Oligosaccharide Analysis Using Anion Attachment in Negative Mode Electrospray Mass Spectrometry

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Eleven different anionic species were able to form adducts with neutral oligosaccharides at low cone voltage in negative ion mode electrospray mass spectrometry. Among them, fluoride and acetate have the ability to significantly enhance the absolute abundance of $[M - H]^-$ for neutral oligosaccharides, which otherwise have low tendencies to deprotonate due to the lack of a highly acidic group. Evidence shows that the source of high abundances of $[M - H]^-$ for neutral oligosaccharides arises from the decomposition of $[M + F]^-$ and $[M + Ac]^-$ with neutral losses of HF and HAc, respectively. The chloride adducts have the best stability among all the adduct species investigated, and chloride adducts consistently appeared in higher abundances relative to $[M - H]^-$. In tandem mass spectrometry (ES-MS/MS) experiments, upon collision induced dissociation (CID), F^- and Ac^- adducts gave purely analyte-related product ions, i.e., no detection of the attaching anion and no incorporation of these anions into decomposition products. Cl^- adducts produced both Cl^- and analyte-related product ions. For the above three anions, CID of adduct species may be used for structural determination of neutral oligosaccharides because, in each case, structurally-informative fragment ions were produced. In the presence of F^- and Ac^- , simultaneous detection of acidic and neutral oligosaccharides was achieved, because the problem of the presence of an acidic group that can impede the deprotonation of a neutral oligosaccharide was minimized. The ratio of Cl^- :non- Cl^- -containing product ions obtained in CID spectra of chloride adducts of disaccharides was used to differentiate anomeric configurations of disaccharides. Density functional theory (DFT) was employed to evaluate the optimized structures of chloride adducts of disaccharides, and it was found that chloride anions favor close contact with the hydrogen from the anomeric hydroxyl group. Multiple hydrogen bonding further stabilizes the chloride adduct. (J Am Soc Mass Spectrom 2005, 16, 60–70) © 2004 American Society for Mass Spectrometry

Carbohydrates, also called saccharides, are the most abundant biological molecules and they are much more heterogeneous in structure than other biological molecules. It is well known that saccharides act as a major energy source (e.g., glucose and starch) and as a material that offers structural solidity (e.g., cellulose). Another interesting feature of saccharides is that they appear to play important roles in cell-surface recognition phenomena [1, 2]. An understanding of the saccharide structure is essential for understanding the varied functions of carbohydrates in biological systems.

Mass spectrometry has emerged as an important technique for oligosaccharide analysis [3–17]. Important innovations came through the introduction of electrospray (ES) and matrix-assisted laser desorption/ionization (MALDI). So far, oligosaccharide studies using mass spectrometry have focused mostly on the positive

mode via adduct formation with H^+ or with metal ions, among which the Group I [18–22], Group II [23] and first row transition metal [24–26] elements primarily have been used. It was found that lithium ions may ionize oligosaccharides more efficiently compared with other alkali metal ions [20], and that fragments derived from cross-ring cleavages may provide linkage information [18, 19]. In addition, the stereochemistry of glycosidic bonds has been studied using tandem mass spectrometry of diastereomeric cobalt-glucosyl-glucose disaccharide complexes [26].

Compared with the extensive number of studies conducted in positive mode mass spectrometry, fewer studies have been done to analyze oligosaccharides in the negative mode, with the exception of the sialylated oligosaccharides which contain at least one neuraminic acid moiety that readily deprotonates. Unlike sialylated oligosaccharides, because of the lack of an acidic group, neutral oligosaccharides exhibit a low tendency to form $[M - H]^-$.

It has been reported [27, 28] that the use of nanoscale electrospray (flow rates of the order of tens of nL/min) can improve sensitivity for neutral oligo-

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saccharides relative to conventional microliter/min flow ES. Pfenninger et al. [29, 30] employed negative mode nanospray together with quadrupole ion trap mass spectrometry to analyze underivatized neutral oligosaccharides from human milk and found that their deprotonated molecules follow distinct fragmentation rules. Chai et al. [31] investigated the fragmentation of neutral underivatized oligosaccharides using negative mode ES-MS with collision induced dissociation (CID) on a Q-TOF instrument. Mulroney et al. [32] showed that ES-MS in the negative ion mode can be used to characterize glycosidic bond linkages in underivatized disaccharides. Wheeler and Harvey [16] investigated the negative mode ES-MS fragmentation spectra of 12 sialylated carbohydrates on a Q-TOF mass spectrometer. Often, sialylated oligosaccharides are detected with the best sensitivity as anions, while the asialylated species perform best in the positive ion mode [33].

