structures of RNase B *N*-glycans (Figure 3A), we found that the number of mannoses present on the α1-6-linked branch of the trimannosyl core (boxed in red, Figure 3A) correlated with the maximum number of mannoses that were still transferred efficiently (red bars in Figure 3B,C). For the transfer of multiple hexoses, this means that any mannose present on the six-branch of the oligomannosidic *N*-glycan may be transferred efficiently to the labeled innermost GlcNAc, compared to the other mannoses present in the molecule. Whether this transfer occurs en bloc or one by one may not be concluded from the present data. Notably, our data give no indication as to the origin of single transferred mannose that results in the fragment ions of composition H1N1-AA and H1N1-AB.

Notably, rearrangement products were not restricted to tandem mass spectrometry by MALDI-TOF/TOF-MS/MS: analysis of the RNase B Man6GlcNAc2-2AB *N*-glycan ($[M + 2H]^{2+}$ at *m/z* 759) by reverse phase-nanoLC−ESI-IT-MS/MS with collisional activation likewise resulted in a hexose rearrangement product at *m/z* 504 (Figure 4). The other 2AB-labeled oligomannosidic *N*-glycans of RNase B were also analyzed which similarly revealed the hexose rearrangement product at *m/z* 504. Rearrangement products with a higher number of hexoses were also visible in some cases, however, at very low intensities (not shown).

In the second part of the study, we addressed the question whether similar hexose rearrangement events occur on tandem mass spectrometry of glycopeptides, as these glycoconjugates are predominantly analyzed in protonated form, similar to the oligomannosidic glycans analyzed above. We started by doing an extensive literature search of glycopeptide tandem mass spectra which showed that similar fragment ions pointing to hexose rearrangement reactions could indeed be found in two tandem mass spectrometric analyses of glycopeptides with oligomannosidic glycans.[27,28] First, a tryptic glycopeptide from RNase B was analyzed by Zhang and Chelius using ion trap multistage tandem mass spectrometry.[28] Using this approach, they fragmented a glycopeptide with a pentamannosidic *N*-glycan. From the observed fragments, they isolated the fragment resulting from the loss of a single mannose. This ion was further fragmented, resulting among others in an ion retaining three mannose residues. Isolation and fragmentation of this ion provided the peptide moiety with a dimannosidic structure, which was subjected to fragmentation, followed by registration of the fragment ions (Figure 5A). Again, while most ions were assigned to glycosidic bond cleavages, two ions at *m/z* 840 and *m/z* 1002 remained unassigned in the original report.[28] On the basis of the above-mentioned indications of hexose rearrangements, these ions may be assigned to $[\text{peptide} + \text{HexNAc} + \text{Hex} + \text{H}]^+$ and $[\text{peptide} + \text{HexNAc} + 2\text{Hex} + \text{H}]^+$, respectively (Figure 5A). Notably, the latter ion corresponds to the loss of an internal *N*-acetylglucosamine, with the retention of the two mannose residues.

Second, the analysis by Nimtz et al.[27] of a tryptic *N*-glycopeptide of a chicken eggshell glycoprotein carrying a Man7 *N*-glycan structure by ESI-quadrupole TOF-MS/MS revealed similar rearrangement candidates: a major ion at *m/z* 920 ($[\text{peptide} + \text{HexNAc} + \text{H}]^+$) arising from chitobiose cleavage (Figure 5B) was accompanied by a signal at *m/z* 1082 which remained unassigned in the original report.[27] This ion may be explained as a hexose rearrangement product ($[\text{peptide} + \text{HexNAc} + \text{Hex} + \text{H}]^+$), as indicated in Figure 5B.

Next to these two studies,[27,28] in which putative rearrangement products could be assigned with confidence as the registered masses of the peaks were indicated, eight other studies were found in the literature which showed candidate hexose rearrangement peaks in 11 tandem mass spectra of glycopeptides with oligomannosidic *N*-glycans (Table 1). The registered mass of the putative rearrangement products was

(27) Nimtz, M.; Conradt, H. S.; Mann, K. *Biochim. Biophys. Acta* **2004**, *1675*, 71–80.
(28) Zhang, S.; Chelius, D. *J. Biomol. Tech.* **2004**, *15*, 120–133.

