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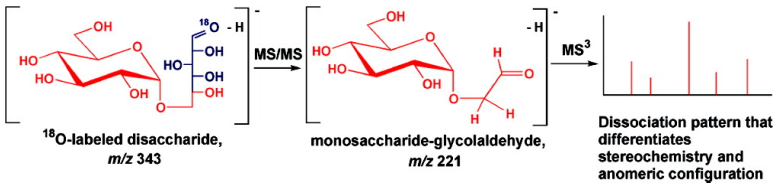

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The Stereochemical Dependence of Unimolecular Dissociation of Monosaccharide-Glycolaldehyde Anions in the Gas Phase: A Basis for Assignment of the Stereochemistry and Anomeric Configuration of Monosaccharides in Oligosaccharides by Mass Spectrometry via a Key Discriminatory Product Ion of Disaccharide Fragmentation, *m*/*z* 221

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Abstract: Mass spectrometry of hexose-containing disaccharides often yields product ions of *m*/*z* 221 in the negative ion mode. Using a Paul trap, isolation and collision-induced dissociation of the *m*/*z* 221 anions yielded mass spectra that easily differentiated their stereochemistry and anomeric configuration, for all 16 stereochemical variants. The ions were shown to be glycopyranosyl-glycolaldehydes through chemical synthesis of their standards. The stereochemistry dramatically affected fragmentation which was dependent on four relative stereochemical arrangements: (1) the relationship between the hydroxyl group at position 2 and the anomeric configuration, (2) a cis relationship of the anomeric position and positions 2 and 3 (1,2,3-cis), (3) a 1,2 trans-2,3 cis relationship, and (4) the relationship between the hydroxyl group at position 4 and the anomeric configuration. After labeling the reducing carbonyl oxygen of a series of disaccharides with ^{18}O to mass-discriminate between their monosaccharide components, it was demonstrated that *m*/*z* 221 anions are comprised of an intact nonreducing sugar glycosidically linked to a 2-carbon aglycon derived from the reducing sugar, irrespective of the linkage position between monosaccharides. This enabled the *location* of the intact sugar to be assigned to the nonreducing side of a glycosidic linkage. Detailed studies of experimental factors necessary for reproducibility demonstrated that the unique mass spectrum for each *m*/*z* 221 anion could be obtained from month-to-month through the use of an internal energy-input calibrant ion that ensured reproducible energy deposition into the ions. The counterparts to these ions for the 2-acetamido-2-deoxyhexoses were *m*/*z* 262 anions, and the anomeric configuration and stereochemistry of these anions could also be reproducibly discriminated for *N*-acetylglucosamine and *N*-acetylgalactosamine. The fragmentation patterns of *m*/*z* 221 anions provide a firm reproducible basis for assignment of sugar stereochemistries in the gas phase.

Introduction

Complex carbohydrates are important in a wide variety of biological processes and display remarkable structural diversity.[1−3] Knowledge of their primary structures is essential for understanding their biological functions. NMR spectroscopy has long been a mainstay in structural carbohydrate chemistry because the dihedral angle dependence of 3-bond *J*-couplings of protons around sugar rings has provided a firm theoretical and practical foundation to assign their stereochemistry and anomeric configuration.[4,5] Even within larger molecules, monosaccharide stereochemistries can be extracted using higher-dimensional NMR experiments, especially effectively after derivatization with isotags.[6−8] Mass spectrometry also has a long history in analysis of complex carbohydrates.[9−12] A strong argument can be made to spotlight new MS approaches because it furnishes structural information at 3 to 4 orders of magnitude greater sensitivity than NMR. However, no firm basis yet exists in mass spectrometry to enable the stereochemistry and anomeric

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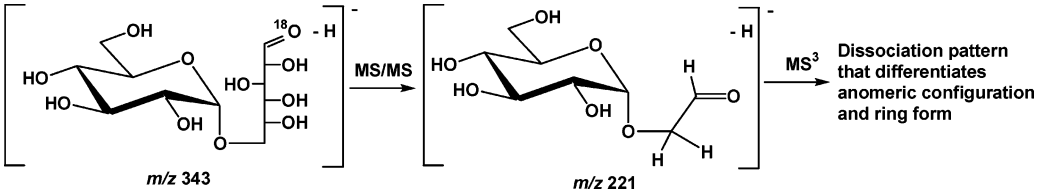
Scheme 1



configuration of monosaccharides to be confidently assigned when they are derived in the gas phase through successive isolation/dissociation events from a larger oligosaccharide molecule. If monosaccharides could be identified with certainty based on some unique physical property in the gas phase, then mass spectrometry could be employed with confidence on par with NMR spectroscopy, at least for establishing the nature of the stereochemistry of monomers derived from larger structures. While the stereochemistry of individual monosaccharides can be determined after hydrolysis/solvolysis through chromatographic means (such as GC−MS),[13,14] this does not permit the *order* of individual monosaccharides having different stereochemistries to be assigned within a larger molecule (i.e., which specific monosaccharides are linked to which). The same argument fundamentally applies to classical "permethylation analysis",[15−17] where neither the anomeric configuration nor the order of monosaccharides can be determined following hydrolysis or reductive cleavage of an oligosaccharide to its monomeric constituents.

To establish such an order requires (1) a controlled disassembly of a larger molecule in the gas phase yielding substructures from different regions of the molecule that are isolable by MS methods and (2) a means to differentiate substructures so that the stereochemistry of individual monosaccharides as gas-phase species can be determined without ambiguity. As part of the stereochemistry of a monosaccharide is inherent in the asymmetry at its anomeric carbon, establishing the anomeric configuration of each sugar is part and parcel of its structural proof.

Fortunately, ion traps and Fourier transform ion cyclotron resonance (FTICR) instruments enable multiple isolation/dissociation steps to be performed[18−21] so that issue 1, above, can be reasonably effectively addressed. At some point, however, differentiation of these substructures is required in a way that can confidently establish the stereochemistry and *location* of monosaccharides within a larger molecule. The best ions to divulge this information have not been conspicuous as yet.

We sought to determine whether disaccharides, the smallest isolable substructures derived from oligosaccharides that still contain a glycosidic linkage between two monosaccharides, would dissociate to yield product ions from which detailed stereochemical information might be obtained about either of its monosaccharides in the gas phase. As disaccharides themselves represent a large number of isomeric molecules, differentiating their structures in the gas phase has not been generally possible. Studies in the positive[22−25] and negative[26−32] ion modes have primarily focused on determination of linkage position in disaccharides; similar information about linkage sites is possible with pyranosyl-1-enes derived from permethylated oligosaccharides[33,34] or from peracetylated disaccharides.[35,36]

Fewer studies have been carried out to address the identification of anomeric configuration in disaccharides.[31,37−40] In the studies cited, emphasis was placed on mass spectral differentiation between two disaccharides having identical sugars and linkage positions, varying only in the anomeric configuration of the nonreducing sugar. Identifying the stereochemistry of one or the other sugars solely by dissociation of a disaccharide in the gas phase, *without knowing the monosaccharide composition in advance*, is a rather more challenging endeavor. It not only implies (1) that key product ions derived from disaccharides must dissociate in a way unique to their stereochemistry but also implies (2) that the origins of individual disaccharide fragments derived by cleavage on either side of the glycosidic linkage be known and (3) that product ions are not isobaric mixtures themselves, containing fragments of sugars originating from either side of the glycosidic linkage.

Bearing these issues in mind, we recently reported that the *m*/*z* 221 anion derived from glucose-containing disaccharides in the negative ion mode was in fact a glucosyl-glycolaldehyde anion (Scheme 1) based on its comparison to synthetic α- and β-glucopyranosyl- and α- and β-glucofuranosyl-glycolaldehydes.[41] Both the anomeric configuration and ring forms of the *m*/*z* 221anions could be clearly assigned by their MS dissociation patterns. It was also demonstrated that the intact monosaccharide

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of these ions was solely derived from the nonreducing sugar of reducing disaccharides based on ^{18}O-labeling of the carbonyl group of the reducing sugar to mass-discriminate between the origins of product ions derived from either side of the glycosidic linkage. Here we report that gas-phase dissociation patterns can be used to discriminate between all 16 stereochemical and anomeric variants of D-aldohexopyranosyl-glycolaldehydes as *m*/*z* 221 anions. The synthetic compounds (222 Da, $[M - H]^-$ *m*/*z* 221) dissociate identically in the gas phase to the *m*/*z* 221 ions isolated from various disaccharides upon fragmentation. Two common 2-acetamido-2-deoxy sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc), were also differentiated in the gas phase as their α- and β-D-glycopyranosyl-glycolaldehyde anions, enabling their anomeric configuration and stereochemistry to be assigned.

Results and Discussion

Monosaccharide Differentiation in the Gas Phase: The Problem. A classic problem in mass spectrometry of oligosaccharides is identification of the stereochemistry and anomeric configuration of its monosaccharide constituents, when these are derived by dissociation of a larger precursor ion in the gas phase. To illustrate this general issue, we studied the dissociation of disaccharides specifically ^{18}O-labeled on the carbonyl group of the reducing sugar ($[M - H]^-$ *m*/*z* 343) so that individual monosaccharide product ions could be mass-discriminated. Disaccharides which contained either a nonreducing glucose (α-D-Glc*p*-(1−6)-D-Glc and β-D-Glc*p*-(1−6)-D-Glc) or a nonreducing galactose (α-D-Gal*p*-(1−6)-D-Glc and β-D-Gal*p*-(1−6)-D-Gal) in both anomeric configurations were ^{18}O-labeled on the carbonyl position of the reducing sugar. In a Paul trap, an ion derived from disaccharides in the negative ion mode having an *m*/*z* of 179 $[M - H]^-$ was invariably observed, representing the nonreducing monosaccharide containing the original glycosidic oxygen. Interestingly, anions having an *m*/*z* 181 were not observed, which indicated a highly selective cleavage on one side of the glycosidic linkage, at least for these reducing disaccharides. Dissociation spectra of the *m*/*z* 179 product ions isolated from the above disaccharides are shown in Figure 1, where these represent MS^3 spectra. Note that monosaccharide anions (*m*/*z* 179) derived from disaccharides having the same nonreducing monosaccharide in either an α or β linkage yielded essentially identical mass spectra (compare, for example, both Figure 1, part A to part B and part C to part D). Similarly, the product ion ratios were only slightly different in comparing the glucose and galactose anions (compare Figure 1, part A to part C or part B to part D). On the basis of the fragmentation of these *m*/*z* 179 anions, it can be concluded that (1) it is impossible to assign the anomeric configuration of the nonreducing monosaccharide using CID in a Paul trap and (2) it is also rather difficult to assign the stereochemistry of either of these sugars. While there was a minor difference observed in product ion ratios between the gluco or galacto product ions in the Paul trap, should either monosaccharide be derived from a completely unknown disaccharide, one would be very hard pressed to assign their stereochemistry with confidence. These studies support the hypothesis that monosaccharide *m*/*z* 179 ions may interconvert from cyclic structures to acyclic variants during acquisition of enough internal vibrational energy to reach threshold dissociation eigenstates[42,43] (Figure 1E). Either the open-chain or cyclic forms as shown may also exist in different charge variants due to proton exchange among hydroxyl groups. Sugar ring opening may be a fundamental limitation in identifying the stereochemistries and anomeric configurations of monosaccharides as (*m*/*z* 179) anions, at least using collision-induced dissociation (CID). Similarly, we have been unable to differentiate monosaccharide anomeric configurations and stereochemistries in the positive ion mode as adducts of either sodium or lithium ions in the gas phase, where the monosaccharide product ions were derived by dissociation of disaccharides (data not shown).

