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Determination of sequence and linkage of tissue oligosaccharides in caprine β-mannosidosis by fast atom bombardment, collisionally activated dissociation tandem mass spectrometry

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Fast atom bombardment, collisionally activated dissociation tandem mass spectrometry (FAB-CAD-MS/MS), combined with *p*-aminobenzoic acid ethyl ester (ABEE) derivatization, were used to confirm the sequence and linkage pattern of subnanomolar amounts of the previously characterized three major thyroid gland oligosaccharides accumulated in caprine β-mannosidosis. Positive ion FAB-CAD-MS/MS of both the $[M + H]^+$ and $[M + Na]^+$ ions from the ABEE derivatized oligosaccharides produced product ions derived from cleavage of the glycosidic bonds which allowed the sequences to be determined. Several fragments resulting from cleavages across the sugar ring permitted the assignment, in some cases, of the linkage positions between the sugar residues. The natriated molecule yielded several fragments of this type which were not observed when the protonated molecule was selected as the precursor ion. Use of these techniques gave the complete sequence and linkage characterization of the disaccharide and complete sequence and partial linkage information for the two higher oligosaccharides.

Keywords: β-Mannosidosis oligosaccharides, sequence and linkage determination, ABEE derivatives.

Fast atom bombardment, collisionally activated dissociation tandem mass spectrometry (FAB-CAD-MS/MS) using a variety of techniques, has now become well established as a means to obtain carbohydrate sequence information from derivatized and underivatized oligosaccharides and glycoconjugates [1–4]. Although FAB-MS without CAD provides molecular weight information, structurally informative fragment ions are often lacking [5]. Moreover, the sensitivity for underivatized oligosaccharides may be poor because of their inherent hydrophilicity and resulting poor surface activity in the FAB matrix. As an alternative to permethylation [6], Sweeley and coworkers [7] introduced the use of the *p*-aminobenzoic acid ethyl ester (ABEE) derivative, which both enhances the FAB response of the oligosaccharide, because it increases the hydrophobicity of the molecule, and at the same time improves the ease of isolation, due to its UV absorption properties. The FAB spectra of ABEE derivatives typically exhibit prominent peaks representing the protonated and/or natriated molecule, but structural information is limited, since only a few peaks representing cleavage around some of the glycosidic bonds are observed [7]. Gillece-Castro and Burlingame [4] have recently demonstrated that FAB-CAD-MS/MS of $[M + H]^+$ or $[M - H]^-$ ions of this same derivative (and several related, more hydrophobic derivatives) in both the positive and negative modes, respectively, can yield information that permit assignment of the complete sequence of N-linked oligosaccharides from various glycopeptides.

FAB-CAD-MS/MS has been used in the past to determine linkages of permethylated, acetylated and underivatized oligosaccharide isomers [6, 8, 9]; in these cases, however, the distinction among linkage isomers was based on differences in the relative abundance of fragment ions, rather than diagnostic cleavages. Alternatively, linkage positions have been determined by fragments formed by cleavages across the sugar ring [2, 3, 10, 11]. Although collisional activation of the $[M + H]^+$ ion of ABEE

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derivatives readily yields sequence related ions, the formation of ions indicative of the linkage pattern has not been investigated in these derivatives.

Several studies [10, 11] have indicated that FAB-CAD-MS/MS of natriated oligosaccharide molecules produce significantly different, and often more abundant, fragments than the analogous protonated molecule. Orlando *et al.* [11] described how many of these ions provide information about the linkage pattern in the oligosaccharide. The observation of differences in fragmentation behavior between protonated and natriated molecules is similar to those reported for FAB-CAD-MS/MS analyses of protonated and metal cationized peptides [12]. In the latter case, however, fragmentation of the natriated molecule is often reduced.

In this investigation we employed ABEE derivatization, FAB-MS and FAB-CAD-MS/MS of the $[M + H]^+$ and $[M + Na]^+$ ions to determine if the linkages as well as sequence could be obtained from small amounts of several model oligosaccharides accumulated in the tissue of β-mannosidosis affected goats.