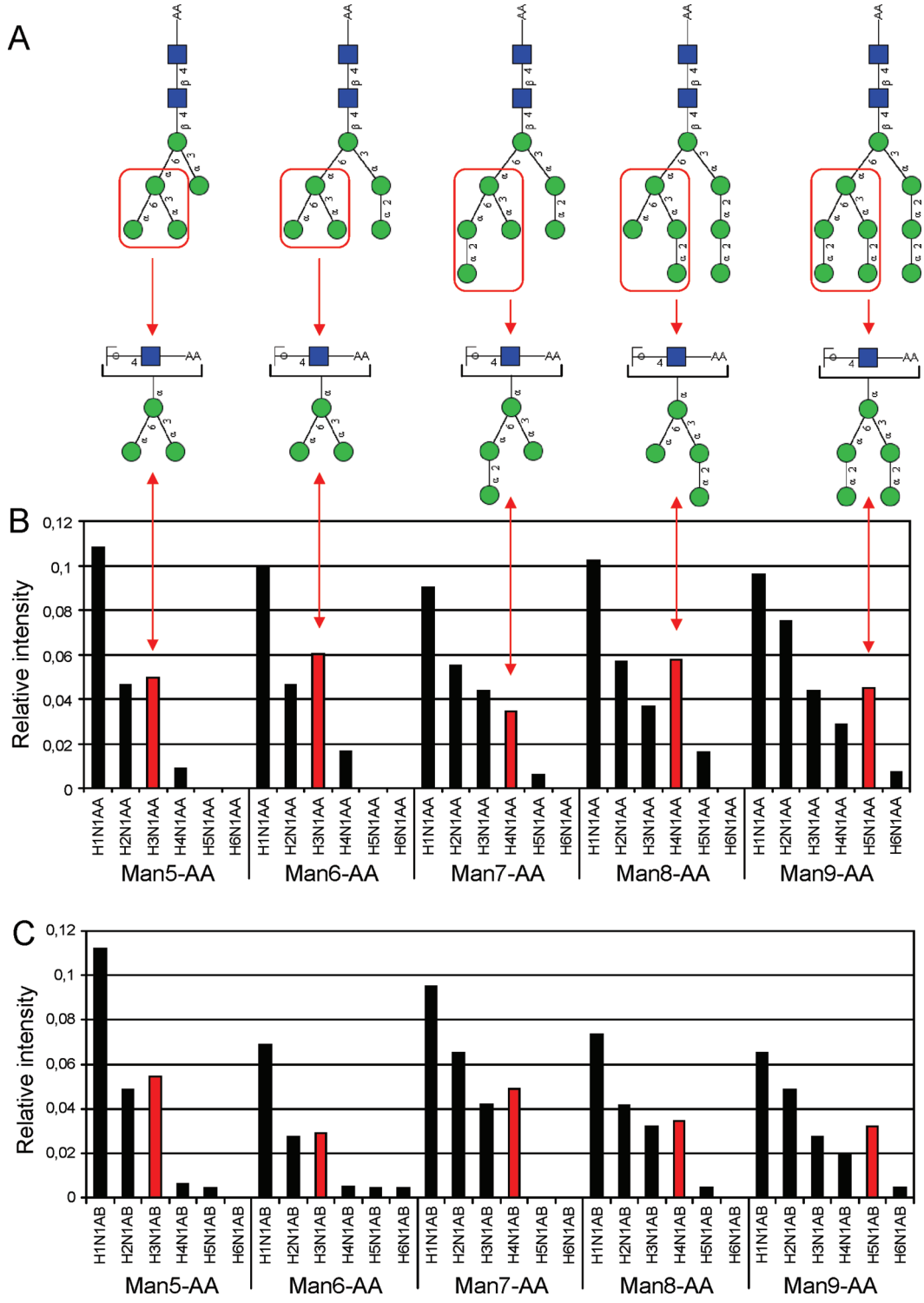

Figure 3. Relative quantitation and schematic representation of hexose rearrangement products. (A) Schematic representation of the major RNase B *N*-glycans. The α1-6-linked branch, which is postulated to undergo hexose transfer reactions particularly efficiently, is boxed in red. Histograms of the relative intensities of hexose rearrangement products observed for AA-labeled (B) and 2AB-labeled (C) RNase B *N*-glycans in MALDI-TOF/TOF-MS of proton adducts support the preferred rearrangement involving the α1-6-linked branch of the oligomannosidic *N*-glycans. Intensities are given relative to the signal of the fluorescently labeled innermost GlcNAc (*m*/*z* 343 and *m*/*z* 342 in parts B and C, respectively).

not provided in these studies. Therefore, masses of the candidate peaks were estimated using graphical interpolation, and the resulting values are given in Table 1. In our literature study, we encountered mannose rearrangement candidates for glycopeptides from six different proteins from different organisms, namely, rat, mouse, cattle, chicken, and cauliflower (Table 1). The study of Zhang and Chelius[28] as well as four other studies applied tandem mass spectrometry to RNase B tryptic glycopeptides resulting in rearrangement candidates (Table 1).[29−32] Two studies were analyzing RNase B tryptic glycopeptides without missed cleavage sites,[28,30] while three studies were looking at glycopeptides with a missed cleavage site.[29,31,32]