Dissociation of Glycopyranosyl-Glycoaldehydes (*m*/*z* 221 Anions) in the Negative Ion Mode Discriminates Both Anomeric Configuration and Stereochemistry of the Ions for All 16 Possible Structural Variants. Another key ion frequently observed during dissociation of disaccharides in the negative ion mode is an *m*/*z* 221 anion. We demonstrated earlier that these ions, derived from 2,4-, or 6-linked disaccharides containing only glucose, were comprised of the intact nonreducing sugar glycosidically linked to a glycolaldehyde molecule. Dissociation of these ions in a Paul trap could be used to distinguish between the anomeric configurations and ring forms of glucosyl-glycoaldehydes.[41] All 16 stereochemical variants of glycopyranosyl-glycoaldehydes were synthesized as standards in order to determine their dissociation patterns in the negative ion mode (Chart 1). In Figure 2, the dissociation patterns of the α- and β-D-glucopyranosyl- and galactopyranosyl-glycolaldehydes under identical dissociation conditions are shown (compounds **1**−**4**, $[M - H]^-$ precursor anion *m*/*z* 221, abbreviated as Hex-GA anions). The mass spectra yielded patterns that clearly differentiated both the anomeric configuration and stereochemistry of the anions. It is worth noting that the ratios of product ions and even the presence of some ions were markedly different between the stereoisomers. For example, in comparing the α- and β-D-glucopyranosyl-glycolaldehyde anions, (Figure 2, part A to part B) there was a major decrease in the intensity of the *m*/*z* 101 product ion and a major increase in the abundance of the *m*/*z* product 161 ion which was solely dependent on the stereochemistry at the anomeric carbon. Likewise, there were major fragmentation differences in comparing the α- and β-D-galactopyranosyl-glycolaldehyde anions (Figure 2, parts C and D). Similarly, only by changing the stereochemistry at the 4-position (Figure 2, proceeding from panel B to panel D, for example), the dissociation was markedly affected. Product ions at *m*/*z* 161, 101, and 113 were significantly diminished in abundance, *m*/*z* 131 becoming the sole dominant product ion with β-D-galactopyranosyl-glycolaldehyde (Figure 2D). Reproducibility of the spectrum as shown in Figure 2A was similar to other glycosylglycolaldehydes and is indicated with error bars that represent the standard deviation of 20 experiments performed over a 2 month period. The reproducibility has been the topic of detailed experimental studies previously described[41] and is briefly discussed later in this article.

In a similar vein, the negative ion spectra of the 12 other aldohexopyranosyl-glycolaldehydes **5**−**16** (Chart 1) showed unique and reproducible differences in ion ratios easily permit-

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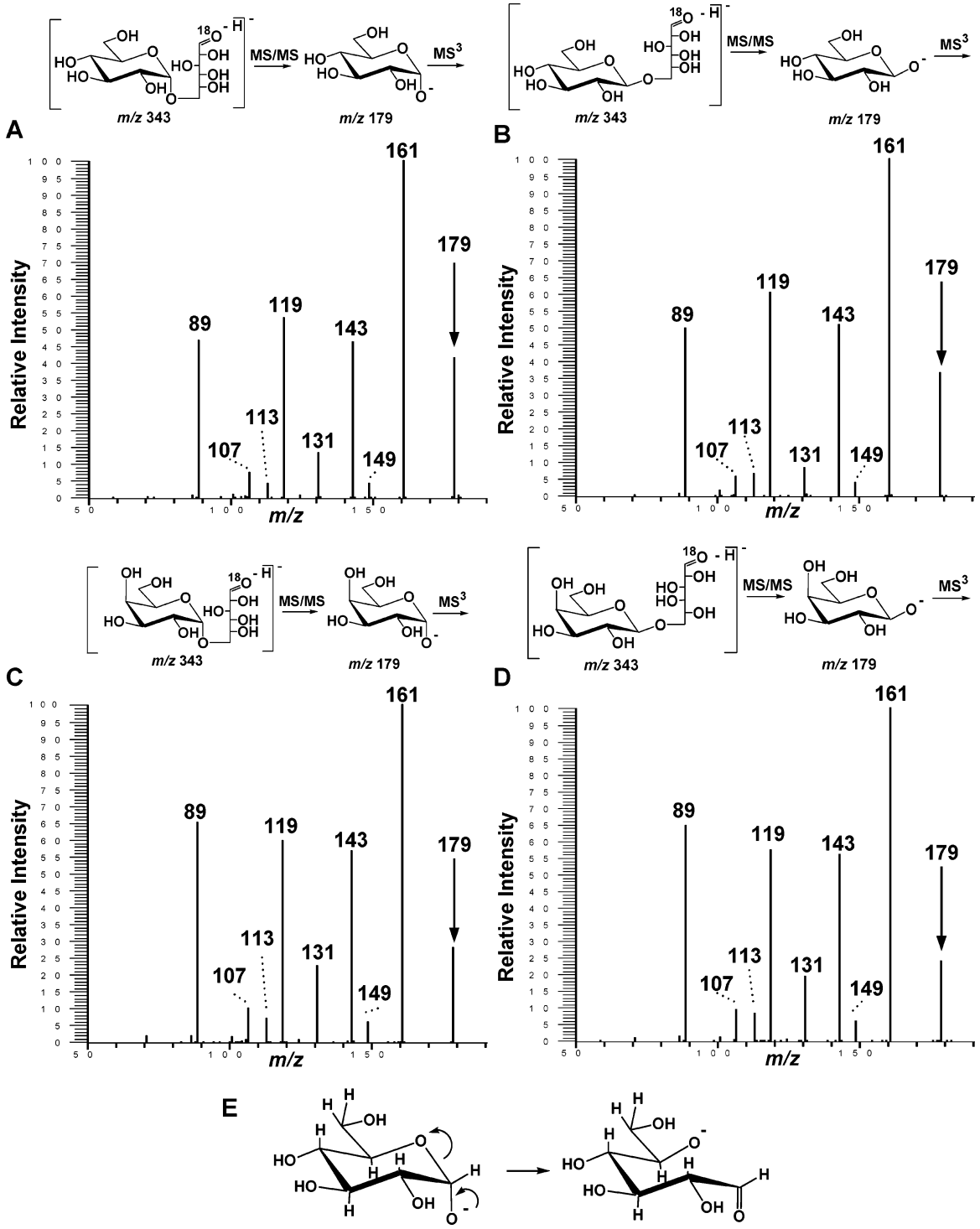
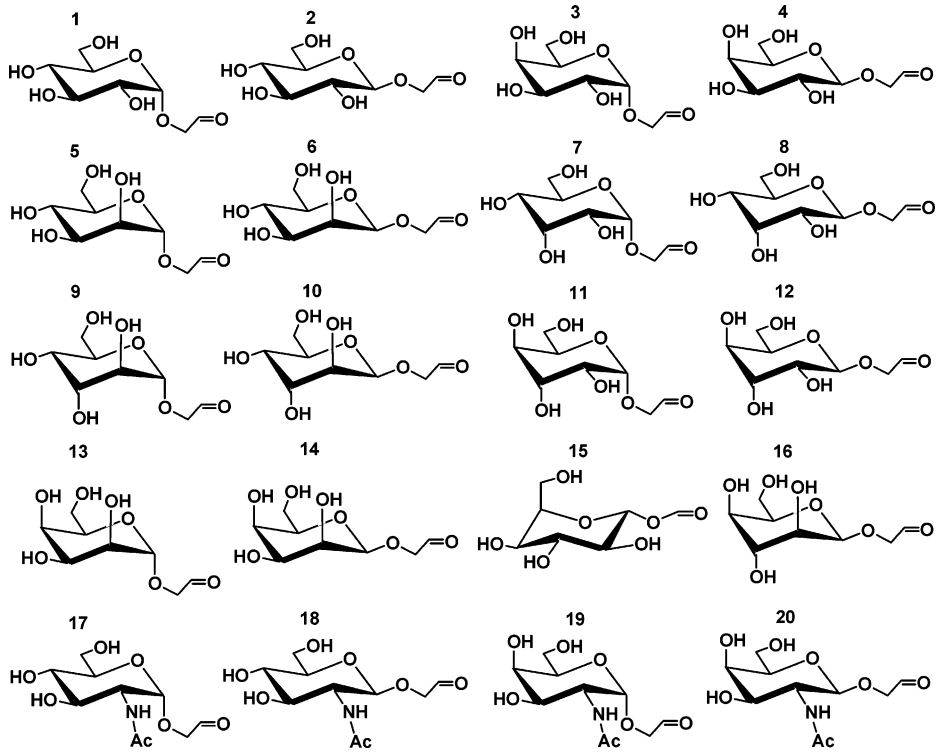


Figure 1. Mass spectrometry of monosaccharide product ions derived from disaccharides ^{18}O-labeled on the carbonyl group of the reducing sugar, illustrating the difficulties in differentiating anomeric configuration and stereochemistry in gas-phase analysis of monosaccharide product anions. A series of four disaccharides were ^{18}O-labeled on the carbonyl group of the reducing sugar, isolated in the gas phase as $[M - H]^-$ *m*/*z* 343 precursor anions, dissociated in the Paul trap, and then the *m*/*z* 179 product ion derived from the nonreducing monosaccharide was isolated and subjected to another dissociation (MS^3). Shown are the MS^3 spectra of *m*/*z* 179 product anions derived from (A) α-D-Glc*p*-(1−6)-D-Glc, (B) *β*-D-Glc*p*-(1−6)-D-Glc, (C) α-D-Gal*p*-(1−6)-D-Glc, and (D) *β*-D-Gal*p*-(1−6)-D-Gal. Structures of the *m*/*z* 343 precursor ions and *m*/*z* 179 product ions for each gas-phase dissociation sequence are shown above individual panels. In panel E is shown the hypothetical sugar ring opening that may occur during acquisition of enough internal vibrational energy needed for gas-phase dissociation.

ting their anomeric configurations and stereochemistries to be assigned (Figures 3−5). A detailed examination of their fragmentation patterns has enabled some empirical rules about their dissociation to be extracted: (1) The abundance of the *m*/*z* 101 anion was strongly dependent on the stereochemical relationship between positions 1 and 2. If these positions contained the aglycon and the 2-hydroxyl group in a cis relationship, the *m*/*z* 101 anion was favored. Hence, the α-gluco-, galacto-, allo-, and gulopyranosides strongly favored the 101 anion over their respective *β* anomers, but the *β*-manno-, altro-, talo-, and idopyranosides favored the 101 anion formation over their respective α anomers.[44] (2) Similarly, the *m*/*z* 113

Chart 1



anion was always more abundant when the aglycon and 2-hydroxyl group were found in a cis relationship for all anomeric pairs.[44] In cases where the 3-hydroxyl group was also cis to the 2-hydroxyl (1,2,3-cis), the 113 anion was even more abundant. This was observed in comparing the 3-epimeric pairs α-allo- and α-gluco-, α-gulo- and α-galacto-, *β*-talo- and *β*-ido-, and to a lesser extent, *β*-manno- and *β*-altropyranosyl-glycolaldehydes. (3) The stereochemistry of the 4-position dramatically affected the ratio of the *m*/*z* 131/161 ion abundances, which was generally favored only for the *β*-anomers. Thus, in proceeding from a 4-equatorial hydroxyl to the 4-axial epimer for *β*-pyranosides, the ratio of 131/161 was markedly increased, as seen in comparing the 4-epimeric pairs *β*-gluco- and *β*-galacto-, *β*-allo- and *β*-gulo, *β*-manno- and *β*-talo-, and *β*-altro and *β*-idopyranosyl-glycolaldehydes. For the α-pyranosides, this was not observed. (4) A preponderance of the *m*/*z* 159 anion relative to *m*/*z* 161 was only found in four cases, where the stereochemistries of positions 1, 2, and 3 were uniquely related. When positions 1 and 2 were trans but positions 2 and 3 were cis, the *m*/*z* 159/161 ratio was dramatically increased. This arrangement only occurs for the *β*-allo and *β*-gulo pair of isomers and for the α-manno and α-talo pair.

