Applications of Tandem Mass Spectrometry in the Structure Determination of Permethylated Sialic Acid-containing Oligosaccharides

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Received February 15, 2005

Sets of sialic acid-containing trisaccharides having different internal and terminal linkages have been synthesized to develop a sensitive method for analysis of the reducing terminal linkage positions. The trisaccharides, sialyl(α2-3)Gal(β1-3)GalNAc and sialyl(α2-3)Gal(β1-X)GlcNAc where X=3, 4 and 6, were synthesized and examined using electrospray ionization (ESI)-collision induced dissociation (CID) tandem mass spectrometry (MS/MS). The compounds chosen for this study are related to terminal groups likely to be found on polylactosamine-like glycoproteins and glycolipids which occur on the surface of mammalian cells. The purpose of this study is to develop tandem mass spectrometral methods to determine detailed carbohydrate structures on permethylated or partially methylated oligosaccharides for future applications on biologically active glycoconjugates and to exploit a faster method of synthesizing a series of structural isomeric oligosaccharides to be used for further mass spectrometry and instrumental analysis.

Key Words : Sialic acid, ESI tandem mass spectrometry, Molecular modeling

Introduction

Cell surface sialic acid residues play important roles in a variety of biological processes. Sialic acids are usually found in terminal positions linked through an α-glycosidic linkage.[1,2] Given their terminal location, it is easy to understand why sialic acids have been implicated in many cell-cell signaling and cell-cell adhesion events. Enzymatic synthesis using sialyltransferase is an alternative and efficient method of preparing sialic acid-containing oligosaccharides. The syntheses of oligosaccharides which are modified at a specific position are gaining interest. The availability of such molecules can provide further insights into their biological functions and might lead to the discovery of novel carbohydrate-based therapeutics.[3,4] Biosynthetically sialyltransferases act on very large and complex glycoproteins and glycolipids, therefore small molecule acceptors, such as linkage isomeric oligosaccharides might be viewed as non-natural. In this study, however, we considered a change of linkage position between sugar units as creating several non-natural acceptors.

Until now, the separation and determination of the biomolecules individual characteristics had to be developed using a variety of instrument analyses. Two of the most important chemical techniques used today for the analysis of biomolecules such as proteins are mass spectrometry (MS) and nuclear magnetic resonance (NMR), the subjects of a 2002 Nobel Prize award. A soft ionization method, such as ESI of mass spectrometry, allows for the analysis of several classes of intact biomolecules including proteins, oligonucleotides and oligosaccharides. Proteins and oligonucleotides are easily protonated because of their basicity, whereas oligosaccharides commonly require chemical modification to increase ionization efficiency. Specifically, acidic oligosaccharides are generally more difficult substances to study using mass spectrometry because of polar and acidic characteristics. Proper methods to derive the substances are required to increase the hydrophobicity and bulkiness around glycosidic bonds and enhance ESI response. In this study, permethylation was introduced to improve sensitivity and to direct the fragmentation along pathways that maximize spectral information content. Collision-induced dissociation (CID) is a process where a small portion of the kinetic energy from the mass-selected incident ion is transformed into vibronic energy upon collision with helium atoms to promote unimolecular decomposition. Differences in the ratio of glycosidic bond cleavage may occur due to ionic considerations and steric hindrance of the absorbance of collision energy, leading to a higher statistical bond cleavage rate for sterically crowded linkages.

Using sialyl transferase (E.C. 2.4.99.1) synthesis, the product trisaccharides obtained were sialyl(α2-3)Gal(β1-3)GalNAc and sialyl(α2-3)Gal(β1-X)GlcNAc where X = 3, 4 and 6. All of the above trisaccharides had the same molecular weight, the only difference being the epimer configuration of the reducing end sugar or linkage position between the second galactose and the third aminosugar-containing hexose. Aminosugar-containing oligosaccharides gave specific fragmentation patterns due partly to charge retention[5,6] and steric hindrance to vibronic degree of motion around glycosidic bonds.[7-9] Extensions of these studies should include an investigation of derived acidic and amino sugar-containing oligosaccharides via ESI CID MS/MS to better understand the carbohydrate fragmentation mechanism resulting from different linkage positions. Our previous reports using FAB (Fast Atom Bombardment) CID MS/MS[7-11] suggested that the rationale for most of the fragmentation (daughter ion) assignment was due to steric factors. To rationalize the results in the ESI CID MS/MS studies, SYBYL and MM4 (2003) molecular modeling

programs were also used to calculate minimum energy structures of the permethylated SGA3 and SGLX series. Earlier, the MM4 calculation was proven to provide results consistent with the conformational properties of oligosaccharides in solution as reflected from their NMR data.[12,13]

Experimental Section

Materials: Gal(β1-3)GalNAc, Gal(β1-3)GlcNAc, Gal(β1-4)GlcNAc, Gal(β1-6)GlcNAc, CMP-sialic acid and sialyl(α2-3)transferase [EC 2.4.99.1] were purchased from Sigma Chemical Co. All other chemicals were reagent grade quality.

