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Dissection of Fragmentation Pathways in Protonated N-acetylhexosamines

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Abstract

Structural characterization of carbohydrates by mass spectrometry necessitates a detailed understanding of their gas phase behavior, particularly for protonated carbohydrates that can undergo complex structural rearrangements during fragmentation. Here we utilize tandem mass spectrometry, isotopic labeling, gas-phase hydrogen/deuterium exchange, and ion mobility measurements to characterize structures of the various product ions of protonated N-acetylhexosamines. Following the facile loss of the reducing end hydroxyl group we identify two primary fragmentation pathways. Detailed mapping of each step in the fragmentation pathway provides new insight into the mechanisms that drive collision induced dissociation of protonated carbohydrates. Several of the smaller fragment ions are mixtures of structural isomers and the relative distributions of these structures reveals information about the stereochemistry of the precursor molecule.

INTRODUCTION

Complex carbohydrate chains in biological matrices, also known as glycans, are found in a diversity of sequences and structures and play a role in a wide range of biological processes¹. Unlike proteins and nucleic acids, glycan synthesis is non-template driven, and their final structures vary greatly as they are ultimately determined by the combined activity of various glycosidases and glycotransferases. Glycan structures are further complicated by multiple potential branching points, each with different potential linkage stereochemistry. Mass spectrometry (MS) has become a primary tool for sensitive characterization of glycans but suffers from the major limitation that many structures are isomeric and therefore cannot be

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Supporting Information Available:

- Table of chemical compositions from accurate masses.
- Table of fragment ion ratios for differentiating HexNAcs.
- CID fragmentation spectrum of ¹⁸O labeled GlcNAc.
- Spectrum of a novel *m/z* 127 fragment ion.
- Solution and gas-phase HDX spectra of GlcNAc fragment ions.
- MSⁿ and IM-MS analysis of isotopically labeled GlcNAc.
- MSⁿ and IM-MS analysis of 3-OMe-GlcNAc.
- Proposed structures of *m/z* 204, 126, and 127 ions.
- LC traces of resolved α/β anomers.
- Collision energy dependence for the fragment ion ratios for distinguishing HexNAcs.

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distinguished through a mass-based measurement. To complicate matters further, glycans can also undergo complex structural rearrangements during fragmentation that may lead to ambiguous and incorrect structural assignment ^{2, 3}.

Historically, MS studies of carbohydrates have focused on metal adducted and/or derivatized (e.g. methylated) species for which tandem mass spectrometry can provide rich structural information ^{3–13}. In contrast, protonated carbohydrates have received less attention due to their: 1) lower intrinsic ionization efficiencies, 2) less informative CID fragmentation spectra as they typically only show glycosidic bond cleavages, and 3) liability to undergo gas phase structural rearrangements ^{2, 3}. At the same time, the glycoproteomics field is tailored to protonated ions and a detailed understanding of the gas phase behavior of protonated carbohydrates could be a great benefit for providing insight into glycans within glycopeptides ^{14, 15}.

The use of additional gas phase analytical approaches coupled to mass spectrometry has added new levels of information that have been effective for studying carbohydrate structure. Isotopic labeling strategies ^{7, 11, 15–19} and gas-phase infrared spectroscopy ^{20–24} together with molecular modelling ^{20, 25–27} have provided valuable insights into the structures of a wide range of carbohydrate ions. Ion mobility spectrometry with mass spectrometry (IM-MS) is fast emerging as a powerful tool for characterizing biological molecules and has been gaining ground in recent years for resolving, identifying, and structurally characterizing glycans ^{25, 28–33}. The IM stage separates ions based on their mobility through a neutral buffer gas, where mobility of the ions is dependent on the charge, size, and the rotationally averaged collision cross section (CCS) of the ion-neutral gas pair. Hydrogen/deuterium exchange (HDX) is another gas phase structural tool that utilizes the natural exchangeability of labile protons within ions. Analyte ions are mixed with a deuterated reagent gas leading to exchange at labile sites ^{34–36}. Since the exchange kinetics are governed by the gas phase conformation of the analyte, it can distinguish structural isomers of small molecules, proteins, and carbohydrates ^{37–41}.

Here we map the CID fragmentation pathways of the biologically relevant protonated N-acetylhexosamines (HexNAcs): GlcNAc, GalNAc, and N-acetylmannosamine (ManNAc) using a combination of MSⁿ, isotopic labeling, IM-MS, and HDX. Our analysis yields new insight into the resulting fragment structures and the relative distributions of the fragment ions provide a reliable approach for differentiating monosaccharides even by conventional MS/MS. This study advances our understanding of the factors that govern protonated carbohydrate fragmentation behavior, which will be critical for reliable analysis of larger and more complex protonated carbohydrates by MS.