Finally, some ions of moderate abundance appeared to be nearly characteristic for one or at most a few stereochemical variants. The *m*/*z* 203 anion, for example, was only found in significant abundance for *β*-D-idopyranosyl-glycolaldehyde, and the *m*/*z* 75 anion only with the α-talo variant. The *m*/*z* 143 anion was similarly found above values of about 1% only in the α-Gul and *β*-Ido variants, and the *m*/*z* 97 anion in the α-talo and to a lesser extent, α-gulo and *β*-ido isomers. Thus, while some dissociation channels were common to many glycopyranosyl-glycolaldehydes, others were unique. It is worth noting that these rules are only *empirical propensities* as for every stereochemistry there were competing alternative dissociation pathways that gave rise to other ions. We have considered potential mechanisms regarding the stereochemical dependence of these dissociation pathways but have specifically refrained from discussing them in this article for three reasons. First, the true origin(s) of each product ion are as yet uncertain and will require detailed studies with isotopomers. Second, little is known and currently little is possible to experimentally verify concerning the conformations of high-energy eigenstates of these ions as they approach and/or exceed their dissociation thresholds. Such measurements may be possible in the future via ion spectroscopy in different electromagnetic wavelength ranges.[45,46] Third, some of these molecules may exist as cyclic hemiacetal variants in the gas phase, as described later in this report.

Dissociation of 2-Acetamido-2-deoxyglycopyranosyl-Glycoaldehydes (*m*/*z* 262 Anions) in the Negative Ion Mode Discriminates Both Anomeric Configuration and Stereochemistry of the Ions for All Four GlcNAc and GalNAc Variants. In Figure 6 is shown the mass spectral differentiation of the anomeric configuration and stereochemistry of two HexNAc-glycolaldehydes for *N*-acetylhexosamines commonly found in mammalian systems. Data are from synthetic molecules with precursor ions ($[M - H]^-$ *m*/*z* 262) indicated above each spectrum. Insets show an expansion of the fragment ion intensities (to the multiplicative values indicated) for the *m*/*z* region from 70 to 200, where the *m*/*z* 112 abundance is off the vertical scale for three of the four insets. It is important to note that the spectra shown in the insets were accumulated with enough scans so that noise levels were less than 0.1% of the base product ion peak. Under identical dissociation conditions,

(44) These same observations applied to idopyranosyl-glycolaldehydes (structures **15** and **16**), even though the α form was found by NMR to be preferentially in the $^1C_4$ conformation and the *β* form was found in the $^4C_1$ conformation.

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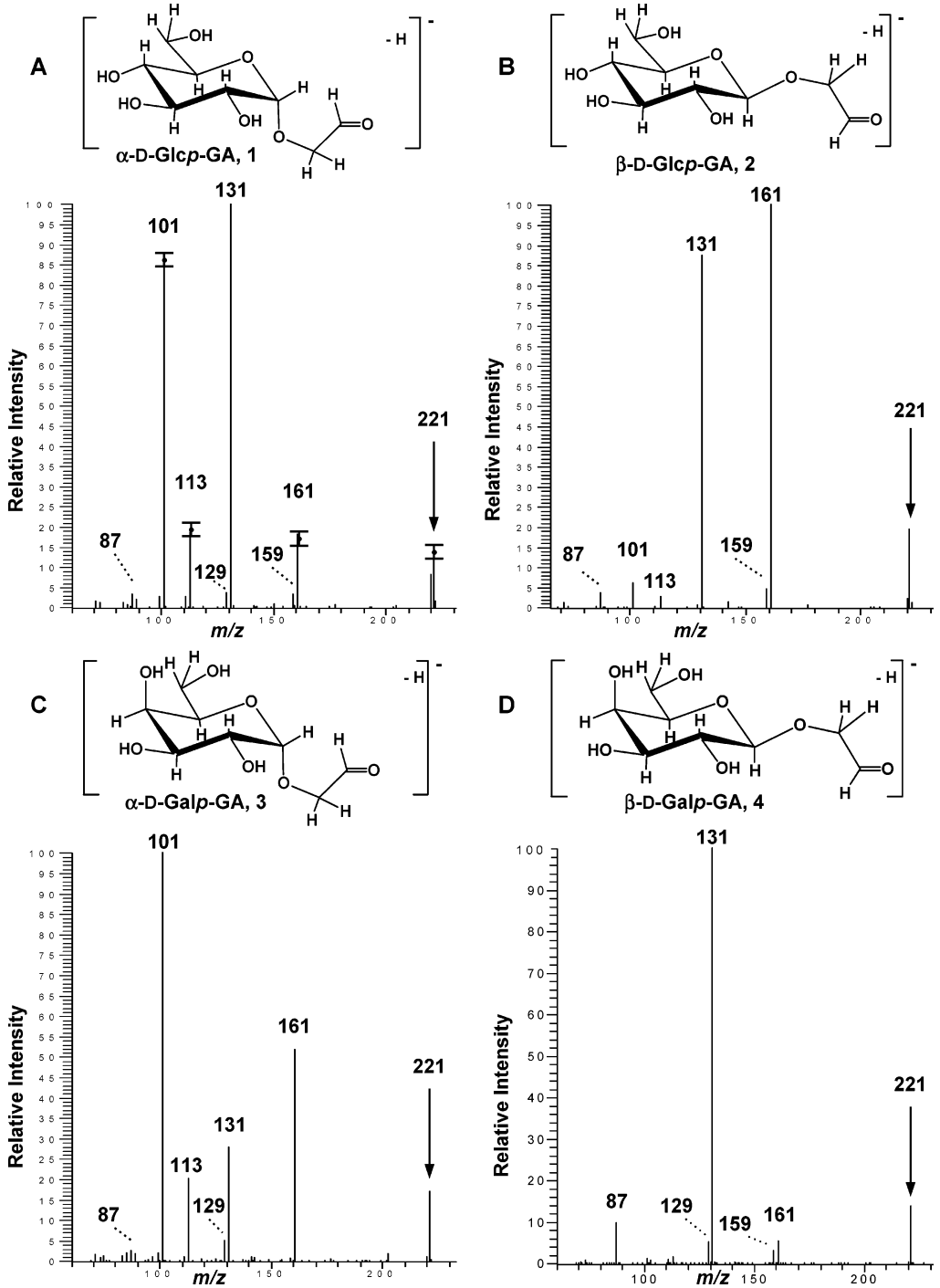


Figure 2. Differentiation of synthetic glucopyranosyl- and galactopyranosyl-glycolaldehyde isomers having different anomeric configurations by MS/MS in the negative ion mode. The $[M - H]^-$, *m*/*z* 221 precursor anions were isolated, and MS/MS spectra were recorded following dissociation in an LCQ Paul trap. Spectra were recorded from (A) α-D-glucopyranosyl-2-glycolaldehyde **1**, (B) β-D-glucopyranosyl-2-glycolaldehyde **2**, (C) α-D-galactopyranosyl-2-glycolaldehyde **3**, and (D) β-D-galactopyranosyl-2-glycolaldehyde **4**. Error bars in panel A illustrate the standard deviation of the indicated major ions calculated using the intensity of *m*/*z* 131 as exactly 100%, for 20 spectra accumulated over a 2 month period.

the HexNAc-glycolaldehydes all showed a base product ion at *m*/*z* 202, and all showed an *m*/*z* 112 product ion of varying relative abundance. Key observations were that the β-D-Gal*p*NAc- and β-D-Glc*p*NAc-glycolaldehydes (Figure 6, insets to panels B and D) showed an *m*/*z* 100 ion of low abundance that was essentially negligible in spectra of their respective α-anomers (Figure 6, insets to panels A and C). Another difference was the respective abundance of their *m*/*z* 140/142 product ions. In the α-linked HexNAc-glycolaldehydes, the *m*/*z* 140 ion was of greater abundance (insets to Figure 6, panels A and C), whereas in the β-glycosides, the *m*/*z* 142 ion was of much greater intensity (insets to Figure 6, panels B and D). In addition the α-linked compounds showed *m*/*z* 128 anions that were of negligible abundance in their respective β-anomers. Two ions having *m*/*z* 184 and 196 were found in the Gal*p*NAc-glycolaldehydes that were of negligible abundance in Glc*p*NAc-glycolaldehydes. The β-Gal*p*NAc- and β-Glc*p*NAc-glycolaldehydes were also easily distinguished by a major difference in their *m*/*z* 112/100 product ion ratios (compare Figure 6, panels B and D), and the α-Gal*p*NAc and α-Glc*p*NAc-glycolaldehydes