Synthesis of trisaccharides: The reaction mixture contained 20 mM disaccharide (Gal(β1-3)GlcNAc, Gal(β1-3)GalNAc, Gal(β1-4)GalNAc and Gal(β1-6)GalNAc) as substrate, 0.50 mM CMP-sialic acid and 0.2 mg/mL α-lactoalbumin with 1 unit of EC 2.4.99.1, 50 mM sodium cacodylate in a total volume of 500 μL. Assay mixtures were prepared in ice and the reaction started by the addition of disaccharide. After incubation (3 hrs, at 37 °C), the reaction was stopped by cooling to 0 °C.

Purification: The incubated sample was applied to a 1 × 100 cm Bio-Gel P2 (100-200 mesh) and eluted with water containing 10% acetic acid. Fractions of 1 mL were collected and the saccharide content in 6 μL aliquots was determined by the phenol-sulfuric acid method.

Permethylation of synthetic trisaccharides: The permethylation technique best suited for this approach was essentially that of Ciucanu and Kerek[14] as modified by Gunnarsson.[15] The method was rapid and gave high yields (98+/−2%) without the formation of the non-sugar products. The trisaccharide samples (1 mg) was dissolved in methylsulphoxide (0.1 mL). Then, finely powdered NaOH (4 mg) and methyl iodide (0.25 mL) were added to the sample solution. Each mixture was stirred (100 r.p.m.) for 6 minutes in a closed vial at 25 °C, respectively. Water (0.5 mL) and chloroform (0.5 mL) were then added and the chloroform layer was washed with water (3 × 5 mL) and dried under nitrogen gas.

Neutralization step: Water (1 mL) containing 1-5% acetic acid (enough to neutralize the NaOH) was added to the reaction mixture. The neutralization of the NaOH was important to avoid methyl ester hydrolysis in the sialic acid residues of oligosaccharides

Instrumentation: All experiments were performed on the ThermoFinnigan LCQ ion trap mass spectrometer (ThermoFinnigan Co., San Jose, CA, USA) using electrospray ionization. Ultra high purity helium was introduced as the buffer and collision gas and dry NF grade nitrogen was used for sample nebulization. Samples were infused by syringe into the source at 5 μL/min. The electrospray source was operated at a voltage of 4.5-5 kV and the capillary heater was set to 200 °C. For the generation of most MS/MS and MS/MS/MS spectra, collision energies were set to −30 ~ −50 eV of maximum at −5 eV increments, though values much lower (20-25%) were sometimes used for optimal transmission of certain ions into the next stage of analysis. The maximum ion collection time was set at 2000 ms, and 2-3 microscans were performed per each individual spectrum. Between 25 and 100 spectra were summed to yield the MS/MS and MS/MS/MS spectra reported here. All experiments were performed in the positive ion mode.

Molecular modeling: Molecular modelings were performed using SYBYL (Tripos Associates Inc., 2003) and specially modified MM4 (University of Georgia, 2003) with force field data and software for carbohydrate molecules. Methoxyl groups were used to replace hydroxyl groups using the graphic portion of SYBYL software. The MM4 program could alter the ring geometry to a low energy form for a particular saccharide shape. This function gave advantages to carbohydrate study using MM4 program. The MM4 (2003) version, automatically provided the anomeric effects that were important for sugars. Also, the lone pairs were treated as if they are atoms because MM4 required lone pairs of electrons on an ether and hydroxyl oxygen atoms to fit the data on alcohols and ethers which were major components of carbohydrate.

