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Carbohydrate Research 313 (1998) 145–155

CARBOHYDRATE RESEARCH

# Matrix-assisted laser desorption/ionization mass spectrometry of neutral and acidic oligosaccharides with collision-induced dissociation

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Received 8 May 1998; revised 17 August 1998; accepted 20 September 1998

**Abstract**

Using ribonuclease B and human $\alpha_1$-acid glycoprotein (AGP) as model glycoproteins, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry with collision-induced dissociation (CID) is validated here as an effective tool for oligosaccharide sequencing. The spectra acquired for high-mannose and complex oligosaccharide structures show characteristic fragments resulting from cleavages of the glycosidic bonds and a few cross-ring cleavages. Esterification of the sialic acid residues is essential in stabilizing the acidic *N*-linked oligosaccharides. An important analytical feature observed in all acquired spectra is the occurrence of cleavages on the same antenna up to the branching point, as deduced from the absence of fragmentation due to the simultaneous cleavages on two or more antennas. 

*Keywords:* MALDI-CID; Human AGP; Esterification of sialic residues; Ribonuclease B; Maltoheptaose

## Introduction

A complete structural characterization of glycans in complex biomolecules (such as glycoproteins) has long been viewed as essential to a better understanding of their roles in biochemical processes. This task is complicated by many methodological difficulties associated with the structural complexity of such biomolecules and a frequent limitation of adequate sample size. Consequently, numerous research efforts have been directed toward the development of sensitive techniques for the determination of carbohydrate sequence, branching and linkage forms.

Toward the isolation of enzymatically or chemically cleaved glycoprotein oligosaccharides, their fractionation, and display into an oligosaccharide map, modern separation methods (such as reversed-phase liquid chromatography, high-pH anion-exchange chromatography, size-exclusion chromatography and, more recently, capillary electrophoresis) have become indispensable. However, such methodologies do not provide structural information without coupling to powerful spectroscopic methods such as high-field nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). While NMR has been highly effective in providing detailed structural information on carbohydrate molecules, milligram sample quantities are typically required. Additionally, interpretation of the NMR data for complex oligosaccharides is far from trivial. An alternative ap-

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0008-6215/98/$ - see front matter 
PII: S0008-6215(98)00264-X

proach to NMR is tandem mass spectrometry (MS-MS), which has been shown useful in aiding complete sequence and branching information on native or derivatized complex carbohydrates that can be ionized by fast atom bombardment (FAB) or electrospray (ES) techniques [1–6]. More recently, matrix-assisted laser desorption/ionization (MALDI) MS has also been found effective at ionizing oligosaccharides [7,8].

The utility of MALDI for the analysis of oligosaccharides has become particularly appealing since the introduction of in-source decay on a magnetic sector instrument [9], post-source decay on a reflectron time-of-flight (TOF) instrument [9–11], a collision-induced dissociation (CID) cell on a magnetic sector instrument fitted with orthogonal TOF analyzer [9], and a CID process in an external source Fourier-transform mass spectrometer equipped with MALDI [12]. These somewhat different approaches have provided the means to fragment the analyzed oligosaccharides, leading, in principle, to the determination of sequence, branching and linkage forms. Most MALDI-based techniques provide adequate analytical sensitivity (in the MS-MS mode) in the nanomole to picomole range.

This paper evaluates the patterns of MS fragmentation for the oligosaccharides cleaved from some glycoproteins that were studied extensively (by a variety of analytical techniques) on previous occasions [13–15]. In using the oligosaccharide examples from model systems (ribonuclease B and $\alpha_1$-acid glycoprotein (AGP)) and linear maltoheptaose as a standard, we wish to validate the use of MALDI-TOF instrumentation, provided with a CID cell, for sequencing studies. These examples provide a representative cross-section of structural types: the high-mannose structures derived from ribonuclease B; desialylated *N*-linked biantennary, triantennary and fucosylated triantennary types derived from AGP; and the same structures from AGP, now with their protected (esterified) sialic residues. The fragmentation patterns are discussed and compared with those observed in previous studies.

## Experimental

*Materials.*—The model glycoproteins, human AGP and ribonuclease B, were obtained from Sigma Chemical (St. Louis, MO). Maltoheptaose and neuraminidase ([EC 3.2.1.18] from *Arthrobacter ureafaciens*) were also obtained from Sigma. The recombinant enzyme *N*-glycosidase F (PNGase F) from *Esherichia coli* [EC 3.2.2.18] was purchased from Boehringer Mannheim (Indianapolis, IN). All remaining chemicals were from Aldrich (Milwaukee, WI).

*Enzymatic cleavages.*—The *N*-linked oligosaccharides studied in this work were enzymatically released from their respective glycoproteins. The glycoproteins to be digested were reconstituted in 0.05 M sodium phosphate buffer (pH 7.5), followed by addition of PNGase F (5 mU/0.1 mg glycoprotein). The samples were subsequently incubated for 24 h at 37 °C.

The enzymatically released oligosaccharides were recovered by applying the digestion mixtures to $C_{18}$ Sep-Pak cartridges (Waters, Milford, MA) that were preconditioned with methanol, acetonitrile, and aqueous methanol (1:19 v/v). The oligosaccharides were eluted with 3 mL of the aqueous methanol solution, and lyophilized or dried under a stream of nitrogen.

Some acidic oligosaccharides were desialylated after reconstitution of the oligosaccharides in 50 mM sodium phosphate buffer (pH 6.0), followed by the addition of neuraminidase (ca. 20 mU for each nanomole of substrates) and incubation for 12 h.

*Esterification of acidic oligosaccharides.*—The sialic acid groups of acidic oligosaccharides were esterified according to the procedure described recently by Powell and Harvey [16]. Briefly, the acidic oligosaccharides dissolved in water were applied to a short column containing Dowex-50W (Sigma Chemicals) resin that had been preconditioned with 1 M sodium hydroxide and water. The eluent was then lyophilized and dissolved in dry dimethylsulfoxide ($Me_2SO$). Methyl iodide was added and the mixture was thoroughly mixed and allowed to react at room temperature for 2 h. The reaction mixture was dried

under a stream of nitrogen and desalted by passing through a short column (made from a 10-mL plastic syringe) containing a mixture of cation- and anion-exchange resins. The eluent was lyophilized and redissolved in deionized water.