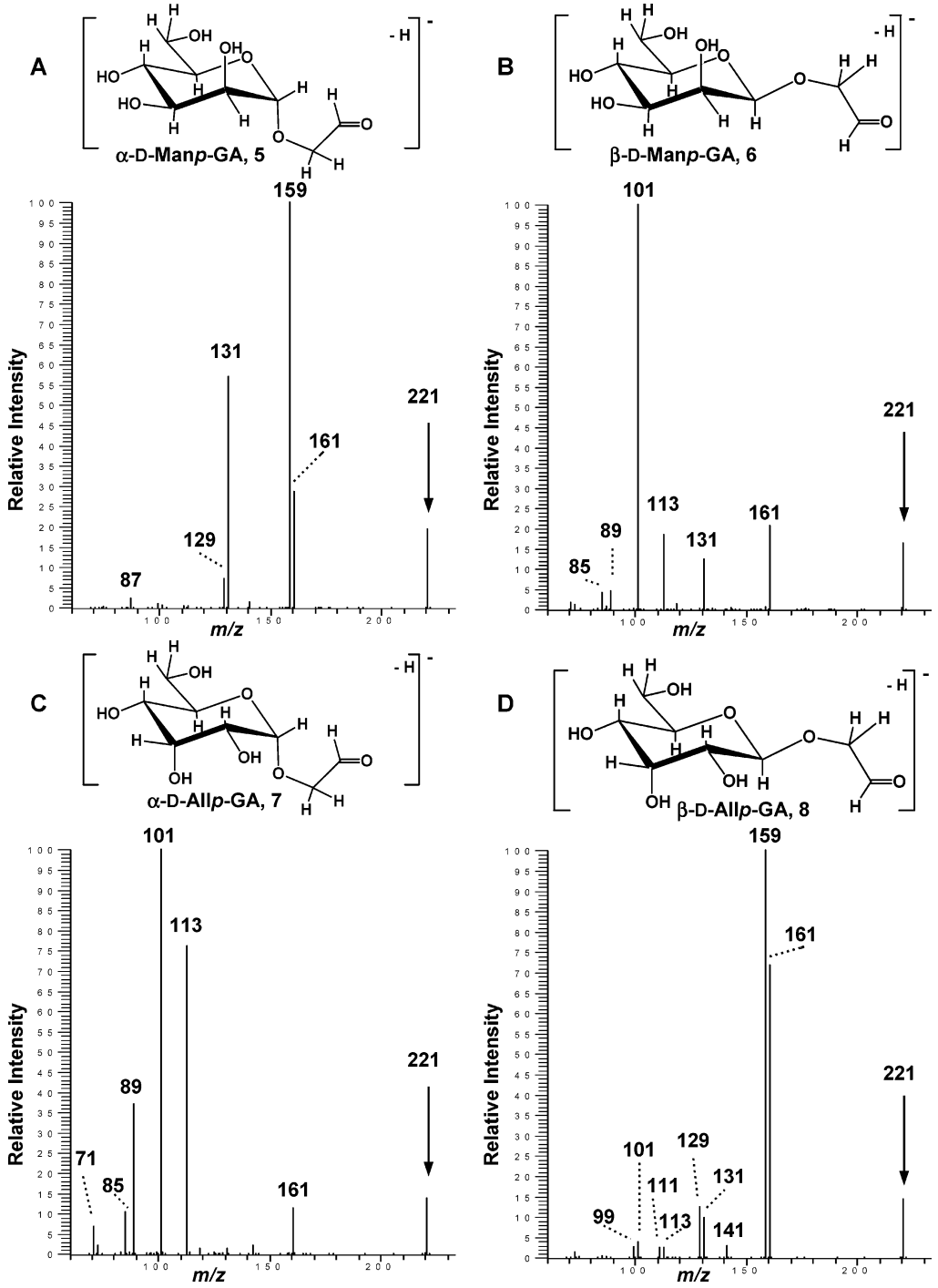


Figure 3. Discrimination of synthetic mannopyranosyl- and allopyranosyl-glycolaldehyde isomers having different anomeric configurations by MS/MS in the negative ion mode. The $[M - H]^-$, *m*/*z* 221 precursor anions were isolated, and MS/MS spectra were recorded in a Paul trap. Spectra were recorded from (A) α-D-mannopyranosyl-2-glycolaldehyde **5**, (B) *β*-D-mannopyranosyl-2-glycolaldehyde **6**, (C) α-D-allopyranosyl-2-glycolaldehyde **7**, and (D) *β*-D-allopyranosyl-2-glycolaldehyde **8**.

were easily discriminated based on a complete lack of the *m*/*z* 196 anion in the spectrum of the Glc*p*NAc form as well as a difference in abundance of their *m*/*z* 112 ions (compare Figure 6 panels A and C). The relative abundances of these ions as compared to the base product ion peak at 100% were reproducibly within a few percent under defined conditions; therefore, the spectra with even moderate signal/noise easily and reproducibly differentiated between the four isomeric HexNAc-glycolaldehydes in the gas phase.

Identification of the Stereochemistry and Anomeric Configurations of Glycopyranosyl-Glycolaldehydes and 2-Acetamido-2-deoxy-glycopyranosyl-Glycolaldehdyes Derived from the Nonreducing Position of Disaccharides by Dissociation in the Gas Phase. A true test as to whether the dissociation spectra of synthetic glycosyl-glycolaldehyde anions could serve as gas-phase standards to assign the stereochemistry and anomeric configuration of monosaccharides derived from larger carbohydrates was to compare their mass spectra to those of *m*/*z* 221 anions derived by dissociation of disaccharides and observe whether one stereochemistry could be clearly identified from among all 16 possible stereochemical variants. Disaccharide precursor ions were fragmented, and their respective *m*/*z*

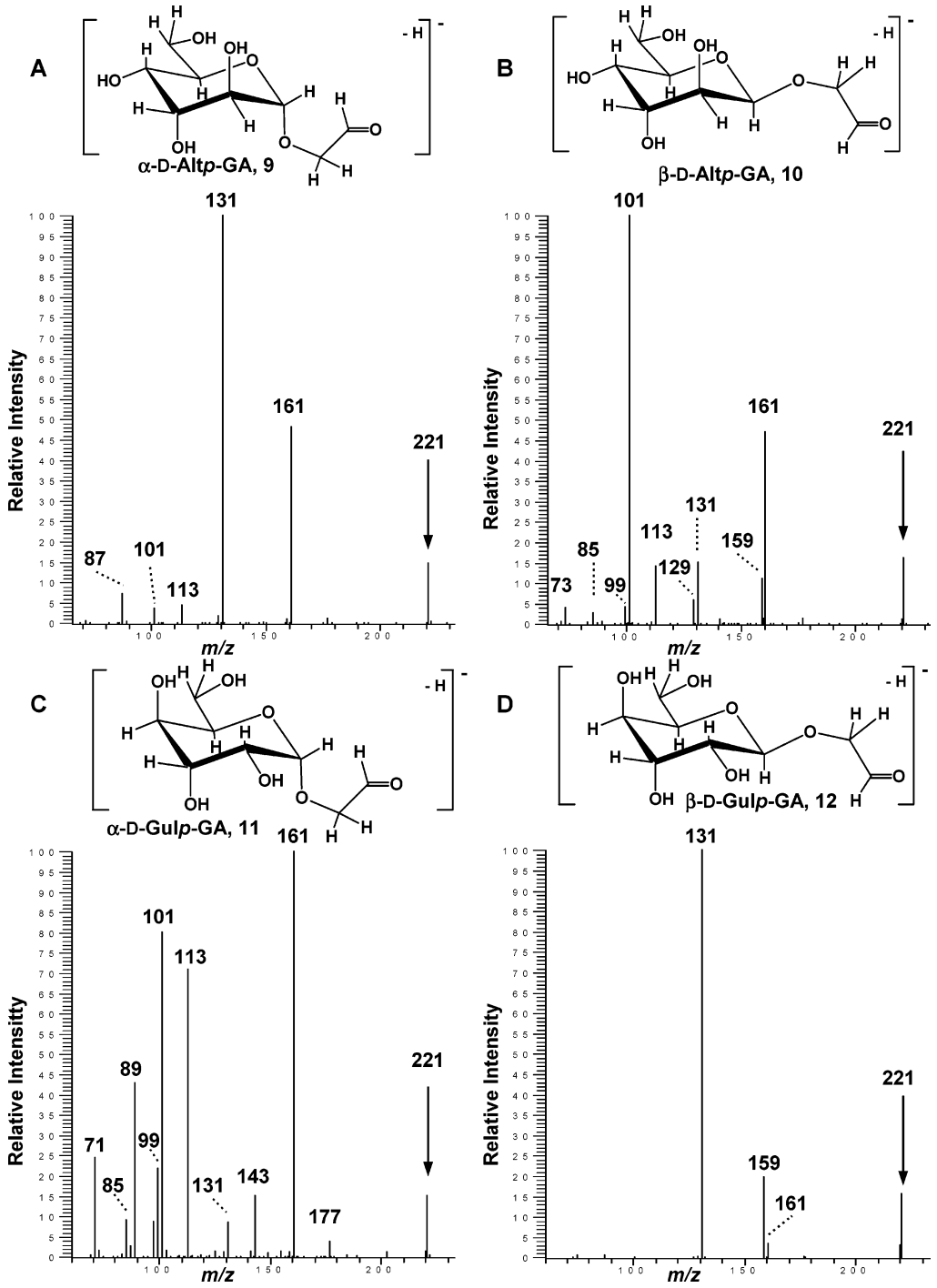


Figure 4. Differentiation of synthetic altropyranosyl- and gulopyranosyl-glycolaldehyde isomers having different anomeric configurations by MS/MS in the negative ion mode. The $[M - H]^-$, *m*/*z* 221 precursor anions were isolated, and MS/MS spectra were recorded in a Paul trap. Spectra were recorded from (A) α-D-altropyranosyl-2-glycolaldehyde **9**, (B) β-D-altropyranosyl-2-glycolaldehyde **10**, (C) α-D-gulopyranosyl-2-glycolaldehyde **11**, and (D) β-D-gulopyranosyl-2-glycolaldehyde **12**.

221 product ions were isolated and further dissociated in an MS^3 experiment (Figures 7 and 8). In the case of 4-linked structures, the *m*/*z* 221 anions were obtained in higher abundance through in-source fragmentation; hence, the spectra shown are MS/MS spectra. Although the *m*/*z* 221 anions could be isolated after MS/MS of 4-linked disaccharides in the gas phase and further fragmented in MS^3 experiments, the spectra required many more scans to obtain good signal/noise as compared to some of the spectra shown in Figures 7 and 8. This was simply due to the low abundance (1−5%) of the *m*/*z* 221 ion generated in the Paul trap from 4-linked disaccharides through resonantly excited CID. In Figure 7, the spectra of *m*/*z* 221 anions derived from four different disaccharides are shown. Disaccharides comprised of two hexoses were ^{18}O-labeled on the carbonyl group of the reducing sugar so that product ions derived from either side of the glycosidic linkage could be mass-discriminated. In the case of structures having a hexose and a HexNAc, there was no need to label them as components derived from either side of the glycosidic linkage already have mass differences.

In Figure 7A, in-source fragmentation of a disaccharide having a nonreducing β-D-mannopyranoside residue (β-D-Man*p*-(1−4)-D-GlcNAc) yielded an *m*/*z* 221 anion that was isolated

