RESEARCH ARTICLE

Linkage and Branch Analysis of High-Mannose Oligosaccharides Using Closed-Ring Labeling of 8-Aminopyrene-1,3,6-Trisulfonate and *P*-Aminobenzoic Ethyl Ester and Negative Ion Trap Mass Spectrometry

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Abstract

A strategy based on negative ion electrospray ionization tandem mass spectrometry and closed-ring labeling with both 8-aminopyrene-1,3,6-trisulfonate (APTS) and *p*-aminobenzoic acid ethyl ester (ABEE) was developed for linkage and branch determination of high-mannose oligosaccharides. X-type cross-ring fragment ions obtained from APTS-labeled oligosaccharides by charge remote fragmentation provided information on linkages near the non-reducing terminus. In contrast, A-type cross-ring fragment ions observed from ABEE-labeled oligosaccharides yielded information on linkages near the reducing terminus. This complementary information provided by APTS- and ABEE-labeled oligosaccharides was utilized to delineate the structures of the high-mannose oligosaccharides. As a demonstration of this approach, the linkages and branches of high-mannose oligosaccharides $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$ cleaved from the ribonuclease B were assigned from MS^2 spectra of ABEE- and APTS-labeled derivatives.

Key words: N-linked oligosaccharides, High-mannose oligosaccharides, 8-Aminopyrene-1,3,6-trisulfonate, *p*-Aminobenzoic acid ethyl ester, Linkage, Electrospray, Closed-Ring Labeling

Introduction

Glycosylation is a predominant post-translational modification as 50 % of mammalian proteins are glycosylated [1–6]. The structural diversity inherent in the sugar moieties of a glycoprotein results in subtle changes in protein shape and charge, ultimately affecting protein function, both temporally and spatially.

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Mass spectrometry is one of the most powerful and versatile tools for structural analysis of carbohydrates [7]. Soft ionization techniques such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are now the most popular ionization methods used in carbohydrate structural analysis [8–38].

Metal ion coordination [13–15], permethylation [16–20, 31–38], reducing-end labeling [21–30], or underivatized glycans [9–12] in combination with tandem mass spectrometry are among reported strategies to produce linkage and branch information. Among these approaches, analysis of permethylated glycans by MALDI-TOF/TOF is quite useful [31–38]. Unfortunately, it requires the use of high-energy

collision-induced dissociation (he-CID), which is not available in many laboratories studying oligosaccharides.

Negative ion spectra of underivatized oligosaccharides could produce many linkage specific fragment ions. However, for oligosaccharides larger than a hexasaccharide, the use of MS^3 or MS^4 is needed. In addition, linkage information on non-reducing end and the linkage information on the 3-linked branch are prone to miss [9–12]. The fragmentation patterns of closed-ring labeled and underivatized oligosaccharides are very similar [9–12, 22]. However, our earlier study of ABEE-labeled oligosaccharides by negative ion ESI showed that MS sensitivity was enhanced and a more clear assignment for the linkages was obtained [22, 28]. Unfortunately, for oligosaccharides larger than a hexasaccharide, linkages near the non-reducing end were not observed. To extend this ABEE approach to larger oligosaccharides, a procedure involving alkaline degradation was introduced prior to labeling and MS^n analysis. Consequently, linkage and branch information near the non-reducing end were obtained [29].

To provide linkage information near the non-reducing end of oligosaccharide, fragmentation near the non-reducing end is required. Trimethyl(p-aminophenyl)ammonium (TMAPA), a fixed-charged derivative, has been studied as a labeling reagent for oligosaccharide analysis [39]. Fragmentation is observed exclusively at the non-reducing terminus with charge retention on the TMAPA label at the reducing terminus. Based on this concept, oligosaccharides were separately labeled with both ABEE and the fixed-charge derivative, 8-aminopyrene-1,3,6-trisulfonate (APTS), before negative ESI-MS/MS analysis. The complementary information provided by ABEE- and APTS-labeled oligosaccharides was utilized to elucidate the structure of oligosaccharides larger than a hexasaccharide. The potential of this combined approach was demonstrated using the high-mannose glycans cleaved from ribonuclease B.

Experimental

Materials

p-Aminobenzoic acid ethyl ester, maltohexaose, kojibiose, and ribonuclease B were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 8-Aminopyrene-1,3,6-trisulfonate was purchased from Fluka (Buchs, Switzerland). Laminarihexaose and isomaltohexaose were purchased from Seikagaku Kogyo (Tokyo, Japan). Peptide-N-glycanase F (PNGase F) was purchased from Roche Biochemicals (Basel, Switzerland). Glacial acetic acid, methanol, and acetonitrile were purchased from J. T. Baker (Phillipsburg, NJ, USA). Deionized (18MΩ) water (Milli-Q water system, Millipore Inc., Bedford, MA, USA) was used in the preparation of the samples and reaction solution.

Preparation of N-Linked Glycans

The N-linked glycans were released enzymatically from ribonuclease B by means of PNGase F digestion and purified by using graphitized carbon cartridges (Alltech Associates, Lancashire, UK) [40, 41] and evaporated to dryness.

Preparation of Closed-Ring Chromophore Labeled Derivatives

For APTS labeling, dried oligosaccharides were derivatized by the addition of 2 μL 0.02 M APTS in 25 % acetic acid and 10 μL deionized water in an Eppendorf tube. The solution was kept at 75 °C for 6 h. The derivatives were purified by passing through a column made of Sephadex G-25 (Sigma) using water as the eluent, followed by lyophilization. For ABEE labeling, dried glycans were derivatized by using the glycosylamine approach [29].

