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Citation	Rapid Communications in Mass Spectrometry, 20(3), 412-418 https://doi.org/10.1002/rcm.2320
Issue Date	2006
Doc URL	http://hdl.handle.net/2115/5844
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Type	article (author version)
File Information	RCMS20-3.pdf



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Structural assignment of isomeric 2-aminopyridine derivatized monosialylated biantennary N-linked oligosaccharides using negative-ion MSⁿ spectral matching †

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書式変更: 英語 (米国)

変更されたフィールド コード

書式変更: 英語 (米国)

書式変更: 英語 (米国)

† Portions of this study were presented at the 53rd ASMS Conference on Mass Spectrometry, 2005 (San Antonio, USA).

Abstract

To investigate the possibility of structural assignment based on negative-ion MSⁿ spectral matching, four isomers of 2-aminopyridine-derivatized (PA) monosialylated oligosaccharides (i.e., complex type N-glycans with an α 2-3 or α 2-6 linked sialic acid on α 1-6 or α 1-3 antennae) were analyzed by using high-performance liquid chromatography/electrospray ion trap time-of-flight mass spectrometry (HPLC/ESI-IT-TOF MS). The negative ion [M-2H]²⁻ is observed dominantly in the MS¹ spectra without the loss of a sialic acid. The MS² spectra derived from it are sufficiently reproducible that MS² spectral matching based on correlation coefficients can be applied to the assignment of these isomers. The isomers containing a sialic acid on α 1-6 or α 1-3 antennae can be distinguished by MS² spectral matching, but the α 2-3 and α 2-6 linkage types of sialic acid cannot be distinguished by their MS² spectra. However, MS³ spectra derived from fragment ions containing a sialic acid (i.e., C₄- and D-type ions) clearly differentiate the α 2-3 and α 2-6 linkage types of sialic acid in their MS³ spectral patterns. This difference might be rationalized in terms of a proton transfer from the reducing-end mannose to the negatively-charged sialic acid. These two moieties are very close in the structural conformations of the precursor C₄-type fragment ions, as predicted by molecular mechanics calculations. Thus, negative-ion MSⁿ (n=2, 3) spectral matching was proven to be useful for the structural assignment of these four monosialylated PA N-glycan isomers.

INTRODUCTION

Electrospray ionization mass spectrometry (ESI-MS)¹ and matrix-assisted laser desorption ionization/mass spectrometry (MALDI-MS)^{2,3} are now routinely used in protein/peptide analysis and are becoming indispensable in oligosaccharide analysis as well. In the past decade, many methods that combine high-performance liquid chromatography (HPLC) or capillary electrochromatography (CEC) (on-line with various mass analyzers) with sequential exoglycosidase digestions or partial hydrolysis, have been proposed and used for analyzing oligosaccharides.⁴⁻⁸ In recent years, tandem or multistage tandem (MSⁿ) mass spectrometry techniques, providing additional information about oligosaccharide structures, are being used extensively for structural analysis of oligosaccharides. However, despite many attempts, it is still difficult and time-consuming for these MSⁿ based techniques to completely assign the oligosaccharide structure (i.e., composition, sequence, anomeric effect, linkage position, and branching pattern) due to the structural diversity of oligosaccharides.

In the structural analysis of oligosaccharides, most efforts have been concentrated on the assignment of bond cleavages and the recognition of diagnostic fragment ions,⁹⁻¹⁷ although some authors have been focusing on the intensity distribution/pattern of fragment ions.¹⁸⁻²⁷ Recently, we reported that positive- and negative-ion MSⁿ (n=2, 3) spectra of non-fucosylated, fucosylated, and sialylated oligosaccharides (complex type N-glycans) derivatized with 2-aminopyridine (PA), obtained using sonic spray ionization ion trap mass spectrometry (SSI-IT MS), are highly reproducible, and that the structural assignment of isomeric PA N-glycans is possible by simply calculating correlation coefficients between their MSⁿ spectra (i.e., MSⁿ spectral matching considering both the m/z values and the relative intensities of the fragment ions).^{28,29} Subsequently, we showed that MS² spectra can be used not only for the assignment of PA N-glycan isomers, but also for their relative quantification when coeluted in a reverse-phase (RP) HPLC/SSI-IT MS analysis.^{30,31} Very recently, we noticed that the use of MSⁿ spectral matching effectively reduces the number of specific sequential exoglycosidase digestions required for the structural analysis of “unknown” (i.e., novel) oligosaccharides not present in an MSⁿ spectral library³².