Biological samples are usually comprised of neutral and acidic oligosaccharides. Most often, they are separated and analyzed independently from one another. Huang and Riggan [34] developed an HPLC/MS method that allows direct analysis of underivatized sialylated (containing acetyl neuraminic acid) and neutral oligosaccharides via online ES mass spectrometry in the positive mode.

The anion attachment approach described in the current paper represents a promising negative ion method for the simultaneous analysis of neutral and acidic oligosaccharides. There have been few studies on the analysis of oligosaccharides in the negative mode via the anion attachment approach. Henion and coworkers [27] observed low abundance acetate adducts alongside larger signals corresponding to multiply deprotonated polysialylated oligosaccharides. Wong et al. [35] reported the use of bisulfate as a dopant for oligosaccharides analysis in MALDI. Both bisulfate adducts, $[M + \text{HSO}_4]^-$, and bisulfate-containing derivatives, $[M + \text{HSO}_4 - \text{H}_2\text{O}]^-$, were observed for neutral oligosaccharides with four or more sugar units. Zhu and Cole [36] investigated the use of chloride anions as additives in ES-MS for the ranking of gas-phase basicities and chloride affinities of monosaccharides, and it was also found that the decomposition of chloride adducts can give glycosidic bond linkage information for disaccharides. Cai et al. [37] found that chloride anions can form stable anionic adducts with neutral oligosaccharides in MALDI-TOF.

The current study is aimed at comparing the use of different anions for anion attachment and oligosaccharide analysis by ES-MS. The ability to distinguish α - and β -linked disaccharides via the anion attachment approach is also explored. Density functional theory calculations were employed to assist us in understanding the optimized anion-disaccharide adduct structures.

Experimental

Chemicals

Maltose, cellobiose, gentiobiose, isomaltose, laminaribiose, trehalose, and raffinose were obtained from Sigma Chemical Co. (St. Louis, MO). 3'-sialyllactose was obtained from Glyko (Novato, CA). Ammonium chloride, ammonium bromide, ammonium fluoride, ammonium bisulfate, ammonium acetate, ammonium trifluoroacetate, ammonium oxalate, ammonium tartrate, ammonium dihydrogen phosphate, sodium nitrate and citric acid were obtained from Aldrich (Milwaukee, WI). All materials were used as received without further purification. Saccharides and salts were prepared separately at 1.0×10^{-3} M in methanol/water = 9:1. The above solutions were mixed in a 1:1 ratio to make a final solution concentration of 5.0×10^{-4} M for both the disaccharides and salts.

Instrumentation

All mass spectrometry experiments were performed on a Quattro II triple quadrupole mass spectrometer (Micromass, Inc., Manchester, UK) equipped with an electrospray source. The ES capillary voltage was maintained at -2.5 kV. Sample solutions were introduced into the ES source at a flow rate of $3.3 \mu\text{L}/\text{min}$ via a syringe pump. Nitrogen gas was used as both nebulizing gas and drying gas to improve ionization efficiency. Argon gas at a pressure of $2.3\text{--}2.5 \times 10^{-4}$ mbar (gauge external to the hexapole collision cell) was used as the collision gas to acquire collision induced dissociation (CID) spectra. Collision energies (in the range of 5–40 eV) are given in the laboratory frame of reference (E_{lab}). In comparison studies of various disaccharides, because all employed disaccharides have the same molecular weights, constant E_{lab} values also mean uniform values in the center-of-mass frame of reference (E_{com}). Mass spectra were acquired and processed using MassLynx software (version 3.0, Micromass).