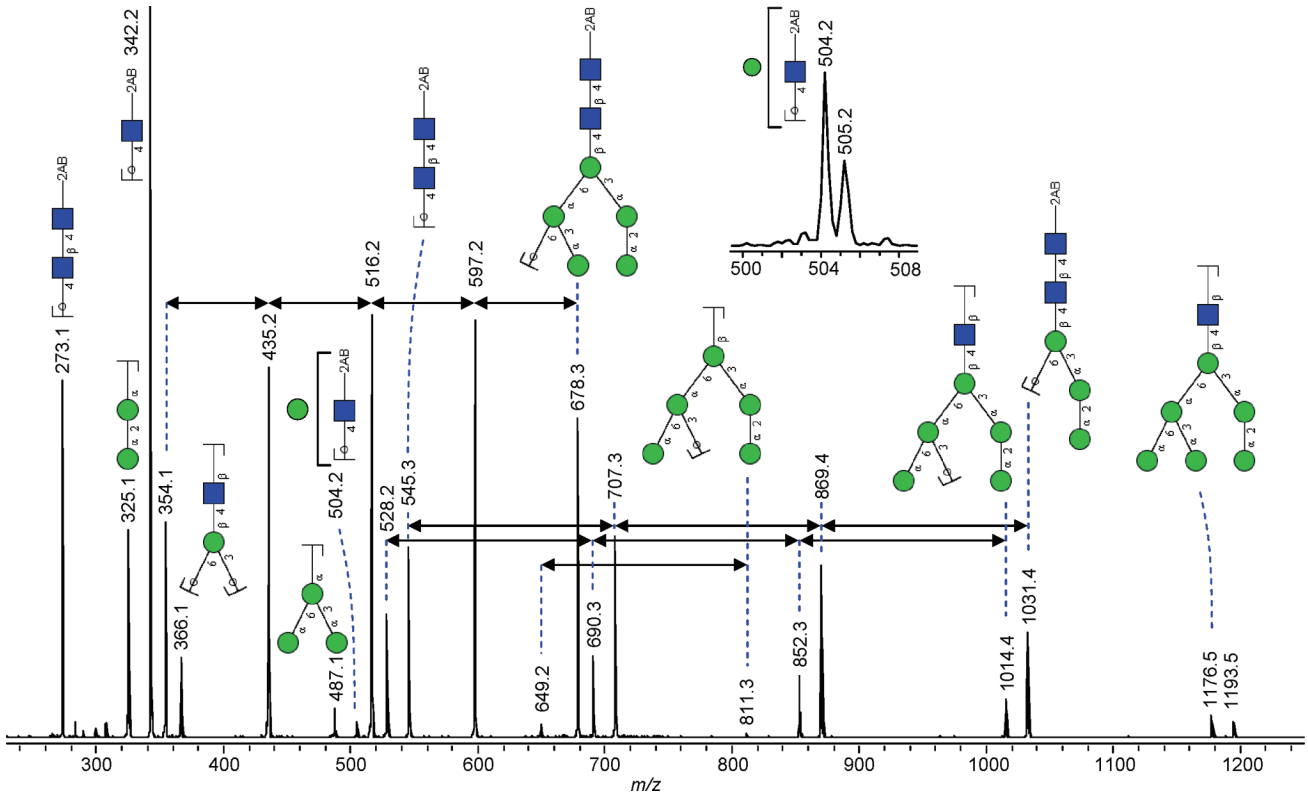

Figure 4. ESI-IT-tandem mass spectrum of a diprotonated, 2AB-labeled oligomannosidic *N*-glycan. Man6GlcNAc2-2AB from RNase B was analyzed by reverse phase-nanoLC−IT-MS/MS ($[M + 2H]^{2+}$ at *m/z* 759). Next to ions arising from glycosidic bond cleavages, a hexose rearrangement product was observed at *m/z* 504. Annotation was performed similar to Figure 1.