In the present study, negative-ion MSⁿ (n=2, 3) spectral matching was applied to the assignment of four isomeric monosialylated PA-oligosaccharides (complex type N-glycans with α 2-3 or α 2-6 linked sialic acid on α 1-6 or α 1-3 antennae) using nanoHPLC/ESI-IT-TOF MS. MS² spectra were derived from the dominant doubly-deprotonated molecules [M-2H]²⁻; MS³ spectra were derived from C₄- (Domon and Costello nomenclature³³) or D-type^{9,10} fragment ions containing a sialic acid. It is shown that MS² spectral matching is useful for distinguishing the positions of sialic acid (i.e., a sialic acid on α 1-6 or α 1-3 antennae), and MS³ spectral patterns clearly differentiate between α 2-3 and α 2-6 linkage types for a sialic acid. This difference of MS³ spectral patterns may be rationalized on the basis of conformational analysis of the precursor C₄-type fragment ions based on molecular mechanics calculations. Although similar MSⁿ spectral analyses of sialylated tri- and tetra-saccharides from human milk^{19,20,25,26,27}, and of two isomeric disialylated N-glycans³⁴ have already been reported, to the best of our knowledge this is the first report that completely distinguished the four types of isomers of monosialylated N-glycans by using negative-ion MSⁿ spectra. Moreover this work provides the first rationalization of the MS³ fragmentation differences of α 2-3 and α 2-6 linked sialic acids in terms of a proton transfer from the reducing-end mannose to the negatively-charged sialic acid.

EXPERIMENTAL SECTION

Materials

Acetonitrile (HPLC/MS grade), methanol (HPLC/MS grades), acetic acid, and ammonium acetate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Water was purified by a Milli-Q system (Millipore Co., Milford, MA). Human serum was obtained from Sigma-Aldrich (St. Louis, MO). CMP-*N*-acetylneuraminic acid (Neu5Ac) was a generous gift of Yamasa (Chiba, Japan). α 2, 3-(*N*)-sialyltransferase (rat, recombinant) was purchased from Calbiochem (Darmstadt, Germany). A ShimPack HRC-ODS-silica column (6.0 mm i.d. \times 150 mm) and a Develosil C30-UG column (2 mm i.d. \times 150 mm) were purchased from Shimadzu (Kyoto, Japan) and Nomura Chemical (Aichi, Japan), respectively. These columns were used for sample preparation and purification.

Preparation of standard sialylated PA-oligosaccharides

PA-oligosaccharides (PA N-glycans) 2A1-200.4 and 200.4 (Takahashi's nomenclature³⁵) were purified from PA N-glycans from human serum by using the Develosil C30-UG column (2.0 mm i.d. x 150 mm), as described previously.³¹ PA N-glycans 1A1-200.4 and 1A2-200.4 were obtained from 2A1-200.4 by acid hydrolysis at 90°C for 5 min with 0.01 M HCl (pH 2.0). PA N-glycans 1A3-200.4 and 1A4-200.4 were also obtained from 200.4 by an α -2-3 sialyltransferase reaction at 37°C for 45 min in 50 mM HEPES buffer (pH 7.0) containing 10 mM MnCl₂, 2 mM CaCl₂, 1% Triton CF-54, 0.1% bovine serum albumin, 30 μ M oligosaccharide acceptor (PA N-glycan 200.4), 600 μ M CMP-Neu5Ac, and 74 mU/mL α -2-3 sialyltransferase. Then, the reaction products were purified again by using the Develosil C30-UG column (2.0 mm i.d. x 150 mm).

Apparatus and analytical conditions

The HPLC/ESI-MS instrument used was a NanoFrontier system (Hitachi High-Technologies, Tokyo, Japan) consisting of a capillary HPLC system based on the AT10PV nanoGR generator³⁶ and an ESI-IT-TOF mass spectrometer.^{37, 38} A Develosil nano-C30 column (130 μ m i.d. and 5 mm in length) (Nomura Chemical, Aichi, Japan) was used for primary trapping and desalting of the samples. A SilicaTip (tip diameter of 10 μ m) (New Objective, Woburn, MA) was used as an electrosprayer. Several types of fused-silica capillary tubes (10, 20, 50 μ m i.d. and 0.36 mm o.d.) (GL Science, Tokyo, Japan) were used for connecting the individual units in the system. Connecting unions and in-line filters were purchased from Upchurch Scientific (Oak Harbor, WA).

The flow rate of the nanoflow pump was set at 200 nL/min. A 1- μ L aliquot of each PA N-glycan sample (about 10 pmol) was injected into the nano-C30 trap column. The solvent used was 50% acetonitrile in 0.1% formic acid aqueous solution. The ESI-IT-TOF MS conditions were as follows: the ESI voltage was -1.7 kV; curtain (nitrogen) gas was used at a flow rate of 0.7 L/min without heating; the scan range was m/z 200-2000; the mass accuracy and resolution were within \pm 50 ppm and more than 8000 FWHM, respectively. Parameters related to MS² and MS³ CID experiments were: CID gas (He) pressure 100mTorr; isolation time 1.5 ms; isolation width 30; CID gain 2.5 (for all MS² spectra

and MS³ spectra of 1A1-200.4 and 1A3-200.3) and 3.1 (for MS³ spectra of 1A2-200.4 and 1A4-200.4); CID time 2.5 ms.