Materials and methods

Oligosaccharides were isolated and purified from β-mannosidosis-affected goat thyroid tissues using methods previously described [13]. The ABEE derivatives of the purified oligosaccharides were prepared by reductive amination with *p*-aminobenzoic acid ethyl ester (ABEE) using the method of Wang *et al.* [7] as modified by Matsuura and Imaoka [14]. The ABEE derivatives were purified by ether extractions and chromatography (SepPac C-18 cartridges, Waters) [14]. FAB-MS of ABEE derivatized oligosaccharides was performed by placing a mixture of approximately 1 µl of an aqueous oligosaccharide solution (100 pmol to 1 nmol μl^{-1}) mixed with 2 µl of thioglycerol matrix on the FAB probe tip. Ions were produced by bombardment with a 6 keV beam of Xe atoms in a JEOL HX-110 double focusing mass spectrometer operating in the positive ion mode. The accelerating voltage was 10 kV and the resolution was set at 3000. For CAD-MS/MS, helium was used as the collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the abundance of the precursor ion $[M + H]^+$ or $[M + Na]^+$, by 50%. A JEOL DA-5000 data system generated linked scans at constant *B/E* ratio. Additional FAB-CAD-MS/MS experiments were conducted on a JEOL HX110/HX110 four sector tandem mass spectrometer. The precursor ion was selected by MS-1 at a resolution of 1000. A floated collision cell (3 kV) located in the third field-free region between MS-1 and MS-2 provided a collision energy of 7 keV. Helium was used as the collision gas and the pressure was adjusted to reduce the precursor ion abundance by 75%. MS-2 was scanned at a constant *B/E* ratio to acquire the MS/MS spectra.

Results and discussion

The linkages and sequences of the three major oligosaccharides which accumulate in the kidney and thyroid tissues of β-mannosidosis affected goats have previously been characterized by several techniques, including MS analysis as their partially permethylated alditol acetates. These compounds were identified as a disaccharide, (Manβ1-4GlcNAc), trisaccharide (Manβ1-4GlcNAcβ1-4GlcNAc) and pentasaccharide (Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc) [13, 15, 16–18].

Following the preparation of the ABEE derivative FAB-MS analysis confirmed the molecular weights of these oligosaccharides by displaying ions representing the protonated molecule and/or the sodium adduct (data not shown). Despite rigorous attempts to desalt the samples, the $[M + Na]^+$ adduct was present in all spectra. The abundance of the natriated molecule in the spectrum relative to the $[M + H]^+$ ion increased with the size of the oligosaccharide. Although a few fragment ions representing cleavage around the glycosidic oxygen were observed in the FAB spectra, complete sequencing was not possible. These results are similar to previous FAB spectra of ABEE oligosaccharides [7] and other glycoconjugates [2, 3]. In order to enhance the formation of structurally informative fragment ions FAB-CAD-MS/MS analyses of both the $[M + H]^+$ (disaccharide and trisaccharide) and the $[M + Na]^+$ (trisaccharide and pentasaccharide) ions were performed.

FAB-CAD-MS/MS of the $[M + H]^+$ ion of the ABEE-disaccharide at *m/z* 533 (Fig. 1) produced a prominent product ion at *m/z* 371 (Y_1), representing loss of hexose by cleavage of the glycosidic bond on the nonreducing side of the oxygen atom with hydrogen transfer. The nomenclature follows that of Domon and Costello [19] where A, B, and C represent fragments with charge retained on the nonreducing end; and X, Y, and Z represent fragments with charge on the reducing (ABEE) end of the compound. Both A and X type fragments are formed by carbon–carbon or carbon–oxygen bond cleavages across the sugar ring. Superscripts indicate the bond(s) cleaved and subscripts refer to the sugar position within the oligosaccharide. Another fragment, at *m/z* 353 (Z_1), is observed which represents cleavage on the reducing side of the glycosidic bond. Secondary fragmentation of the Y_1 fragment with subsequent loss of EtOH (−46) and $COOCH_2CH_2$ (−72, −COOEt with H transfer) yielded peaks at *m/z* 325 and 299, respectively. In addition to these sequence-related ions, some of the product ions formed by bond cleavages across the sugar residues gave diagnostic linkage information. The fragment ion at *m/z* 399 ($^{1,5}X_1$) is a frequently observed type of cross-ring fragment [2, 3, 19] that does not provide significant linkage data, but does help confirm the sequence given by the glycosidic cleavage fragments. On the other hand, the fragment ion at *m/z* 277 (designated $^3X_0$, since