*Sample and matrix preparation for MALDI-TOF studies.*—The MALDI matrix used in this study was arabinosazone, a new matrix reported recently by our laboratory [8]. The samples were prepared by spotting 1.0 μL of esterified or desialylated samples on a polished stainless-steel sample plate, followed by the addition of 1.0 μL of the matrix, dissolved in ethanol/water (3:1 v/v) at a final concentration of 10 mg/mL. The spot was allowed to dry at room temperature.

*Instrumentation.*—Mass spectra were acquired on a Voyager-DE™ RP Biospectrometry™ Workstation (PerSeptive Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser (337 nm). The instrument can operate in both the reflector and linear modes and has delayed ion-extraction capability. Moreover, the instrument is equipped with a collision cell, with argon being used as the collision gas. The precursor ion was selected by the timed ion selector, with a mass window of ca. 45 $m/z$. The CID spectra were acquired at 25 kV accelerating voltage, while the reflectron voltage was decreased successively by 10%, allowing the detection of certain fragments at a time. Generally, 10–12 segments were acquired at 100 scans for each segment and the composite CID spectrum was a combination of all acquired segments, as generated by the instrument software. The instrument was calibrated externally with the maltose ladder. All acquired spectra were smoothed by applying the 19-point Savitzky-Golay routine [17].

## Results and discussion

It has been observed in the FAB mass spectra of *N*-linked oligosaccharides that sequence-specific fragments are often absent, or are in low abundance, suggesting some influence of the carbohydrate composition and structure on the fragmentation process [18,19]. In contrast, a similar effect was not observed in the case of linear oligosaccharides. It thus appears that highly branched oligosaccharides tend to fragment less than linear structures under the same conditions. Moreover, branched fragments tend to occur in higher abundance than the linear ones [18,19].

To evaluate fully the effect of carbohydrate composition and branching on the fragmentation of oligosaccharides by MALDI-CID, a series of linear and branched oligosaccharides was studied in this work. The branched oligosaccharides first included the esterified, acidic *N*-linked oligosaccharides cleaved from human AGP. The desialylated forms of these oligosaccharides were also included together with high-mannose oligosaccharides for a comparison. Since the parent ion dissociation can be accomplished through either a post-source decay or a CID process with our instrument, we briefly compared the merits of these procedures. As the CID approach appeared universally more effective, especially for low-mass fragments, collision-induced fragmentation was utilized for the reminder of this study.

*Linear neutral oligosaccharides.*—The CID spectrum of maltoheptaose, the corresponding structure and its fragmentation are shown in Fig. 1. The nomenclature for fragmentation utilized here was developed by Domon and Costello [20] for the identification of the oligosaccharide fragments in mass spectrometry. For a linear oligosaccharide with a repeating structural unit, each fragment can result from a loss of a hexose unit at either end of the molecule. The presence of all expected fragments with almost the same abundance indicates the ease of fragmentation of such a linear structure. Moreover, the rarely observed $A_i$ fragment, which is formed in two steps involving a ring-opening at the reducing end followed by a retro-aldol rearrangement [21], is also observed in high abundance (Fig. 1). Cross-ring cleavages are known to occur predominantly at the molecules' reducing end [21,22]. This behavior is due to the fact that ring-opening, which is believed to occur in the case of such fragmentation, becomes hindered at the non-reducing end through the presence of the protected anomeric oxygen. Neverthe-

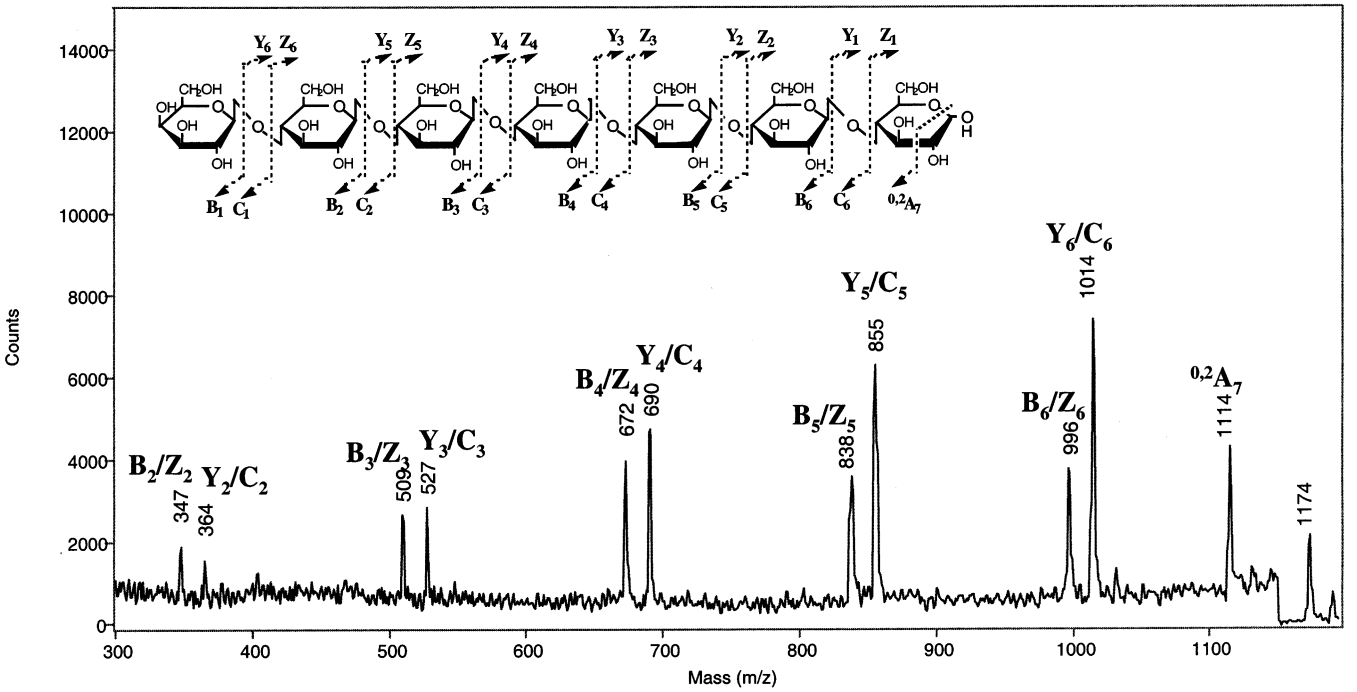


Fig. 1. MALDI-CID spectrum of maltoheptaose. The $m/z$ values correspond to the monoisotopic masses of cationized compounds.