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Molecular mechanics (MM) calculations

Conformational energy calculations for fragment ions were performed using the MOE program code (Chemical Computing Group, Montreal, Canada) and the MM method using MMFF94S force fields. During the energy calculation the negative charge was assumed to be localized on the carboxyl group of sialic acid, and all glycosidic bond angles were rotated (for the α 2-6 linkage type of sialic acid, the C5-C6 bond angle of galactose was also rotated). At every 60-degree rotation of each bond angle, the conformation was optimized.

RESULTS AND DISCUSSION

Reproducibility of MSⁿ spectra

The monosialylated PA N-glycans (1A1-200.4, 1A2-200.4, 1A3-200.4, and 1A4-200.4) studied here are illustrated in Scheme 1, together with the abbreviations for their sub-components used in this study. The N-glycans are isomers with a sialic acid (Neu5Ac) at different positions (i.e., on α 1-6 or α 1-3 antennae) with a different linkage type (i.e., α 2-3 or α 2-6 linked to the terminal galactose). Previously, we reported that the monosialylated PA N-glycan isomers 1A1-200.4 and 1A2-200.4 can be distinguished by using negative-ion MS² spectra obtained using semi-micro HPLC/sonic spray ionization (SSI)-IT MS, but a complete assignment of these four isomers was not discussed.²⁹ Figure 1 shows the MS¹ spectrum of 1A1-200.4 obtained by a nanoHPLC/ESI-IT-TOF MS. The [M-2H]²⁻ ion (m/z 1003.9) is predominantly observed, accompanied by low-abundance chloride-adduct ions [M-H+Cl]²⁻ (m/z 1021.9) and [M+2Cl]²⁻ (m/z 1039.9). The other isomers also show similar MS¹ spectra (data not shown).

Figure 2 shows MS² (Fig. 2A) and MS³ (Fig. 2B) spectra of 1A1-200.4 in three runs. The CID conditions used are described in the Experimental section. The MS² spectra (Fig. 2A) were derived from the [M-2H]²⁻ precursor ion (m/z 1003.9). The cross-ring cleavage ions ^{2,4}A₆ (m/z 782.7, doubly charged)

and $^{1,3}A_{3\alpha}$ (m/z 423.9) are observed as intense peaks in the MS^2 spectra. A $C_{4\beta}$ -type fragment ion (m/z 835.2, Neu5Ac-Gal-GlcNAc-Man) is also relatively intense. A D-type double-bond cleavage ion (m/z 688.0, Gal-GlcNAc-Man-Man) and a dehydrated fragment ion (D-H₂O)₂ are observed at low abundance. These D-type ions provide useful information regarding the α 1-6 antennae structure.^{9, 10, 29} The $C_{4\beta}$ -type fragment ion containing sialic acid was selected as precursor ion for the MS^3 spectra (Fig. 2B). These MS^3 spectra show a very characteristic pattern wherein the $B_{3\beta}$ -type fragment ion (m/z 655.1, Neu5Ac-Gal-GlcNAc) becomes a strong base peak and the $B_{1\beta}$ -type fragment ion (m/z 289.8, Neu5Ac) is observed as a very weak peak; a proposed fragmentation pathway leading to this pattern is discussed below. In addition, these data demonstrate that the MS^2 (Fig. 2A) and MS^3 (Fig. 2B) spectra are reproducible. The average of the self-correlation coefficients between MS^2 spectra in three runs was 0.98, and that of MS^3 spectra in three runs was 0.99, where the relative intensities of the recognized 13 (MS^2) and 5 (MS^3) fragment ions were considered in the correlation coefficient calculations.³¹ The other isomers showed similarly high reproducibility (data not shown). Such high reproducibility of MS^n spectra is essential for application of MS^n spectral matching for differentiation of structural isomers, as previously discussed.^{28, 29}

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MS^2 and MS^3 spectral matching among the four monosialylated PA N-glycan isomers