IgG Fc glycopeptides with complex-type *N*-glycans showed hexose rearrangement products similar to those observed for glycopeptides with oligomannosidic *N*-glycans. Figure 6 shows a tandem mass spectrum (nanoLC−ESI-IT-MS/MS) of the tryptic Fc glycopeptide of IgG1 purified from human plasma that carries a neutral biantennary, digalactosylated *N*-glycan lacking core fucose ($[M + 2H]^{2+}$ at *m/z* 1406). While most ions could be assigned to glycosidic bond cleavages resulting in B-ions and Y-ions that may eliminate water and ammonia, a low-intensity fragment ion at *m/z* 1554 corresponded to a theoretical composition of a singly protonated peptide moiety carrying a HexNAc and a hexose residue. Similar fragment ions corresponding to $[peptide + HexNAc + Hex + H]^+$ were observed in fragment ion analysis of other tryptic Fc glycopeptides of human IgG1, IgG2, and IgG4 (data not shown). While human IgG Fc N-glycosylation is particularly well-characterized, hexose attachment to the innermost GlcNAc has not been reported yet. As such *N*-glycan modifications have to our knowledge not been reported in mammals, we assume that the $[peptide + HexNAc + Hex + H]^+$ fragment ion observed for human IgG glycopeptides arose from hexose rearrangements, as they have been described above for oligomannosidic glycopeptides. Moreover, a similar ion of theoretical composition peptide + GlcNAc + Hex was observed on fragmentation of a Fc glycopeptide with a core-α1-3-fucosylated, core-xylosylated trimannosyl *N*-glycan exhibiting a bisecting GlcNAc (Hex3HexNAc5dHex1Pent1) that was derived from a glyco-engineered human monoclonal IgG2 produced in genetically modified tobacco plants (signal at *m/z* 1522 in Figure 7 of Rouwendal et al.;[33] Table 1). These findings indicate that hexose rearrangements may represent a general phenomenon in collisional fragmentation of protonated IgG glycopeptides.

DISCUSSION

Hexose rearrangements were observed in tandem mass spectrometry of proton adducts of both reductively aminated *N*-glycans and tryptic *N*-glycopeptides. Strikingly, the transfer of higher numbers of hexoses to the conjugated innermost GlcNAc of reductively aminated glycans was occurring rather efficiently in MALDI-TOF/TOF-MS. This may be due to the open-ring form of the innermost GlcNAc, resulting in enhanced flexibility of this residue and may point to a specific role of the protonated secondary amine group of the AA- and 2AB-labels in catalyzing a fragmentation/rearrangement reaction, as postulated by Harvey et al.[14] Mo et al. have not observed hexose rearrangement products on the MALDI postsource decay MS of an RNase B Man8GlcNAc2 *N*-glycan labeled with 4-aminobenzoic acid 2(diethylamino)ethyl ester, which points to a role of the label in

(29) Liu, T.; Li, J.-D.; Zeng, R.; Shao, X.-X.; Wang, K.-Y.; Xia, Q.-C. *Anal. Chem.* **2001**, *73*, 5875–5885.
(30) Pitchayawasin, S.; Isobe, M. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1424–1433.
(31) Henning, S.; Peter-Katalinic, J.; Pohlentz, G. *J. Mass Spectrom.* **2007**, *42*, 1415–1421.
(32) Temporini, C.; Perani, E.; Calleri, E.; Dolcini, L.; Lubda, D.; Caccialanza, G.; Massolini, G. *Anal. Chem.* **2007**, *79*, 355–363.
(33) Rouwendal, G. J.; Wuhrer, M.; Florack, D. E.; Koeleman, C. A.; Deelder, A. M.; Bakker, H.; Stoopen, G. M.; van, Die, I.; Helsper, J. P.; Hokke, C. H.; Bosch, D. *Glycobiology* **2007**, *17*, 334–344.