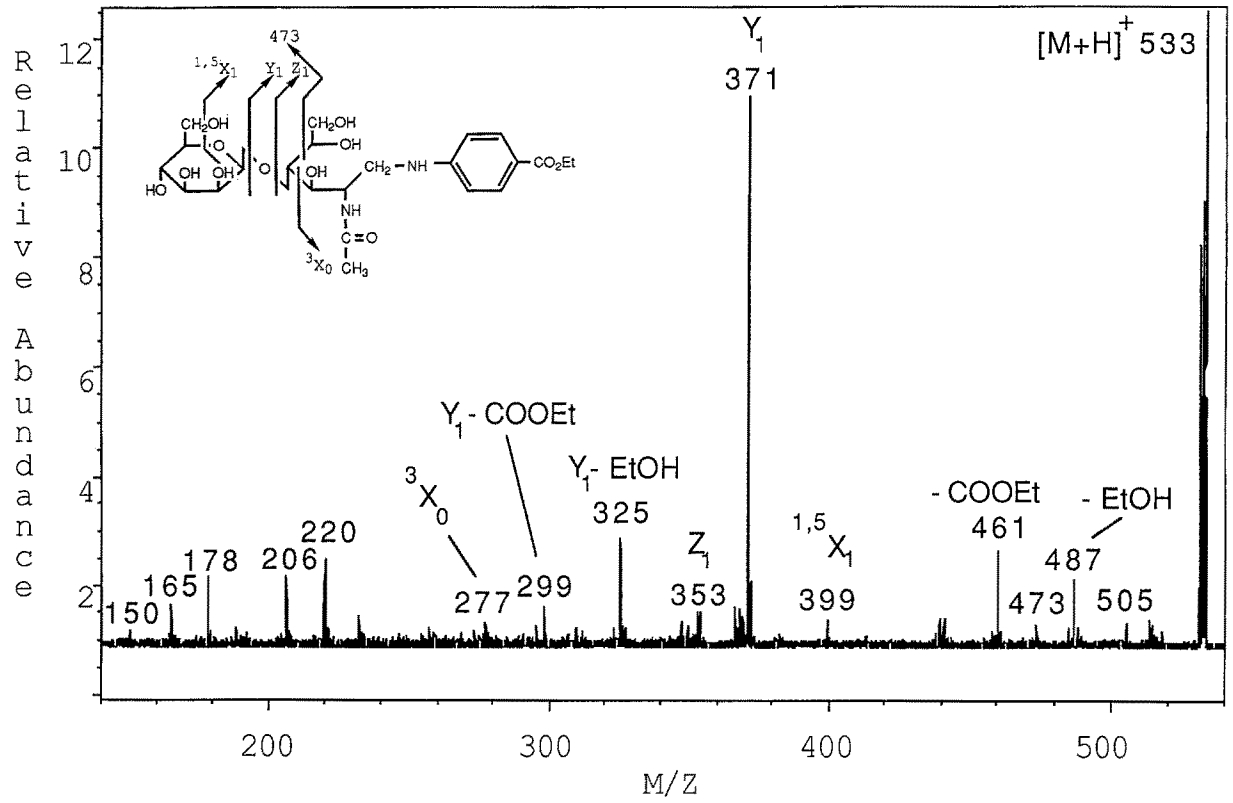

Figure 1. FAB-CAD-MS/MS of the $[M + H]^+$ ion of the disaccharide Manβ1-4GlcNAc. The ions are labeled according to the nomenclature of Domon and Costello [19]. Fragments labeled $-$COOEt represent the loss of COOEt with a proton transfer ($-COOCH_2CH_2$, -72 mass units).