Electrospray Mass Spectrometry

All mass spectrometry experiments were performed using a Finnigan LTQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) under negative ion electrospray. Samples were infused by syringe pump at a rate of 200 nL/min. The heated capillary was maintained at 275 °C for all experiments. For mass-analyzer collision-induced dissociation (CID), relative collision energy of 35 %–50 % was used in MS^2 experiments.

Results and Discussion

The Linkage Analysis of APTS-Labeled Oligosaccharides

A typical negative ESI mass spectrum of APTS-labeled oligosaccharide (spectrum not shown) is characterized by the doubly and triply charged molecular ions along with the sodiated adducts. The $[M - 3 H+Na]^{2-}$ ion was chosen as the precursor ion because it produced more linkage related cross-ring cleavage fragments when compared to MS^2 analysis of the $[M - 2 H]^{2-}$ and $[M - 3 H]^{3-}$ ions (data not shown).

Kojibiose, laminarihexaose, maltohexaose, and isomaltohexaose are 1-2, 1-3, 1-4, and 1-6 linked linear oligosaccharides, respectively. They were closed-ring labeled with APTS and studied by negative ion ESI-MS^2 (Figures 1 and 2). The results revealed that the fragmentation patterns of APTS-labeled oligosaccharides occurred primarily via charge-remote fragmentation. As a result, linkage information starting at the non-reducing end was provided. A summary of the observed linkage specific fragment ions is shown in Table 1. Owing to the lack of a standard containing an internal 1-2 linkage, ions specific for internal 1-2 linkages were not assigned.

Analysis of Man₅GlcNAc₂ Based on ABEE and APTS-Labeling

For oligosaccharides larger than a hexasaccharide, such as Man$_5$GlcNAc$_2$, the linkages near the reducing-end
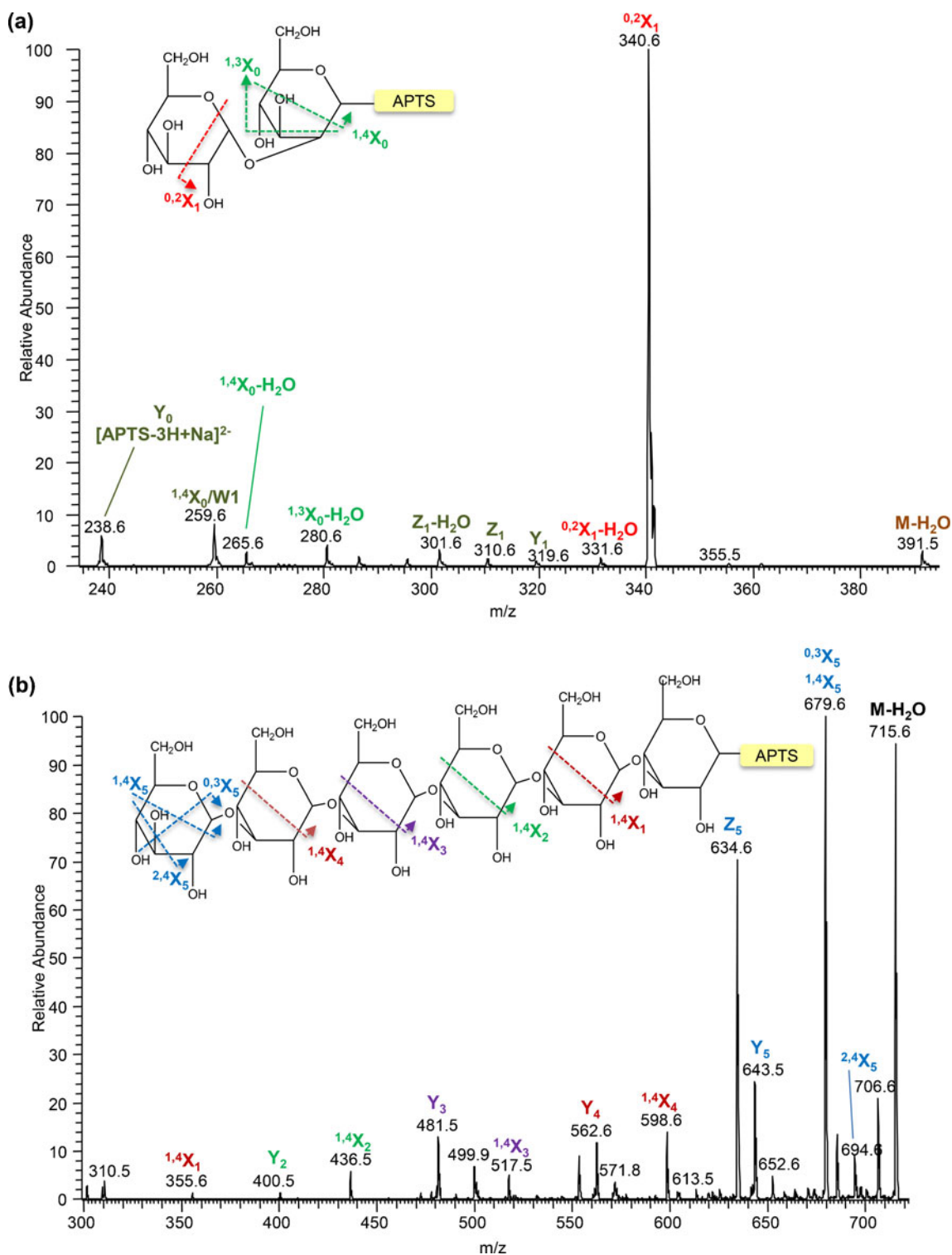
Figure 1. MS^2 negative ESI mass spectra of APTS-labeled oligosaccharides. **(a)** MS^2 spectrum of APTS-labeled kojibiose ([M − 3H + Na]$^{2-}$, m/z 400.5). **(b)** MS^2 spectrum of APTS-labeled laminarihexaose ([M − 3H + Na]$^{2-}$, m/z 724.5). The specific fragment ions were assigned based on the nomenclature proposed by Domon and Costello [43]