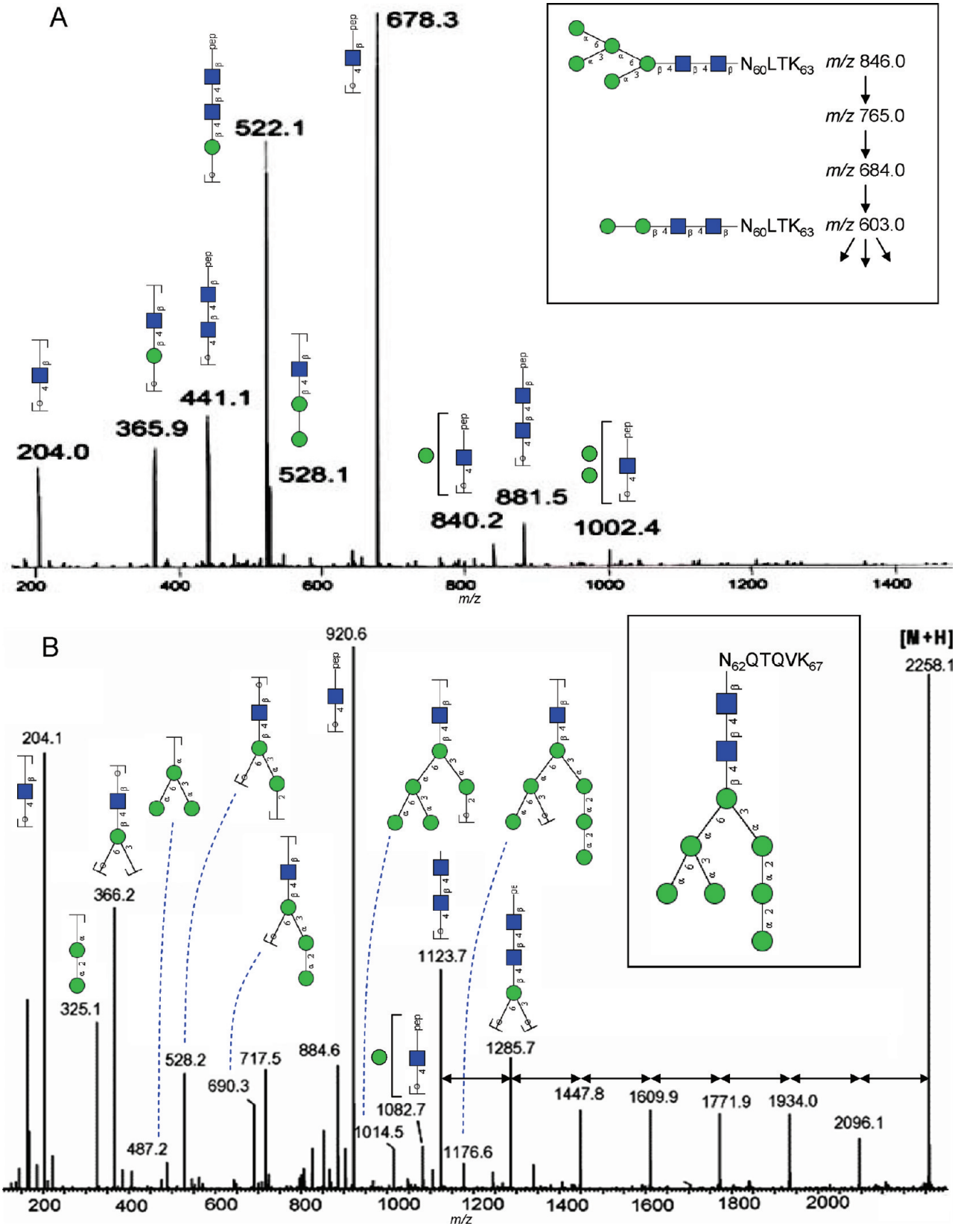

Figure 5. Examples for glycopeptide tandem mass spectra from literature that show indications for hexose rearrangements. (A) Nano-ESI-MS^5 spectrum of the tryptic glycopeptide from bovine RNase B.[28] The double-charged glycopeptide carrying a pentamannosidic *N*-glycan was selected as a precursor. In a series of four ion selection/fragmentation cycles, a dimannosidic glycopeptide was generated and fragmented, as shown in the box. (B) NanoLC−ESI-quadrupole-TOF-MS/MS of a tryptic glycopeptide from chicken egg shell glycoprotein carrying a heptamannosidic *N*-glycan.[27] Part A was reprinted with permission from Zhang and Chelius (2004).[28] Copyright 2004 The Association of Biomolecular Resource Facilities. Part B was reprinted with permission from Nimtz et al. (2004).[27] Copyright 2004 Elsevier. Blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose; white circle, hexose; pep, peptide moiety; #, loss of water and/or ammonia.

Table 1. Putative Hexose Rearrangement Products Observed Tandem Mass Spectra of Glycopeptides from Literature[a]