less, other cross-ring fragmentations such as X's [20] were not detected here, suggesting the lack of high collision energy needed for such fragmentations. However, the collision cell used here provided sufficient energies for the cleavage of glycosidic bonds. Note that all observed masses are due to the sodiated ion form (i.e., $[M+Na]^+$) as a result of the sodium-rich matrix used [8].

*Branched neutral oligosaccharides.*— Branching of oligosaccharides causes a decrease in the number of fragmentations, as can be seen in the CID spectrum of oligomannose 5 cleaved from ribonuclease B (Fig. 2). The dominant fragments observed in this case were at $m/z$ 1156.6 and 1037.1, resulting from the cross-ring fragmentation at the reducing end and the loss of the terminal *N*-acetylglucosamine residue, respectively. In agreement with the previously reported FAB studies [23], the loss of terminal *N*-acetylglucosamine was expected to be very favorable, thus resulting in the high abundance of the fragment. This effect also accounts for the difference in abundance between the B fragments and Y fragments. We have observed many other fragmentations in the CID spectrum of Man5, as summarized in Fig. 2. Designation 'x' is used to indicate the occurrence of fragmentation at either of the equivalent (a or b) antennas, while '/' indicates multiple fragmentation. If more than one structure is assignable to the same $m/z$, the structural alternative resulting in a fragment with more branching points is postulated to be the correct fragment. This prediction is based on the aforementioned observation and the fact that branched molecules tend to be more resistant to fragmentation [22]. Moreover, if a fragment can be obtained by either single or multiple fragmentation, single fragmentation is automatically assumed to be the source of such a fragment, since multiple fragmentation requires higher energy. However, this is not true if the multiple fragmentation path results in a fragment with higher branching points. Note that the differences in abundance between $m/z$ 1037.16 and 1054.71, and between 832.86 and 852.013, are due to the fact that B ($m/z$ 1037.16 and 832.86) fragmentation requires less energy than C ($m/z$ 1054.71 and 852.013) fragmentation, which involves cleavage of the glycosidic bond followed by H-transfer and protonation [20]. Moreover, the abundance of a fragment is a function of the number of cleavages required to obtain specific fragmentation. As a

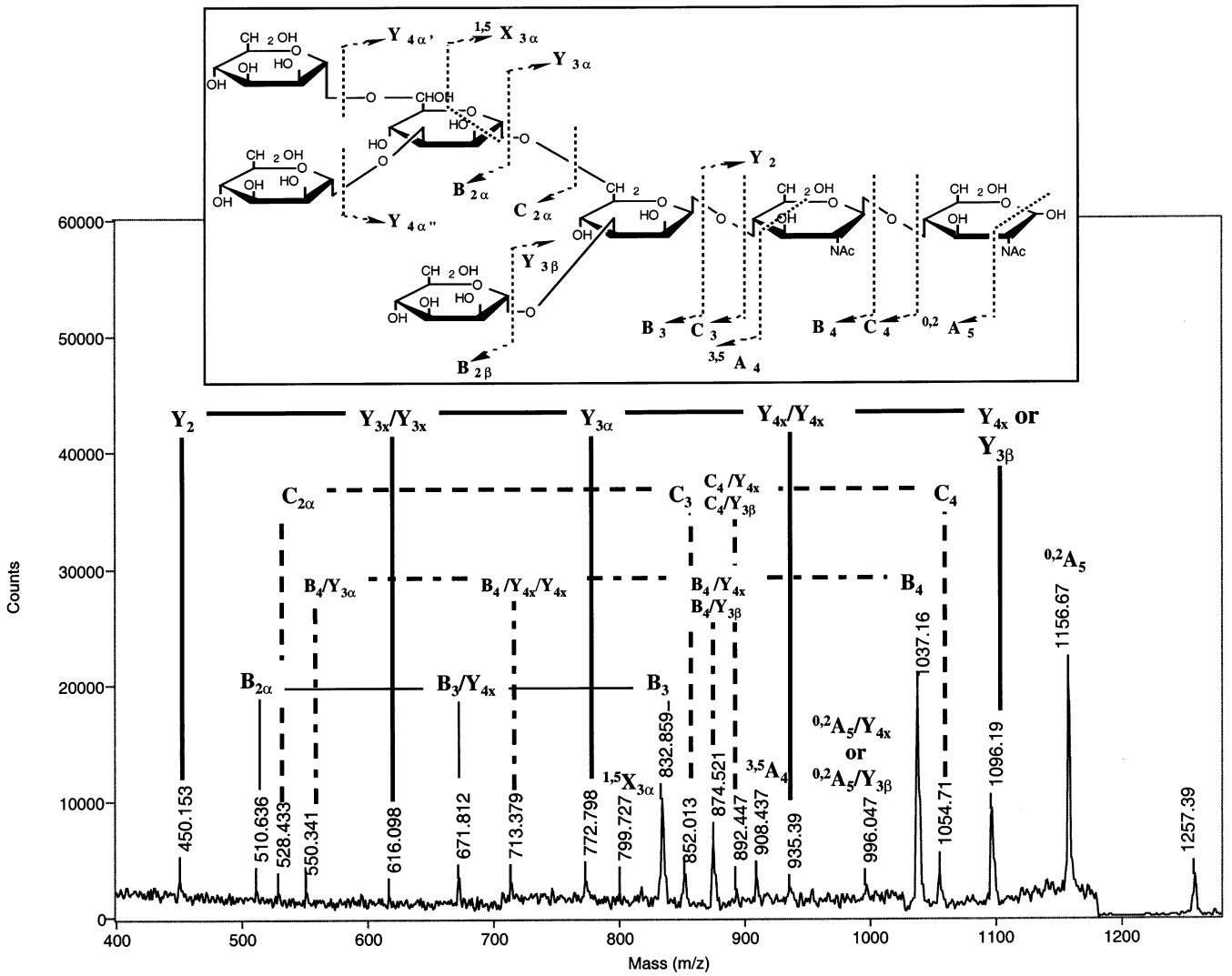


Fig. 2. MALDI-CID spectrum of *N*-linked oligomannose 5 derived from ribonuclease B. The *m*/*z* values correspond to the monoisotopic masses of cationized compounds.

result, the fragments resulting from multiple cleavages are usually in lower abundance than those originated from single cleavages.