could not be determined based on the spectrum of APTS labeling (Figure 3b). A strategy incorporating both APTS and ABEE labeling was employed to assign all linkages of Man$_5$GlcNAc$_2$.
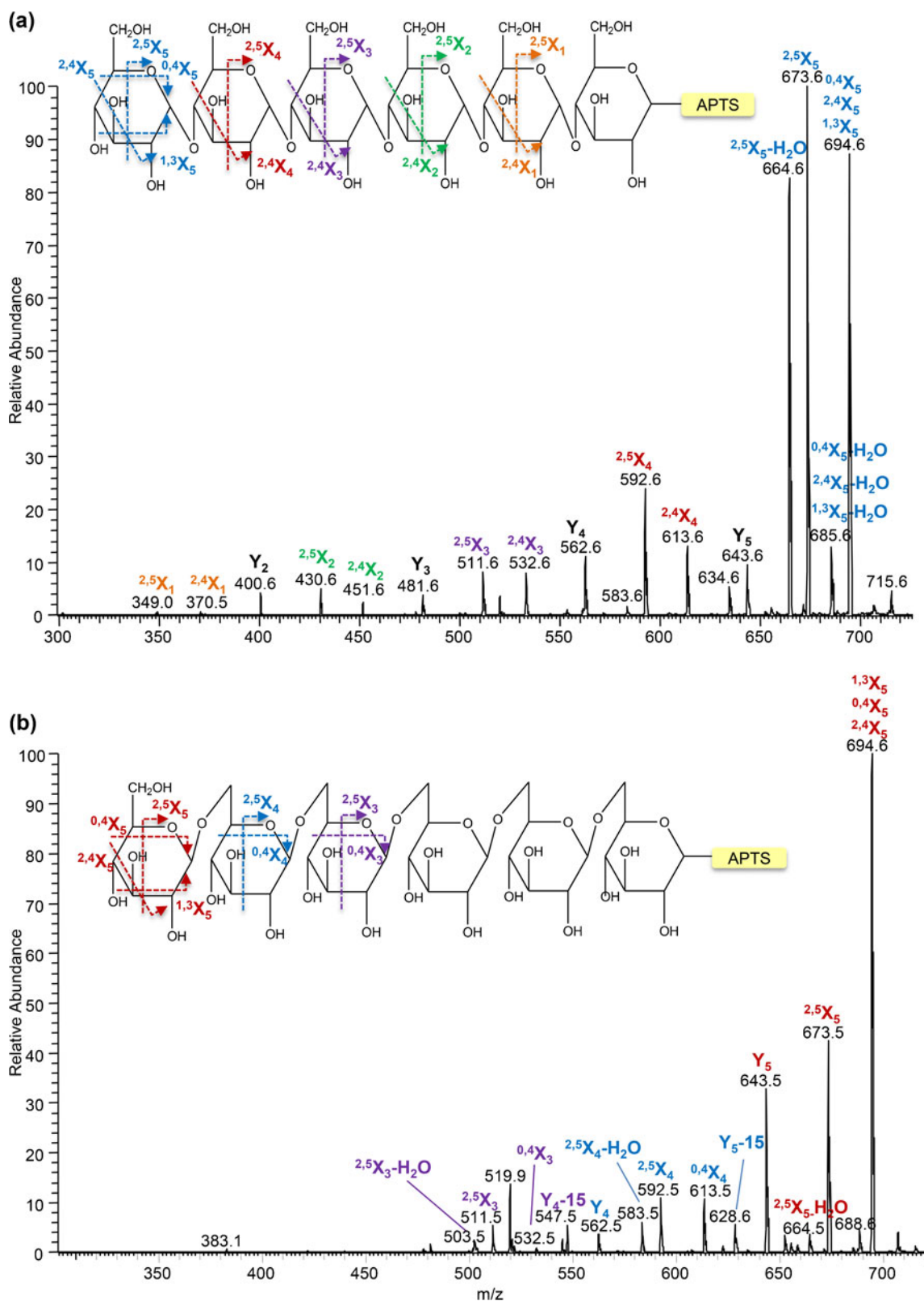
Figure 2. **(a)** MS^2 spectrum of APTS-labeled maltohexaose ([M − 3H + Na]$^{2-}$, m/z 724.6). **(b)** MS^2 spectrum of APTS-labeled isomaltohexaose ([M − 3H + Na]$^{2-}$, m/z 724.5)

The MS^2 spectrum of ABEE-labeled Man$_5$GlcNAc$_2$ is shown in Figure 3a. It is noted that the linkage assignment of ABEE-labeled Man$_5$GlcNAc$_2$ has previously been reported [29]. The fragment ions ^{0,2}A$_5$-18 (m/z 1114.2), ^{0,2}A$_4$ (m/z

