Determining Anomericity of the Glycosidic Bond in Zn(II)-Diethylenetriamine-Disaccharide Complexes Using MS^n in a Quadrupole Ion Trap

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Zinc-diethylenetriamine (Zn-dien) *N*-glycoside complexes of four 1,4 and four 1,6 linked disaccharides are prepared. Each reaction mixture is ionized by electrospray and the resulting species $[Zn(dien)(disaccharide) - H]^+$ is allowed to undergo collision-induced dissociation in a quadrupole ion trap. An MS^3 analysis is used to differentiate α versus β anomericity of the glycosidic bond in the disaccharide moiety. In addition, the MS^2 and MS^3 spectra can be used together to determine the linkage position of this glycosidic bond. (J Am Soc Mass Spectrom 1999, 10, 269–272) © 1999 American Society for Mass Spectrometry

Oligosaccharides displayed on cell surfaces and on the surfaces of proteins are known to play significant roles in a variety of biological processes [1–4]. For example, it is the blood group antigen(s) displayed on the surface of blood cells which determines blood type [4]. Other glycans serve as transport and secretion signals for proteins, "on–off" switches for enzyme activity, and promoters of cell/cell and cell/matrix adhesion. The proper functioning of such glycans depends in large part on the subtle yet specific differences between the monomeric units, the location and type of the linkages between them, and the anomericity of the glycosidic bonds.

It is the latter parameter which often presents the greatest challenge because there are relatively few techniques available for differentiating between α versus β configuration of each linkage. Enzymatic assays [5–7] are the current method of choice for determination of linkage configuration in oligosaccharides. A limitation of the method, however, is that in certain cases glycosides present at the reducing end of an oligosaccharide are, in fact, not cleaved by their respective glycosidase. Such an occurrence can be because of steric hindrance from neighboring sugar chains or to strict linkage position selectivity of the glycosidase. ^{1}H–NMR and ^{13}C–NMR spectra [7–9] have also been used to assign linkage configuration; however, such analyses require reference spectra of similar compounds and larger amounts of sample are required. Finally, oxidation with chromium trioxide [7, 10] has been used to differentiate α versus β linked polysaccharides, but a lengthy procedure is involved which includes acetylation, oxidation, deacetylation, methylation, and GLC of the oligosaccharide. Moreover, this procedure is best applied to oligosaccharides containing entirely α *or* β linkages. There are a greater number of techniques available for the determination of linkage position but these methods, too, can present problems. In fact, there is no one method currently available which is able to provide unambiguous determination of linkage position and configuration in all cases.

A significant body of research has been published on the use of mass spectrometry alone or in conjunction with traditional chemical methods to probe carbohydrate structure [11–30]. Our lab has focused on differentiating stereochemical features of oligosaccharides through metal ion coordination of the saccharide followed by mass spectrometry or tandem mass spectrometry (MS^n) [23–30]. We have previously shown that mass spectrometry has potential for differentiating cobalt complexes of glucosyl-glucose disaccharide complexes using fast atom bombardment (FAB) and kinetic energy release (KER) measurements [26]. The method presented in this paper utilizes electrospray ionization with an ion trap instrument and extends the analysis to glucosyl-galactose and galactosyl-galactose disaccharides. In addition, the method involves an inexpensively prepared derivatizing agent, $Zn[NH_2(CH_2)_2NH(CH_2)_2NH_2]_2Cl_2$, and only a short reaction time is required for the derivatization procedure.

Experimental

Reagents

Glucosyl-β1,4-glucose was purchased from ICN Biomedicals (Aurora, OH). Mannosyl-α1,6-mannose and

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galactosyl-β1,4-galactose were purchased from V-labs (Covington, LA). All other disaccharides were purchased from Sigma (St. Louis, MO). $Zn(dien)_2Cl_2$ (dien = diethylenetriamine) was obtained from Dr. Steven Pedersen, Department of Chemistry, University of California at Berkeley. Reagent grade methanol was purchased from Fischer (Fair Lawn, NJ). NMR spectra were supplied with saccharides purchased from V-labs. All reagents were used as received.