only one bond in the ring is broken) suggests that the terminal hexose cannot be attached through the 3 position. An additional small fragment at *m/z* 473, representing cleavage between C-4 and C-5 of the *N*-acetylglucosamine residue and loss of a $C_2H_5O_2$ fragment, indicates that the linkage of the terminal hexose must be through the 4 position. The disaccharide can thus be characterized as Hexose 1-4-*N*-acetylhexosamine (Hex1-4HexNAc). Some other structurally noninformative peaks are present in the spectrum which arise from cleavage within the ABEE residue. Among these are the peaks at *m/z* 150, 165 $[H_2N\phi COOEt]^{+\cdot}$, and 178 $[CH_2{=}N^+H\phi COOEt]$, which retain charge on the lost ABEE fragment, and peaks at *m/z* 461 (loss of COOEt with H transfer) and 487 (loss of EtOH).

FAB-CAD-MS/MS of the $[M + H]^+$ ion (*m/z* 736) of the ABEE-trisaccharide is shown in Fig. 2. Product ions at *m/z* 574 (Y_2) and 556 (Z_2) show that the residue at the nonreducing terminal is hexose. That the middle residue is an *N*-acetylhexosamine is clearly indicated by glycosidic cleavage fragments at *m/z* 371 (Y_1) and *m/z* 353 (Z_1). A complementary set of product ions at *m/z* 366 (B_2) and *m/z* 384 (C_2) with charge retention on the nonreducing end of the molecule do not indicate the sequence of the two nonreducing sugars, but they do confirm their total weight. By difference from the $[M + H]^+$ ion at *m/z* 736 it can be deduced that the reducing end sugar is another *N*-acetylhexosamine. Two cross-ring fragments, designated $^{1,5}X_2$ and $^{1,5}X_1$, at *m/z* 602 and 399, do not provide a great deal of structural insight, but they support the sequence assignment. Note that although the intensity of the $^{1,5}X_2$ ion is rather low in the spectrum (Fig. 2), the secondary loss of 72 mass units from this ion ($-$COOEt + H) to form the fragment at *m/z* 530 is indicative of its presence. An additional fragment at $^4X_0$ (*m/z* 676) shows that the second sugar is linked to the four position of the reducing sugar. The absence of analogous fragments from the middle sugar does not permit the complete linkage of the terminal middle hexose to be determined. Thus, the trisaccharide can be partially characterized as Hex1-?HexNAc1-4HexNAc.

A significantly different FAB-CAD-MS/MS spectrum is obtained from the $[M + Na]^+$ ion of the same ABEE derivatized trisaccharide (Fig. 3). Here analogous fragment ions were observed, but these were usually natriated (e.g., $Y_1 + Na - H$, $^{1,5}X_1 + Na$). For simplicity, the fragments are described using the same nomenclature as above, without including the addition of sodium. The spectrum revealed the presence of the natriated product ions at *m/z* 596 (Y_2), 578 (Z_2) and 393 (Y_1). These represent cleavages of the glycosidic bonds, allowing the sequence to be determined as Hex-HexNAc-HexNAc. A complementary set