Results and Discussion

The ESI CID MS/MS spectra of methylated isomeric trisaccharides, SGA3 (sialyl(α2-3)Gal(β1-3)GalNAc), SGL3 (sialyl(α2-3)Gal(β1-3)GlcNAc), SGL4 (sialyl(α2-3)Gal(β1-4)GlcNAc) and SGL6 (sialyl(α2-3)Gal(β1-6)GlcNAc) are shown in Figure 1 at −35 eV collision energy level. The spectra showed the same $[M+Na]^+$ ion at m/z 879 as expected, as well as common fragment ions at m/z 504 and m/z 398 except SGL4. At −50 eV collision energy level, SGL4, did not show m/z 398 ion. The samples with structures sialyl(α2-3)Gal(β1-3)GalNAc (SGA3) and sialyl(α2-3)Gal(β1-X)GlcNAc (SGLX) where X=3 and 4 have been found in nature except for the X=6 compound. The major fragment ion at m/z 504 [Y_2 ion + Na^+] was formed by the loss of a methylated sialic acid with cleavage of the glycosidic bond between sialic acid and galactose according to the a-type pathway,[16] which was characterized by a hydrogen transfer from the sialic acid to the methylated galactose, generating an oxonium ion on the methylated sialic acid. The m/z 504 ion contained the linkage in question. From the m/z 504 peak, there was a loss of the methylated internal sugar with cleavage of the glycosidic bond between the non-reducing monosaccharide with charge retention and the internal galactose gave m/z 398 [B_1+Na^+].

The m/z 620 [C_2 ion + Na^+] of SGA3 and m/z 603 [B_2 ion + Na^+] of SGL3 were the molecular weights of sodium ion adducted methylated disaccharides. Those peaks did not appear in methylated SGL4 or SGA6 (Figure 1). Compared with the spectra of the four methylated saccharides, SGA3 exhibited a relatively intense peak at m/z 620 at each collision energy level. The SGL3 exhibited a relatively intense peak at m/z 603 which was diagnostics for the remnant reducing aminosugar moiety from the nonreducing terminal loss of 1-3 linked methylated sugars. Egge *et al.*[7]