Table 1. Specific Linkage Fragment Ions for APTS Closed-Ring Labeled Linear Oligosaccharides

Linkage	Fragments of the terminal linkage	Fragments other than the reducing end
1-2	$^{0,2}X$, $^{0,2}X-H_2O$	N/A
1-3	$^{2,4}X$, $^{0,3}X$ (or $^{1,4}X$)	$^{1,4}X$
1-4	$^{2,4}X$ (or $^{1,3}X$ or $^{0,4}X$), $^{2,4}X-H_2O$ (or $^{1,3}X-H_2O$ or $^{0,4}X-H_2O$), $^{2,5}X$, $^{2,5}X-H_2O$	$^{2,4}X$, $^{2,5}X$
1-6	$^{0,4}X$ (or $^{1,3}X$ or $^{2,4}X$), $^{2,5}X$, $^{2,5}X-H_2O$	Y-15, $^{0,4}X$, $^{2,5}X$, $^{2,5}X-H_2O$

929.2), and $^{0,2}A_4$-18 (*m/z* 911.2) indicated that the first two linkages were 1-4 linkages. The mass difference between C_3 (*m/z* 827.1) and $C_3/Z_{3\beta}$-H (*m/z* 647.1) ion represents a 3-linked Hex connected to the first branch. The fragment ions $C_3/Z_{3\beta}$-H-18 (*m/z* 629.2), $^{0,3}A_3$ (*m/z* 575.1), and $^{0,4}A_3$ (*m/z* 545.3) revealed that the first branch has a 3-,6-linked sugar.

The linkages near the non-reducing terminal were deduced from the MS^2 spectrum of APTS-labeled Man$_5$GlcNAc$_2$ (Figure 3b). The fragment ions at *m/z* 816.6 ($^{0,4}X_{4\alpha'}$), *m/z* 795.6 ($^{2,5}X_{4\alpha'}$) and *m/z* 786.6 ($^{2,5}X_{4\alpha'} - H_2O$) suggested that one of the terminal mannose moieties binds by a 1-6 glycosyl bond. In addition, two series of terminal 1-3 linked characteristic ions were observed. The ions at *m/z* 735.2 ($^{2,4}X_{4"}$ /$Y_{4\alpha'}$) and *m/z* 720.6 ($^{0,3}X_{4"}$ /$Y_{4'}$) represent a 1-3 linked terminal mannose with the loss of a monosaccharide. The ions at *m/z* 574.6 ($^{2,4}X_{3\beta}/Y_{3\alpha}$) and *m/z* 560.5 ($^{0,3}X_{3\beta}$ /$Y_{3\alpha}$) represent a 1-3 linked terminal mannose with the loss of a trisaccharide. The $C_{2\alpha}$ ion (*m/z* 503.1) in Figure 3a and $Z_{3\alpha}$ ion (*m/z* 595.1) in Figure 3b suggested that a trisaccharide is connected to the 6-arm of the first branch. Therefore, these two ions were assigned as the cross-ring cleavage ions from the 3-linked mannose connected to the first branch. The 1-6 linked terminal and the second 1-3 linked terminal sugar were assigned as connected to the 6-arm of the first branch. As a result, a second branch point connected to the 6-arm of the first branched sugar was assigned as a 1-3 and 1-6 linked mannose.

Analysis of Man$_6$GlcNAc$_2$ Based on ABEE and APTS-Labeling

The MS^2 spectrum of ABEE-labeled Man$_6$GlcNAc$_2$ is shown in Figure 4a. The assignment is similar to Man$_5$GlcNAc$_2$, except that the mass difference between C_3 (*m/z* 989.2) and $C_3/Z_{3\beta} - H$ (*m/z* 647.1) ion (342 Da) suggested that a disaccharide was connected to the 3-branch.

The MS^2 spectrum of APTS-labeled Man$_6$GlcNAc$_2$ is shown in Figure 4b. In addition to the terminal 1-3 and 1-6 linked mannoses as in Man$_5$GlcNAc$_2$, a 1-2 linked terminal mannose was assigned by the characteristic ions $^{0,2}X_{4\beta}/Y_{3\alpha}$ (*m/z* 624.3) and $^{0,2}X_{4\beta} - H_2O/Y_{3\alpha}$ (*m/z* 614.8). The characteristic ion $^{2,5}X_{3\alpha}$ (*m/z* 715.4) suggested that the second branch did not contain a 1-2 linked mannose, therefore, the 2-linked terminal mannose was assigned as the terminal sugar of the 3-linked

disaccharide in the first branch. The observed 1-3 linked internal fragments $^{1,4}X_{3\beta}/Y_{3\alpha}$ (*m/z* 560.7) was assigned as the 3-linked disaccharide connected to the first branch. Other assignments were similar to Man$_5$GlcNAc$_2$.

Analysis of Man$_8$GlcNAc$_2$ Based on APTS and ABEE-Labeling

The Man$_8$GlcNAc$_2$ is a mixture of three structural isomers with a molar ratio of~84 : 6 : 10 [42]. Because of the much higher abundance of one isomer (84 %), the fragmentation is expected to be predominated by this isomer.

The MS^2 spectrum of ABEE-labeled Man$_8$GlcNAc$_2$ (Supplemental Figure S1) is similar to Man$_6$GlcNAc$_2$ (Figure 4a). The mass difference between C_4 (*m/z* 1313.2) and $C_4/Z_{3\beta} - H$ (*m/z* 809.2) ion (504 Da) suggested that the 3-arm was substituted with a trisaccharide. In addition, the fragment ion $C_{3\alpha}$ (*m/z* 665.2) indicated that a tetrasaccharide was connected to the 6-arm. Other assignments were the same as in Man$_5$GlcNAc$_2$, and the predicted structure is shown in Figure 5a-1.