Figure 3 compares MS^2 spectra of the four isomers 1A1-200.4 (A), 1A3-200.4 (B), 1A2-200.4 (C), and 1A4-200.4 (D), acquired under the same CID conditions as those of Fig. 2. A structural difference between 1A1-200.4 and 1A3-200.4 is the linkage type of sialic acid (i.e., α 2-3 or α 2-6 linked sialic acid) on the same α 1-3 antenna. At a glance, it can be seen that the MS^2 spectra derived from the dominant precursor ion $[M-2H]^{2-}$ (m/z 1003.9) are very similar. The MS^2 spectral matching value (i.e., correlation coefficient) is 0.98, which is equal to the average of the self-correlation coefficients; this means that these isomers cannot be distinguished from their MS^2 spectra. However, MS^3 spectra derived from each $C_{4\beta}$ -type fragment ion (m/z 835.2, Neu5Ac-Gal-GlcNAc-Man) are quite different. As described above, 1A1-200.4 with an α 2-6 linked sialic acid (Neu5Ac) provides an abundant $B_{3\beta}$ -type fragment ion (m/z

655.1, Neu5Ac-Gal-GlcNAc) and a very low-abundance B_{1β}-type fragment ion (*m/z* 289.8, Neu5Ac). In contrast, 1A3-200.4, with an α2-3 linked sialic acid, shows the opposite pattern. Thus, the α2-3 and α2-6 linked sialic acids can easily be distinguished by their MS³ spectral patterns. There is another characteristic difference between the MS³ spectra of these isomers, namely, the three-bond cleavage fragment ion Y_{6β}/ ^{1,3}A_{4β} (indicated as ^{1,3}A_{3β}(*)) is only observed for 1A3-200.4 (Fig. 2B). This might imply that the cross-ring cleavage ^{1,3}A_{3β}(*) was followed by a neutral loss of the sialic acid, which occurs readily for the α2-3 linked sialic acid.

Similarly, another isomeric pair, that of 1A2-200.4 and 1A4-200.4, have a different linkage type of sialic acid (i.e., α2-3 or α2-6 linked sialic acid) on the same α1-6 antenna. Additionally, like the first-mentioned isomeric pair of 1A1-200.4 and 1A3-200.4, MS² spectra (Fig. 3C and 3D) of 1A2-200.4 and 1A4-200.4 are very similar to one another (correlation coefficient 0.97), and thus it is difficult to differentiate them from one another. However, MS³ spectra derived from each D-type two-bond cleavage ion (*m/z* 979.3, Neu5Ac-Gal-GlcNAc-Man-Man), which is relatively abundant in this case compared to the first pair of isomers (Fig. 3A and 3B), are quite different from one another, especially in the intensity pattern of the characteristic fragment ions of the B_{1α}-type (*m/z* 289.8, Neu5Ac), C_{4α}-type (*m/z* 835.2, Neu5Ac-Gal-GlcNAc-Man), and ^{1,3}A_{3α}(*) (or Y_{6α}/ ^{1,3}A_{4α}). Thus, these two isomers can also be distinguished via their MS³ spectral patterns, as can the other pair of 1A1-200.4 and 1A3-200.4.

Consider next the difference between the two pairs of isomers, discussed separately above. For this purpose the MS² spectral matching values (correlation coefficients) between (1A1-200.4, 1A3-200.4) and (1A2-200.4, 1A4-200.4) were calculated. The values obtained were 0.75-0.79, which are clearly different from the values of 0.98 (for 1A1-200.4 vs. 1A3-200.4) and 0.97 (for 1A2-200.4 vs. 1A4-200.4), discussed above. Thus, we conclude that the four monosialylated PA N-glycan isomers can be distinguished from one another by a combination of MS² and plus MS³ spectra derived from a fragment ion containing a sialic acid; the MS² spectra provide the positional information for the sialic acid (e.g., α1-6 or α1-3 antennae), and the MS³ spectra determine the linkage type for sialic acid (e.g., α2-3 or α2-6 linked to the terminal galactose).

[It is appropriate here to](#) refer briefly to previous articles discussing similar MSⁿ spectral analyses of isomeric sialylated oligosaccharides. Viseux et al.^{19, 20} analyzed positive ESI CID MS² spectra of permethylated sialylated tetra-saccharides (LST-a (α 2-3), LST-b(α 2-6), and LST-c(α 2-6)) from human milk. They reported a diagnostic E₃-type fragment ion derived from a B₃-type ion, but did not observe a clear difference between B₁- and B₃-type fragment ion intensities. Yamagaki and Nakanishi^{26, 27} obtained negative/positive MALDI PSD spectra of sialylated trisaccharides (α 2-3 and α 2-6 sialyllactoses and α 2-3 and α 2-6 sialyl-N-acetylglucosamines), and found a clear difference in the B₁-type fragment ion intensity of these α 2-3 and α 2-6 isomers. Later, Sato et al.²⁵ confirmed the difference in positive MALDI PSD spectra of α 2-3 and α 2-6 sialyllactoses [derivatized with 2-aminobenzamide](#). Wheeler and Harvey³³ reported negative-ion MSⁿ (n=2, 3) spectra derived from the two isomeric disialylated N-glycans (2A1-200.4 and 2A4-200.4 of Takahashi's nomenclature³⁵) with α 2-3 or α 2-6 linked sialic acid on both α 1-6 and α 1-3 antennae. They showed that the fragment ion (*m/z* 306.1) [that](#) originated only from α 2-6 linked sialic acid can be used as a diagnostic ion to distinguish [the](#) α 2-3 or α 2-6 linkage types [for](#) sialic acid. Although this was confirmed by Meisen et al using Ar as a CID collision gas,³⁹ the diagnostic fragment ion (*m/z* 306.1) was not observed in this study using negative ESI-IT-TOF MS and low-energy CID using He gas. This diagnostic fragmentation evidently depends on the CID type used.