Density Functional Theory Calculations

Individual disaccharide structures were generated using PC SPARTAN Plus version 2.0 (Wavefunction, Inc., Irvine, CA) and energy minimized using the MMFF94 force field to obtain low energy starting structures. To build the chloride adduct structures, the chloride ion was placed arbitrarily away from the disaccharide, and the preliminary structure was refined using a MMFF94 force field. The input structures were submitted to geometry optimization at the B3LYP/6-31G* level in Gaussian 98, Revision A.3 [38] (Gaussian, Inc., Pittsburgh, PA). For all adduct calculations, the multiplicity was set to 1 and the charge state was set to -1 .

Table 1. Adduct formation and decomposition of cellobiose with inorganic and organic anions^a

Anion	Formula	<i>m/z</i>	Gas phase basicity (kJ/mol) ^f	[M – H] ^{–d}	[M + anion] ^{–d}	Upon Anion ^e	CID ^c Analyte-related ^e
Deprotonated cellobiose	C ₁₂ H ₂₁ O ₁₁ [–]	341	~1372	+	nd	nd	*
Fluoride	F [–]	19	1529 ± 0.84	++	++	nd	*
Chloride	Cl [–]	35/37	1372.8 ± 0.84	+	+++	*	*
Bromide	Br [–]	79/81	1331.8 ± 0.84	+	+++	*	nd
Nitrate ^(b)	NO ₃ [–]	62	1329.7 ± 0.84	+	+++	*	nd
Bisulfate	HSO ₄ [–]	97	1265 ± 10	++	+++	*	nd
Dihydrogen phosphate	H ₂ PO ₄ [–]	97	1351 ± 21	+	+++	*	nd
Acetate	CH ₃ COO [–]	59	1427 ± 8.4	++	++	nd	*
Trifluoro-acetate	CF ₃ COO [–]	113	1328 ± 8.4	+	+++	*	nd
Oxalate	HOOC-COO [–]	89	N/A	++	+++	*	nd
Tartrate	HOOC-CH(OH)-CH(OH)-COO [–]	149	N/A	++	++	*	nd
Citrate	HOOC-CH ₂ -C(OH)(COOH)-CH ₂ -COO [–]	191	N/A	++	++	*	nd

^aAmmonium salts are used as the anion source.^bSodium salt is used as the anion source.^cAll MS/MS CID experiments were performed under the same conditions. Cone voltage was set at 15 V, collision energy at 30 eV (*E*_{LAB}), and the collision gas pressure at 2.4 × 10^{–4} mbar.^dIndicates intensity of [cellobiose – H][–] peak: + indicates absolute intensity < 10⁵, ++ indicates 10⁵ < absolute intensity < 10⁶, +++ indicates 10⁶ < absolute intensity < 10⁷.^e*Stands for appearance, nd for not detected.^fFor the gas phase basicities of anions, see reference [41].

Results and Discussion

Most oligosaccharides are not charged in aqueous solutions near neutral pH, unlike peptides and oligonucleotides, so the ionization efficiency is intrinsically low. In the positive mode, many reports have demonstrated that certain metal cations, especially Group I cations [18–22], can aid the ionization of oligosaccharides via formation of metal adducts. Moreover, the decomposition of such adducts may give richer information about the oligosaccharide structure than that of the protonated molecule. However, the number of reports detailing anion attachment approaches in the negative mode is far fewer than those published on cation attachment in the positive ion mode. In this work, the first investigation was focused on comparing different anions for neutral oligosaccharide analysis.

Anion Selection

Using cellobiose (Glcβ1-4Glc) as a model oligosaccharide, eleven anionic species, i.e., chloride, bromide, fluoride, bisulfate, acetate, trifluoroacetate, oxalate, tartrate, dihydrogen phosphate, nitrate, and citrate, were tested for their ability to form adducts with this disaccharide. Setting the cone voltage at 15 V, all the tested anions could form observable adducts with cellobiose, and the obtained signal intensities are shown in Table 1. The ES-MS/MS studies investigating decompositions of [cellobiose + anion][–] adducts (Figure 1 and Table 1) show that F[–] and Ac[–] adducts gave purely disaccharide-related product ions,

whereas Cl[–] adducts produced both Cl[–] and disaccharide-related product ions. All the other tested anions gave only the respective anions as product ions (e.g., see bisulfate, Figure 1e), hence, they are not useful for detailed disaccharide structure determinations. For these reasons, the studies that follow focus on the behavior of F[–], Ac[–], and Cl[–] as anionic adducts of cellobiose.