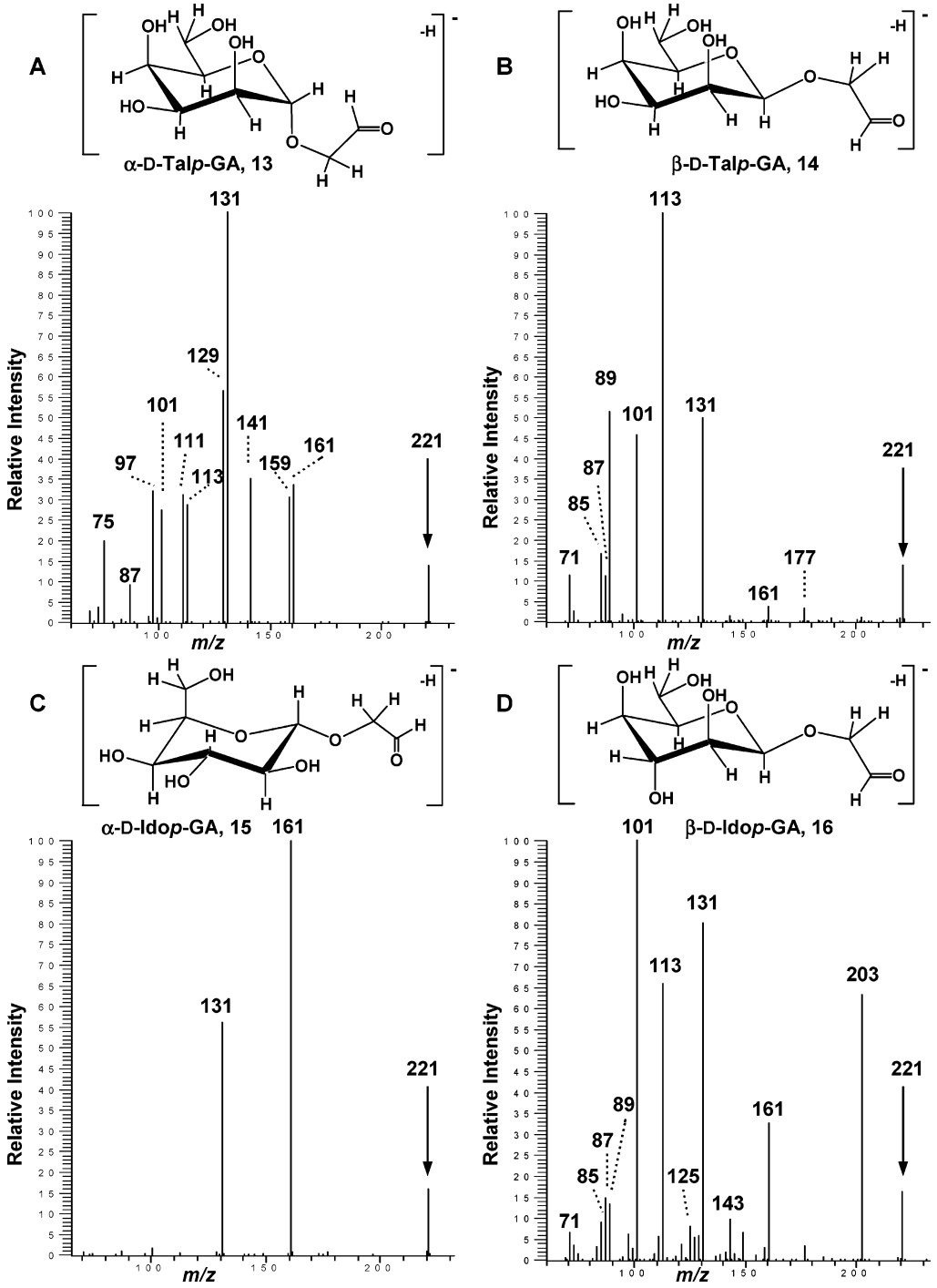


Figure 5. Discrimination of synthetic talopyranosyl- and idopyranosyl-glycolaldehyde isomers having different anomeric configurations by MS/MS in the negative ion mode. The $[M - H]^-$, *m*/*z* 221 precursor anions were isolated, and MS/MS spectra were recorded in a Paul trap. Spectra were recorded from (A) α-D-talopyranosyl-2-glycolaldehyde **13**, (B) *β*-D-talopyranosyl-2-glycolaldehyde **14**, (C) α-D-idopyranosyl-2-glycolaldehyde **15**, and (D) *β*-D-idopyranosyl-2-glycolaldehyde **16**.

and further dissociated in the Paul trap to furnish a characteristic mass spectrum. Comparison of this mass spectrum to all 16 variants of the synthetic glycopyranosyl-glycolaldehyde anions shown in Figures 2−5 clearly indicated a match with just one of them (Figure 3B), the *β*-D-mannopyranosyl-glycolaldehyde **6**. In Figure 7B, the MS^3 spectrum of an ion having *m*/*z* 221 derived from a disaccharide anion (*β*-D-Gal*p*-(1−6)-D-GlcNAc is shown. Comparison of this spectrum to all 16 variants of the synthetic glycosyl-glycolaldehydes (Figures 2−5) again clearly showed a match to just one of them (Figure 2D), the *β*-D-galactopyranosyl-glycolaldehyde **4**. The structures shown in Figure 7, parts C and D, were disaccharides comprised of only hexoses, which were ^{18}O-labeled on the carbonyl of the reducing sugar. For both structures, *m*/*z* 221 ions were isolated (no *m*/*z* 223 ions were observed).[47] Again, the fragmentation patterns of these ions matched their respective glycosyl-glycolaldehyde anion spectra. For Figure 7C, the spectrum of the *m*/*z* 221 anion derived from α-D-Man*p*-(1−6)-D-Man matched that of synthetic

(47) ^{18}O-labeling was carried out with disaccharides solely comprised of hexoses so that an anion comprised of a nonreducing sugar attached to a 2-carbon fragment derived from the reducing sugar (*m*/*z* 221) could be differentiated from a potential anion containing a reducing sugar attached to a 2-carbon fragment derived from the nonreducing sugar (*m*/*z* 223).

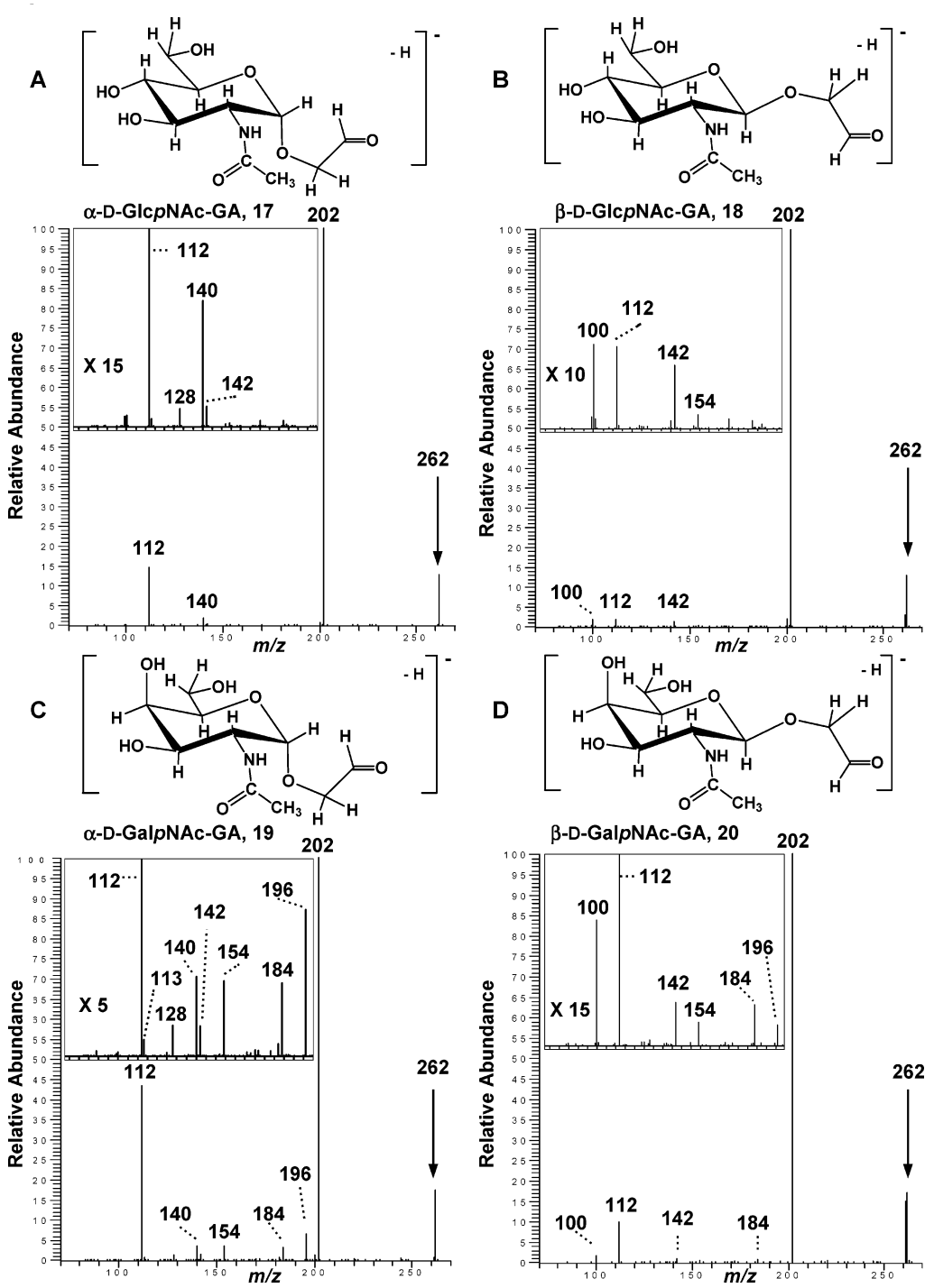


Figure 6. Differentiation of synthetic glycosyl-glycolaldehyde isomers of the *N*-acetylhexosamines GlcNAc and GalNAc, having different anomeric configurations by MS/MS in the negative ion mode. The $[M - H]^-$, *m*/*z* 262 precursor anions were isolated, and MS/MS spectra were recorded in a Paul trap. Spectra were recorded from (A) (2-acetamido-2-deoxy-α-D-glucopyranosyl)-2-glycolaldehyde **17**, (B) (2-acetamido-2-deoxy-*β*-D-glucopyranosyl)-2-glycolaldehyde **18**, (C) (2-acetamido-2-deoxy-α-D-galactopyranosyl)-2-glycolaldehyde **19**, and (D) (2-acetamido-2-deoxy-*β*-D-galactopyranosyl)-2-glycolaldehyde **20**. Insets illustrate vertical expansions of the *m*/*z* 70−200 regions, to the multiplicative extent indicated in each, so the *m*/*z* 112 product ion is off-scale for panels A, C, and D. Shown in insets are multiple-scan spectra acquired with high signal/noise.

α-D-mannopyranosyl-glycolaldehyde **5** (Figure 3A), whereas in Figure 7D, derived from α-D-Gal*p*-(1−6)-D-Glc, the spectrum of the *m*/*z* 221 anion matched that of synthetic α-D-galactopyranosyl-glycolaldehyde **3** (Figure 2C). Similar results are shown in Figure 8, parts A and B, where the mass spectra of *m*/*z* 221 anions derived from the ^{18}O-labeled disaccharides *β*-D-Gal*p*-(1−6)-D-Gal and *β*-D-Gal*p*-(1−4)-D-Glc both matched the spectrum of the synthetic *β*-D-galactopyranosyl-glycolaldehyde **4** (Figure 2D). The spectra of the *m*/*z* 221 anions in Figure 7 and Figure 8, parts A and B, combined with ^{18}O-labeling of their precursor disaccharides, yielded results in full accordance with data from a set of *m*/*z* 221 ions derived from six different glucose-containing disaccharides previously reported.[41] That is, the *m*/*z* 221 anions could be used to assign the anomeric