Few cross-ring fragmentations were observed in the CID spectrum of Man5, as shown in Fig. 2. This result is in disagreement with the results reported recently by Küster et al. [24] who observed a large number of cross-ring fragments. This difference is explainable by the use of a different collision gas (xenon, compared with argon used in this study). The use of a heavier gas should result in a higher center-of-mass collision energy, thus favoring the occurrence of the high-energy requiring cross-ring fragmentation. The fragments observed in the CID spectrum of Man5 are depicted in Fig. 2 according to the type of fragmentation. It is demonstrated that all possible Y and B fragments are observed, in addition to a few C fragments which originated from the cross-ring fragmentation (A and X).

The other *N*-linked oligosaccharides studied in this report were of the complex type, featuring those having *N*-acetylglucosamine and galactose attached to the mannose core. These types of oligosaccharides can be di-, tri- or tetrabranched, in addition to fucosylated. The CID spectra of desialylated dibranched (biantennary), tribranched (triantennary) and fucosylated tribranched structures, cleaved from human AGP, were acquired. Fig. 3 depicts a CID spectrum of the desialylated biantennary along with the short-hand structures. An in-

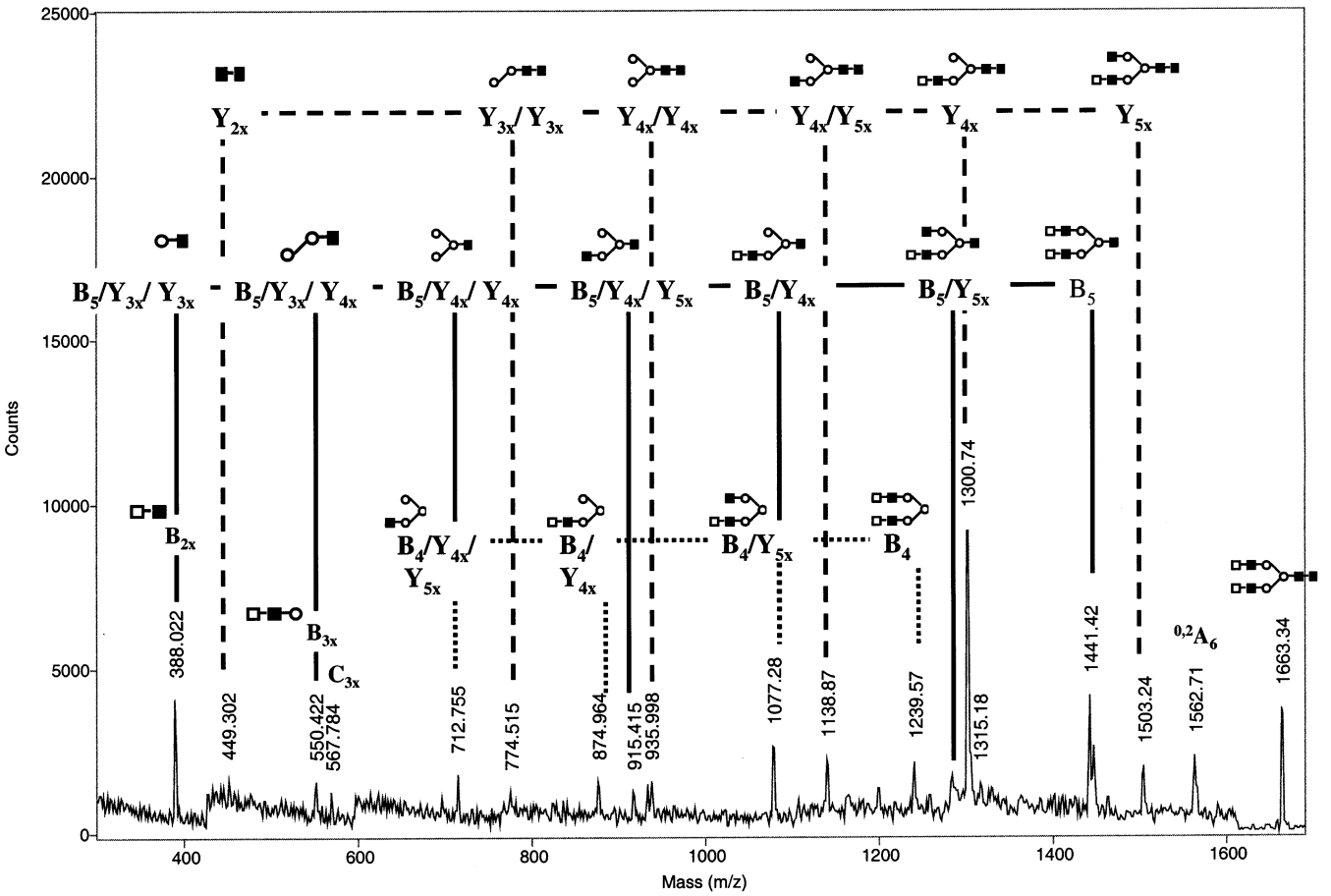


Fig. 3. MALDI-CID spectrum of an *N*-linked biantennary oligosaccharide derived from human AGP. The *m*/*z* values correspond to the monoisotopic masses of the cationized compounds. Symbols: ○, mannose; ■, *N*-acetylglucosamine; □, galactose.