Anion Concentration Study

The effect of anion concentration was evaluated next. Figure 2 shows the variations of absolute abundances of [M – H][–] in response to changing anion concentrations from 5 × 10^{–5} to 5 × 10^{–3} M while holding cellobiose concentration constant at 5 × 10^{–4} M with the cone voltage fixed at 20 V. The ratios of anion versus cellobiose concentration were 1:10, 3:10, 1:1, 10:3, and 10:1 for every anion investigated. For OH[–], F[–] and Ac[–] as attaching anions, the absolute abundance of [M – H][–] increased linearly with rising anion concentration up to 5 × 10^{–4} M. Further augmentation of the anion concentration did not result in a clear increase in abundance of [M – H][–] for any of the anions tested; for Cl[–], the absolute abundance of [M – H][–] barely changed over the entire range. Overall, this experiment indicated that an anion concentration of 5 × 10^{–4} M gave the optimal signal intensity for solutions containing cellobiose at this same concentration, so the anion versus disaccharide ratio was set at 1:1 for subsequent experiments.

Figure 1. ES-MS/MS spectra of: (a) deprotonated cellobiose $[M - H]^-$; anionic adducts of cellobiose employing the following precursor ions: (b) $[M + F]^-$; (c) $[M + Ac]^-$; (d) $[M + Cl]^-$; and (e) $[M + HSO_4]^-$. Collision energy in the central hexapole was set at 30 eV (E_{LAB}).

Comparison of Anions and Influence of Cone Voltage

When attaching anions are present, the cone voltage value exhibited a large impact on the appearance of mass spectra. Figure 3a shows the absolute abundances of $[M - H]^-$ as a function of cone voltage. Five different solutions were tested, i.e., pure cellobiose (5×10^{-4} M), and cellobiose mixed with ammonium salts of OH^- ,

CH_3COO^- , F^- , and Cl^- (cellobiose and accompanying salt were each prepared at 5×10^{-4} M in all cases). For the pure cellobiose solution, the best sensitivity for $[M - H]^-$ was obtained using cone voltages from 20 to 40 V. At cone voltages below 20 V, the transmission of all ions from the source to the quadrupole was low, while at cone voltages higher than 40 V, decomposition of $[M - H]^-$ became substantial. Addition of base to sample solutions is often practiced to aid the formation

Figure 2. Absolute abundances of deprotonated cellobiose, $[M - H]^-$, versus anion concentration. Cellobiose was held at a constant final concentration of 5×10^{-4} M, anion concentration was prepared at 5×10^{-5} , 1.5×10^{-4} , 5×10^{-4} , 1.5×10^{-3} or 5×10^{-3} M. The cone voltage was set at 20 V. Each point was the average of three measurements and error bars represent the standard deviation.

of $[M - H]^-$ for analytes which do not readily undergo deprotonation. In this study, the effect of base addition to the solution was tested. It was found that the abundance of $[M - H]^-$ in the presence of OH^- was increased approximately 10- to 20-fold relative to that of the pure cellobiose solution run under the same conditions. The influence of F^- addition was different from that of base addition. In the presence of F^- , using a cone voltage of 10 V, the signal of $[M - H]^-$ was about 10 times higher than that obtained from pure cellobiose solution. Most notably, with a cone voltage setting of 20 V, the abundance of $[M - H]^-$ was the highest achieved over all the experiments performed in this study, i.e., 100 times higher than found with the pure cellobiose solution under the same instrumental conditions. With further increases in the cone voltage, the abundance of $[M - H]^-$ decreased due to the decomposition of $[M - H]^-$. It should be noted that the presence of F^- gave a higher intensity peak for $[M - H]^-$ than the addition of OH^- . The general trend and tendencies observed during Ac^- addition were similar to those observed for F^- addition except that the absolute abundance of $[M - H]^-$ did not reach the same high values at cone voltages of 20 to 30 V. In contrast, the presence of Cl^- cannot increase the $[M - H]^-$ signal intensity; instead, a high intensity $[M + Cl]^-$ peak is observed (Figure 3b). It can thus be concluded that the presence of OH^- , F^- , or Ac^- can each increase the peak intensity of $[M - H]^-$, with F^- addition offering the most dramatic increase, followed by Ac^- , then OH^- . Of course, increases in $[M - H]^-$ abundance will benefit MS/MS studies aimed at structural elucidation of oligosaccharides.