EXPERIMENTAL METHODS

Reagents

Deuterated ammonia (ND₃, 99%) and ¹⁸O (98%) water were purchased from Cambridge isotope labs (Tewksbury, MA, USA). GlcNAc, ManNAc, and GalNAc, were purchased from Sigma Aldrich (St. Louis, MO, USA). ¹³C and ¹⁵N labeled GlcNAc were purchased from

Omicron (South Bend, IN, USA). 3-OMe-GlcNAc (3-O-methyl-GlcNAc) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Ion trap mass spectrometry

Carbohydrates were resuspended in LC-MS grade optima water and diluted to a working concentration of 50 μ M in 0.1% formic acid. ^{18}O labeling of the reducing end hydroxyl of GlcNAc was performed as described previously ⁴². Samples were analyzed on a LTQ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) by direct infusion at a rate of $\sim$ 5 μ L/min. The protonated precursor ion was subjected to a CID using a range of normalized collision energies (CE) of 13 – 35. Full (MS1) scans were acquired followed by selection of the most intense fragment ions for further fragmentation with up to five rounds of MS/MS (MS⁵).

Ion mobility spectrometry

Ion mobility measurements were performed on a Synapt G2-Si Q-TOF mass spectrometer (Waters). 5 μ M HexNAcs in 0.1% formic acid were directly infused and ions of interest were generated using a cone voltage optimized for each ion. Ions were mass selected using the quadrupole to avoid any artifacts of ion decay in, during, or after the IM stage ⁴³. Measurements using N₂ (flow rate of 90 mL/min) as a drift gas were performed with a traveling wave (TW) velocity of 550 m/s and height of 4.3 V, while with He as the drift gas (flow rate of 120 mL/min) the TW velocity and height were 500 m/s and 24 V, respectively. The ion current was kept below 10e5 counts/sec to avoid detector saturation, which may lead to apparent ATD broadening. ATDs were fit to Gaussian distributions using custom scripts in Excel (Microsoft, Redmond WA).

Hydrogen/deuterium exchange

Gas-phase HDX experiments were performed on a Waters Synapt G1 quadrupole-time-of-flight (Q-TOF) mass spectrometer as described previously ³⁹. Carbohydrate solutions ($\sim$ 200 μ M in 0.1% formic acid) were infused at a flow rate of 10 μ L/min. Protonated ions were subjected to deuterium exchange in the transfer cell for various times using ND₃ at a flow of 0.5 mL/min. An elevated cone voltage was used to generate various fragment ions at the source which were then mass selected with the quadrupole to avoid possible spectral overlap due to water loss occurring during HDX. Fully deuterated samples were infused in 99% D₂O with 0.1% formic acid, and all lines and the source inlet were pre-equilibrated with pure D₂O to remove residual water. Data was batch converted using scripts in UniDec ⁴⁴ and deuterium uptake was analyzed using HX-Express v2 ⁴⁵.

RESULTS AND DISCUSSION

Ion-trap experiments

We first compared the CID spectra of the ions generated from each of the three protonated HexNAcs (m/z 222). Though all three HexNAcs are compositionally identical, GalNAc and ManNAc differ from GlcNAc at a single stereocenter (Fig. 1). To test whether the initial fragmentation of protonated HexNAc occurs at the reducing end we examined ^{18}O labeled GlcNAc (m/z 224). Spectra from both an ion trap and a Q-TOF instrument showed a

resulting m/z 204 ion indicating that the initial loss of water occurs primarily from the reducing (Fig. S1) consistent with previous reports^{10, 11, 16, 17}.

The subsequent m/z 204 was further fragmented along with all subsequent fragment ions showing the transitions: $204 \rightarrow [186, 168, 144, 138, 126]$; $186 \rightarrow [168, 144, 138, 126]$; $168 \rightarrow [150, 138, 128, 98]$; $144 \rightarrow [126, 109, 99, 98]$; $138 \rightarrow 96$ (Fig. 1A–E). Interestingly, MS³ of m/z 126 isolated directly from m/z 204 led solely to an abundant m/z 84 ion, while fragmentation of m/z 126 isolated from either m/z 186 or m/z 144 showed a complex mixture of ions (Fig. 1F, G). Comparisons of the relative abundances of each fragment ion (generated under identical conditions) reveal distinct fragmentation footprints for each HexNAc, most notably in the ratio of the relative abundances of m/z 138 and m/z 144 fragments (Fig. 1B). Additionally, GalNAc had a notable transition from $168 \rightarrow 150$, which was observed at only very low abundance in GlcNAc or ManNAc (Fig. 1C). Comparison of the $204 \rightarrow 186 \rightarrow 144$ MS⁴ and $204 \rightarrow 186 \rightarrow 144 \rightarrow 126$ MS⁵ product ions also reveal characteristic differences between GalNAc vs. GlcNAc/ManNAc including a skewed ratio of the m/z 109:108:99:98 ions (Fig. 1D, G). All experiments were repeated using the high resolution (Orbitrap) mass analyzer to achieve high mass accuracy, which enabled assignment of the chemical formulas (Table S1).