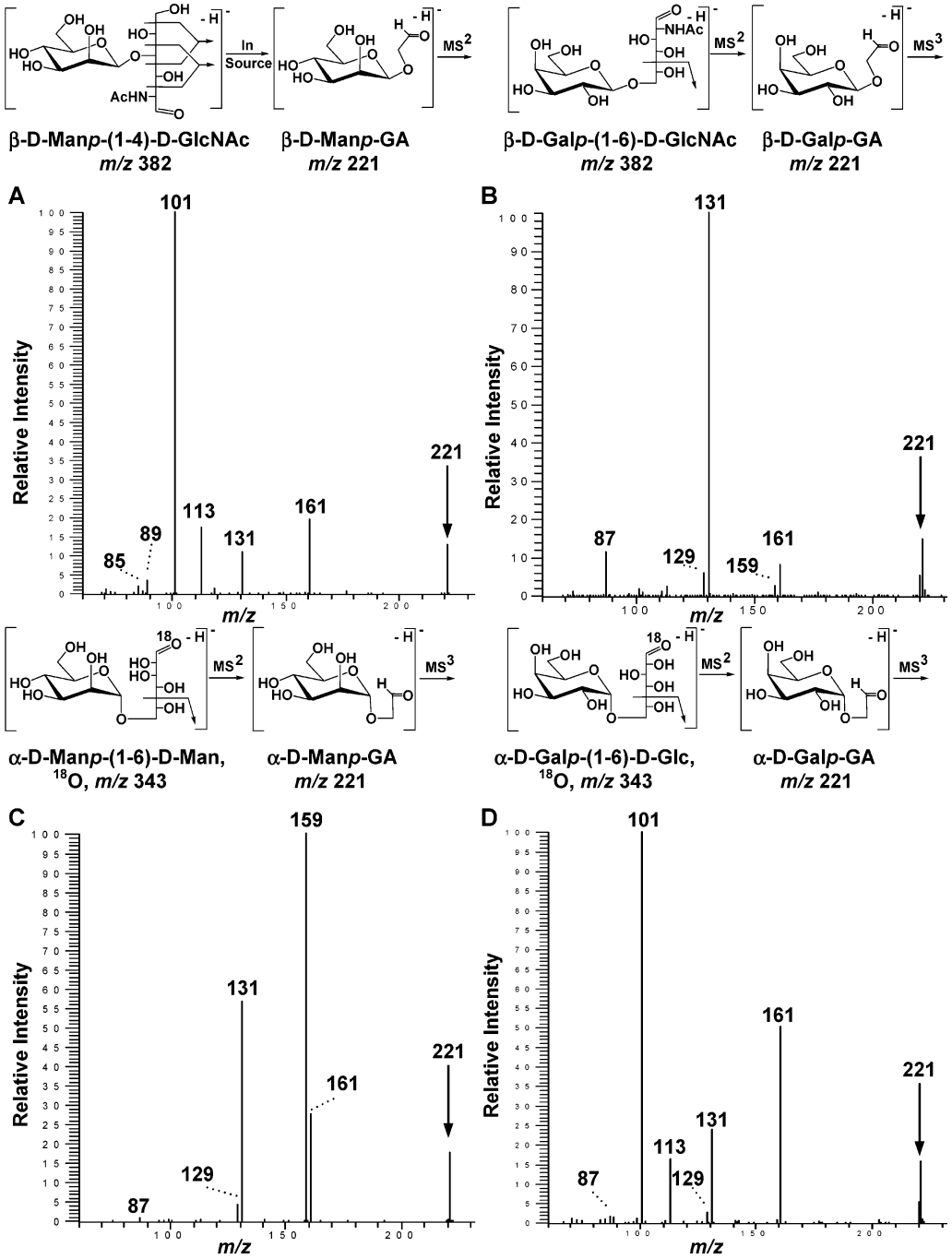


Figure 7. Dissociation spectra of *m*/*z* 221 product ions derived from various disaccharide precursor ions in the negative ion mode. Shown are the dissociation spectra of the *m*/*z* 221 product ion derived from (A) *β*-D-Man*p*-(1−4)-D-GlcNAc (*m*/*z* 382 → *m*/*z* 221→ spectrum), (B) *β*-D-Gal*p*-(1−6)-D-GlcNAc (*m*/*z* 382 → *m*/*z* 221→ spectrum), (C) α-D-Man*p*-(1−6)-D-Man, ^{18}O-labeled on the carbonyl of the reducing Man (*m*/*z* 343 → *m*/*z* 221 → spectrum), and (D) α-D-Gal*p*-(1−6)-D-Glc, ^{18}O-labeled on the carbonyl of the reducing Glc (*m*/*z* 343 → *m*/*z* 221 → spectrum). Structures of the precursor and product ions are indicated above each mass spectrum. The open-chain form of the reducing sugar was drawn solely to illustrate the fragmentation position; no implication regarding the relative abundance of open-chain or cyclic form(s) of the reducing sugar in the gas phase is intended, nor are any inferences drawn concerning dissociation mechanisms.

configuration and stereochemistry of the nonreducing aldohexopyranoside in these disaccharides, by comparison to the complete series of aldohexopyranosyl-glycolaldehyde standards.

Disaccharides having a nonreducing HexNAc residue fragmented in the negative ion mode to yield *m*/*z* 262 product ions, with no *m*/*z* 221 anions. Spectra of the *m*/*z* 262 anions derived from these disaccharides also matched those of synthetic standards. As illustrated in Figure 8, parts C and D, dissociation spectra of the *m*/*z* 262 anions isolated in the gas phase from *β*-D-Glc*p*NAc-(1−2)-D-Man and *β*-D-Gal*p*NAc-(1−4)-D-Gal matched the negative ion spectra of synthetic *β*-D-Glc*p*NAc-glycolaldehyde **18** (Figure 6B) and *β*-D-Gal*p*NAc-glycolaldehyde **20** (Figure 6D), respectively. Admittedly all 16 stereochemical variants of the 2-acetamido-2-deoxyhexopyranosyl-glycolaldehydes would need to be prepared to unambiguously assign their stereochemistry, but it is relevant that the dissociation of each isolated *m*/*z* 262 anion matches those of the appropriate synthetic standards and enables the anomeric configuration and stereochemistry of these two 2-acetamido-2-deoxyhexoses to be differentiated.

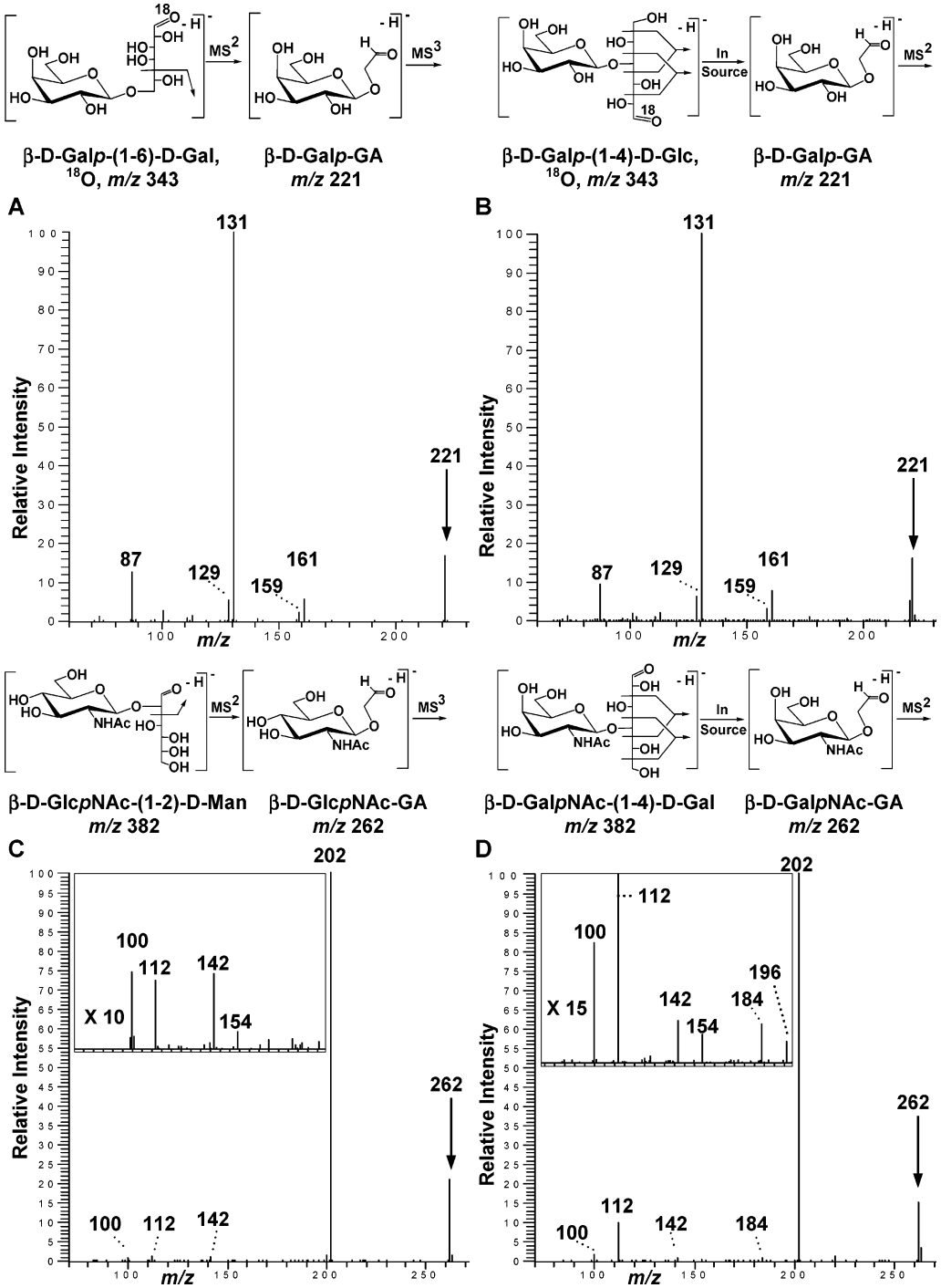


Figure 8. Dissociation spectra of *m*/*z* 221 and *m*/*z* 262 product ions derived from various disaccharide precursor ions in the negative ion mode. Shown are the dissociation spectra of the *m*/*z* 221 or *m*/*z* 262 product ion derived from (A) *β*-D-Gal*p*-(1−6)-D-Gal, ^{18}O-labeled on the carbonyl of the reducing Gal (*m*/*z* 343 → *m*/*z* 221→ spectrum), (B) *β*-D-Gal*p*-(1−4)-D-Glc ^{18}O-labeled on the carbonyl of the reducing Glc (*m*/*z* 343 → *m*/*z* 221→ spectrum), (C) *β*-D-Glc*p*NAc-(1−2)-D-Man (*m*/*z* 382 → *m*/*z* 262 → spectrum), and (D) *β*-D-Gal*p*NAc-(1−4)-D-Gal, (*m*/*z* 382 → *m*/*z* 262 → spectrum). Structures of the precursor and product ions are indicated above each mass spectrum. The open-chain form of the reducing sugar was drawn solely to indicate the fragmentation position(s); no implication regarding the relative abundance of open-chain or cyclic form(s) of the reducing sugar in the gas phase is intended, nor are any inferences drawn concerning dissociation mechanisms.

It is also important to emphasize that these ions were always comprised of an intact nonreducing sugar with the 2-carbon aglycon derived from the reducing sugar, as previously established using glucose-containing disaccharides.[41] In considering the potential of isolating various disaccharides through multistep disassembly from larger oligosaccharides in the gas phase, it is highly relevant to also be able to assign the *location* of an experimentally determined monosaccharide stereochemistry to one specific side of the glycosidic linkage in disaccharides.

Assignment of the Linkage Position of Disaccharides Based on Dissociation Patterns in the Negative Ion Mode. It has been possible to assign the linkage position of disaccharides in the negative ion mode based on previous studies carried out using sector instruments,[26−28] FTICR instruments,[29] in-

source decomposition,[30,31] ion traps,[41] and triple-quadrupole instruments.[41] Our results with the hexose-containing disaccharides reported in Figures 7 and 8 showed the same fragmentation products for different linkages in approximately the ratios previously reported[41] and enabled the linkage position between two sugars in disaccharides to be assigned (data not shown). With disaccharides having a nonreducing HexNAc and a reducing hexose, dissociation of the hexose occurred to give fragmentation patterns similar to hexose-containing disaccharides with product ions having a mass increment of +41 due to the mass difference of the acetamido group as compared to a hydroxyl group for the nonreducing sugar. With disaccharides having the reducing HexNAc, linkages could also be ascertained, but some of the product ions contained the +41 mass increment due to the presence of the acetamido group. This data has not been presented in detail herein as it was confirmatory to other reports and the key focus of this investigation was the dissociation properties of the glycosyl-glycoaldehyde anions. It is important to point out that the *m*/*z* 221 anions (for hexose-containing disaccharides) were observed as product ions from fragmentation of 2-, 3-, 4-, and 6-linked disaccharides, although for 3-linked species, their abundance was less than 1% of the base product ion peak when dissociated by resonant excitation in a Paul trap. They were obtained in higher abundance for 3-linked disaccharides (1−2%) using in-source fragmentation but were still too low in abundance for isolation and subsequent dissociation of the *m*/*z* 221 anion. They have been obtained in higher abundance using in-source fragmentation with triple-quadrupole instruments and under higher-energy collision conditions.[31,41]