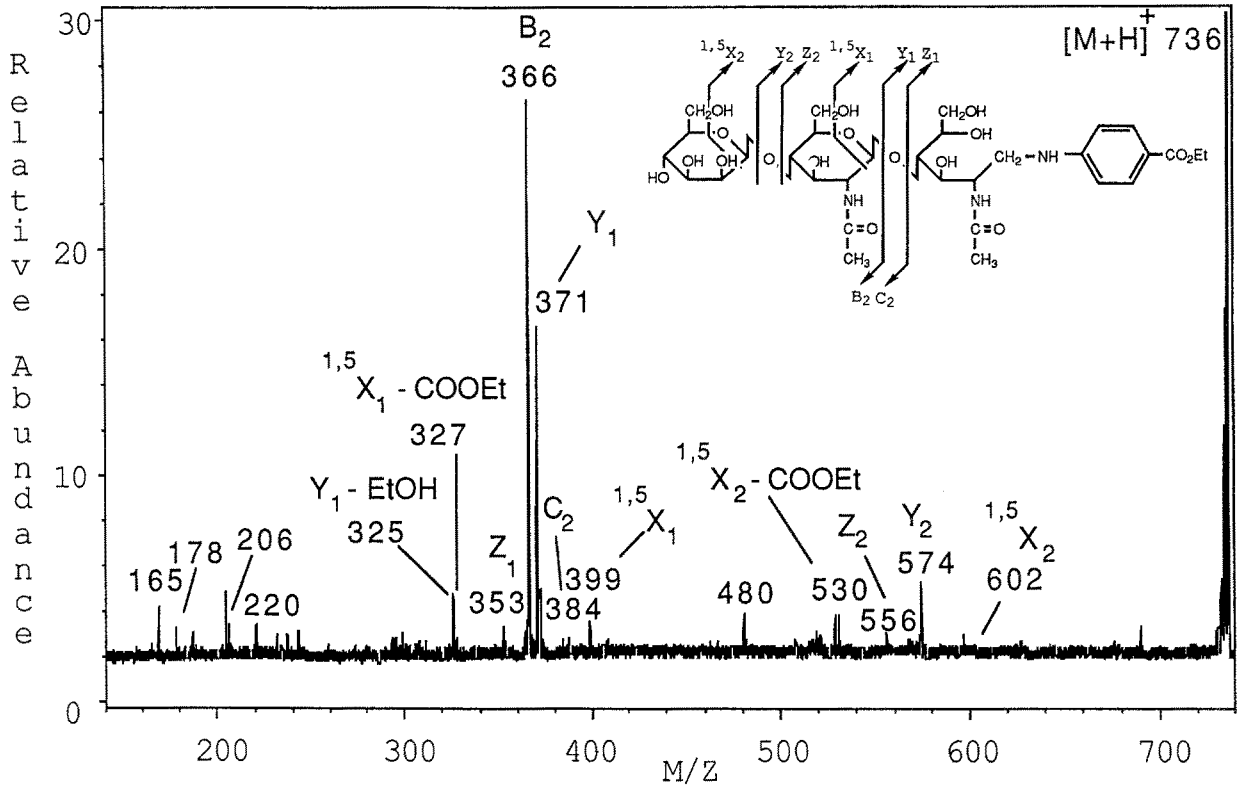

Figure 2. FAB-CAD-MS/MS of the $[M + H]^+$ ion of the ABEE derivative of the trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc.