The MS^2 spectrum of APTS-labeled Man$_8$GlcNAc$_2$ is shown in Figure 5b. Three terminal (two 1-2, one 1-3 linked) and three internal mannoses (1-3, 1-6, 1-2 linked) were observed (Supplemental Table S1). The characteristic ion $^{1,4}X_{3\beta}/Y_{3\alpha}$(*m/z* 560.5) corresponds to an internal 1-3 linked mannose with the loss of the 6-arm of the first branch. It suggested that a trisaccharide was connected to the first branch through a 1-3 linkage. This assignment is consistent with the assignment based on the $C_4/Z_{3\beta} - H$ ion (*m/z* 809.2) observed in ABEE derivative. The ions at *m/z* 705.6 ($^{0,2}X_{5\beta}/Y_{3\alpha}$) and *m/z* 696.6 ($^{0,2}X_{5\beta} - H_2O/Y_{3\alpha}$) correspond to a terminal 1-2 linked mannose with a loss of the 6-arm. It suggested that the terminal linkage of the 3-linked trisaccharide was a 1-2 linkage (Figure 5a-2). The ions at *m/z* 897.6 ($^{2,4}X_{4\alpha"}/ Y_{4'}$) and *m/z* 883.4 ($^{0,3}X_{4\alpha"}/ Y_{4'}$) correspond to a terminal 1-3 mannose with loss of a disaccharide. It suggests that Man$_8$GlcNAc$_2$ contained three terminal mannoses (Figure 5a-3). The fragment ions $^{0,2}X_{5\alpha' or \ 5\beta}$ (*m/z* 1029.7) and $^{0,2}X_{5\alpha' \ or \ 5\beta}-H_2O$ (*m/z* 1020.7) resulted from a terminal 1-2 linked mannose suggested that the terminal linkage of the 6-arm was a 1-2 linkage (Figure 5a-4).

The fragment ion $^{1,4}X_{3\beta}/Y_{3\alpha}$ suggested that position C6 of the 3-linked internal mannose was not substituted
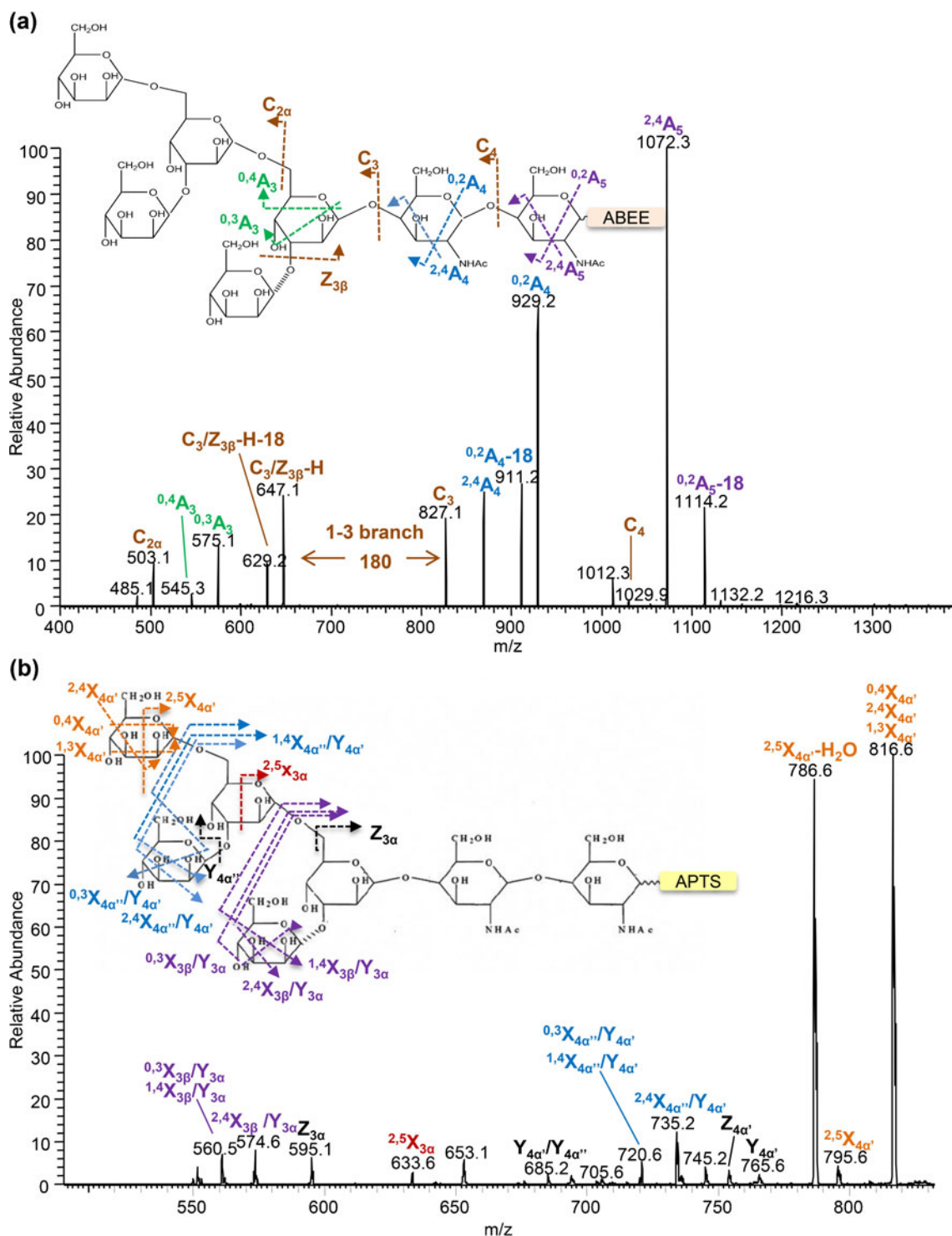
Figure 3. MS² negative ESI mass spectra of ABEE- and APTS-labeled Man₅GlcNAc₂. **(a)** MS² spectrum of ABEE-labeled Man₅GlcNAc₂ ([M − H]⁻, *m/z* 1380.2→). **(b)** MS² spectrum of APTS-labeled Man₅GlcNAc₂ ([M − 3 H + Na]²⁻, *m/z* 846.5→)