teresting feature noticeable in Fig. 3 is the absence of any *m*/*z* values corresponding to a simultaneous cleavage of monosaccharide moieties on different antennas. This result suggests that dissociation of the glycosidic bond occurs on the same antenna only up to a branching point; thereafter, the cleavages from another antenna commence. This behavior was also observed in electrospray low-energy CID [25]. As an example, *m*/*z* of 1339, which can only correspond to the simultaneous cleavage of monosaccharide moieties on both antennas, was not detectable. This pattern of cleavage was more noticeable for the triantennary and fucosylated triantennary structures as well as for the esterified acidic *N*-glycans (see below). Once again, the ions requiring high energy for the cross-ring fragmentation were not detected, except for the $^{0.2}A_5$ fragment. While the information deduced from our CID spectra reveals some details of the monosaccharide sequence and branching, the absence of a cross-ring fragmentation limits the linkage determination. The *m*/*z* values of 1077, 915, 712, 550 and 388 corresponded to two fragments and, if the proposed pattern of fragmentation is correct, both fragments should exist simultaneously.

The CID spectrum of a triantennary oligosaccharide was more complex than that of a biantennary oligosaccharide, due to the higher degree of branching. The major fragment observed in the case of desialylated triantennary oligosaccharides is at *m*/*z* 1664.87, resulting from a loss of one of the branches (Fig. 4). Generally, the fragments observed here correspond to the B or Y types, or both types of fragmentation, while no high-energy, cross-ring cleavages are detected except for the $^{0.2}A_6$ and $^{0.2}A_6/Y_{4x}$ fragments (Fig. 4). As in the case of the biantennary structure, the cleavages appeared to occur on the same antenna up to the branching point, just before cleavages on the other branches start occur-

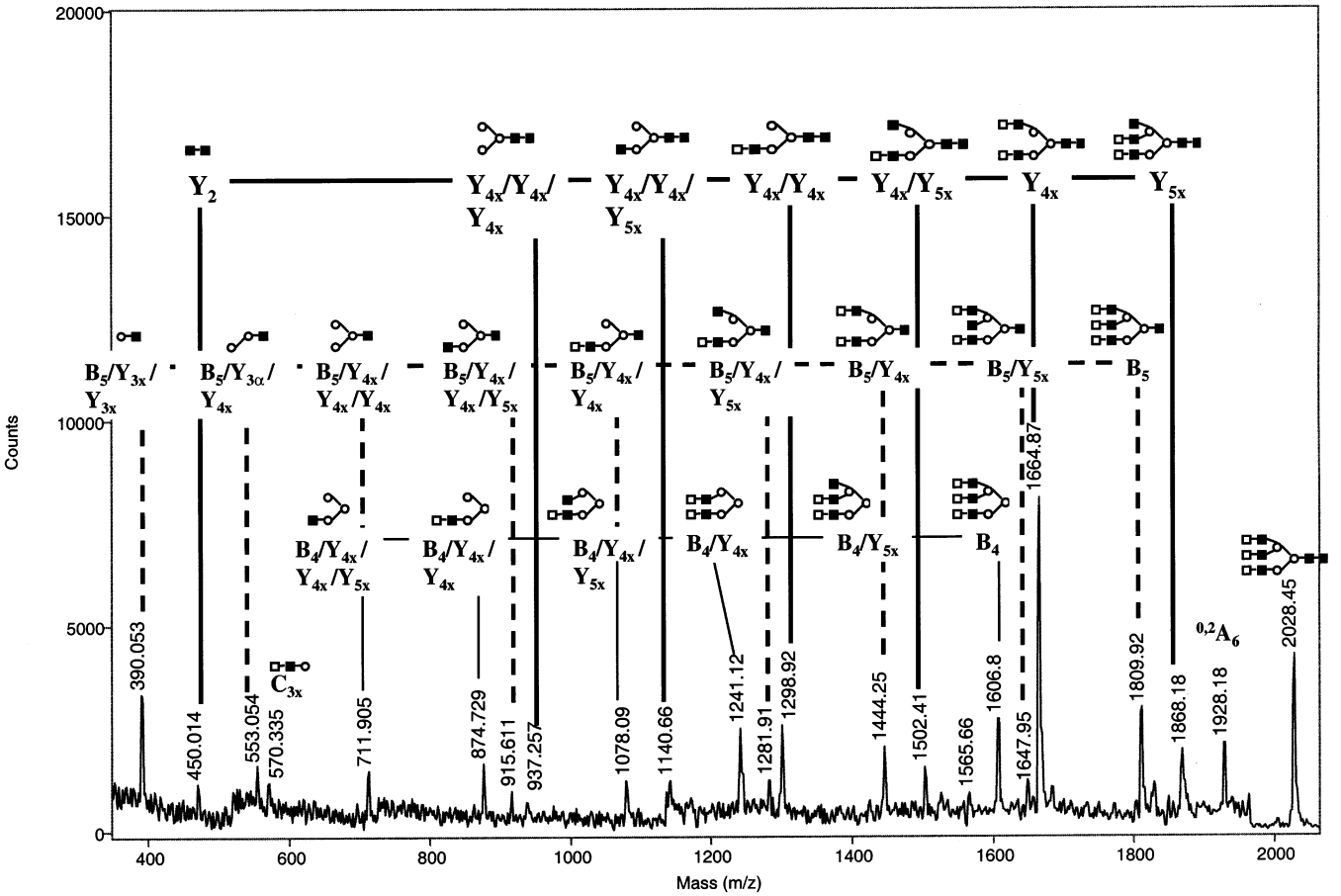


Fig. 4. MALDI-CID spectrum of an *N*-linked triantennary oligosaccharide derived from human AGP. The *m*/*z* values correspond to the monoisotopic masses of cationized compounds. Symbols as in Fig. 3.

ring. This behavior was evidenced more clearly by the absence of *m*/*z* at 1704, 1542 and 1339, which correspond to the simultaneous cleavages of monosaccharides on different antennas.