glycopeptide species	potential hexose rearrangement peak	proposed assignment	ref
tryptic glycopeptide from chicken eggshell glycoprotein with a Hex7HexNAc2 *N*-glycan	peak at *m/z* 1082 in deconvoluted tandem mass spectrum obtained by LC−ESI-quadrupole TOF-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Nimtz et al.,[27] Figure 1A; see Figure 5B
tryptic glycopeptide from bovine RNase B with a Hex5HexNAc2 *N*-glycan	peaks at *m/z* 840 and *m/z* 1002 in MS^5 experiment performed on a $[pep + HexNAc2Hex2 + 2H]^{2+}$ using chip-based infusion-ESI-IT-MS/MS	$[pep + HexNAc1Hex1 + H]^+$ and $[pep + HexNAc1Hex2 + H]^+$	Zhang and Chelius,[28] Figure 4C; see Figure 5A
tryptic glycopeptides from bovine RNase B with one missed cleavage site containing Hex5HexNAc2 and Hex6HexNAc2 *N*-glycans	peaks at approximately *m/z* 1083 in tandem mass spectra obtained by capillary electrophoresis−ESI-IT-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Liu et al.,[29] Figure 4A,B
tryptic glycopeptide from bovine RNase B with one missed cleavage site containing a Hex5HexNAc2 *N*-glycan	peaks at approximately *m/z* 1083 in tandem mass spectra obtained by nano-ESI-quadrupole-TOF-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Henning et al.,[31] Figure 2
tryptic glycopeptide from bovine RNase B containing a Hex6HexNAc2 *N*-glycan	peak at approximately *m/z* 840 in tandem mass spectrum obtained by LC−ESI-quadrupole-TOF-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Pitchayawasin and Isobe,[30] Figure 4
tryptic glycopeptide from bovine RNase B with one missed cleavage site containing a Hex5HexNAc2 *N*-glycan	peaks at approximately *m/z* 1111 in tandem mass spectra obtained by nano-ESI-quadrupole-TOF-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Temporini et al.,[32] Figure 6A
tryptic glycopeptide from cauliflower xyloglycan endotransglycosidase 16A with a Hex6HexNAc2 *N*-glycan	peak at approximately *m/z* 2765 in deconvoluted tandem mass spectrum obtained by LC−ESI-quadrupole-TOF-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Hendriksson et al.,[37] Figure 5
tryptic glycopeptide from rat brain Thy-1 with a Hex5HexNAc4dHex2 *N*-glycan	peaks at approximately *m/z* 1472 and *m/z* 1634 in tandem mass spectra obtained by LC−ESI-IT-MS/MS	$[pep + HexNAc1Hex1 + H]^+$; $[pep + HexNAc1Hex2 + H]^+$	Itoh et al.,[38] Figure 3A
tryptic glycopeptide from rat brain Thy-1 with a Hex5HexNAc2 *N*-glycan	peak at approximately *m/z* 980 in tandem mass spectrum obtained by LC−ESI-IT-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Itoh et al.,[38] Figure 5A
tryptic glycopeptide from murine cyclooxygenase 2 with a Hex9HexNAc2 *N*-glycan	peak at approximately *m/z* 1988 in tandem mass spectra obtained by nano-ESI-quadrupole-TOF-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Nemeth et al.,[39] Figure 3B
trytpic glycopeptide from rat brain LAMP glycoprotein with a Hex5HexNAc2 *N*-glycan	peak at approximately *m/z* 1123 in tandem mass spectrum obtained by LC−ESI-IT-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Itoh et al.,[40] Figure 5A1
tryptic glycopeptide from rat brain Kilon glycoprotein with a Hex5HexNAc2 *N*-glycan	peak at approximately *m/z* 1081 in tandem mass spectrum obtained by LC−ESI-IT-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Itoh et al.,[40] Figure 8A1
tryptic glycopeptide from glycoengineered human IgG1 expressed in tobacco with a Hex3HexNAc5dHex1Pent1 *N*-glycan	peak at *m/z* 1522 in tandem mass spectrum obtained by LC−ESI-IT-MS/MS	$[pep + HexNAc1Hex1 + H]^+$	Rouwendal et al.,[33] Figure 7

[a] For hexose rearrangement candidates which were not labeled with a registered mass, the mass was determined by graphical interpolation and showed good agreement with the theoretical mass of the hexose rearrangement products (deviation below 2 Da).

inducing hexose rearrangement reactions.[34] Notably, the rearrangement reactions for AA-labeled and 2AB-labeled oligomannosidic *N*-glycans were less pronounced in ESI-IT-MS/MS of the diprotonated forms (Figure 4) than in MALDI-TOF/TOF-MS of the single protonated species (Figure 1C), and rearrangements with transfer of multiple hexoses were restricted to MALDI-TOF/TOF-MS, which may indicate a role of the charge state in determining rearrangement events.

The ions at *m/z* 505, *m/z* 667, *m/z* 829, and *m/z* 991, that are interpreted as rearrangement products, show a mass difference of 41 Da when compared to the series of GlcNAc2-AA (*m/z* 546), Man1GlcNAc2-AA (*m/z* 708), Man2GlcNAc2-AA (*m/z* 870), and Man3GlcNAc2AA (*m/z* 1032), respectively (Figure 1C). However, no conclusive explanation for such a loss of a 41 Da functional

(34) Mo, W.; Takao, T.; Sakamoto, H.; Shimonishi, Y. *Anal. Chem.* **1998**, *70*, 4520–4526.