(otherwise, a fragment ion would appear two hexoses higher than *m/z* 560.5). Therefore, the internal 1-6 linked mannose was assigned to the second branched sugar (Figure 5a-5). The second linkage of 3-arm was assigned

as an internal 1-2 linkage (Figure 5a-6). Because of the lack of a standard with internal 1-2 linkage, the assignment was based on two sources of indirect evidence. The 1-6 linkage was ruled out as described
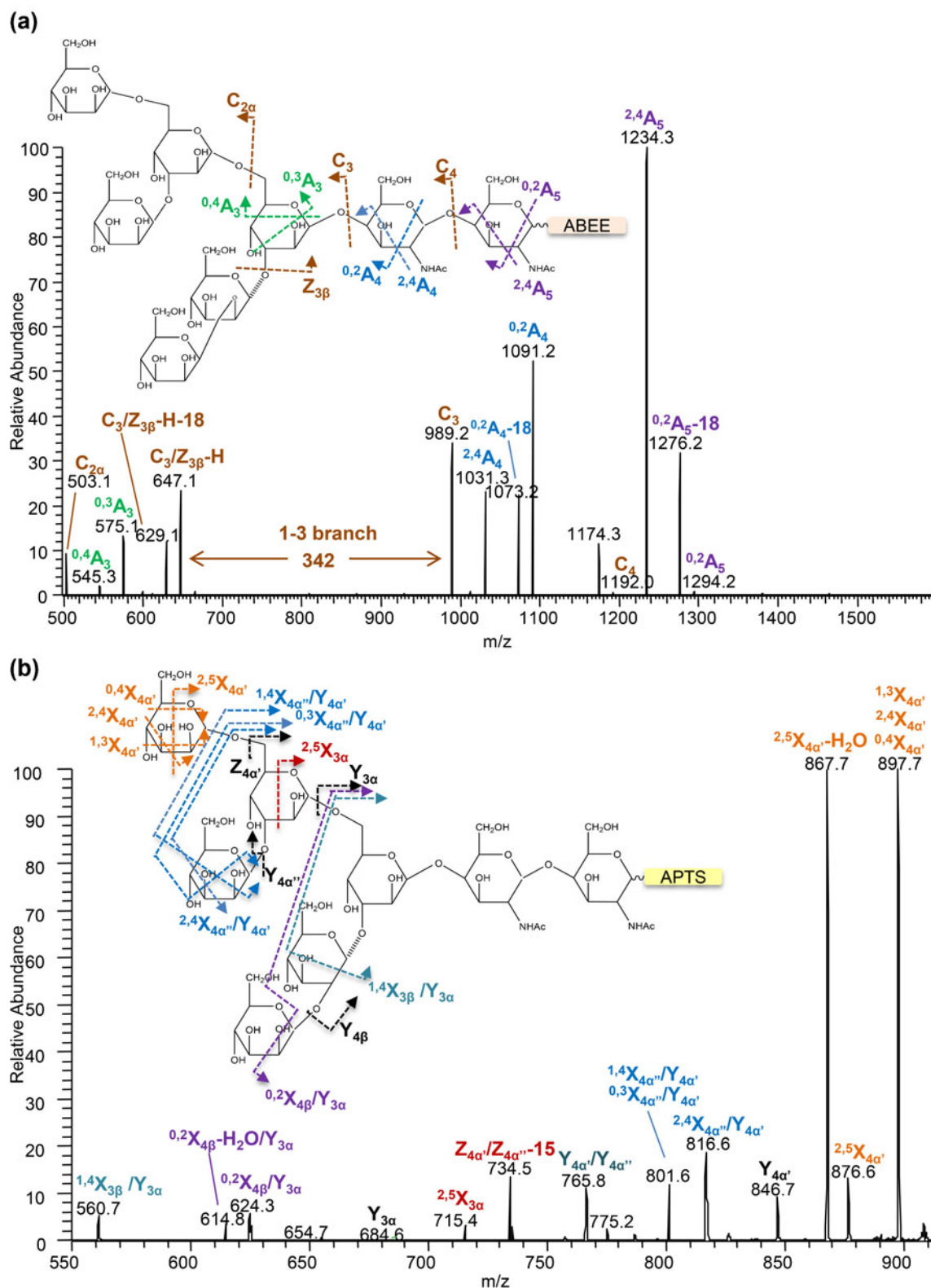
Figure 4. MS^2 negative ESI mass spectra of ABEE- and APTS-labeled Man$_6$GlcNAc$_2$. **(a)** MS^2 spectrum of ABEE-labeled Man$_6$GlcNAc$_2$ ([M − H]$^-$, m/z 1542.3→). **(b)** MS^2 spectrum of APTS-labeled Man$_6$GlcNAc$_2$ ([M − 3 H + Na]$^{2-}$, m/z 927.5→)

in the first sentence of this paragraph. Due to the absence of fragments derived from either internal 1-4 or