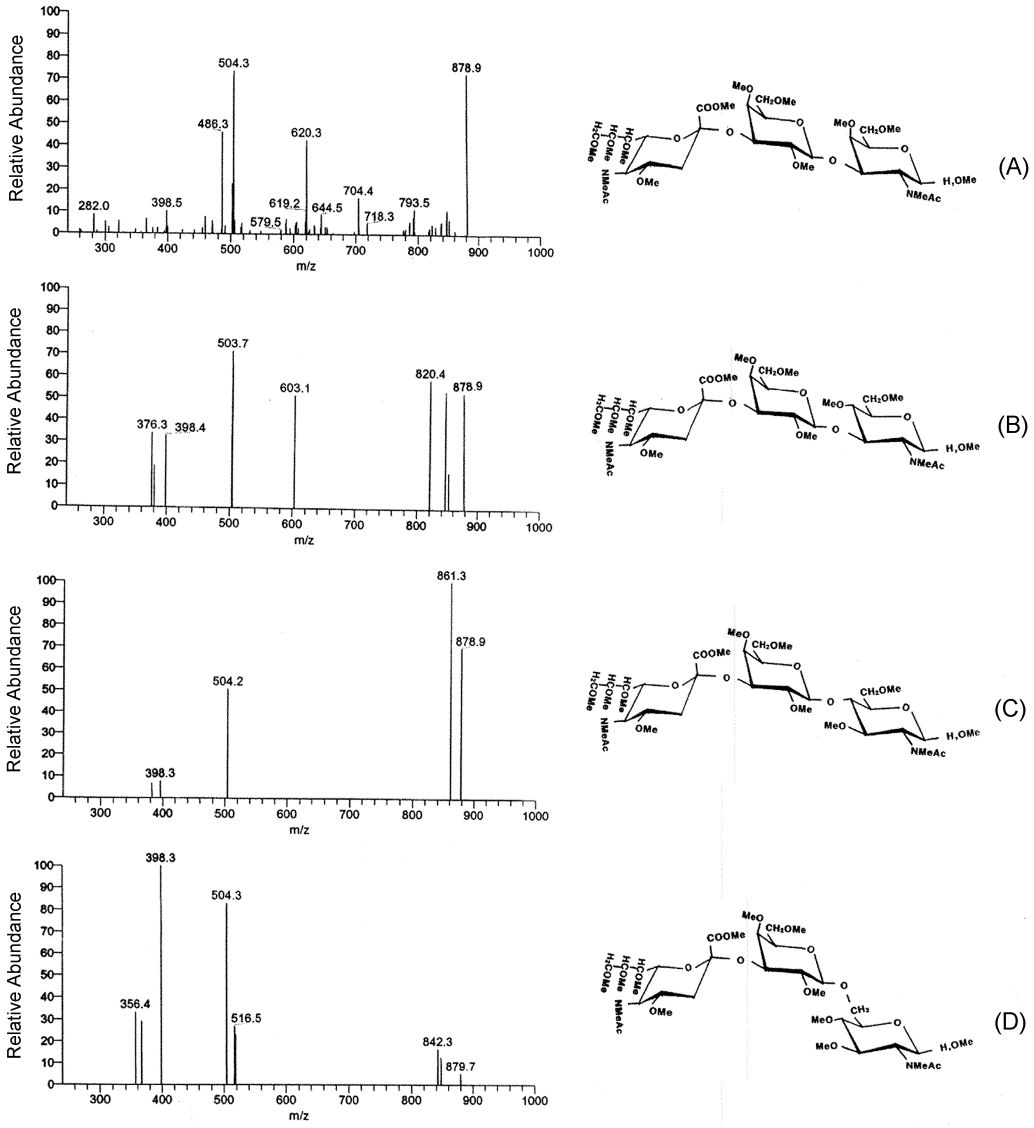


Figure 1. ESI CID tandem spectra of m/z 879 for permethylated sialyl(α2-3)Gal(β1-3)GalNAc(A) and sialyl(α2-3)Gal(β1-X)GlcNAc where X=3(B), 4(C) and 6(D) at –35 eV.

proposed a similar observation for a 1-3 specific ion. The ratios m/z 504/879 were switched when comparing methylated SGL4 and methylated SGL6 at several levels of collision offsets. The m/z 861 ion (loss of water from molecular ion) and m/z 842 peak (loss of CH_3OH from molecular ion) were fingerprints for 1-4 and 1-6 linkages in the same class of molecules, respectively. Further loss of the methanol moiety from sialic acid yielded an unsaturated, partially methylated sialic acid ion at m/z 356 of SGA6. In Figure 1, the relative intensity of the molecular ions with respect to the daughter ions in the acetic aminosugar-containg trisaccharide series at –35 eV collision offset and 0.8 mTorr argon gas were as follows:

methylated SGA3 (80%) > methylated SGL4 (70%) > methylated SGL3 (60%) > methylated SGL6 (10%).

A particularly interesting relationship was found between the collision offset energy from MS/MS analysis and the linkage position in permethylated SGA3 and SGLX. The

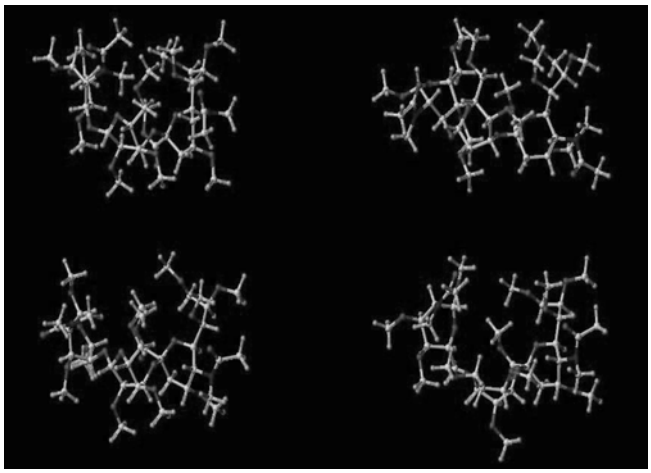


Figure 2. Optimized molecular modeling structures of SGL6, SGL3, SGL4 and SGA3 (clockwise). White circles with four connected atoms denote carbon atoms, red circles with two short bonds indicate oxygen atoms with lone pairs electrons, blue circles are nitrogens, and light blue circles are hydrogen atoms.

survival ratio of a molecular ion at each collision energy from −30 to −50 eV at −5 eV increments gave a depiction of the relative stability among the three linkage positions (β1-3, 4 and 6). Parent ion survival ratios of permethylated isomeric trisaccharides in each collision energy were a strong indicator of linkage position in tandem mass spectra in which the order of bond stability was β1-3 > β1-4 > β1-6. We expected that methylated SGL3, β1-3 linkage containing compound, would be cleaved easier than β1-4 linkage containing compound (methylated SGL4) or C-4 epimer form (methylated SGA3) because of the nearby methoxyl group on C4 position and the bulky CH_2OCH_3 group on C5 of the ring. Unexpectedly, the β1-6 linkage showed the highest rate of cleavage in the sialic acid-containing trisaccharides among three linkages. To rationalize the results we observed in the ESI CID MS/MS studies, SYBYL and MM4 molecular modeling programs were used to calculate minimum energy structures of the permethylated SGA3 and SGLX (Figure 2). This was performed on the basis of thermodynamic considerations that the most stable equilibrium state of a system was the one with the lowest free energy. The above relative intensity results correlated the freedom of motions around glycosidic bonds of molecular modeling study for these isomeric saccharides (Figure 2). In the linkage-isomeric oligosaccharides, the compounds containing the β1-6 linkage (SGL6) were always more labile because of the propensity for charge retention on the nearby amino group on GalNAc or GlcNAc. The exceptional glycosidic cleavage could probably be related to bond energies. In ESI CID MS/MS, vibronic