Structural analysis of fragment ions by IM-MS and HDX

We next compared the various fragments of the HexNAcs by ion mobility. The primary ions (m/z 204) and all major fragment ions were assayed for their arrival time distributions (ATDs) using either N₂ or He as the drift gas (Fig. 2A, B). The ATD of the m/z 204 ion is distinct from all three HexNAcs. Comparison of the subsequent fragment ions reveals where the various structures differ among the HexNAcs. In the case of the m/z 186, 168, and 144 ions, the mobility profiles from GlcNAc and ManNAc were indistinguishable, but GalNAc was distinct. The minor m/z 150 product was observed by IM-MS for GalNAc, but the low signal to noise ratio makes reliable detection of this fragment difficult for GlcNAc and ManNAc. The mobility profiles of the m/z 126 species varied for each HexNAc, which was most apparent in N₂ (Fig. 2B). The ATDs for the m/z 138 and 84 were indistinguishable from all three HexNAcs.

We observed a minor m/z 127 ion from all three monosaccharides. Though this ion initially is unresolvable in the ion trap data, it could be resolved from the heavier isotopic peak of m/z 126 (Fig. S2). High mass resolution data revealed that this was a unique fragment ion whose chemical formula is consistent with the loss of water and the entire N-acetyl group (Table S1). GlcNAc and ManNAc yielded similar ATDs for m/z 127 whereas GalNAc was distinct (Fig. 2A, B). Unfortunately, due to the relatively low abundance and overlap with the heavier isotopic peak of the m/z 126 ion, we were not able to obtain a reliable fragmentation spectrum of m/z 127 through MSⁿ.

As a secondary method for probing the HexNAc fragment ion structures we applied gas-phase HDX (Fig. 2C). Deuterium uptake kinetics of m/z 204 show a rapid exchange of one of the four labile hydrogens followed by gradual deuterium uptake which is distinct for each HexNAc, consistent with previous findings³⁹. The HDX profile of the m/z 186 ion shows fewer exchanges with very similar profiles for GlcNAc and ManNAc that are distinct from

GalNAc. A similar trend was observed with m/z 144, showing similar profiles for GlcNAc and ManNAc that are different from GalNAc. The signal for the m/z 168 ion was heavily suppressed under HDX conditions and could not be reliably measured beyond the first 2 timepoints, which showed a rapid uptake of a single deuterium in all HexNAcs. The m/z 138 and m/z 150 ions showed no observable deuterium uptake throughout the time course under these exchange conditions. The m/z 127 ion showed similar kinetics for all HexNAcs. Interestingly, the kinetics for m/z 126 were similar for GalNAc and ManNAc, but different for GlcNAc.

Additional HDX experiments were performed to probe the total number of labile protons in each fragment structure. HexNAcs were either infused in D_2O or subjected to exhaustive gas-phase HDX at elevated pressure of ND_3 (Fig. S3). Additionally, we performed a third experiment combining infusion in D_2O and gas-phase HDX to probe for structural transitions that render non-labile protons from the precursor ion, that won't be deuterated in the D_2O solution, into labile protons in the resulting fragment ion that can be exchanged in the gas phase. The mass shifts revealed the maximum number of exchangeable hydrogens (in parentheses) for: m/z 222(6), 204(4), 186(3), 168(2), 144(4), 150(0), 138(1), 126(3), 127(2), 84(3).