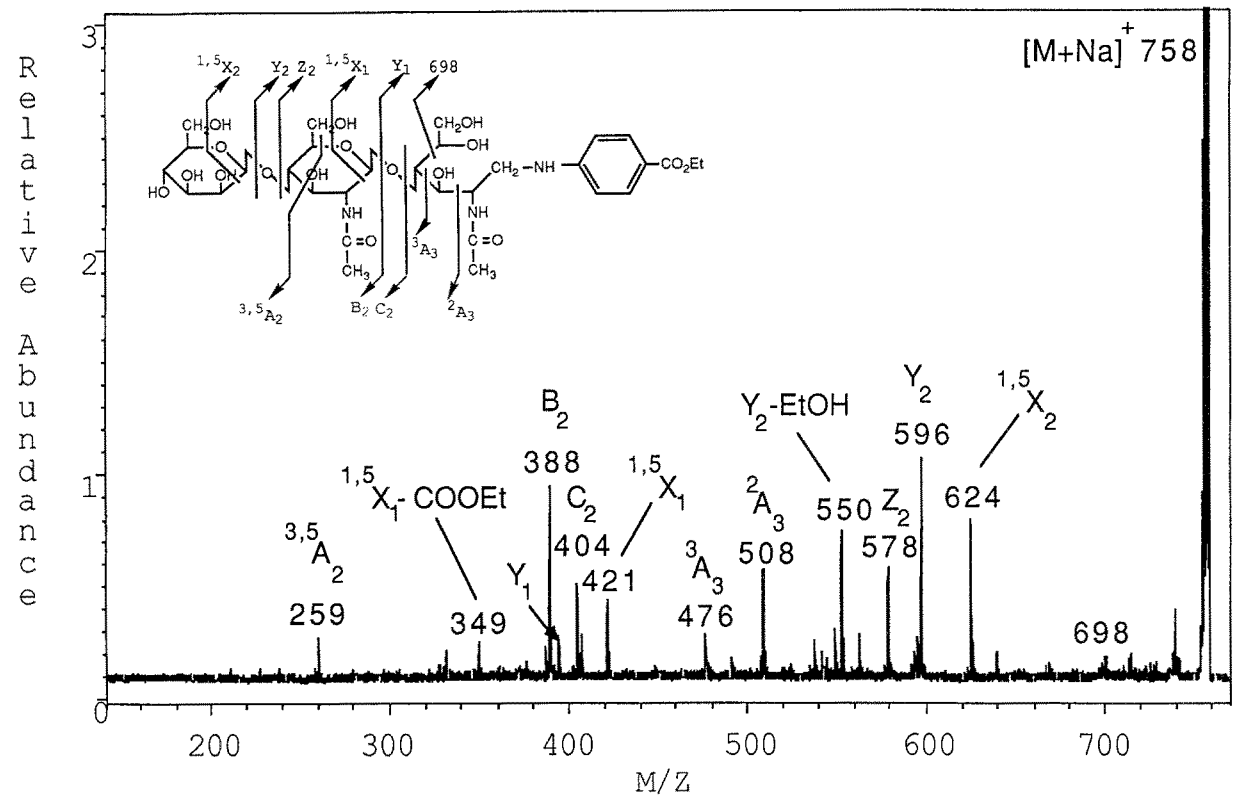

Figure 3. FAB-CAD-MS/MS of the $[M + Na]^+$ ion of the ABEE derivative of the trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc. Ring cleavage peaks (A ions), not seen in the spectrum of the $[M + H]^+$ ion (Fig. 2), provide additional linkage information.