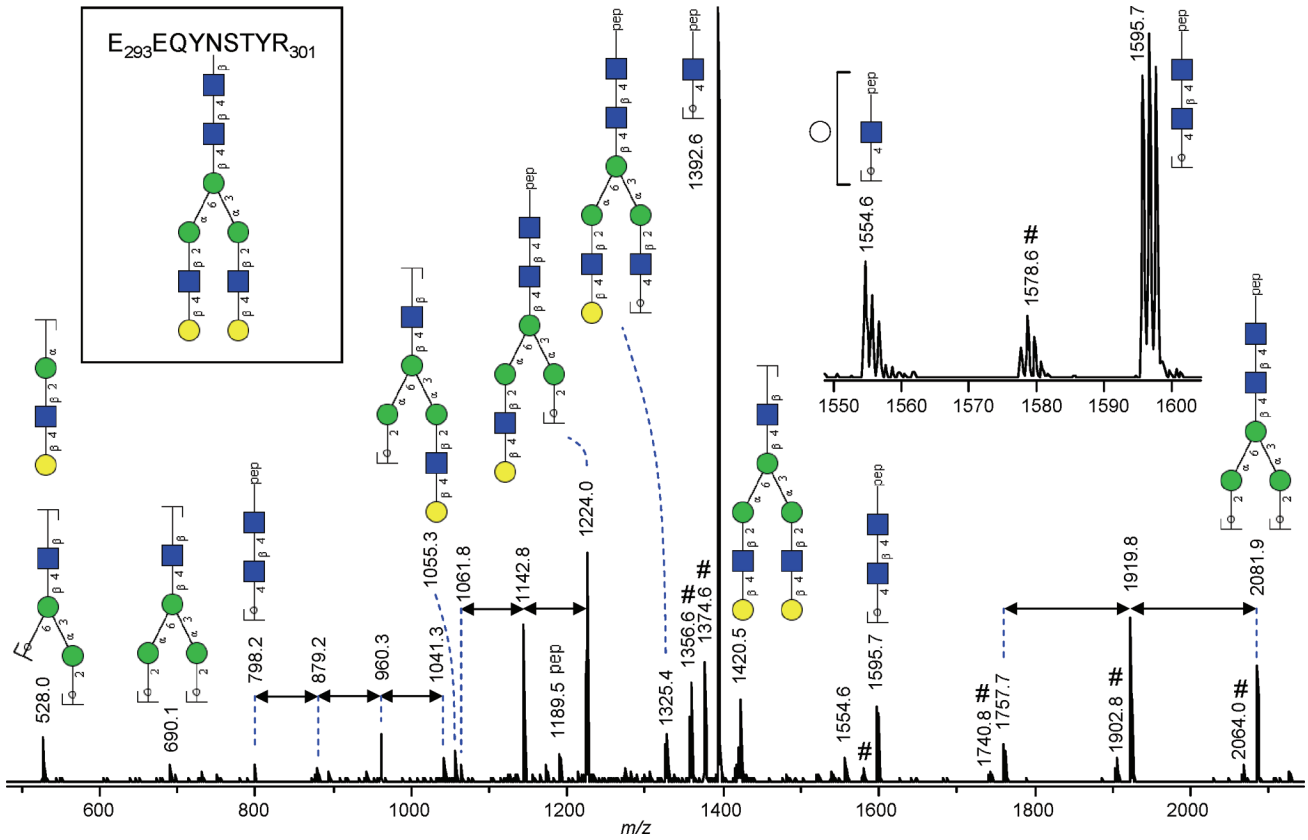

Figure 6. NanoLC−ESI-IT-MS/MS of a tryptic Fc glycopeptide of IgG1 from human plasma (precursor $[M + 2H]^{2+}$ at *m/z* 1406). The attached oligosaccharide is a nonsialylated biantennary, noncorefucosylated *N*-glycan. Annotation was performed similar to Figure 5.

group could be found: it may theoretically correspond to a C2N1H3 unit (even electron) or a C2O1H1 unit (odd electron).

We observed a correlation between the size of the 6-branch of the RNase B oligomannosidic *N*-glycans and the number of hexoses which is still transferred efficiently to the fluorescently labeled GlcNAc. This suggests that the α1-6-linked mannose of the trimannosyl core, together with its substituents, may easily act as a donor in the transfer of multiple hexoses to the secondary amine group of the tag, probably due to the pronounced flexibility of the 6-branch. We would assume that this transfer of multiple mannoses occurs en bloc and is part of a rearrangement reaction that leads to the cleavage of the chitobiose core. However, transfer one by one of the mannoses cannot be ruled out at the current stage. Moreover, while the preferred transfer of the 6-antenna substituents seems to result in multiply mannosylated rearrangement products, the current data do not allow us to specify the origin of the single mannose included in the H1N1-AA and H1N1-AB fragment ions.

Isotope labeled standards may provide a key in future experiments to establish preferred migration paths in hexose rearrangements. Isotope labeling approaches may likewise be necessary to elucidate the transfer paths in fucose rearrangements.[19] Other derivatization approaches like permethylation, in contrast, are expected to suppress most of the rearrangement reactions, as has been shown for fucose rearrangements,[19] and may therefore only provide a limited contribution to the elucidation of rearrangement paths and mechanisms.