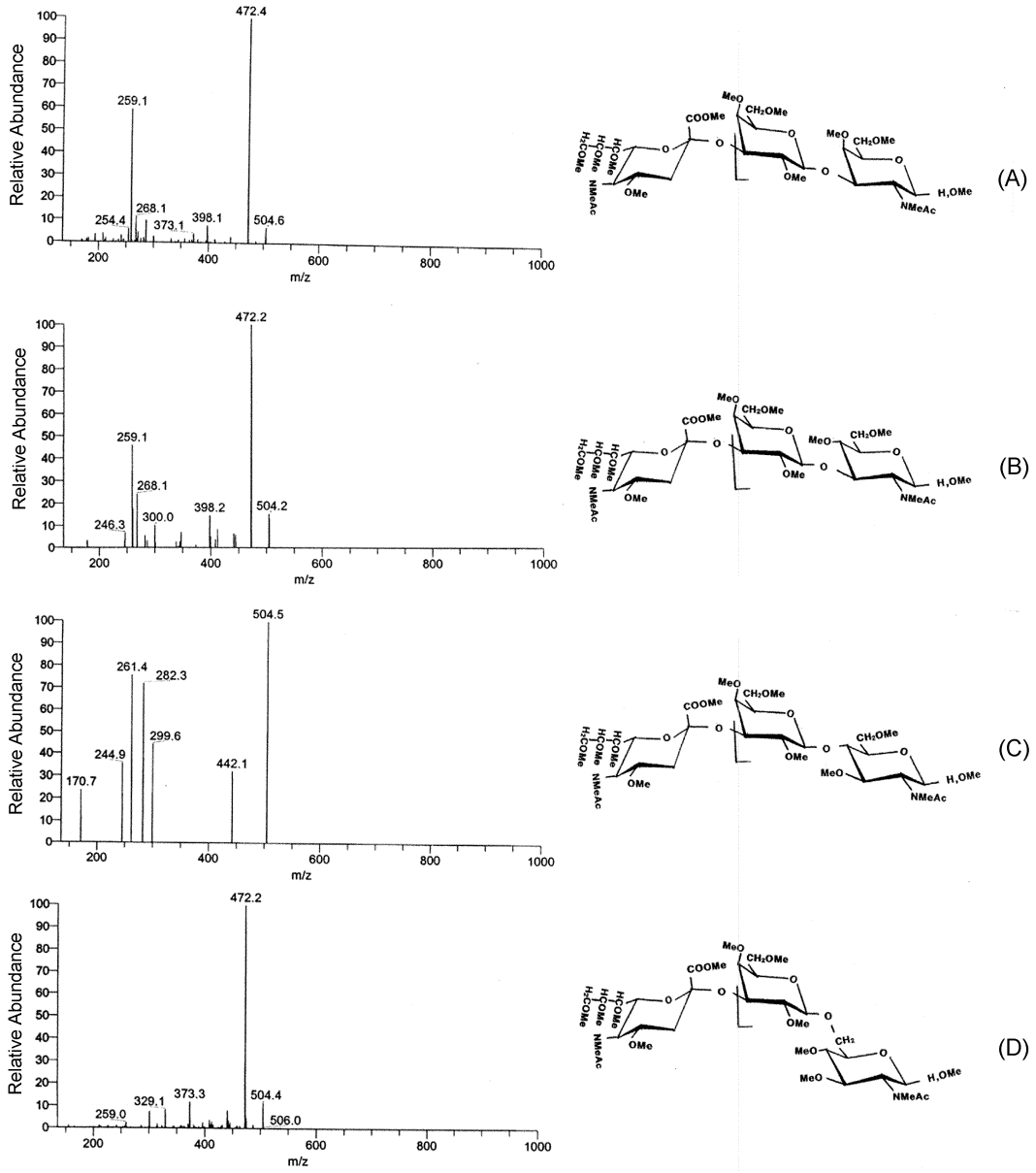
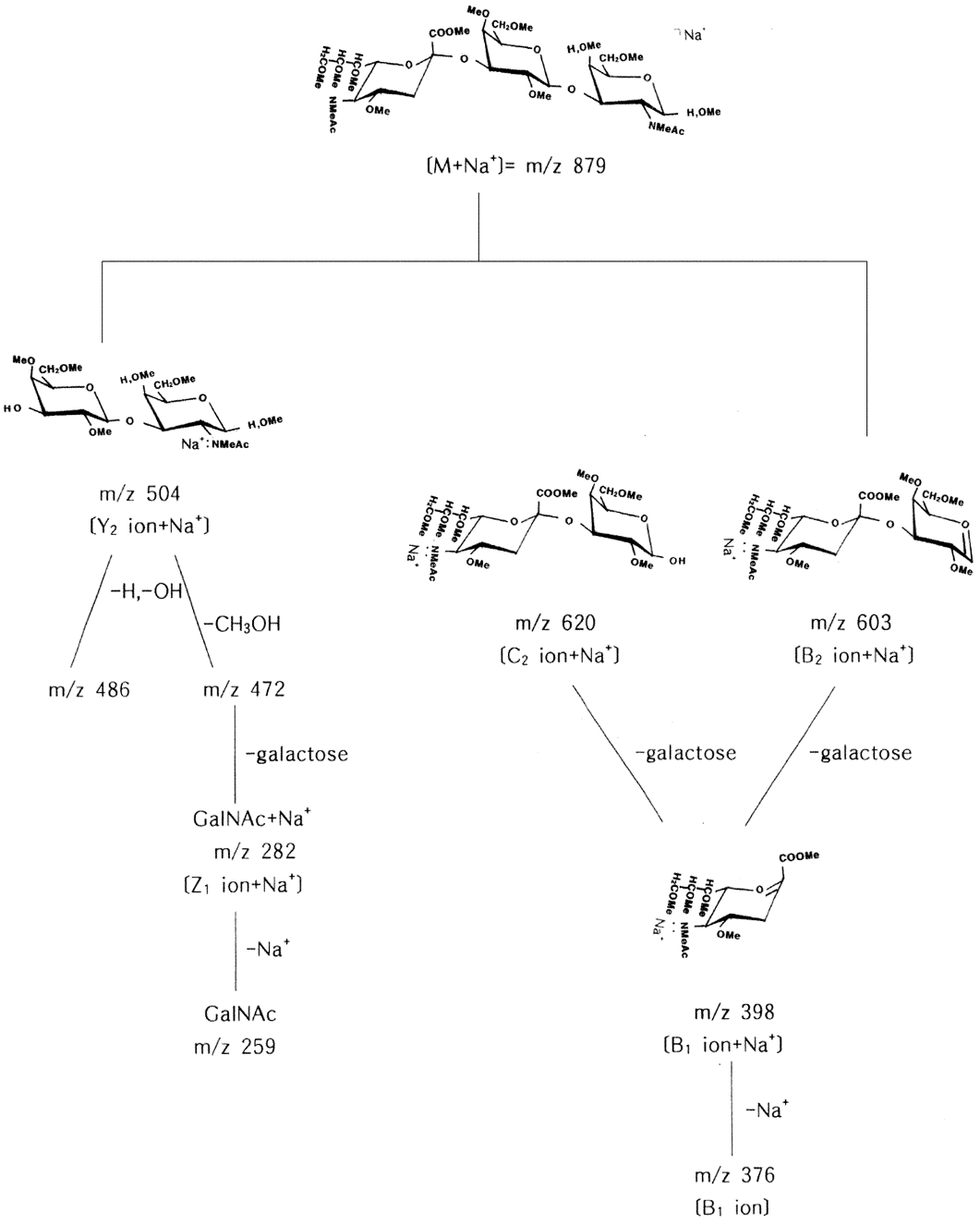