Fragmentation pathways of C₄- type fragment ions of α 2-3 and α 2-6 linked sialic acids

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Finally, [an attempt was made](#) to explain the characteristic patterns of MS³ spectra seen in Fig. 3A and 3B [based on](#) the conformations predicted by molecular dynamics calculations (see the [Experimental section](#)). Figures 4A and 4B illustrate the optimized conformations of C₄-type fragment ions with α 2-3 or α 2-6 linked sialic acid. The averaged stabilization energies at these configurations were 199 kcal/mol (α 2-3 type) and 173 kcal/mol (α 2-6 type), respectively. The reliability of the optimized conformations was also checked by fixing the position of Gal. Overlaying conformations accumulated up to 99% of [the](#) Boltzmann distribution at 298 K. As [can be](#) seen in the overlaid conformations illustrated in the lower

part of Fig. 4, both of the C_{4β}-type fragment ions have a relatively rigid conformation centered at Gal. The most important point in these optimized conformations is the distance between the negative charge localized on the carboxyl group of Neu5Ac (this assumption may be considered reasonable) and Man on the reducing end of the C_{4β}-type fragment. This interaction is so close for the C_{4β}-type fragment ion with α2-6 linked Neu5Ac that intra-molecular hydrogen (H)-bonds can be formed between the carboxyl group of Neu5Ac and the hydroxyl group on the C4 and/or C3 positions of Man, so that a proton transfer from Man to Neu5Ac can easily occur. Then, the result of moving the negative charge to the Man residue finally initiates cleavage of the glycosidic bond between Man and GlcNAc. This rationalization of the fragmentation mechanism is based on a charge-driven reaction, as discussed previously.^{15, 40, 41} Thus, we can propose an explanation of why the B_{3β}-type fragment ion (*m/z* 655.1, NeuAc-Gal-GlcNAc) is abundant and the B_{1β}-type fragment ion (*m/z* 289.8, NeuAc) is observed at very low abundance (Fig. 3A). Conversely, for the C_{4β}-type fragment ion with α2-3 linked Neu5Ac, the distance between Neu5Ac and Man is so large that the occurrence of such a proton transfer is unlikely. As a result, the negative charge localized on the carboxyl group of Neu5Ac initiates B_{1β}-type fragmentation, and the MS³ spectral pattern in this case thus becomes the opposite of that for the α2-6 linked Neu5Ac isomer (Fig. 3B).

In a similar manner, the D-ion fragmentations in Figs. 2C and 2D can be rationalized because the α2-3 linked Neu5Ac is still far removed from both the α1-6 linked Man (i.e., Man in Fig. 4) and reducing-end Man residues, while the α2-6 linked Neu5Ac is very close to the α1-6 linked Man residue as for the cases shown in of Figs. 4A and 4B (data not shown). We believe that the proposed formation of intra-molecular hydrogen bonds and the proton transfer, predicted from the conformational analysis based on molecular mechanics calculations, is reasonable and could be also applicable to other glycoside bond cleavages in fragment ions.

CONCLUSIONS

The four isomers of 2-aminopyridine-derivatized (PA) monosialylated oligosaccharides (complex type N-glycans) were analyzed using nanoHPLC/ESI-IT-TOF MS in the negative-ion mode. It was shown that the negative-ion MS² and MS³ spectra are reproducible; this is an essential element for MSⁿ spectral matching. In particular, it was shown that MS² spectral matching is useful for distinguishing the position of sialic acid (i.e., on α 1-6 or α 1-3 antennae), and MS³ spectra clearly differentiate the α 2-3 or α 2-6 linkage type of the sialic acid. Thus, it was concluded that the four monosialylated N-glycan isomers can be distinguished from their negative-ion MS² and MS³ spectra by combining them with the MSⁿ spectral matching method. It was also shown that, based on conformational analysis based on molecular dynamics calculations, the difference in the MS³ spectra derived from C_{4 β} -type fragment ions with α 2-3 and α 2-6 linked sialic acid could be rationalized based on a proposed proton transfer from the reducing-end mannose to the sialic acid.