Sample Preparation

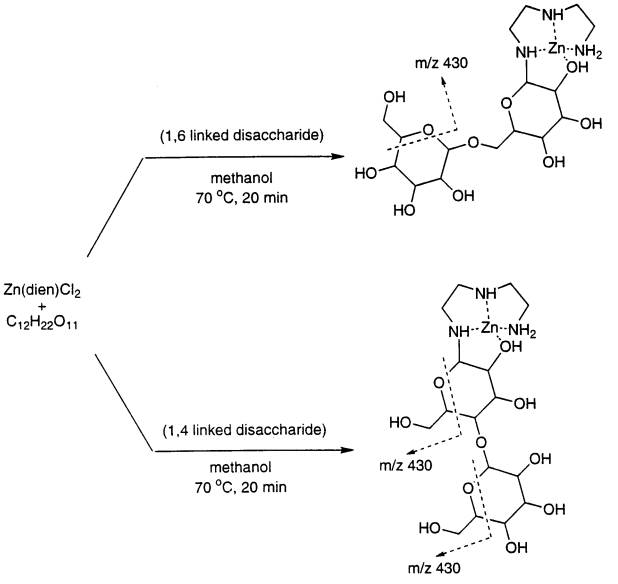
N-glycoside complexes were prepared by allowing 1.0 mg (2.8 μmol) of disaccharide and 0.5 mg (1.4 μmol) of $Zn(dien)_2Cl_2$ to react in 50 μL of methanol at 70 °C for 20 min. Samples were prepared for analysis by diluting 1 μL of the crude reaction mixture to 500 μL in 100% methanol.

Mass Spectrometry

Samples were directly infused at a flow rate of 2 μL/min into a Finnigan LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA), and ions were produced by electrospray ionization. The automatic gain control was set to allow a maximum of 5×10^7 and 3×10^7 ion counts into the trap for MS and MS^n, respectively. CID was performed on the ion of interest by using the He gas present in the trap with the *q* value set at 0.25 and an ion activation time of 30 ms. The amplitude of the supplementary rf applied across the end caps was set at 0.6–0.8 V and 0.5–0.7 V for MS^2 and MS^3, respectively. Each spectrum represents the average of 20–50 scans; each scan is composed of three "microscans."

Results and Discussion

N-glycoside complexes of eight disaccharides were prepared as shown in Scheme **1**. Several species were present in solution as evidenced by the mass spectra obtained for the crude reaction mixtures. However, the ion of interest at *m*/*z* 490, which corresponds to the $[Zn(dien)(disaccharide) - H]^+$ complex, was present with no apparent isobars and in sufficient abundance (typically around 30% relative abundance) for MS^2 analysis. This ion at *m*/*z* 490 was selected and allowed to undergo collision-induced dissociation (CID) as described above. Typical MS^2 spectra are shown in Figure 1 (A, B) for the 1,4 and 1,6 linked disaccharide complexes, respectively. A summary of the product ions observed from MS^2 of *m*/*z* 490 for each of the eight complexes is provided in Table 1. These product ions correspond to cross ring and/or glycosidic cleavages and the relative abundance of each ion is 3% or greater. A criterion of 3% has been established for all data collected on diastereomeric differentiation [29]. This value is based on multiple acquisitions of different metal ligated oligosaccharides and we have determined



Scheme 1. Synthetic route to Zn(dien)(disaccharide) *N*-glycoside complexes and possible cleavage pathways leading to *m*/*z* 430 from *m*/*z* 490 for 1,4 and 1,6 linked disaccharide complexes.

that ions of lower relative abundance are not reproducible, or are included in the noise level background.

As seen in Table 1 there is a product ion at *m*/*z* 460 corresponding to a cross ring cleavage of CH_2O for the 1,6 linked disaccharide complexes examined, whereas there is no such ion for the 1,4 linked disaccharide complexes. Unfortunately, this ion is extremely weak (typically 3%–5% abundant), and in the absence of other diagnostic ions would not be suitable for unambiguous differentiation of stereochemistry for an unknown sample. In addition, there are no differentiating features in the MS^2 spectra pertaining to anomeric configuration. At this point the possibility of using MS^3 as a second check on linkage information obtained by MS^2 as well as an indicator of anomeric configuration was explored.