N-Glycopeptide fragmentation is known to depend largely on the type of tandem mass spectrometer and fragmentation mode applied. A comparative study on glycopeptide fragmentation using CID and ETD on ion traps, quadrupole fragmentation applying a range of different energies, MALDI-TOF/TOF-MS with postsource decay and high-energy fragmentation, as well as infrared multiphoton fragmentation (IRMPD) and 157 nm photodissociation should provide deeper insights in rearrangements occurring in glycopeptide fragmentation.[11,35,36]

One may speculate as to the acceptor site of the transferred hexoses in tandem mass spectrometry of glycopeptides: the innermost GlcNAc as well as various functional groups of the peptide moiety may possibly serve as acceptor structures. The contribution of these different candidate acceptor sites is expected to depend on the sequence and structure of the individual peptide moiety as well as on the structure of the glycan part. Strikingly, while many tandem mass spectra of glycopeptides with complex-type *N*-glycans are reported in literature, products of rearrangement reactions of complex-type *N*-glycans were only found for IgG tryptic Fc glycopeptides. This indicates that particular peptide sequences, including the IgG tryptic Fc glycopeptide, possibly together with the charge state and charge distribution of the glycopeptide, may stimulate rearrangement reactions by providing mobile protons and hexose acceptor groups.

(35) Seipert, R. R.; Dodds, E. D.; Clowers, B. H.; Beecroft, S. M.; German, J. B.; Lebrilla, C. B. *Anal. Chem.* **2008**, *80*, 3684–3692.
(36) Zhang, L.; Reilly, J. P. *J. Proteome Res.* **2009**, *8*, 734–742.
(37) Henriksson, H.; Denman, S. E.; Campuzano, I. D.; Ademark, P.; Master, E. R.; Teeri, T. T.; Brumer, H., III *Biochem. J.* **2003**, *375*, 61–73.
(38) Itoh, S.; Kawasaki, N.; Harazono, A.; Hashii, N.; Matsuishi, Y.; Kawanishi, T.; Hayakawa, T. *J. Chromatogr., A* **2005**, *1094*, 105–117.
(39) Nemeth, J. F.; Hochgesang, G. P., Jr.; Marnett, L. J.; Caprioli, R. M. *Biochemistry* **2001**, *40*, 3109–3116.
(40) Itoh, S.; Hachisuka, A.; Kawasaki, N.; Hashii, N.; Teshima, R.; Hayakawa, T.; Kawanishi, T.; Yamaguchi, T. *Biochemistry* **2008**, *47*, 10132–10154.

Monosaccharide rearrangements seem to be closely associated with the fragmentation event. In this study we observed rearrangement reactions occurring at very different time scales and charge states (MALDI-TOF/TOF-MS versus ESI-IT-MS/MS), which is in line with an earlier report.[19] Hence, we do not expect that the choice of specific mass spectrometric instruments and settings will allow the suppression of these rearrangement reactions in tandem mass spectrometry using collision-induced dissociation of protonated glycoconjugates.

Ions resulting from internal residue loss are generally identified on the basis of their unique composition which may not be explained by glycosidic bond cleavages alone. Hexose rearrangements may, however, result in ions of nonspectacular composition and may, therefore, be mistaken as a conventional fragment arising from glycosidic bond cleavage. Hence, we predict that the actual prevalence of rearrangement reactions with internal residue loss may be much higher than currently indicated. In the case of multistage tandem mass spectrometry, as applied by Zhang and Chelius,[28] the accumulation of "hidden" rearrangement products of nonspectacular composition in the course of multiple fragmentation events may actually lead to an accumulation of rearrangement products in the multistage fragment spectrum (see Figure 5A).

We studied a range of tandem mass spectra of *N*-glycopeptides from the literature for putative hexose rearrangement products (Table 1). Unfortunately, the candidate rearrangement peaks, which we saw in many literature tandem mass spectra, were in most cases not labeled with the registered mass. This makes reinvestigation and interpretation of tandem mass spectra particularly difficult. There is clearly a custom of not giving masses of peaks that could not be assigned by the investigators. We would like to stimulate the use of mass labels for assigned peaks as well as nonassigned peaks.

Awareness of the possibility of hexose rearrangements in fragmentation of glycopeptides is expected to be important for *de novo* glycopeptide structural analysis, which may be performed by manual interpretation or with the aid of software tools. Notably, software for the interpretation of glycopeptide MS/MS data should be able to deal with the fact that some fragments might not directly represent structural motifs of the intact glycopeptide but rather correspond to rearrangement products. These signals should, therefore, not be mistaken as indicator of the presence of minor isomers in glycopeptide mixtures.

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