Acknowledgments

This work was supported in part by the National Project on Functional Glycoconjugate Research Aimed at Developing New Industry of the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported in part by SENTAN, JST (Japan Science and Technology Agency).

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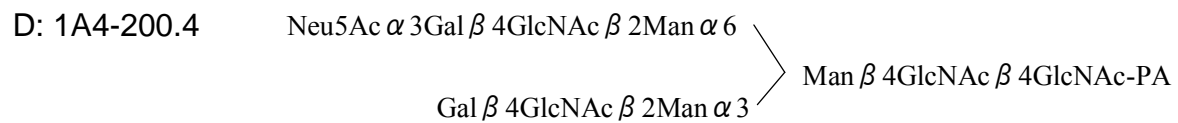
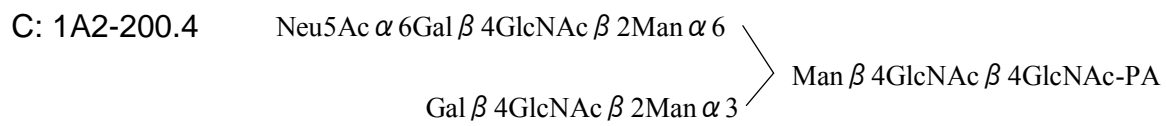
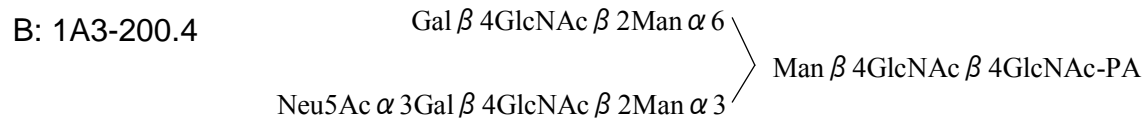
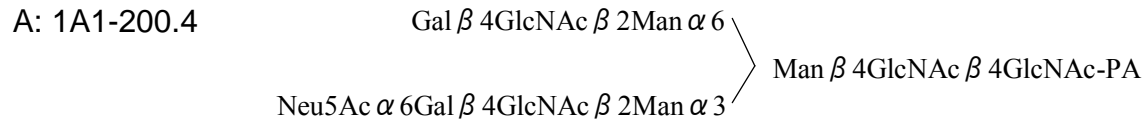
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Scheme 1. Structures of isomeric monosialylated PA N-glycans (molecular mass 2009.75 Da)



Man : Mannose (\circ); Gal: Galactose (\bullet); GlcNAc: *N*-acetyl-glucosamine (\square);
 Neu5Ac: *N*-acetyl-neuraminic acid (sialic acid) (\blacklozenge); PA: 2-aminopyridine

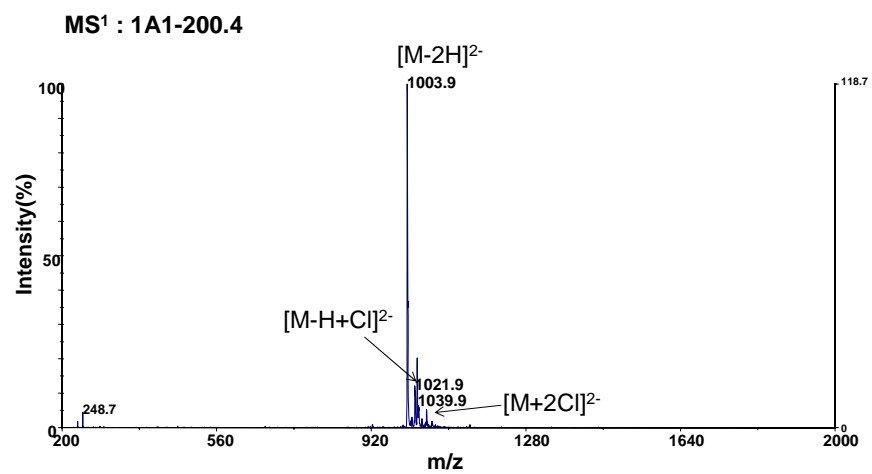


Figure 1. MS¹ negative ion spectrum of monosialylated PA N-glycan (1A1-200.4).