1-3 linked mannose on the 3-arm, the second linkage was assigned as a 1-2 linkage. This assignment was also
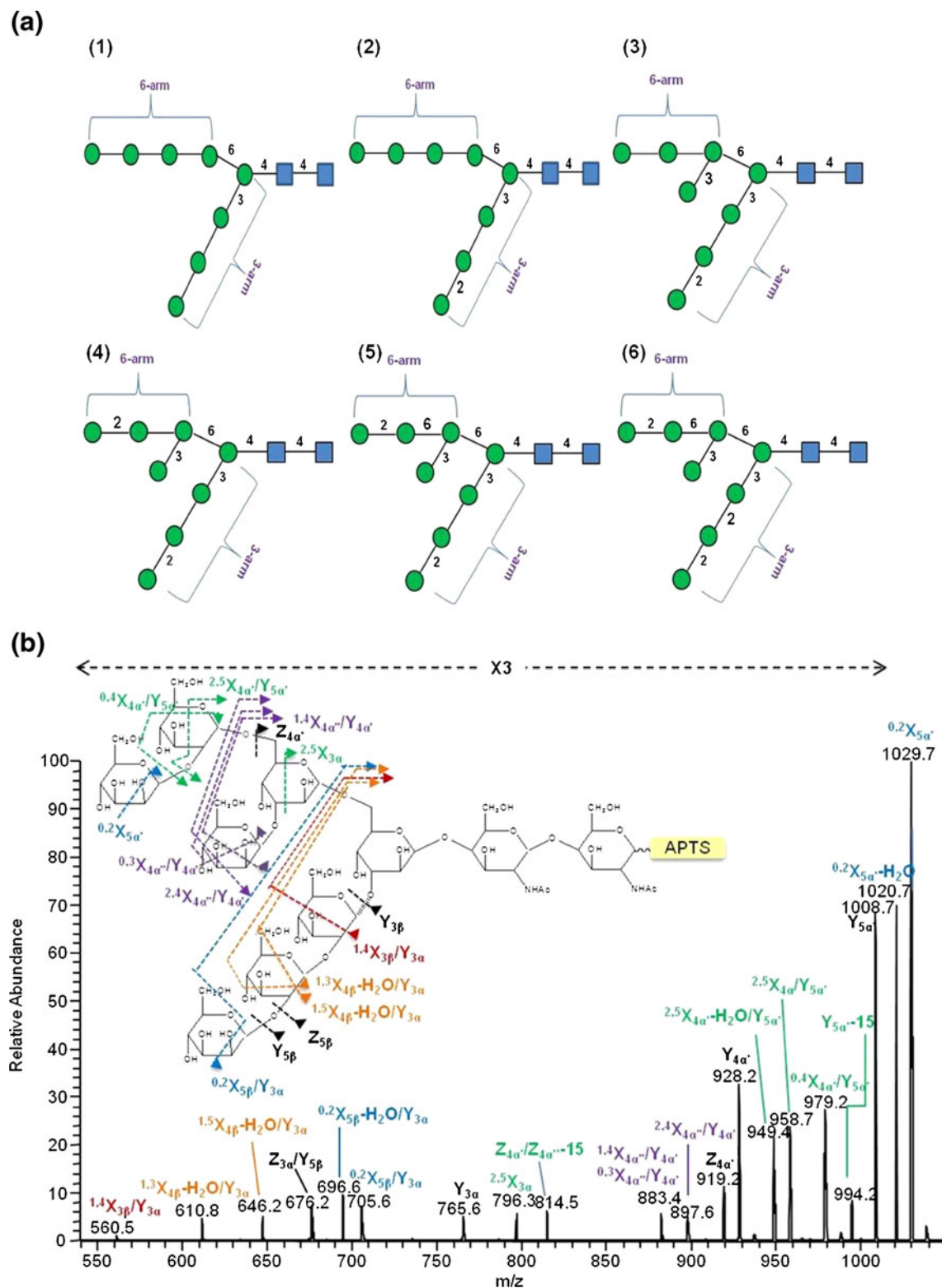
Figure 5. **(a)** Schematic representation of predicted structure of $Man_8GlcNAc_2$ (1)–(6) based on the product ions of APTS-labeled $Man_8GlcNAc_2$. **(b)** MS^2 spectrum of APTS-labeled $Man_8GlcNAc_2$ ($[M - 3H + Na]^{2-}$, m/z 1089.5→)

supported by observation of the $^{1,3}X_{4\beta} - H_2O/Y_{3\alpha}$ (m/z 646.7) and $^{1,5}X_{4\beta} - H_2O/Y_{3\alpha}$ (m/z 610.2) ions, which differ from the characteristic ions of internal 1-3, 1-4, and 1-6 linkages and were tentatively assigned as ions specific to internal 1-2 linkage. As a result, the linkages and branches of $Man_8GlcNAc_2$ were assigned.