Figure 3. ESI CID MS/MS/MS of m/z 504 for permethylated sialyl(α2-3)Gal(β1-3)GalNAc(A) and sialyl(α2-3)Gal(β1-X)GlcNAc where X=3(B), 4(C) and 6(D) at −40 eV.

energy absorbed from collision kinetic energy imparted to molecules and distributed among several modes included normal, bending, and rotation. Also, hydroxyl group blocking by permethylation could stabilize the ring and restrict the rotation of sugar rings. Vibronic normal and bend modes were strongly connected to rotation, thereby providing spectra with strong dependence on freedom of motion at the glycosidic bonds. Structures with weaker charge centers and more freedom of motion such as methylated SGA3 would more readily dissipate energy absorbed from collision events due to lowered probability of populating the reaction coordinate for glycosidic bond cleavage. The methylated SGA3 and SGLX which had polar sialic acids had strong intramolecular hydrogen bonds between the N-acetyl-CH_3 proton in GalNAc or GlcNAc and the carboxyl group in sialic acid and were stabilized energetically (Figure 2). They did not have linear structures but had bended structures. Methylated acidic and amino-sugar-containing trisaccharides were good examples for suspecting an ionic effect to the cleavage events due to charges on the carboxyl group of sialyl moiety and on the N-acetyl groups of amino sugar and sialic acid. Acidic trisaccharides (SGA3, SGL3, SGL4, and SGL6), free from a dominant charge center, could provide two or three charge



Scheme 1. Possible fragment pathway of permethylated sialyl(α2-3)Gal(β1-3)GalNAc and sialyl(α2-3)Gal(β1-X)GlcNAc where X=3, 4 and 6.

centers and credence for the effect of ionic effects and steric hindrance to saccharide cleavage events.