Dissociation pathways of similar Zn(II) *N*-glycoside complexes of the monosaccharides glucose, galactose, mannose, and talose have been previously investigated using tandem mass spectrometry on a quadrupole ion trap [30]. Specifically, ^{13}C and ^2H isotopically labeled analogs of the glucose complex were used to determine the atoms lost for a particular cross ring cleavage. The results of these labeling studies indicated that cross ring cleavage occurred beginning with C6 and continuing sequentially along the carbon backbone; i.e., the loss of CH_2O includes C6, a loss of $C_2H_4O_2$ includes C5–C6 and so on. We hypothesized that a similar mechanism might be occurring in the disaccharide complexes. Furthermore, we noted that if this were in fact the case, a 1,4 linked disaccharide complex would have two pathways available to lose a $C_2H_4O_2$ neutral to form *m*/*z* 430, whereas a 1,6 linked dissacharide would only have one such pathway as depicted in Scheme **1**. This would lead to very different populations of ion species at *m*/*z* 430 for 1,4 versus 1,6 linked disaccharide complexes.

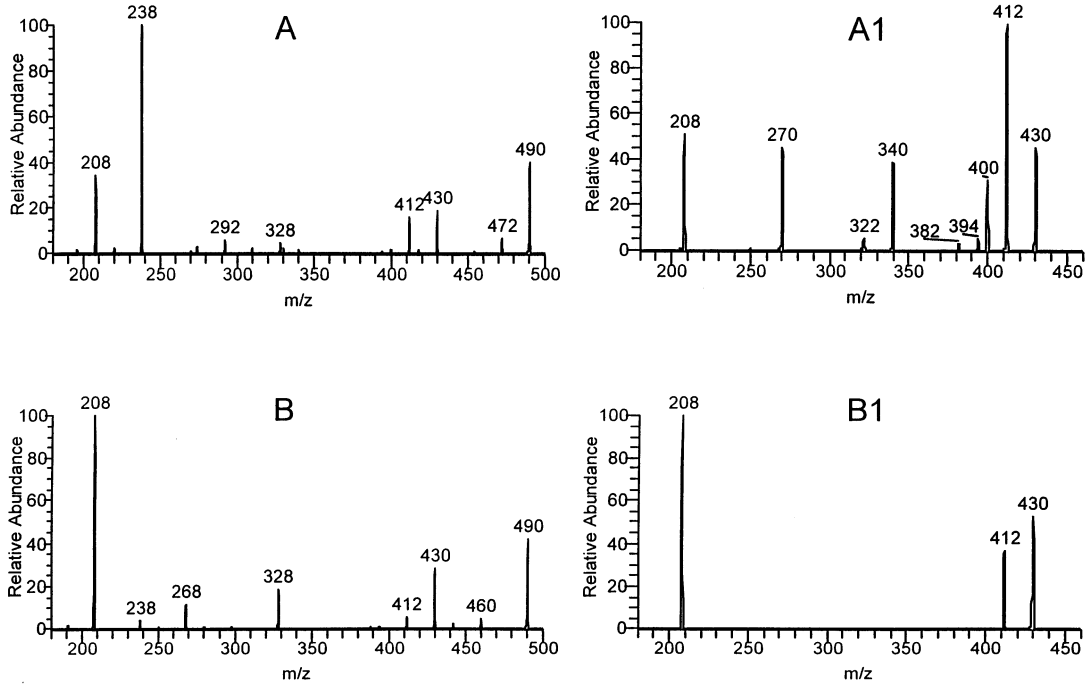


Figure 1. **(A)** MS^2 spectrum of [Zn(dien)(Galβ1,4Gal) − H]$^+$ (m/z 490) representative of 1,4 linked disaccharide complexes. **(A1)** MS^3 spectrum of [Zn(dien)(Galβ1,4Gal) − H]$^+$ (m/z 490 → 430) representative of β linked disaccharide complexes. **(B)** MS^2 spectrum of [Zn(dien)(Glcα1,6Glc) − H]$^+$ (m/z 490) representative of 1,6 linked disaccharide complexes. **(B1)** MS^3 spectrum of [Zn(dien)(Glcα1,6Glc) − H]$^+$ (m/z 490 → 430) representative of α linked disaccharide complexes.

When subsequently selected and allowed to undergo CID these distinct ion populations might dissociate by unique pathways, and differences in the MS^3 spectra would then be observed. As seen in Table 1, each disaccharide complex (m/z 490) does dissociate through a neutral loss of $C_2H_4O_2$ to give product ion(s) at m/z 430. This product ion was therefore selected and allowed to further undergo CID in order to obtain the MS^3 spectra as summarized in Table 2 for each of the eight disaccharide complexes. Typical MS^3 spectra for α versus β linked disaccharides are shown in Figure 1(A1, B1).