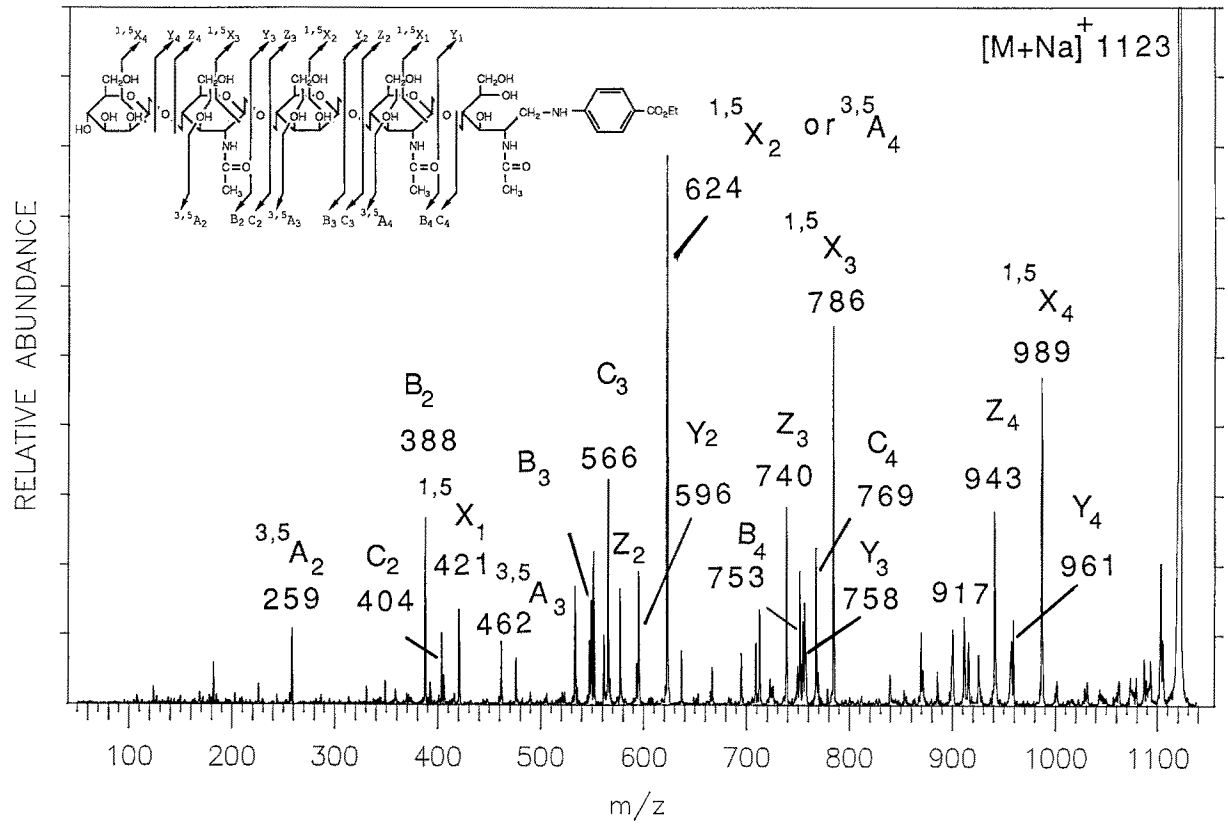

Figure 4. FAB-CAD-MS/MS of the $[M + Na]^+$ of the ABEE-pentasaccharide Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc, acquired on a four-sector tandem mass spectrometer. For simplicity, a number of peaks are unlabeled. These are secondary fragments for the most part and include, for example, $^{1,5}X_n$ $-$COOEt + H (-72) at *m/z* 349, 552, 714 and 917.

of fragment ions with charge retention on the nonreducing end of the molecule at *m/z* 388 (B_2) and 404 (C_2) again indicates the composition of the nonreducing terminal sugars. The sequence is further supported by the ions at *m/z* 624 ($^{1,5}X_2$) and 421 ($^{1,5}X_1$). Cross-ring fragment ions giving linkage information are also present. The product ions $^3A_2$ (*m/z* 508), $^3A_3$ $-$H (i.e., $^3A_3$ + Na $-$ 2H, *m/z* 476) and *m/z* 698 suggest that the second sugar is linked to the four position of the reducing sugar. The terminal nonreducing end sugar is shown to be linked either through the 4 or 6 positions of the middle sugar by the $^{3,5}A_2$ ion at *m/z* 259. Thus, the trisaccharide can be partially characterized as Hex1-4HexNAc1-4HexNAc or Hex1-6HexNAc1-4HexNAc by the FAB-CAD-MS/MS spectrum of the natriated molecular ion alone. One striking difference in the spectra of the $[M + H]^+$ and $[M + Na]^+$ precursor ions is the presence in the latter of cross-ring fragments with charge retention on the nonreducing end of the molecule (A_n ions). These useful fragments are not commonly encountered in positive ion mode FAB-CAD-MS/MS spectra of oligosaccharides [4, 18].