The ability of F^- and Ac^- to increase the abundance of $[M - H]^-$ is proposed to result from the decomposition of the corresponding adducts involving loss of the conjugate acid of the respective anion. For the F^- example, Figure 3b shows that, at a cone voltage of 10 V, $[M + F]^-$ was the predominant peak relative to $[M - H]^-$. After increasing the cone voltage to 20 V, the peak intensity of $[M + F]^-$ dropped off quickly while the peak intensity of $[M - H]^-$ climbed dramatically. Combining the information derived from Figure 3a that, in the absence of F^- , the intensity of $[M - H]^-$ did not increase significantly upon changing the cone voltage from 10 to 20 V, it can be inferred that in the presence of F^- , the high abundance of $[M - H]^-$ is attributable to the decomposition of $[M + F]^-$ via HF

Figure 3. Ion abundance vs. cone voltage of deprotonated molecules and anionic adducts of cellobiose arising from five different solutions, i.e., pure cellobiose solution (5×10^{-4} M), and cellobiose mixed with ammonium salts of OH^- , CH_3COO^- , F^- , and Cl^- (cellobiose and the salt were each prepared at 5×10^{-4} M in all cases). (a) Absolute abundances of deprotonated cellobiose $[M - H]^-$ versus cone voltage; (b) absolute abundances of deprotonated cellobiose $[M - H]^-$ and cellobiose adducts $[M + A]^-$ with various anions (A^- = anion) versus cone voltage. Each point was the average of three measurements and error bars show the standard deviation.

loss. This deduction is supported by CID studies that show a substantial peak corresponding to loss of neutral HF from $[M + F]^-$ (Figure 1b). At a cone voltage of 20 V (Figure 3), the internal energy of $[M + F]^-$ was high enough to cause the adduct to undergo substantial decomposition to form $[M - H]^-$, but low enough to prevent consecutive decomposition of $[M - H]^-$ resulting in the highest abundance of $[M - H]^-$ in the whole study. Under such optimized conditions, employing addition of NH_4F at 5×10^{-5} M, cellobiose displayed a limit of detection (S/N = 3) of 1.3×10^{-8} M [flow rate of 1.7 μ L/min for an average of 1 min acquisition time (22 fmol consumed)].

The stability of anionic adducts varied for the different attaching anions (Figure 3a). Chloride adducts appeared to be the most stable adduct species. Notably, under all the conditions tested, chloride adduct was always the predominant species compared to $[M - H]^-$ in agreement with previous studies [39, 40]. The underlying reason for this phenomenon has been attributed to a close matching of the proton affinity of the deprotonated molecules ($[M - H]^-$) with that of Cl^- .

CID of Anionic Adducts of Disaccharides for Linkage Differentiation

ES-MS/MS product ion spectra of $[M + F]^-$ and $[M + Ac]^-$ of cellobiose gave purely disaccharide-related product ions, while $[M + Cl]^-$ produced both Cl^- and disaccharide-related product ions. The decomposition of fluoride adducts is a straight-forward process, with F^- showing strong attraction for one proton leading to loss of HF, thus leaving $[M - H]^-$ and the possibility for consecutive decompositions. The decomposition of chloride adducts revealed a competition between Cl^- formation and disaccharide-related product ion formation. This contrast can be explained by considering that F^- has a higher gas-phase basicity (Table 1) [41] than $[M - H]^-$ of the disaccharide, so F^- has a higher tendency to attract the proton of the disaccharide. On the other hand, Cl^- has a gas-phase basicity similar to that of $[M - H]^-$ of the disaccharide, so there is a more even competition to capture the proton during decomposition [36, 39].