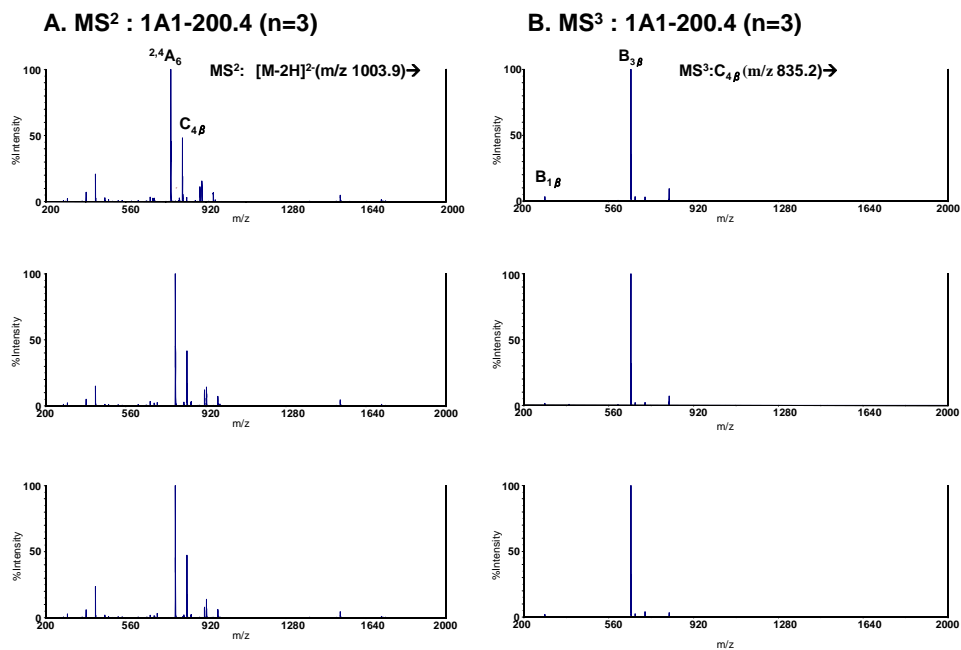


Figure 2. Repeatability ($n=3$) of MS^2 and MS^3 spectra of monosialylated PA N-glycan (1A1-200.4).

The MS^2 and MS^3 spectra were derived from the $[M-2H]^{2-}$ ion (m/z 1003.9) and the fragment ion $C_{4\beta}$ (m/z 835.2), respectively. Averaged correlation coefficients for MS^2 spectra and MS^3 spectra are 0.98 and 0.99, respectively.