The CID spectrum of the *N*-linked fucosylated triantennary oligosaccharide was also acquired. The spectrum, which is more complex than those of the other two *N*-linked complex oligosaccharides, is illustrated in Fig. 5. Three major fragments were observed at *m*/*z* 2030.19, 1810.50 and 1662.63. The *m*/*z* at 2030.19 corresponds to the loss of a fucose residue, while that at 1810.50 can result from either the loss of an antenna, or from the loss of a fucose and *N*-acetylglucosamine at the reducing end. The *m*/*z* at 1662.63 corresponds to the loss of an antenna and a fucose residue (for fragments and structures, see Fig. 5). The *m*/*z* at 1810.50 might suggest the attachment of a fucose residue to *N*-acetylglucosamine at the reducing end, yet the observation of *m*/*z* values corresponding to $B_4$ and $B_5$ fragments, which include a fucose residue (Fig. 5), supports the known structure of a fucosylated triantennary oligosaccharide derived from human AGP, where the fucose residue is attached to one of the *N*-acetylglucosamines on the antennas via a $(1\rightarrow3)$ linkage (see Fig. 5) [26]. Generally, all major fragments observed in the CID spectrum of triantennary oligosaccharide (Fig. 4) were also present in the spectrum of the fucosylated triantennary oligosaccharide (Fig. 5). An interesting feature of the CID spectrum of the fucosylated triantennary oligosaccharide is the presence of two sets of Y and B fragments, corresponding to the presence and absence of the fucose residue with a difference of 16 in *m*/*z* (Fig. 5). These fragments result from the loss of a hexose residue instead of deoxyhexose residue, and their presence in spectra of unknown oligosaccharides will distinguish these two residue types. Just as with the biantennary and

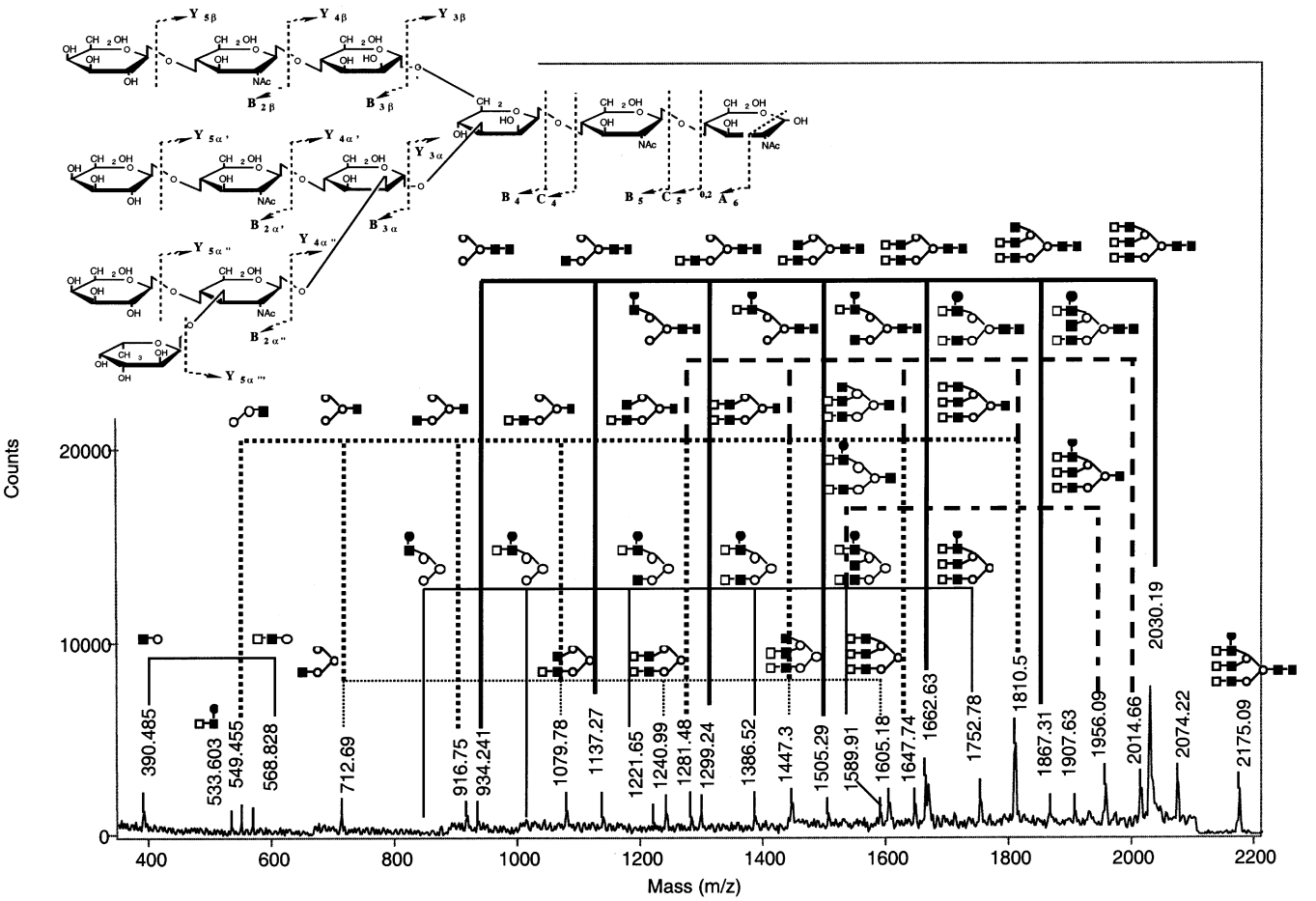


Fig. 5. MALDI-CID spectrum of an *N*-linked fucosylated triantennary oligosaccharide derived from human AGP. The $m/z$ values correspond to the monoisotopic masses of cationized compounds. Symbols as in Fig. 3.

triantennary oligosaccharides, we believe that fragmentation of the fucosylated triantennary structures also occurs on the same antenna up to the branching point, just before cleavages on a second antenna become initiated. This prediction was, once again, supported by the absence of fragments corresponding to simultaneous cleavages on different antennas.