Analysis of isotopically labeled GlcNAc

We repeated MS^n and IM-MS using isotopically labeled GlcNAc at either: C(1) carbon “GlcNAc[$^{13}C(1)$]”, C(2) carbon “GlcNAc[$^{13}C(2)$]”, N-acetyl carbonyl carbon “GlcNAc[$^{13}C(CO)$]”, or at the C(6) carbon along with a ^{15}N at the N-acetyl nitrogen “GlcNAc[$^{13}C(6)/^{15}N$]”. The fragments, m/z 204, m/z 186, and m/z 168 formed from protonated HexNAc showed the expected increase in their masses due to the heavy isotope labels (Fig. 3A). The m/z 144 ion was observed at higher masses in all cases except in the GlcNAc[$^{13}C(CO)$] spectrum, indicating that this structure has lost the acetyl group. Meanwhile, in GlcNAc[$^{13}C(6)/^{15}N$], m/z 144 was observed at m/z 146, therefore both C(6) and the N are present in this structure. The m/z 138 ion shows an increase of 1 Da in each labeled sample and thus the C(1), C(2), and carbonyl carbons are intact within the m/z 138 structure. However, only one of the two labels is retained in GlcNAc[$^{13}C(6)/^{15}N$] indicating a loss of either the C(6) carbon or the nitrogen. The chemical compositions obtained by accurate mass analysis (Table S1) is consistent with a nitrogen being present and therefore the m/z 138 structure loses the C(6) carbon.

The isotopic labeled MS/MS experiments were particularly insightful for revealing the structures of the m/z 126 ion (or equivalent), which shifted primarily to 127 in each labeled GlcNAc. However, CID of the 186 or 144 species yielded a m/z 126 peak for GlcNAc[$^{13}C(CO)$], but a m/z 128 for GlcNAc[$^{13}C(6)/^{15}N$] (Fig. 3A). Similar product ions were generated from either 204 or 186/168 precursors when analyzed on a Q-TOF (Fig. S4A). All structures retain the C(1) and C(2) carbons as seen from the 1 Da mass increase in GlcNAc[$^{13}C(1)$] and GlcNAc[$^{13}C(2)$]. The nitrogen atom is retained as evidenced by the chemical composition from the accurate mass (Table S1). Therefore, the m/z 126 ion must be a combination of at least two distinct structures. The primary m/z 126 fragment from 204 has the acetyl group intact, explaining the m/z 127 peak with GlcNAc[$^{13}C(CO)$], and lacks

the C(6) carbon, explaining the m/z 127 with GlcNAc[$^{13}\text{C}(6)/^{15}\text{N}$]. Due to the loss of C(6) this structure will be referred to as the 5-membered ring 126 ion. Another m/z 126 species, a secondary product of either 186, 168 or 144, retains the C(6) carbon and loses the acetyl group. This structure will be referred to as the 6-membered ring 126 ion.

Further fragmentation of the m/z 126 also supports the presence of at least two very different structures. Unlabeled HexNAc fragmented to an abundant m/z 84 fragment through $204 \rightarrow 126$, while several unique fragment ions formed through $204 \rightarrow 186 \rightarrow 144 \rightarrow 126$ (Fig. 1F, G). The isotopically labeled data are consistent with the 5- and 6-membered 126 ions producing either the m/z 84 or the 98 and 108 equivalent fragments, respectively (Fig. S4B). These spectra also reveal that the m/z 84 shows no mass increase in GlcNAc[$^{13}\text{C}(\text{CO})$] and only a single label from GlcNAc[$^{13}\text{C}(6)/^{15}\text{N}$]. Just as with the 5-membered 126 ion, the chemical formula from accurate mass is consistent with retention of the nitrogen, and therefore the acetyl carbonyl and C(6) carbons are absent in the m/z 84 structure.

IM-MS was utilized to track the distinct m/z 126 structures from GlcNAc[$^{13}\text{C}(6)/^{15}\text{N}$] and GlcNAc[$^{13}\text{C}(\text{CO})$]. The ATD of the 5-membered ring (m/z 127 peak) from GlcNAc[$^{13}\text{C}(6)/^{15}\text{N}$] matched that of GlcNAc[$^{13}\text{C}(\text{CO})$], showing a seemingly uniform distribution in He (Figs. 3B). Conversely, the 6-membered ring structure (m/z 128 peak) from GlcNAc[$^{13}\text{C}(6)/^{15}\text{N}$] and (m/z 126 peak) from GlcNAc[$^{13}\text{C}(\text{CO})$] both showed a much broader ATD. Deconvolution of the broad ATDs for the 6-membered ring structure revealed that the approximate arrival times and distribution widths of each component were similar from both GlcNAc[$^{13}\text{C}(6)/^{15}\text{N}$] and GlcNAc[$^{13}\text{C}(\text{CO})$] (Fig. S4C).