Positive ion FAB-CAD-MS/MS of the $[M + Na]^+$ precursor ion from the ABEE derivatized pentasaccharide was obtained on a four-sector tandem mass spectrometer (Fig. 4). Comparative analyses of the previous compounds on two-sector and four-sector mass spectrometers showed that both instruments produced similar spectra, though the signal/noise ratio and accuracy of product ion mass assignments was superior in the latter due to better precursor ion resolution and transmission. These differences were most significant in the FAB-CAD-MS/MS spectra of the ABEE-pentasaccharide.

The spectrum of the $[M + Na]^+$ ion revealed the presence of fragment ions at *m/z* 961 (Y_4) and *m/z* 943 (Z_4), indicating that hexose is the nonreducing terminal sugar. From the ions at *m/z* 758 (Y_3) and *m/z* 740 (Z_3) it follows that the next sugar in the sequence is a HexNAc. The B_2 and C_2 products at *m/z* 388 and 404 support this conclusion. The set of glycosidic bond cleavage fragments present at *m/z* 596 (Y_2) and *m/z* 578 (Z_2), along with their complements at *m/z* 566 (C_3) and *m/z* 550 (B_3), provide evidence for a hexose in the next position. The sequence ion series from the nonreducing terminus (giving Hex-HexNAc-Hex) is followed by differences of 203 mass units. The next sequence ion, at *m/z* 393 (Y_1), is not abundant, but its complement at *m/z* 769 (C_4) is intense, indicating that the penultimate sugar is a HexNAc (thus, a Hex-HexNAc-Hex-HexNAc sequence). This together with the $[M + Na]^+$ ion at *m/z* 1123, allows the reducing terminal sugar to be determined as HexNAc and the full sequence to be elucidated as

Hex-HexNAc-Hex-HexNAc-HexNAc. Ring cleavages at *m/z* 989 ($^{1,5}X_4$), 786 ($^{1,5}X_3$), 624 ($^{1,5}X_2$) and 421 ($^{1,5}X_1$) are in accord with this sequence. It is clear that other cross-ring fragments which provide additional linkage information are found less frequently in the spectra of larger ABEE-oligosaccharides. Nevertheless, ions in the $^{3,5}A_n$ series indicated that the nonreducing terminal Hex and HexNAc were both either 1-4 or 1-6 linked ($^{3,5}A_2$ at *m/z* 259 and $^{3,5}A_3$ at *m/z* 462) and that the HexNAc in turn was linked to either the 4 or 6 position of the adjacent sugar (Hex) in this oligosaccharide. The ion at *m/z* 624 probably represents $^{3,5}A_4$ as well as the isobaric $^{1,5}X_2$ fragment, which would indicate the penultimate sugar is also 4 or 6 linked. Thus, the pentasaccharide linkages can at least be characterized partially by FAB-CAD-MS/MS of the $[M + Na]^+$ ion. In comparison, only sequence information can be determined by FAB-CAD-MS/MS of the $[M + H]^+$ ion of this pentasaccharide (data not shown).

The combination of ABEE derivatization and FAB-CAD-MS/MS allows for easy isolation and rapid characterization of small quantities of oligosaccharides. Significant structural information can be obtained from small quantities of oligosaccharides isolated from biological sources using these methodologies. In particular, FAB-CAD-MS/MS yields product ions from both the $[M + H]^+$ and $[M + Na]^+$ ions that represent cleavages around the glycosidic bonds, permitting sequence determinations. Fragments representing charge retention on both the reducing and nonreducing ends are observed. The formation of the latter is especially enhanced when the $[M + Na]^+$ precursor ion is selected. Among the most important of these product ions are those formed by cleavage across the sugar rings, which allow the linkages to be determined. In favorable cases, complete structural characterization can be obtained from cross-ring fragments. Further studies are required to determine if this approach is applicable to a wider variety of structural types. However, it is clear that complete linkage determinations, especially of larger oligosaccharides, will still require traditional methods involving the preparation of partially permethylated alditol acetates and analysis by GC/MS.

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