As mentioned previously, F^- , Ac^- , and Cl^- adducts may undergo decompositions to produce structurally-informative fragment ions. Fragmentation of these three adducts leads to similar CID spectra (Figure 1b–d), with the appearance of the dissociated anion, Cl^- (Figure 1d), and the variable abundance of deprotonated cellobiose (m/z 341) as the main differences. Moreover, the fragmentation patterns were quite similar to that of $[M - H]^-$ (Figure 1a) which indicates that the first step of the decomposition for each adduct was the loss of the conjugate acid of the anion prior to consecutive decomposition of $[M - H]^-$.

To widen the applicability of findings to other disaccharides, F^- was employed to form adducts with a

variety of glucopyranosyl-glucose disaccharides with different linkage positions, and the decomposition of each adduct was studied (Figure 4). The fragmentation patterns of $[M + F]^-$ adducts were quite similar to the fragmentation patterns of $[M - H]^-$ upon in-source decomposition reported by Mulroney et al. [32], which reinforces the notion that, in each case, the first step of decomposition of $[M + F]^-$ is loss of HF. In addition, the CID product ion spectra of $[M + F]^-$ adducts show great similarity with those of $[M + Cl]^-$ except for the appearance of Cl^- in the latter [36]. Thus, the decomposition of $[M + F]^-$ can also be used for linkage position differentiation by inspection of the peaks characteristic of cross-ring cleavages appearing at m/z values higher than m/z 179, and by the intensity ratio of m/z 161 versus m/z 179 (glycosidic bond cleavages at either side of the oxygen). The high signal-to-noise ratios in CID spectra obtained using $[M + F]^-$ as precursor ions was largely due to the high abundances of the precursor adducts as compared with $[M - H]^-$, and to the lack of competition during the first decomposition step (loss of HF). $[M + Cl]^-$ ions appeared as the highest abundance precursor adducts, but decompositions yielded Cl^- in competition with structurally-informative fragments.

Anion Attachment for Simultaneous Analysis of Neutral and Acidic Oligosaccharides

In the oligosaccharide pool from biological sources, it is often the case that neutral and acidic oligosaccharides coexist. Most reports treat the analyses of neutral and acidic oligosaccharides separately. Because F^- and Ac^- addition can increase the abundance of $[M - H]^-$ ions at cone voltages higher than 20 V, these anions are good candidates for use in the simultaneous analysis of neutral and acidic oligosaccharides by negative mode mass spectrometry.

Raffinose (Gal α 1-6Glc β 1-2Fru) was chosen as a model neutral oligosaccharide and 3'-sialyllactose (Neu5Ac α 2-3Gal β 1-4Glc) as a model acidic oligosaccharide; the simultaneous detection of these compounds is demonstrated in Figure 5. As shown in a previous section, a cone voltage between 20 and 40 V allowed the detection of $[M - H]^-$ for oligosaccharides, so for this study, the cone voltage was set at 40 V. With the two oligosaccharides present in equal concentrations (5×10^{-4} M), Figure 5a shows that raffinose (mol. wt. = 503) was barely detectable compared with 3'-sialyllactose (mol. wt. = 632) methanol:water (9:1). Figure 5b shows that the signal intensity of raffinose was enhanced to 1/5 of the intensity of 3'-sialyllactose with the addition of base (5×10^{-4} M NaOH). Figure 5c and d show that the response of deprotonated raffinose was about 2/3 that of 3'-sialyllactose upon addition of F^- or Ac^- (5×10^{-4} M). F^- or Ac^- addition was thus superior to OH^- in that the response was better, and the caustic nature of OH^- addition may affect analyte stability in solution.

Figure 4. ES-MS/MS spectra of fluoride adducts of various glucopyranosyl-glucose disaccharides having different glycosidic bond linkages: (a) trehalose (Glc α 1-1Glc); (b) laminaribiose (Glc β 1-3Glc); (c) cellobiose (Glc β 1-4Glc); and (d) gentiobiose (Glc β 1-6Glc). The collision energy was fixed at 30 eV (E_{LAB}).