The MS/MS/MS spectra of specific fragmentions helped to suggest a simple fragment pathway as shown in Scheme 1. The MS/MS/MS spectra (Figure 3) of m/z 504 ions at −40 eV were also much more stable in the series SGL4 > SGL3 > SGA3 > SGL6. From the m/z 504 ion, loss of CH_3OH brought m/z 472 peak. The m/z 259 peaks of permethylated SGA3 and SGL4 were the methylated aminosugar, methylated GalNAc and GlcNAc, respectively.

Scheme 1 was suggested based on the assumption that the deprotonation and Na^+ ion adduct were localized at the most acidic carboxyl or N-acetyl group of the sialic acid portion and also the same group at the reducing end. This process occurred via an electron pair rearrangement which either took place from the reducing end in the pyranose or open chain form, but the ring-opening is, in contrast to ring-fragmentation, not necessary. As indicated in Scheme 1, a proton transfer had to occur prior to electron-pair rearrangement for acidic saccharides. The Na^+ ion also adducted near the lone pairs of the N-acetyl-CH_3 group of sialic acid and also aminosugars such as GalNAc and GlcNAc.

Without a permethylation process neutralization step, $R(OH)(O^-)_{n-1} + CH_3I \rightarrow R(OCH_3)_{n-1}(OH) + I^-$, sodiated but partially methylated ion appeared at m/z 865 $[R(OCH_3)_{n-1}(OH) + Na^+]$ which yielded ions from one hydroxy group instead of methoxyl group. The ESI CID MS/MS of unsaturated partially methylated SGA3, SGL3, SGL4 and SGL6 at −35 eV are shown in Figure 4. Compared to fully methylated trisaccharides (Figure 1), the unsaturated, partially methylated SGA3 and SGLX also exhibited the same cleavage fragment patterns but different survival peak ratios according to the linkage positions