As an additional control, we examined GlcNAc O-methylated at the C(3) position (3-OMe-GlcNAc). By MS/MS the precursor ion (m/z 236) formed 218 (O-methylated equivalent of 204) and interestingly all other ions showed the same patterns as from unlabeled GlcNAc, including an abundant m/z 186 ion (Fig. S5A). No m/z 200 ion was observed, which would correspond to a second water loss, if the methylated oxygen were retained at the C(3) position. IM-MS yields indistinguishable ATDs of m/z 186 generated from 3-OMe-GlcNAc and GlcNAc in both He and N_2 (Fig. S5B). While the energetics of the fragmentation may be different, the similarity in the product m/z 186 structures indicate that water loss from m/z 204 occurs primarily through the loss of the hydroxyl at C(3). This is consistent with previous investigations of the predominant fragmentation products of permethylated carbohydrates, despite that derivatization is likely to influence fragmentation pathways^{17, 42}.

Fragment ion structures and predominant fragmentation pathways

The combination of the data presented here provides new insights into the fragmentation pathways of protonated HexNAcs including many structural details of the ions generated. The detailed fragmentation map is summarized in Figure 4 with key steps described below. After the initial loss of the reducing end hydroxyl, protonated HexNAc ions dissociate through two primary pathways: **I**) further loss of water to form m/z 186, or **II**) ring cleavage to generate a 5-membered m/z 126 ion. Classically the m/z 204 ion has been depicted as an oxonium ion through loss of the reducing end hydroxyl⁴, though more recently a range of possible structures have been proposed^{11, 25, 26}. While the 204 ion is likely an ensemble of

several structures, the differences observed for the m/z 204 ion for each HexNAc by IM-MS and HDX kinetics indicate that the stereochemical centers at C(2) and C(4) that distinguish each HexNAc are intact (Fig 2). Furthermore, the 204 ion has exactly 4 labile protons (Fig. S3), consistent with either the oxonium, O(6)-C(1), or O(4)-C(1) bridge structures (depicted in Fig. S6A).

Through pathway I, the 204 ion loses a water to form m/z 186 which can undergo further dissociation to form m/z 168, 150, 144, 138, and the 6-membered ring 126. MS/MS, IM-MS, and HDX all agree that m/z 186 ion from GalNAc is different from that of GlcNAc and ManNAc (Fig. 1B, 2A–C). This distinction is consistent with the C(2) stereochemistry being lost in the transition of m/z 204 to 186. Additionally, the comparison with the 3-OMe GlcNAc data indicates the loss of the hydroxyl specifically occurs from C(3) to form m/z 186 (Fig. S5). This evidence suggests a double bond forms between C(2) and C(3) upon loss of water and the only remaining difference between GalNAc and GlcNAc/ManNAc is the stereochemistry at C(4). If instead, the C(2) stereochemistry remained intact, then GlcNAc and GalNAc would be similar while differing from ManNAc (Fig. S7A). In all cases the m/z 186 ion bears three labile protons consistent with the maximum observed by HDX (Fig. S3). Interestingly, the overall ATD by IM-MS was relatively broad, and there was a subpopulation of m/z 186 that only exchanged one deuterium. This indicates that while the predominant structure has a C(2)-C(3) double bond, other minor structures may co-exist under these experimental conditions.

Subsequent loss of water from m/z 186 can occur from either the C(4) or the C(6) carbon to form m/z 168, presumably with the ring gaining aromaticity (depicted in figure S3). The IM-MS profiles of GalNAc for this ion differ from ManNAc and GlcNAc (Fig. 2A, B). Though the deuterium uptake data is limited by poor signal, it does indicate that GalNAc is the outlier (Fig. 2C). Since the m/z 186 fragment of GalNAc differs from ManNAc/GlcNAc specifically at the C(4) position (Fig. S7A), we predict that C(4) stereochemistry governs whether hydroxyl is lost from C(4) or C(6). The different resulting m/z 168 structures explain the trends observed by IM-MS, HDX, and the differing fragmentation of m/z 168 in GalNAc vs. GlcNAc/ManNAc (Fig. 2C).

Further dissociation of 168 generates either 138 (predominantly for GlcNAc/ManNAc) or 150 (predominantly for GalNAc). The isotopically labeled GlcNAc MSⁿ data reveals that the 138 ion retains the acetyl group but loses the C(6) carbon (Fig. 3A). This fragment appears identical by every measure for all HexNAcs, and therefore retains no stereochemistry at C(2) or C(4). A single, relatively slow exchanging proton is consistent with the single exchangeable proton on the amide (Fig. S3). Further dissociation of m/z 138 to 96 is consistent with loss of the acetyl group (Fig. 1E). The 150 fragment is likely formed by a final dehydration step from m/z 168. Consequently, this fragment should be structurally indistinct for all three HexNAcs. No mass increase was observed by HDX, consistent with no labile protons present in this structure.