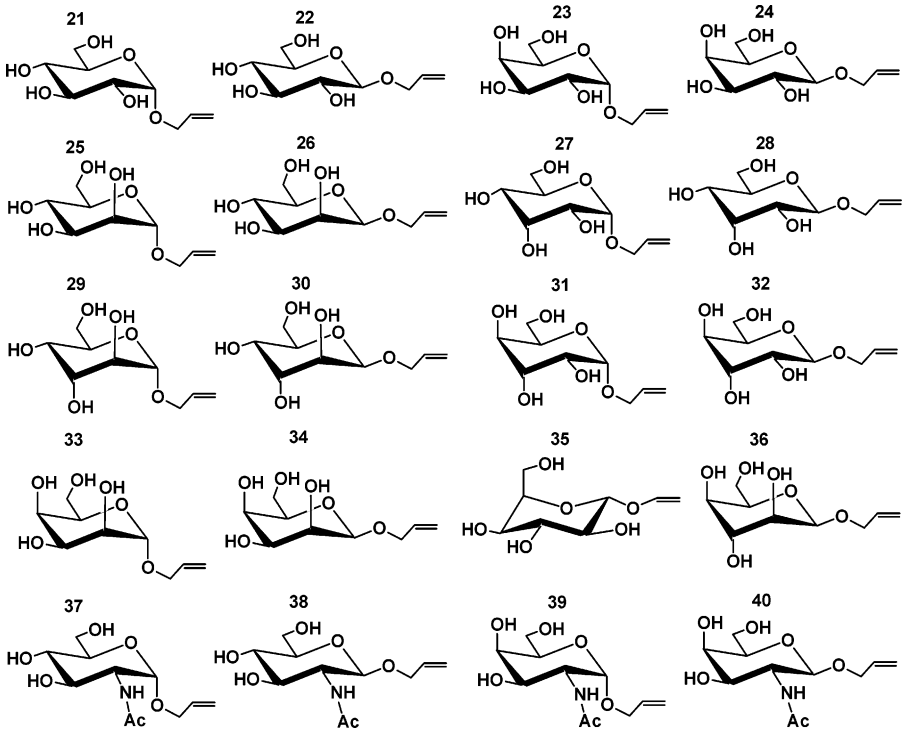
Mass Spectral Reproducibility. In a previous report,[41] issues relevant to reproducible acquisition of mass spectra in a Paul trap were described in detail, so they will only be briefly described here. Spectral reproducibility is crucial for identification of ions as "spectral fingerprints" and is particularly relevant for discrimination between isobaric variants having identical elemental compositions. Shown in Figure 2A is the variability of the spectrum of α-D-glucopyranosyl-glycolaldehyde **1**, where error bars show a standard deviation of peak heights over the collection of 20 independent spectra obtained over a 2 month period. Spectral reproducibility could be maintained from day-to-day and over several months by controlling four key parameters: (1) Isolation width needed to be kept constant and of moderate width (3 Finnigan "amu" units) purposefully to improve reproducibility. (2) Frequent calibration of the resonance excitation frequency was essential. Normally, after calibration of the instrument, this frequency drifts and can do so in the LCQ at a rate different than that of the mass measurement frequencies. If this drifts by 0.2−0.5 amu, it will slightly affect the ratios of product ions and either requires recalibration or can be finely adjusted by slightly shifting the excitation resonance frequency to manually optimize the total product ion current. (3) The most important issue involves defining the energy input into an ion, where the ratio of the most abundant product ion (base) peak to the remaining precursor ion was chosen as the most reliable criterion for reproducible energy deposition into ions. We selected synthetic α-D-glucopyranosyl-glycolaldehyde as an "internal energy-input tuning ion" and routine standard, in a similar fashion to Bristow et al.,[48] and kept its base product ion/precursor ion ratio (100:18) as constant as possible over the long term to ensure consistent energy deposition into this ion and other isomeric *m*/*z* 221 precursor ions. Thus, over a period of months, the collision energy needs to be slightly adjusted to attain the same ratio of base product ion/precursor ion. (4) The number of ions in the trap needed to be approximately the same for comparison of a standard to an unknown (within an order of magnitude) to have best reproducibility. This was tested systematically from 5×10^3 to 3×10^6 ions in the trap; the overall number of ions in the trap slightly affected dissociation patterns, regardless of other parameter settings. It is worth noting that for these 16 isomeric variants, the differences in dissociation were marked enough that this was not a significant factor in their discrimination. Issues 2 and 3, above, were most essential to control on an ongoing basis to yield reproducible spectra as illustrated in Figure 2A.

Possible Cyclization of *m*/*z* 221 Anions in the Gas Phase: Evidence for Cyclic Structures and Internal Hemiacetal Formation of Glycosyl-Glycolaldehydes Based on NMR Spectroscopy in Solution. Although NMR spectroscopy was not the primary focus of this investigation, during the synthesis of the glycopyranosyl-glycolaldehydes, 20 allyl-glycopyranosides (Chart 2) were necessarily prepared. The allyl glycosides all showed NMR spectra characteristic of one allyl signal set, one anomeric proton signal, and one set of sugar ring protons, which were completely assigned (see the Supporting Information). However, NMR spectra of synthetic glycosyl-glycolaldehydes prepared from the allyl glycopyranosides indicated that several of them exist in cyclic forms through formation of a hemiacetal between the glycolaldehyde carbonyl group and the sugar 2-hydroxyl group. In Figure 9 is shown the downfield region of the NMR spectrum of β-D-galactopyranosyl-glycolaldehyde. The anomeric protons of the β-D-galactopyranosyl moiety were observed as three sets of doublets in varying proportions (with $^3J_{1,2} = 8$ Hz for each of them). In addition three sets of the glycolaldehyde H-1′, H-2′a, and H-2′b signals were observed, the H-1′ signals being found downfield (Figure 9). Above the spectrum is shown the ratios of the cyclic and open-chain isomers that contribute in solution to the overall NMR spectrum. Form A was the open-chain hydrate, which showed similar couplings of the glycolaldehyde H-1′ to both H-2′a and H-2′b. Form B had one large coupling of the glycolaldehyde H-1′ to one H-2′ signal (an antiperiplanar arrangement) and one small coupling to another H-2′ signal (a gauche arrangement). Form C had two small couplings of H-1′ to both H-2′a and H-2′b (both gauche). The NMR spectra of all the glycopyranosyl-glycolaldehydes in D_2O (Supporting Information) showed that some of them existed in cyclic forms in equilibrium with the open-chain hydrate (like the β-Gal version shown in Figure 9), whereas some existed solely, or at least highly predominantly, as the open-chain hydrate of the glycoaldehyde. The 2-acetamido-2-deoxyhexopyranosyl-glycolaldehydes only existed as open-chain hydrates in solution.

Although the open-chain hydrates of the synthetic glycosyl-glycolaldehydes exist in D_2O solution as evaluated by NMR, the hydrate ions (*m*/*z* 239) were not abundant species found in the gas phase in the negative ion mode when nanospray from

(48) Bristow, A. W. T.; Nichols, W. F.; Webb, K. S.; Conway, B. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 2374−2386.

Chart 2



methanol solution was carried out (a maximum of ~20% of the *m*/*z* 221 anion was ever observed).[49] Taken together, the data indicate that the *m*/*z* 221 anions may exist in multiple cyclic/acyclic forms in the gas phase through internal hemiacetal formation as is characteristic of reducing sugars. Cyclization might confer upon these ions unique gas-phase equilibria that in part contribute to the stereochemical dependence of their spectral differences. This adds another layer of complexity in considering possible mechanisms of their dissociation.

Summary and Conclusions

The utility of repeated isolation/dissociation steps in a multistage disassembly of oligosaccharides using ion traps or FTICR instruments has now enabled substructures to be isolated on a routine basis.[18−21] The *m*/*z* 221 anion, isolated as a substructure in the negative ion mode from disaccharides or potentially from larger oligosaccharides using MS^n, appears particularly useful and worthy of further detailed study for four reasons: (1) The stereochemistry and anomeric configuration of all 16 variants of the aldohexopyranosyl-glycolaldehydes can be reproducibly differentiated by their dissociation "signatures" in a Paul trap in the negative ion mode. This has not been possible using the *m*/*z* 179 anions of monosaccharides derived from disaccharide *m*/*z* 341 precursors, where neither the anomeric configuration nor the stereochemistry can typically be ascertained (Figure 1). (2) The *m*/*z* 221 anions derived from reducing disaccharides contain the intact nonreducing sugar and a 2-carbon aglycon derived from the reducing sugar, irrespective of the anomeric configuration or linkage position. This is based on our observations up to this point with 14 different 2-, 4-, and 6-linked disaccharides, where ^{18}O-labeling of the reducing carbonyl group has been invaluable for mass-discrimination of product ion fragments originating from either side of the glycosidic linkage (ref 41 and herein). Many more isotopically labeled reducing disaccharides will need to be investigated in order to draw any firm general conclusions about the origins of the *m*/*z* 221 anions. Moreover, at least in the Paul trap using CID, 3-linked disaccharides give rise to *m*/*z* 221 anions in only trace abundance, although higher energy dissociation conditions and in-source fragmentation can give rise to somewhat larger quantities, as high as 12% of the base product peak.[28,31,41] (3) Neither of the carbons of the glycolaldehyde portion of the *m*/*z* 221 ions are chiral. This limits the number of possible stereochemical and/or positional variants of the monosaccharide-glycoaldehydes. With the next largest fragment typically derived from disaccharides in the negative ion mode (*m*/*z* 251), one more chiral carbon and two linkage positions of the aglycon (the 2- or 3-positions of D- or L-glyceraldehyde) contribute to 4 times the number of overall stereochemical variants. (4) Our preliminary data with glucosyl-glycolaldehydes indicates that pyranosides can also be differentiated from furanosides,[41] at least for this sugar.

The *N*-acetylhexosamines GalNAc and GlcNAc can be discriminated as *m*/*z* 262 anions (HexNAc-glycolaldehydes) as can their anomers. This should be useful in analysis of glycoprotein and other HexNAc-containing oligosaccharides.

Assignment of both the stereochemistry and anomeric configuration of the nonreducing monosaccharide component of reducing disaccharides was previously only possible using NMR spectroscopy or, with some sugars, through glycosidase treatments. Differences in fragmentation of all 16 glycopyranosyl-glycolaldehyde variants provide a fundamental spectral basis and spectral standards for the potential assignment of monosac-

(49) Another ion, *m*/*z* 237, became evident in small quantities and more abundant in increasing quantities as the source temperature was increased from 120 to 250 °C. We have tentatively assigned this as an artifact resulting from oxidation of the carbonyl to a carboxyl group that can occur in the source if the temperature of the heated capillary is too high.