Figure 6. (a) Schematic representation of predicted structure of Man$_9$GlcNAc$_2$ (1)–(5) based on the product ions of APTS-labeled Man$_9$GlcNAc$_2$. (b) MS^2 spectrum of APTS-labeled Man$_9$GlcNAc$_2$ ([M − 3 H + Na]$^{2-}$, m/z 1170.3→)

In this analysis of Man$_8$GlcNAc$_2$, it is assumed that the mass spectrum was dominated by the most abundant isomer (84 %). However, it should be cautious with this assumption, because many characteristic ions are of low abundance and the mass spectrum is normalized out of one dimensional plot.

Analysis of Man₉GlcNAc₂ Based on APTS and ABEE-Labeling

The fragment ion $C_{3\alpha}$ (*m/z* 827.1) in MS^2 spectrum of ABEE-labeled Man₉GlcNAc₂ (Supplemental Figure S2) suggested that a pentasaccharide was connected to the 6-arm of the first branch. Other assignments were the same as in Man₈GlcNAc₂, and the predicted structure is shown in Figure 6a-1.

The MS^2 spectrum of APTS-labeled Man₉GlcNAc₂ is shown in Figure 6b. Three terminal 1-2 linked and four internal mannoses (one 1-6, one 1-2, and two 1-3) were observed (Supplemental Table S2). The characteristic ion $^{1,4}X_{4\alpha'}/Y_{4\alpha''}$ (*m/z* 883.5) corresponds to an internal 1-3 linked mannose (second from the terminus) with the loss of a disaccharide. This ion suggested that the 6-arm of the first branched sugar had a second branched sugar, and that a disaccharide was linked to the second branch through a 1-3 linkage (Figure 6a-2). The ions at *m/z* 705.6 ($^{0,2}X_{5\beta}/Y_{3\alpha}$) and 696.6 ($^{0,2}X_{5\beta} - H_2O/Y_{3\alpha}$) were produced from a terminal 1-2 linked mannose with a loss of a pentasaccharide from the 6-arm. It indicated that the terminal linkage of the 3-linked trisaccharide was a 1-2 linkage (Figure 6a-3). The fragment ions $^{0,2}X_{5\alpha'}/Y_{4\alpha''}$ (*m/z* 949.2) and $^{0,2}X_{5\alpha'} - H_2O/Y_{4\alpha''}$ (*m/z* 940.4) correspond to a terminal 1-2 linked mannose with the loss of a disaccharide. The fragment ions $^{0,2}X_{5\alpha' \text{ or } 5\beta}$ (*m/z* 1110.7) and $^{0,2}X_{5\alpha' \text{ or } 5\beta} - H_2O$ (*m/z* 1102.1) correspond to a terminal 1-2 linked mannose with no loss of the sugar. These two series of terminal 1-2 linked fragment ions suggested that the two termini of the second branched sugar were 1-2 linked (Figure 6a-4). The determination of two unassigned linkages in Figure 6b-4 was the same as in Man₈GlcNAc₂. As a result, the linkages and branches were assigned (Figure 6a-5)

Analysis of Man₇GlcNAc₂ Isomers

The Man₇GlcNAc₂ is a mixture of three isomers with similar abundance. The structures of the isomers and the MS^2 spectrum of APTS-labeled Man₇GlcNAc₂ were shown in Supplemental Figure S3. Many product ions were observed and some linkage specific fragments suggested that more than one isomer was present in the sample analyzed. For example, the fragments at *m/z* 898.2 ($^{2,4}X_{4\alpha''}/Y_{4\alpha'}$), *m/z* 883.1 ($^{0,3}X_{4\alpha''}/Y_{4\alpha'}$) and at *m/z* 816.8 ($^{2,4}X_{4\alpha''}/Y_{4\alpha'}$), *m/z* 801.8 ($^{0,3}X_{4\alpha''}/Y_{4\alpha'}$) correspond to a terminal 1-3 linked mannose with the loss of a monosaccharide (isomer I) and a disaccharide (isomer III) from the 6-arm, respectively.

The linkage specific fragments were assigned and summarized in Supplemental Table S3. As can be seen, linkage specific fragments related to the three isomers were observed. However, it should be mentioned that without knowing the structure of the isomers, some fragments could be assigned to different structures. For example, fragments labeled with (X) could be assigned as the linkage specific fragments with an assumptive structure of X. As a result,

when this approach is used to analyze an unknown sample, purification before MS^2 analysis is highly recommended.

Conclusions

Negative-ion ESI-MS/MS analysis of APTS closed-ring labeled oligosaccharides showed that linkages can be assigned for oligosaccharides up to six sugar residues. Unlike the ABEE labeling, the linkage information is mainly derived from the non-reducing end. This complementary information produced by ABEE- and APTS-labeled oligosaccharides was utilized to delineate the linkages and branches of larger oligosaccharides. The analysis of N-linked glycans from ribonuclease B showed that the linkages and branches for Man₅GlcNAc₂, Man₆GlcNAc₂ Man₈GlcNAc₂ and Man₉GlcNAc₂ could be deduced from the combined information obtained from MS^2 of ABEE- and APTS-labeled oligosaccharides. It is worth to mention that low-energy MS^2 instead of high-energy MS^2 or MS^n is used in this study. As a result, this approach can be adopted by laboratories equipped with triple quadrupole, quadrupole time-of-flight (Q/TOF) or ion trap mass spectrometer. High-mannose glycans studied in this approach are well characterized oligosaccharides. There could be many other structural isomers. Isomers not examined in this approach may present identical or very similar mass spectra. Therefore, the assignments may not be fully conclusive when this approach is applied to true unknown.

Acknowledgments

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