*Esterified acidic oligosaccharides.*—A major problem encountered in the MALDI mass spectrometry of acidic oligosaccharides in positive-ion mode is the loss of sialic acids or the detection of multiple peaks resulting from ion formation between the free acid and the salts present in samples. These multiple peaks cause a substantial decrease in sensitivity of the MALDI technique for acidic oligosaccharides to the extent that, in some cases, the signal is totally masked by the baseline noise. This problem was recently solved by Powell and Harvey [16] who acquired the MALDI spectra of acidic oligosaccharides and gangliosides as their methyl esters, according to the procedure of Norgard-Sumnicht et al. [27]. In our work, the acidic oligosaccharides derived from human AGP were also esterified and their MALDI-CID spectra were acquired.

Esterified oligosaccharides derived from human AGP were subjected to MALDI-CID. Five classes of oligosaccharides are known to exist in human AGP, including disialylated biantennary, trisialylated triantennary, fucosylated trisialylated triantennary, tetrasialylated tetraantennary, and fucosylated tetrasialylated tetraantennary structures [26]. The CID spectra of the two structures existing in relatively high concentrations, namely disialylated biantennary and trisialylated triantennary, were studied in some detail. Just as with the biantennary oligosaccharides, the CID spectrum of the esterified disialylated biantennary structure was simple (Fig. 6). The CID spectrum of

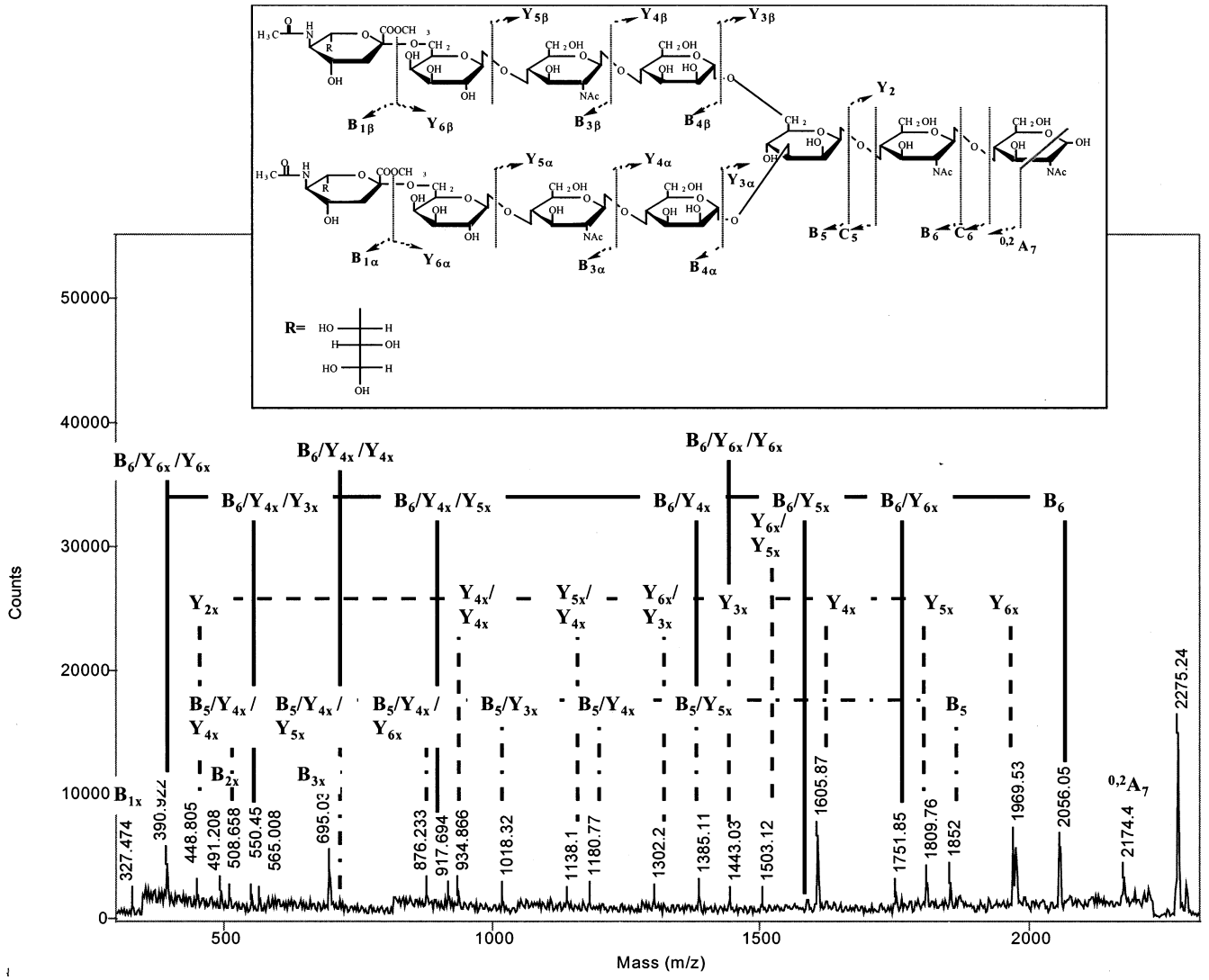


Fig. 6. MALDI-CID spectrum of esterified, disialylated *N*-linked biantennary oligosaccharide derived from human AGP. The $m/z$ values correspond to the monoisotopic masses of cationized compounds.

esterified disialylated biantennary oligosaccharide further supports the observation that cleavages of the glycosidic bond in branched oligosaccharides take place on the same antenna up to the branching point, before any residues on the second antenna become cleaved. This was supported by the absence of $m/z$ values, such as those corresponding to a loss of two esterified sialic acid groups. The fragments observed in the CID spectrum are illustrated in Fig. 6 and are grouped according to a fragment type. Similarly to the previous cases, no cross-ring fragmentations were detected, except for the $^{0,2}A_7$ fragment.