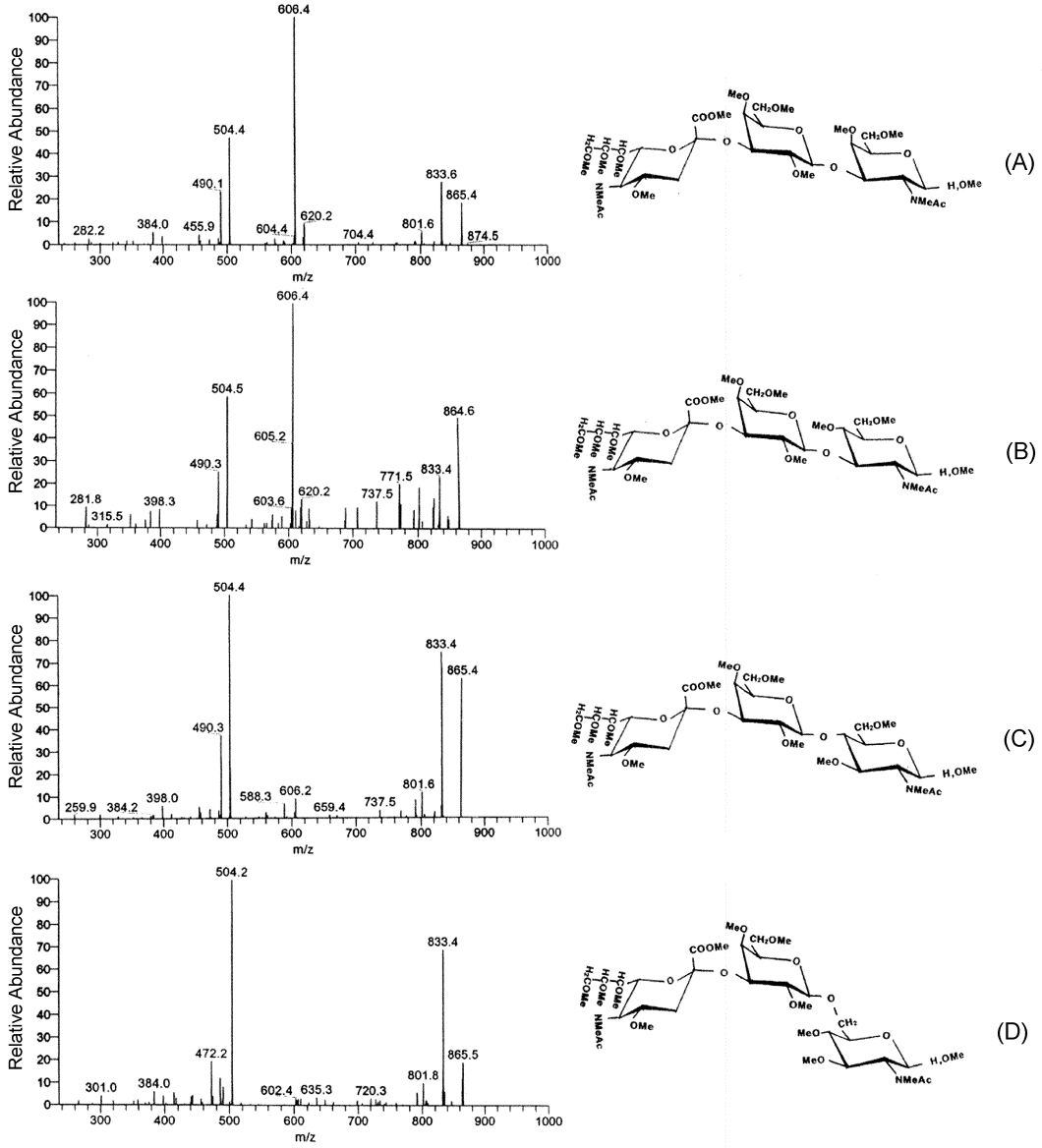


Figure 4. ESI CID tandem spectra of m/z 865 for partially methylated sialyl(α2-3)Gal(β1-3)GalNAc(A) and sialyl(α2-3)Gal(β1-X)GlcNAc where X=3(B), 4(C) and 6(D) at −35 eV.

(Figure 4).

In contrast to Figure 1, the molecular ion of partially methylated trisaccharides (m/z 865) was much more stable in SGL3 than SGA3 or SGL6. Compared with the spectra of the other unsaturated, partially methylated saccharides, those of SGA3 and SGL3 (which contain the β1-3 linkage) at each collision energy level from −30 to −50 eV at −5 eV increments exhibited a relatively intense peak at m/z 606 that was diagnostic for the remnant methylated sialyl(α2-3)galactose moiety. Partially methylated SGL4 and SGL6 had an identical cleavage pathway. Significant differences in the daughter ion ratios contributed to discernment between β1-4 and β1-6 linkages. Methods for detailed characterization of these recognition structures such as sialyl(α2-3)Gal(β1-X)GlcNAc were important in modern structural cell biology to derive structure/function relationships particularly in the postgenomic era, in order to understand posttranslational glycosylation and its function.[1,9] Previously, we demonstrated a distinction of chain type and also blood group type of asialo oligosaccharides such as $Le^{a/x}$ and $Le^{b/y}$ through a positive ESI CID MS/MS method.[7] The present extension of the previous study,[7] ESI CID MS/MS, in conjunction with suitable permethylated procedures and justified with molecular modeling, yielded a sensitive method to analyze the reducing terminal linkage position in a set of four synthetic, linkage-isomeric trisaccharides.

Conclusion

Carbohydrate composition and structure strongly influenced the abundance of fragment ions observed in the ESI CID tandem mass spectra (Figures 1 and 4). Chemically derived biomolecules also might be beneficial for increasing fragmentation during electrospray ionization. Linkage-specific fragments had been observed for oligosaccharides with N-acetyl residues or carboxyl group of acidic hexose residues; a structural feature that appeared to enhance cleavage at the adjacent glycosidic bond. In the sialic acid containing oligosaccharides, SGA3 and SGLX series, the compound containing β1-6 linkage was always more labile because of the propensity for charge retention on a carboxyl group of sialic acid and the nearby amino group on GlcNAc or GalNAc. In the molecular modeling comparison, the β1-4 linkage compound was always intermediate in stability and the β1-3 linkage-containing compound the most stable. Using ESI CID tandem mass spectrometric experiments and molecular modeling, it had been suggested that significant differences in glycosidic bond cleavage occurred due to ionic considerations from acidic and N-acetyl groups and also from contributions from steric hindrance of the absorbance of collision energy, leading to a higher bond cleavage for sterically crowded linkages.

Complementary information for linkage positions of oligosaccharides will be obtained using a different ionization mode, such as a positive or negative mode and several sample derivatizations such as peracetylation or cation adducts of intact samples. This will be possible by creating a database with a number of sets of synthetic isomeric oligosaccharides.

Acknowledgement. This work was supported by the Korea Science and Engineering Foundation [R04-2002-000-20042-0]. The support of the Program for Cultivating Graduate Student in Regional Strategic Industry was also acknowledged.

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