An alternate dissociation pathway from m/z 186 is the loss of the acetyl group to generate m/z 144. The absence of the acetyl carbon was evident from the MSⁿ of the isotopically labeled GlcNAc (Fig. 3A). This would leave the stereochemistry at C(4) and C(6) intact and

a free amine at the C(2) position. The subtle difference observed in the ATDs and HDX kinetics between GalNAc and ManNAc/GlcNAc are consistent with stereochemistry retained at C(4) (Fig. S7B). The fact that only 3 labile protons were observed when infused in D₂O (Fig S3), is attributed to the mechanism of amine formation through proton transfer from the nearby methyl group as described previously¹⁵. Further HDX in the gas phase shows deuteration of all 4 labile protons. By MSⁿ, the m/z 144 breaks down into m/z 126 consistent with a loss of water, and further fragments to the characteristic m/z 98/99/109 ions observed for 6-membered rings (Fig. 1G, S4B, and discussed below).

Pathway II occurs through a structural rearrangement of m/z 204 to a 5-membered ring (Fig. 5). This transition was only observed from m/z 204 and not from any downstream fragment ion in pathway I (i.e. m/z 186). The 5-membered ring structure has been proposed previously to occur via a retro Diels-Alder reaction resulting in loss of C(3) and C(4) ring carbons¹⁵. Our isotopically labeled MSⁿ and IM-MS results (Fig. 3A, B) reveal that it is actually the C(6) carbon that is lost in this step. We propose that the reaction is initiated from a O(4)-C(1) bridge structure (Fig. S6B), where a proton transfer from O(4) to the carbonyl initiates a rearrangement resulting in loss of the C(5) and C(6) carbons as an ethylene diol. This type of fragmentation is similar to previous studies showing the loss of C(5) and C(6) carbons in the CID of metal-adducted HexNAcs⁷. Subsequent fragmentation of the 5-membered 126 ion results in a dominant m/z 84 ion, consistent with a loss of the acetyl group, just as seen with the 186 → 144 transition.

The 6-membered m/z 126 can form through pathway I, either through the loss of the acetyl group from m/z 168, or a water loss from m/z 144. The isotopically labeled GlcNAc data reveal that this 6-membered structure retains C(1), C(2) and C(6) but not the acetyl group. IM-MS of isotopically labeled GlcNAc in He shows multiple ATDs for the 6-membered m/z 126 ion (Fig. S4C) that can be attributed to the hydroxyl group being at either C(4) or C(6) carbons. Just as with m/z 168, there are two possible structures for the 6-membered 126, whose distributions vary in GalNAc vs. GlcNAc/GalNAc. This is evidenced by the distinct product ion patterns of the 204 → 186 → 144 → 126 (compare GlcNAc and ManNAc vs. GalNAc in Fig. 1G). Lastly, HDX shows the m/z 126 ions picking up either 2 or 3 Da, consistent with the number of labile protons in the distinct structures (Fig. S3). The fact that there are three resolvable m/z 126 structures whose distributions differ for each HexNAc would explain the variability in the ATDs of the m/z 126 ions generated directly from m/z 204 (Fig 2B).

Evidence for a third minor pathway was observed forming a m/z 127 ion (Fig. S2). This accurate mass corresponds to the loss of the entire N-acetyl group and a water. Presumably this transition can occur either from either 204 or 186 resulting in structures with two hydroxyls at either C(3), C(4), or C(6) (Fig. S6C). IM-MS showed distinct ATDs of m/z 127 from GalNAc compared to GlcNAc/ManNAc. The stereochemistry at C(4) may impact the distribution of the m/z 127 ion structures that form, similar to what is observed with the m/z 168 or the 6-membered m/z 126 structures. However, HDX showed no differences in uptake from all HexNAcs (Fig. 2C). Since the m/z 127 ion was such a minor product and had little impact on the observed fragmentation patterns it was not examined further.

Anomeric influence on fragmentation patterns and ion structures

Recently Gray and colleagues have reported that carbohydrate ions retain information about the stereochemistry at their reducing end (anomerity) even after the loss of their reducing end hydroxyl²⁵. This ‘memory effect’ was attributed to the formation of unique ion structures, including bridged structures, also supported by molecular modelling^{11, 26}. To ascertain whether protonated HexNAc fragment ions also contain “anomeric memory”, we performed LC-MS/MS of the three HexNAcs to resolve the α/β anomers (Fig. S8). The spectra from the transition ($222 \rightarrow 204 \rightarrow$) obtained for GlcNAc and GalNAc from either anomers showed no major differences, however, the relative abundances of the products in the two anomers of ManNAc were notably different (Fig. 5A). The α anomer of ManNAc fragmented primarily to m/z 186 while the β anomer produced an abundant m/z 126. For all three HexNAcs, the MS⁴ ($222 \rightarrow 204 \rightarrow 186 \rightarrow$) spectra were indistinguishable for both anomers, indicating that any anomeric memory is lost in fragments downstream of the 204 (Fig. 5A).