As shown in Table 2 there is a product ion at m/z 400 representing a loss of formaldehyde from m/z 430 for all of the complexes possessing a β glycosidic linkage, whereas there is no such product for the corresponding α linked isomers. Thus, differentiation of the α versus β configuration of the glycosidic linkages in these isomeric disaccharides is achieved. It is important to emphasize that differentiation of this particular parameter required an MS^3 analysis, because the MS^2 spectra recorded for complexes of α and β linked isomers were essentially identical. In addition, the MS^3 analysis does provide a second check on the preliminary determination of linkage position by MS^2. As seen in Table 2, MS^3 spectra of 1,4 linked disaccharide complexes possess a product ion at m/z 394 corresponding to two losses of water from the precursor m/z 430. This product ion at

Table 1. Cross-ring and glycosidic cleavage products observed for Zn(dien)(disaccharide) *N*-glycoside complexes, m/z 490

	Cross-ring and glycosidic cleavages present (m/z)					
	$-CH_2O$ (460)	$-C_2H_4O_2$ (430)	−(G)[a] (328)	−(G)/ $C_2H_4O_2$[b] (268)	−(G)/ $C_3H_6O_3$ (238)	−(G)/ $C_4H_8O_4$ (208)
Glcα1,4Glc		X	X			X
Glcβ1,4Glc		X	X			X
Galβ1,4Glc		X	X		X	X
Galβ1,4Gal		X	X		X	X
Glcα1,6Glc	X	X	X	X	X	X
Glcβ1,6Glc	X	X		X		X
Galα1,6Glc	X	X	X	X	X	X
Manα1,6Man	X	X		X	X	X

[a](G) = glycosidic cleavage = loss of $C_6H_{11}O_5$.
[b](G)/$C_2H_4O_2$ = loss of $C_6H_{11}O_5$ and $C_2H_4O_2$.

Table 2. Results from MS^3 of m/z 490 → 430 → for Zn(dien)(disaccharide) *N*-glycoside complexes

	Neutral losses observed					
Disaccharide	H_2O	CH_2O	$2H_2O$	$C_3H_6O_3$	Unk[a]	$C_8O_7H_{14}$
Glcα1,4Glc	412		394			208
Glcα1,6Glc	412					208
Galα1,6Gal	412					208
Manα1,6Man	412					208
Glcβ1,4Glc	412	400	394		270	208
Galβ1,4Glc	412	400	394	340	270	208
Galβ1,4Gal	412	400	394	340	270	208
Glcβ1,6Glc	412	400				208

[a]Unknown composition.

m/z 394 does not occur for the 1,6 linked disaccharide complexes. Thus, these linkage positions are distinguished from one another. Overall, these observations demonstrate the utility of the MS^n capabilities of the ion trap in differentiating various stereochemical features of the saccharides studied.

Although MS^3 investigation of the geneology m/z 490 → m/z 430 → was guided by a certain logic, these experimental observations are currently somewhat empirical. Because the experimental conditions within the ion trap involve long (millisecond) reaction times and multiple collision conditions for each stage of mass spectrometry analysis, rationalizing the MS^3 results is not trivial. As we have hypothesized, it is possible that during MS^2 the product ion at m/z 430 formed from m/z 490 is actually a mixture of isomeric species. The specific isomeric products formed for the α versus the β (and 1,4 versus 1,6) linked *N*-glycoside precursors could, in fact, be structurally quite different because of the time available for product rearrangement before MS^3 analysis. If this were the case, it would not be surprising that in the subsequent MS^3 analysis these products would tend to dissociate through entirely different pathways. However, such complex rearrangements of atoms could only be detected and studied through isotopic labeling studies which is the subject of future experiments.

As shown in Table 1, the disaccharides used for this preliminary study were composed primarily of glucose and galactose monomeric units. We expect future experiments to focus on investigating whether the observations presented in this paper can be extended to oligosaccharide complexes containing mannose or other types of monosaccharide units such as *N*-acetyl hexosamines. In addition, *N*-glycoside complexes of other linkage types will also be examined. These experiments are part of an overall goal to develop a set of tools and methodology for complete oligosaccharide structure elucidation.

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