Chloride Adducts for Differentiating Anomeric Configuration of Disaccharides

A study was undertaken to investigate whether ES-MS/MS of chloride adducts could be used as a means to differentiate stereoisomeric disaccharides. Two pairs of disaccharides were obtained: gentiobiose (Glc β 1-6Glc) and isomaltose (Glc α 1-6Glc), plus maltose (Glc α 1-4Glc) and cellobiose (Glc β 1-4Glc). These isomeric pairs differ only in the anomeric configuration at the glycosidic bond, and the anomers constituting each pair are not readily distinguishable based solely upon relative abundances of oligosaccharide-related product ions in ES-MS/MS spectra. Upon low-energy CID, there are two lowest energy possibilities for chloride adducts to undergo decompositions. One pathway is to simply form chloride (Pathway 1), while the other pathway is loss of HCl to leave $[M - H]^-$ (Pathway 2) which may undergo

consecutive decomposition. For each disaccharide pair, Figure 6a and b show that the ratio of Cl^- /non-Cl product ions is heavily dependent on the CID conditions. In both figures, as the collision energy was increased, the ratio of Cl^- /non-Cl product ions rose. At low collision energy (E_{lab}), the ratio of Cl^- /non-Cl product ions was below 1, which indicated that Pathway 1 was less favorable than Pathway 2. At high collision energy, the ratio of Cl^- /non-Cl product ions was above 1, which indicated that Pathway 1 had become more favorable than Pathway 2. Considering the numerous available electropositive hydrogens (e.g., each hydroxyl hydrogen on the disaccharides), it is reasonable to assume that a distribution of structures of chloride adducts exists, with Cl^- attached to various hydroxyl hydrogens, including conformations exhibiting multiple hydrogen bonding [40]. However, in order

Figure 5. ES-MS spectra of a solution containing raffinose ($\text{Gal}\alpha 1\text{-6Glc}\alpha 1\text{-2Fru}$) and 3'-sialyllactose, each at 5×10^{-4} M: (a) in methanol:water (9:1); (b) with 5×10^{-4} M OH^- added; (c) with 5×10^{-4} M F^- added as ammonium salt; and (d) with 5×10^{-4} M Ac^- added as ammonium salt.

to decompose via loss of neutral HCl in preference to departure of Cl^- , chloride may be required to be located near to the most acidic proton (the hydroxyl hydrogen at the anomeric reducing end). At low collision energy, it is conceivable that energy was consumed in reorienting the Cl^- closer to the most acidic hydrogen via movement along the potential energy surface of the ion-dipole complex. This mechanism can account for the tendency to lose neutral HCl at low collision energy. At high collision energy, however, it is rationalized that the excess energy present tended to favor Cl^- departure before it could reach the most acidic proton, leading to a higher rate of Cl^- loss vs. HCl loss at high collision energy.

For the 1-6 linkage disaccharides tested, the $\text{Cl}^-/\text{non-Cl}$ ratio was higher for the α isomer (isomaltose) than for the corresponding β isomer (gentiobiose) at every collision energy (Figure 6a). Conversely, for the 1-4 linkage disaccharides tested, the $\text{Cl}^-/\text{non-Cl}$ ratio was always higher for the β isomer (cellobiose) than for the corresponding α isomer (maltose, Figure 6b). The

differentiation of anomeric configurations is thus possible based upon the different tendency to form Cl^- versus $[\text{M} - \text{H}]^-$ and consecutive decompositions.

Density Functional Theory Calculations

Computer modeling is a valuable approach to visualize the interaction between a neutral analyte and the attaching anion. Efforts have been made to study halide adducts of olefins [42], chloride adducts of acetylene [43], and halide adducts of α -D-glucose [40]. The aim in the present study was to gain insight into the probable attaching position of chloride anion onto cellobiose and maltose disaccharides. Carbohydrates have both extensive hydrogen bonding possibilities and anomeric effects, so modeling results are sensitive to the level of calculation. B3LYP/6-31G* was chosen because it has been used previously to study the minimum energy conformation of monosaccharides [44–49], and it is a higher level calculation compared to the HF/6-31G* level. The HF/6-31G* level of theory was employed