LC-IM-MS was also performed to elucidate any differences in ion structure attributed to anomeric memory. Interestingly, the ATDs of the m/z 204 fragment ions from different anomers exhibited slight differences, most notable in ManNAc (Fig. 5B, top panels). GlcNAc also showed a slightly broader ATD for the m/z 204 obtained from the α anomer. The ATDs of the m/z 186 fragments from both anomers showed no differences (Fig. 5B, bottom panels), again consistent with anomeric memory being lost after the m/z 204 ion. These results are consistent with the notion that the m/z 204 ion is an ensemble of different structures (e.g. chemical structures, protomers, pucker conformations), which can differ depending on the anomeric configuration of their precursor ion^{11, 25, 26}. This is best exemplified in ManNAc, where the m/z 204 structure that is primarily adopted from the β -ManNAc precursor mainly dissociates through pathway II to the 5-membered m/z 126, whereas the α -ManNAc predominantly goes through pathway I to form m/z 186.

Memory of stereochemistry is retained in secondary product ions

Several of the small fragment ions were found to retain information about the precursor ion’s stereochemistry, even after multi-stage MSⁿ. Distinct structures for the m/z 186 and 144 ions for GalNAc compared to GlcNAc or ManNAc is expected as these ions retain the C(4) and C(6) stereocenters (Fig. 4). However, the m/z 168 and 127 fragments presumably do not retain any stereocenters, yet differences are observed for these ions with GlcNAc vs. GalNAc (Fig. 1, 2). Such differences can be attributed to the relative distribution among the two possible structures suggested for m/z 168 (Fig. 4). Similarly, m/z 126 is composed of at least three structures whose distributions are distinct for all three HexNAcs and this distribution can reveal stereochemical and anomeric information about the precursor. These observations suggest that the consequences of “stereochemical memory” on carbohydrate fragmentations is not limited to the reducing end²⁵, but is also present within secondary fragment ions.

Fragmentation propensities for differentiating carbohydrates

CID fragmentation propensities have proven useful for effectively differentiating carbohydrates^{5, 7, 9}, whereas fragmentation of the protonated glycopeptides typically

provide only limited structural information on the glycans³. The distribution of the product ions, m/z 126, 138, 144, 168 generated from m/z 204 has been shown to be useful for distinguishing GalNAc vs. GlcNAc in protonated glycopeptides¹⁴. The fragmentation map (Fig. 4) offers some insight into how the propensities of m/z 138 and 144 can be attributed to the stereochemistry at the C(4) carbon that influences the $186 \rightarrow 168$ transition. Having the C(4) and C(6) hydroxyls on the same side of the ring (in GalNAc) may disfavor the formation of m/z 168 along with the subsequent m/z 138. As a result, the loss of the acetyl group would instead become a favored pathway, leading to a higher abundance of m/z 144 (Fig. 4).

Since the critical fragmentation step for differentiating C(4) stereochemistry ($186 \rightarrow 168$ vs. $186 \rightarrow 144$) is within pathway I, the relative intensities of m/z 138 and 144 alone should enable efficient differentiation of GlcNAc and GalNAc. To test this, we compared the ion intensity ratios for the various LC-resolved anomers of each HexNAc (Table S2). The $138/(138+144)$ ratios were 0.81 ± 0.04 for GlcNAc and 0.37 ± 0.05 for GalNAc, and were not affected by the anomeric state of the precursor ion. The ratio for ManNAc (0.73 ± 0.11) was within error of GlcNAc, as expected due to their identical stereochemistry at C(4). In contrast, using the $(138+168)/(126+168)$ metric as described by Halim et al.¹⁴, is complicated by the fact that m/z 126 can be formed through either pathway I or II. As such it can result in significantly different ratios for different anomers (Table S2).

We also examined the effect of collision energy (CE) on the resulting ion ratios. On the ion trap instrument the $138/(138+144)$ ion ratio was consistent over a wide range of CE (Fig. S9). Using beam-type CID on a Q-TOF instrument, the ratios varied considerably as the CE is altered. This difference likely stems from the mechanisms of ion activation. With beam type CID multiple collisions can lead to activation of product ions resulting in secondary fragmentation events, whereas product ions in ion traps generally do not undergo further excitation^{18, 46}. Therefore, using the 138 and 144 ion ratios within an ion trap provides a robust method for defining C(4) stereochemistry that is largely independent of CE, and entirely independent of other factors such as the precursor ion anomericity.