Structural assignment of the fragments acquired with the esterified trisialylated triantennary oligosaccharide is more complex than that for the biantennary oligosaccharides (Fig. 7) because of a higher degree of branching. The two major peaks observed in the CID spectrum of the esterified trisialylated triantennary oligosaccharide are at $m/z$ 2645.65 and 2280.67. The signal at $m/z$ 2645.65 corresponds to the fragment resulting from the loss of an esterified sialic acid residue, while the fragment at 2280.67 corresponds to the loss of an antenna. The signals at $m/z$ 1810.9 and 1604 are assumed to result from the loss of monosaccharide residues after an initial loss of an antenna, since the same signals were detected in the CID spectrum of the esterified disialylated biantennary oligosaccharide (com-

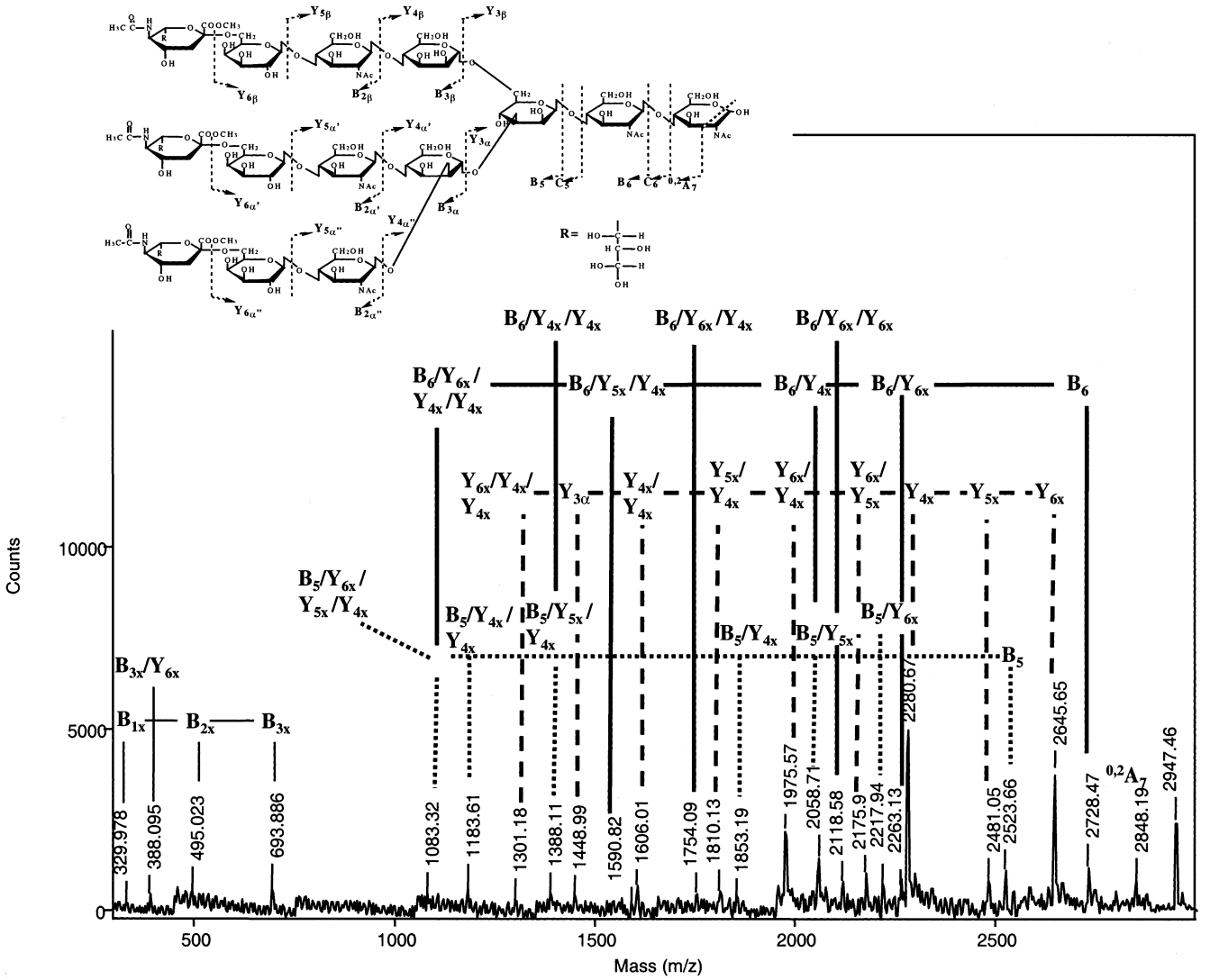


Fig. 7. MALDI-CID spectrum of esterified, trisialylated *N*-linked triantennary oligosaccharide derived from human AGP. The $m/z$ values correspond to monoisotopic masses of the cationized compounds.

pare Figs. 6 and 7). Note that for both esterified oligosaccharides, a consistent signal is observed at ca. $m/z$ 693, corresponding to a fragment consisting of *N*-acetylglucosamine, galactose and the esterified sialic acid residues. Once more, the cleavage occurring on the same antenna is evident in the CID spectrum of esterified acidic, trisialylated triantennary structure (Fig. 7).

## Conclusions

The MALDI mass spectra acquired here through CID in a reflectron-type instrument permitted determination of the isobaric monosaccharide sequence and the branching points. This procedure appears more valuable in the acquisition of representative cleavage fragments than the post-source decay approach, but less informative than the more sophisticated Fourier-transform MS [12] that allows more energetic dissociation. The lack of extensive cross-ring fragmentation in the spectra acquired in this work hinders the determination of linkages. Nevertheless, the spectra obtained through the described procedure facilitate a sequence determination from the characteristic $m/z$ ratios. Determination of the linkage forms becomes feasible through the combined use [28] of selective glycosidases and MALDI-CID.

Acquisition of spectra on the sialylated oligosaccharide structures is greatly facilitated

through a prior esterification of acidic oligosaccharides. The molecules, stabilized through esterification, yield readily discernible spectra due to the sequence and branching patterns.

A potentially significant observation of this work has been a consistent dissociation of the glycosidic bonds on the same antenna, up to a branching point. This phenomenon, noticeable with all branched oligosaccharides investigated in this work, and especially with the tetra-antennary structures, could be due to the fact that a lower energy is needed to fragment an antenna that has already lost a monosaccharide residue.

## Acknowledgements

This work was supported by Grant No. GM 24349 from the National Institute of General Medical Sciences, US Department of Health and Human Services.

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