Figure 6. The relative ratio of $\text{Cl}^-/\text{non-Cl}$ product ions versus collision energy (laboratory frame of reference) for anomeric pairs: (a) gentiobiose ($\text{Glc}\beta 1\text{-6Glc}$) and isomaltose ($\text{Glc}\alpha 1\text{-6Glc}$); (b) maltose ($\text{Glc}\alpha 1\text{-4Glc}$) and cellobiose ($\text{Glc}\beta 1\text{-4Glc}$).

elsewhere to calculate relaxed potential energy surfaces for 12 analogs of disaccharides [50]. In the latter study, analogs were made by replacing glucose with tetrahydropyran; thus, hydrogen bonding effects were ignored. It was found that the torsional energy and the simple bulk of ring structures were major factors in determining favored disaccharide conformations.

In this study, the structures of chloride adducts with cellobiose and maltose were optimized at the B3LYP/6-31G* level using Gaussian 98, Revision A.3 [38]. The initial adduct structures were built using PC SPARTAN Plus version 2.0 on a template of cellobiose or maltose optimized by a MMFF94 conformer search. The chloride anion was placed away from the disaccharide and another MMFF94 conformer search was performed. The conformer search gave several geometries depending upon the initial placement of the anion. The adduct structures of different geometries were further optimized by B3LYP/6-31G* calculations with the parameters set at the same criteria. Four pathways were checked for force and displacement convergence. Only certain input structures can give four-pathway convergence (maximum energy, RMS energy, maximum dis-

placement, and RMS displacement), which marks a successful optimization procedure. Figure 7 shows the optimized structures for chloride adducts of cellobiose and maltose. Computer modeling suggests that Cl^- exhibits different favored binding positions for the two anomeric configurations. But, each time, the binding involved the hydrogen of the anomeric hydroxyl group in the reducing ring. This may be rationalized by considering that the electronegative chloride interacts with the most acidic hydrogen in the disaccharide molecule. Indirect evidence for this assertion was also given by the observation that the chloride adduct had a similar product ion spectrum as compared to $[\text{M} - \text{H}]^-$, hence the first step in chloride adduct decomposition was $[\text{M} - \text{H}]^-$ formation via loss of HCl ; the least energy would be consumed in the latter loss when Cl^- was bound to the most acidic proton of the disaccharide. In addition, the optimized structures indicated that multiple hydrogen bonding was contributing to the high stability of the chloride adducts.

Conclusions

Fluoride and acetate anions were found to have the ability to significantly increase $[\text{M} - \text{H}]^-$ formation for neutral oligosaccharides, even in the presence of acidic oligosaccharides which may severely suppress the formation of $[\text{M} - \text{H}]^-$ from neutral oligosaccharides in ES mass spectrometry. Addition of these anions may find application in the analysis of oligosaccharide mixtures present in biological samples that contain both neutral

Figure 7. Computer modeling structure of the chloride adduct optimized at B3LYP/6-31G* level using Gaussian 98, Revision A.3. (a) chloride adduct with cellobiose ($\text{Glc}\beta 1\text{-4Glc}$); (b) chloride adduct with ($\text{Glc}\alpha 1\text{-4Glc}$) maltose. In each case, the reducing end is on the right.

and acidic oligosaccharides. Chloride adducts, however, gave even higher signals than $[M - H]^-$. The ES-MS/MS product ion spectra of fluoride, acetate, and chloride adducts with oligosaccharides showed great similarity with the ES-MS/MS spectrum of $[M - H]^-$ of the same disaccharide, so the decomposition of these anionic adducts of disaccharides was shown to be initialized by loss of the conjugate acid of the anion to form $[M - H]^-$. The chloride adducts also tended to decompose by forming Cl^- as product ions. The B3LYP/6-31G* level optimized chloride adduct structure showed that the proton involved in HA loss most probably comes from the anomeric hydroxyl group in the reducing ring. In addition, the abundance ratio of Cl^- /non- Cl^- product ions from disaccharide adduct decompositions was shown to be useful for differentiating the anomeric configuration of glycosidic bond linkages.

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