The fact that there are two predominant pathways of fragmentation from m/z 204 offers potential approaches to further define monosaccharide identifies from fragment ion distributions. Zhu et al have used the ratio of m/z 126 to either m/z 186 or 168 to differentiate between ManNAc and GlcNAc⁹. In hindsight, their approach was effective because the stereochemistry at C(2) strongly influences whether m/z 204 dissociates via pathway I or II, thus changing the abundance and predominant structure of m/z 126 (6-membered versus 5-membered) relative to all the fragments obtained through pathway I (m/z 138, 144, 168, 186). However, this approach is complicated by the fact that the m/z 204 ion also retains anomeric memory, which can strongly influence the degree of fragmentation via pathway II. Further characterization of the structural factors governing the competition between pathway I vs. II, may provide other useful diagnostic ion ratios revealing information about the original carbohydrate structure. Since nearly all biologically relevant glycans contain HexNAcs, this can provide a relatively simple approach for expanding the scope of glycan structural information obtained from simple MS/MS data.

CONCLUSIONS

The combination of comparative MSⁿ, IM-MS, HDX, and isotopic labeling provided the means to resolve the various isomeric fragment ion structures from protonated HexNAcs. Beyond mapping the fragmentation pathways, we find that the distribution of structures at a given fragment *m/z* reveals information regarding the precursor's stereochemistry. While this study is limited to the fragmentation pathways and propensities of protonated HexNAcs, it provides a starting point and a viable approach for characterizing the behaviors and differentiating isomeric fragment ions of larger oligosaccharides. Identifying monosaccharides and linkage stereochemistry within protonated glycans can fill the gap of information currently lacking in glycoproteomic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

MSⁿ analysis of HexNAcs. MSⁿ spectra for GlcNAc (top), ManNAc (middle), and GalNAc (bottom) are shown. The numbers above each panel indicate the masses: (A) *m/z* 204, (B) *m/z* 186, (C) *m/z* 168, (D) *m/z* 144, (E) *m/z* 138, (F, G) *m/z* 126, selected for each stage of MSⁿ shown by the arrows. Structures of each monosaccharide are shown on the left.

Figure 2.

IM-MS analysis of HexNAcs using helium (**A**) or nitrogen (**B**) as the drift gas. The arrival time distributions (ATDs) for each fragment ion are shown as dots for GlcNAc (blue), ManNAc (green), and GalNAc (red). The lines show the Gaussian fits and numbers within each plot represent the Gaussian peak width (FWHM). Dashed lines are added to help illustrate slight differences in ATDs. **C**) HDX-MS analysis of HexNAcs. Deuterium uptake for various fragment ions is shown as a function of incubation time with ND₃. The points

and error bars represent the average and standard deviations from triplicate measurements.

*The m/z 127 ion was mass resolved from the second isotopic peak of the m/z 126 ion.

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Figure 3.

Analysis of isotopically labeled GlcNAc at either C(1) (tan), C(2) (green), acetyl carbonyl (blue), or both C(6) and the nitrogen (purple). **A)** MSⁿ spectra of unlabeled and each isotopically labeled GlcNAc with mass selection steps shown above each panel. The position of ^{13}C and ^{15}N labels are shown on the structure of GlcNAc on the top left. **B)** IM-MS analysis of the m/z 126, m/z 127, or m/z 128 ions from $^{13}\text{C}(\text{CO})$ and $^{13}\text{C}(6)/^{15}\text{N}$ GlcNAc shown as described in figure 2.

Figure 4.

Fragmentation pathway of $[\text{GlcNAc}+\text{H}]^+$. After the initial loss of water from the reducing end fragmentation can proceed through two distinct pathways: **I**) further loss of water and a range of subsequent fragment ions; or **II**) ring cleavage to form a stable 5-membered ring structure.

Figure 5.

Anomeric effects in HexNAc fragmentation. **A**) MSⁿ spectra of the *m/z* 204 ion and *m/z* 186 ions from the α (left) and β (right) anomers of GlcNAc (blue), ManNAc (green), and GalNAc (red). **B**) IM-MS analysis of the *m/z* 204 and *m/z* 186 ions shown as described in figure 2 from the α (orange dashed lines) and β (blue solid lines) anomers. The LC-MS traces are shown in figure S8.