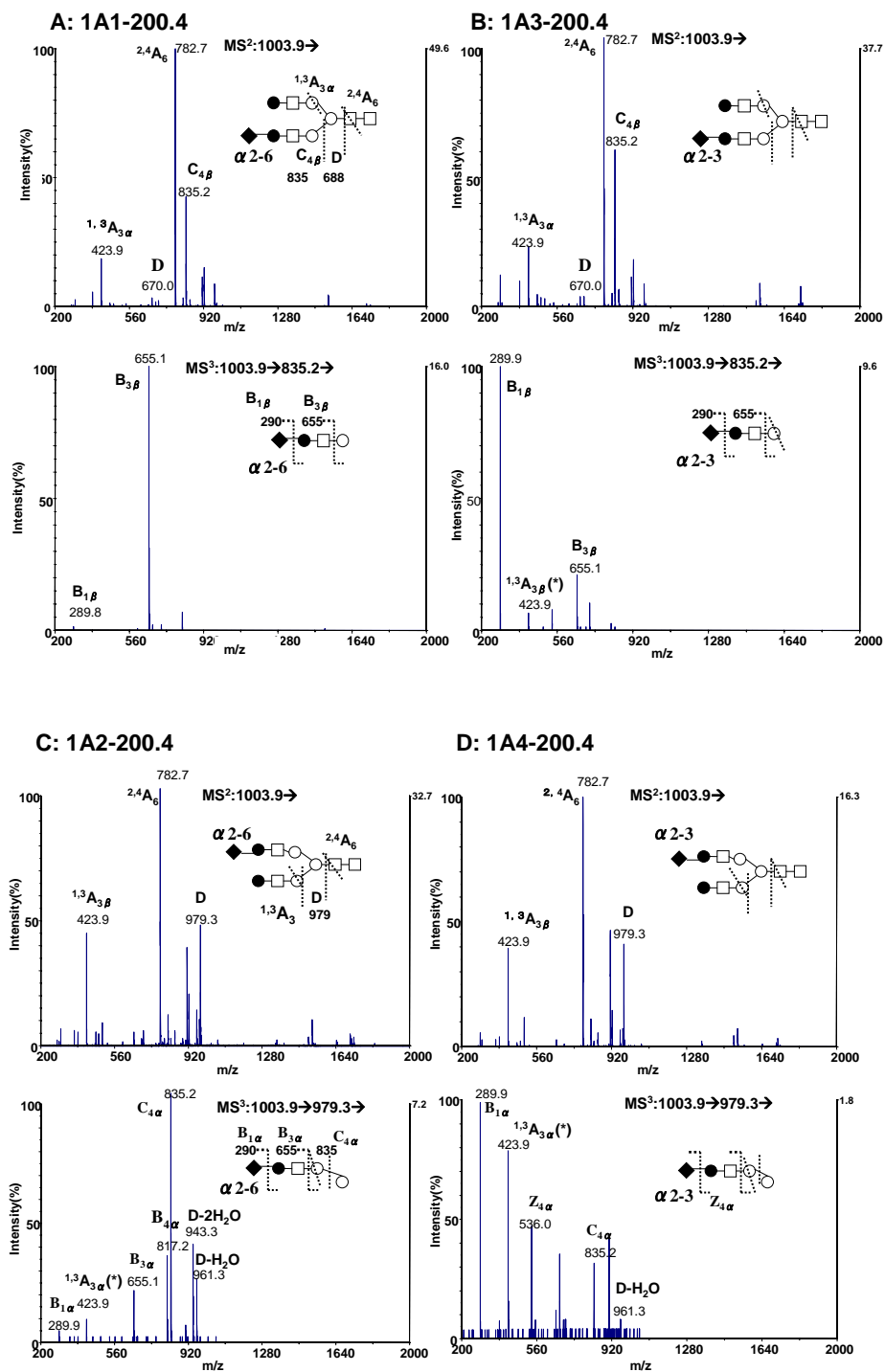


Figure 3. MS² and MS³ spectra of four isomeric monosialylated PA N-glycans 1A1-200.4(A), 1A3-200.4 (B), 1A2-200.4 (C), and 1A4-200.4 (D).

Only major fragment ions are annotated in the MS² and MS³ spectra. ^{1,3}A_{3β}(*) indicates a cross-ring cleavage followed by loss of neutral sialic acid. The symbols used to represent each monosaccharide are defined in Scheme 1.

A: C_{4β} (Neu5Ac α6Gal β4GlcNAc β2Man)

B: C_{4β} (Neu5Ac α3Gal β4GlcNAc β2Man)

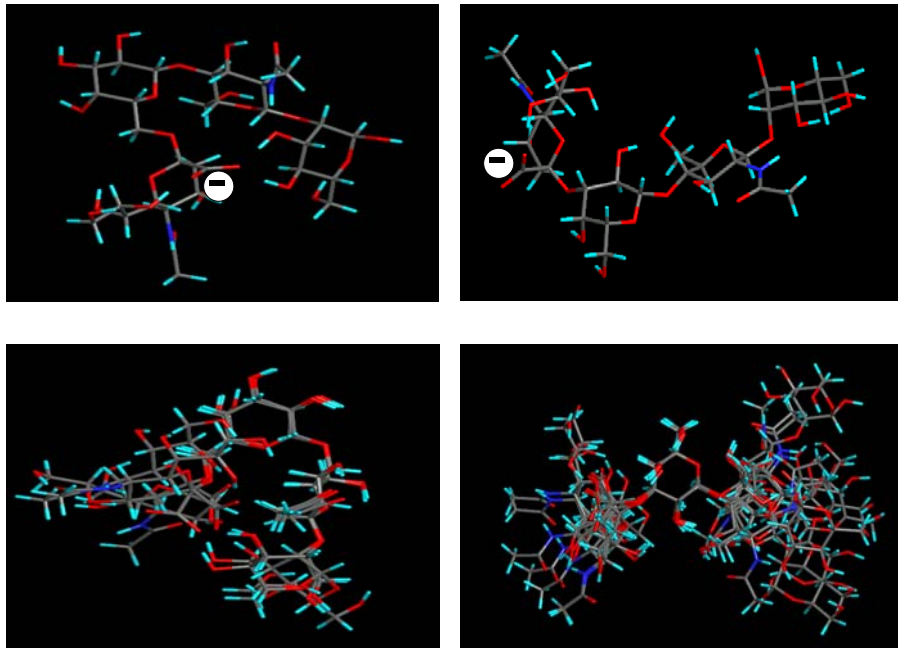


Figure 4. Conformations of C_{4β} fragment ions containing α2-6 (A) and α2-3 (B) linked sialic acids derived from molecular mechanics calculations.

A negative charge (large white circle with minus (-)) was assumed to be localized on the carboxylate group of sialic acid. Overlaid conformations (lower part), centered at the position of the galactose residue, demonstrate the reliability of the calculated conformations. Both conformations of C_{4β} fragment ions are relatively rigid. Note the close distance between Neu5Ac and Man for the C_{4β} fragment ion with α2-6 linked sialic acid (A), and the long separation of these two moieties for the C_{4β} fragment ion with α2-3 linked sialic acid (B).