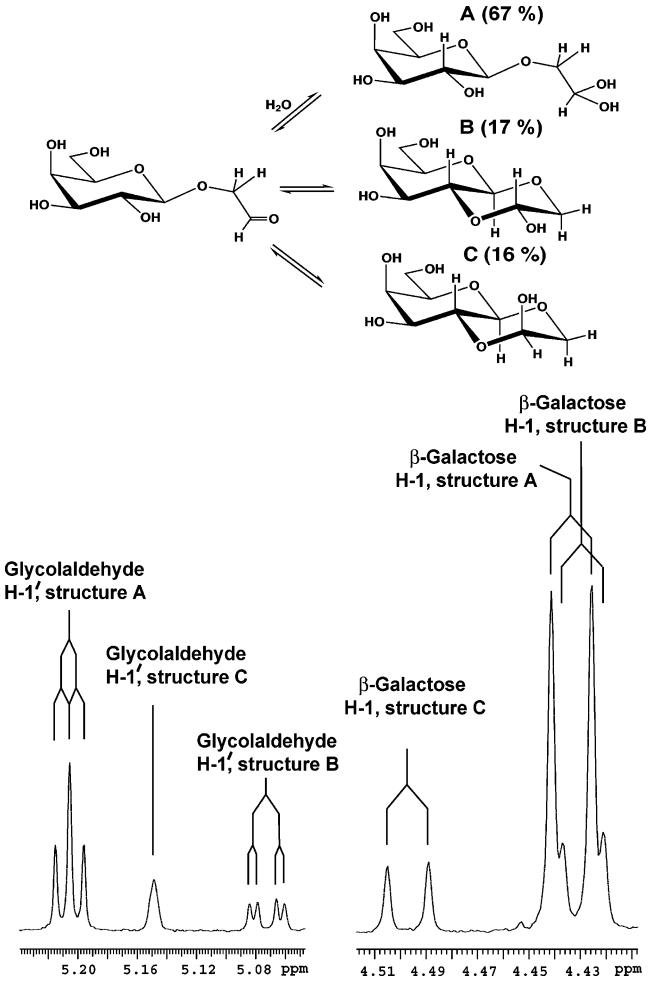


Figure 9. NMR spectroscopy (500 MHz, D_2O) of β-D-galactopyranosyl-2-glycolaldehyde **4**, illustrating the equilibrium between the acyclic hydrate form (A) and the two cyclic variants (B and C) as shown in the structural diagram. Downfield regions of the NMR spectrum show the glycolaldehyde H-1′ signals (from 5.05 to 5.22 ppm) and the β-Gal*p* H-1 signals (from 4.41 to 4.51 ppm) for the three different species in equilibria, integrated to give the percentages shown along with each structure. Not all sugar stereochemistries showed cyclization; notably, the 2-acetamido-2-deoxy-hexose-glycolaldehydes were incapable of cyclization at the 2-position and showed no other variants but the open-chain hydrates.

charides within larger oligosaccharides through multistep disassembly to disaccharide substructures. At this point in time, additional methods other than CID to dissociate larger oligosaccharides would also be desirable so that useful sets of disaccharide substructures, especially overlapping disaccharides, could be isolated. This would enable more effective use to be made of the information that can be garnered from the glycosyl-glycolaldehyde anions.

Experimental Section

Mass Spectrometry. Mass spectrometry was performed to MS^2 or MS^3 using a Thermoquest (Finnigan) classic LCQ instrument of Paul trap design equipped with a Finnigan nanospray source. The LCQ was operated using automatic gain control, with a typical needle voltage of 2.8 kV and a heated capillary temperature of 120 °C for glycoside-glycolaldehydes (200 °C for allyl glycosides) with the sample introduced at 0.4 μL/min. The MS^n experiments were carried out with a relative collision energy that ranged from 27% to 34% and an activation *q* value of 0.25. The activation time was 30 ms. A total of 20−100 scans were accumulated for each spectral acquisition. Samples were introduced in methanol or methanol/water mixtures having up to 5% water or ^{18}O water. High-resolution MS measurements were carried out using the same solvent mixture in the negative ion mode on a Thermoquest 7 T FTICR instrument interfaced to a front end linear ion trap equipped with a nanospray source (Finnigan LTQ-FTICR), located in the Research Resources Center at the University of Illinois at Chicago Medical Center.

^{18}O-Labeling of Reducing Disaccharides. Disaccharides were obtained from Sigma or V-labs. Initially, labeling of the carbonyl oxygen of the reducing sugar of disaccharides was carried out essentially as described,[23,41] a procedure that utilized 0.5 μL of acetyl chloride added to the disaccharide dissolved in 100 μL of $H_2{}^{18}O$ (Isotec). However, a time-course was performed to evaluate labeling in the absence of acetyl chloride. It was found that after 3 days in $H_2{}^{18}O$ alone, labeling occurred essentially completely in the absence of the acetyl chloride. The following method was therefore used: disaccharide (1 mg) was dissolved in 100 μL of $H_2{}^{18}O$ (Isotec), and after 3 days the solution was frozen. This stock could be stored essentially indefinitely and was diluted just prior to use 100−1000-fold in methanol prior to nanospray.

Nuclear Magnetic Resonance Spectroscopy. NMR spectroscopy was carried out at a field strength of 500 MHz (^1H frequency) on a Varian Inova instrument. Acquisitions were performed on synthetic compounds in D_2O at 25 °C with a trace of acetone as an internal chemical shift standard at $\delta = 2.225$ ppm and are reproducible to within 0.002 ppm. $^1H-^1H$ *J*-couplings are accurate to within 0.2 Hz. Assignments were either made through decoupling experiments in 1D experiments or through 2D gCOSY[50] correlations. In some cases, a presaturation pulse was applied to the HOD peak to diminish its intensity.

Synthesis of Allyl Glycopyranosides. Monosaccharides were obtained from Sigma or ICN Biomedicals. Allyl glycosides **21**−**40** (Chart 2) were prepared by basic Fischer-type glycosidation as shown in the first step of Scheme 2. Monosaccharides (100 mg) were dissolved in 10 mL of allyl alcohol by heating to about 90 °C for 5−15 min. The sample was cooled to room temperature, trifluoroacetic acid (Aldrich, 0.385 mL) was added to 1 M, and the mixture was heated in a Teflon-capped tube under argon to 105 °C for 26 h. The sample was concentrated under high vacuum and taken up in 0.5 mL of water followed by 4.5 mL of acetonitrile. Resultant allyl glycosides of all four configurations and ring forms were separated by normal phase HPLC. First, separation was performed in about 10 batches on a column (2.15 cm × 60 cm) of glycopak N (Waters) eluted with 95/5, v/v, acetonitrile (Mallinckrodt, ChromAR grade)/water at 5 mL/min, with detection by UV at 200 nm using a Waters 486 variable wavelength detector. This yielded from 1 to 3 pure allylglycosides, either pyranosides or furanosides. When two isomeric compounds were not separated by the above column, they were further fractionated by normal phase separation on a Shodex DC-613 (6 mm × 150 mm) column either converted to the Cs^+ or Li^+ form,[51] eluted with 95/5, v/v, acetonitrile/water at 0.6 mL/min, detecting at 200 nm. If required, isolation of individual allyl-glycopyranosides on columns other than the glycopak N is presented in the Supporting Information. Some of the allyl glycopyranosides have been previously prepared with accompanying NMR data (**21**−**24**, **37**, **38**,[52,53] **25**,[54] **26**,[55] **32**,[56] **39**, **40**[57]). Our NMR

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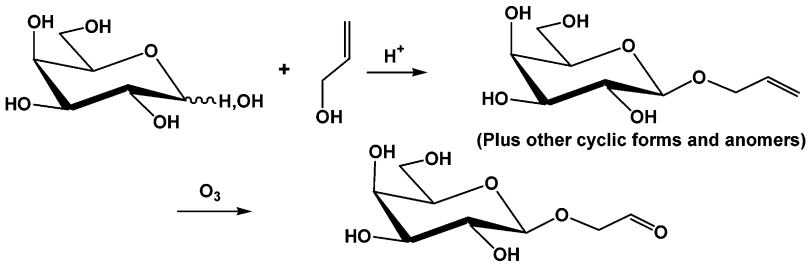
Scheme 2



results were consistent, although in many of these reports NMR spectra were acquired at lower field. At 500 MHz, additional splittings of the allyl group protons due to small couplings of the H-1′a to H-1′b and both H-1′a and H-1′b to H-3′a and H-3′b were observed, and more assignments and endocyclic *J*-couplings could be made. Also, in one case (for **32**[56]) the solvent used was CD_3OD rather than D_2O; thus, chemical shifts were somewhat different, but endocyclic *J*-couplings were essentially the same. Three further points were worthy of note regarding NMR spectra of the allyl-glycopyranosides. First, for allyl-α-D-idopyranoside **35** the predominant conformation was the $^1C_4$ form (it may also exist to an undefined extent in a skew form or interconvert between the two forms), which was not the case for the other allylglycosides (Chart 2). Second, characteristic for all the allyl glycopyranosides was the upfield shift of the H-5 signal of the β-glycopyranosides relative to the H-5 of their respective α-glycopyranosides. Third, many of the allyl furanosides were encountered during HPLC purification steps, and their NMR spectra clearly distinguished them from pyranosides based on the previous assignment of methyl glycofuranoside *J*-couplings by Angyal.[58] In the interest of comparing and consolidating the higher-field data for all the allyl glycopyranosides in this report, NMR spectra and tabulated NMR data for **21**−**40** are presented in the Supporting Information. No previous reports have presented MS/MS spectra of allyl glycopyranosides in the negative ion mode; for **21**−**40** these are presented in the Supporting Information along with quantitative peak data using the Paul trap and high-resolution MS data using the LTQ-FTICR.

Synthesis of Glycopyranosyl-Glycolaldehydes. A few glycopyranosyl-glycolaldehydes have been previously prepared,[41,53,59−61] but for only two structures has NMR data been presented (for **1** and **2**).[41,59] MS data in the negative ion mode has only recently been presented for **1** and **2**.[41] In the other reports,[53,60,61] products were directly converted to Schiff base derivatives without characterization. The glycopyranosyl-glycolaldehydes **1**−**20** (Chart 1) were prepared from the pure allyl glycopyranosides **21**−**40** (Chart 2) by ozonolysis of the allyl double bond as shown in step 2 of Scheme 2. Allyl glycosides (10 mg) were dissolved in 2 mL of D_2O. Ozone, produced from pure O_2 gas using a commercial ozone generator, was bubbled through the sample for 6−8 min. Samples were immediately analyzed by NMR to check for reaction completion, freeze-dried to remove formaldehyde, then stored frozen in aqueous solution. Ozonolysis was a clean, essentially quantitative reaction during this (optimized) reaction period, as judged by the complete disappearance of starting compound. Similar high reaction yields (albeit under different reaction conditions) have been previously reported for preparation of some glycosyl-glycolaldehydes from their respective allyl glycosides.[53] Aliquots were removed for NMR and MS analyses. NMR spectra were often more complex than expected, because the glycosyl-glycolaldehydes were often present in three forms: the open-chain hydrate and two additional six-membered cyclic forms, as indicated for the β-D-galactopyranosyl-glycolaldehyde in Figure 9. Some of the glycosyl-glycolaldehydes were only found in the open-chain hydrate form; the HexNAc-glycolaldehydes, for example. Wherever possible, complete sets of *J*-couplings and assignments for each form are reported in tables in the Supporting Information. MS/MS spectra of the glycopyranosyl-glycolaldehydes in the negative ion mode are presented in Figures 2−6. Quantitative relative MS/MS ion abundances from the Paul trap and high-resolution MS data using the LTQ-FTICR is presented in the Supporting Information. Compounds **1**−**20** will be available through the University of Colorado technology transfer office, 12635 East Montview Blvd., Suite 350, Aurora, Colorado 80045, U.S.A.

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Supporting Information Available: NMR spectra of **1**−**40**, tabulated NMR assignments, *J*-couplings, and chemical shifts for **1**−**40**, mass spectra (negative ion mode) of **21**−**40**, intensity data for mass spectral peaks for **1**−**40**, high-resolution MS data for **1**−**40**, additional HPLC columns, if necessary, and counterions used for normal phase purification of **21**−**40**. This material is available free of charge via the Internet at http://pubs.acs.org.

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