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Fragmentation of negative ions from *N*-linked carbohydrates. Part 5: Anionic *N*-linked glycans

David. J. Harvey [a,*], Pauline M. Rudd [b]

[a] *Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*
[b] *National Institute for Bioprocessing Research and Training, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland*

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Dedicated to Catherine Costello to honour her receipt of the Field and Franklin Award and in recognition of her major contribution to the mass spectrometry of carbohydrates and related compounds.

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^{13}C-label

ABSTRACT

The negative ion CID spectra of singly ($[M-H]^-$) and doubly ($[M-2H]^{2-}$) charged sialylated bi-, tri- and tetra-sialylated *N*-glycans are described with particular reference to ions that define the linkage of the sialic acids. The singly charged ions generally gave prominent deprotonated molecular ions but, because the proton mainly arose from the sialic acid moieties, the fragmentation was not as diagnostic as that of neutral glycans where deprotonation occurs from one of several hydroxyl groups. Nevertheless, ions defining most structural features such as the location of fucose residues and differentiation between triantennary isomers were still present. Ions in the high mass end of the spectra of glycans with $\alpha2\rightarrow6$-linked-sialic acids were generally dominated by $^{0,2}A_7$ cleavage products rather than the $^{2,4}A_7$ ions that dominate the spectra of the neutral glycans. The ion at m/z 306 ($^{0,4}A_2$–CO_2) was most useful in defining the $\alpha2\rightarrow6$-linkage. The antenna-specific D and $[D-18]^-$ ions were usually present but of low intensity. Glycans with $\alpha2\rightarrow3$-linked-sialic acids produced spectra that more closely resembled those of the neutral compounds. Doubly charged ions fragmented mainly to singly charged products following loss of sialic acids. Neutralization by linkage-specific derivatization and consequent formation of $[M+anion]^-$ ions generally restored the abundant antenna-specific fragments. Singly charged sulfated glycans fragmented in a similar manner to the sialylated compounds but the lower end of the spectra were dominated by B_1 (sulfated GalNAc) and B_2 fragments rather than the cross-ring fragments common to the corresponding spectra of the sialylated compounds.

1. Introduction

Fragmentation of negative ions ($[M-H]^-$ or $[M+anion]^-$) from neutral *N*-linked glycans (those attached to asparagine in the consensus sequence Asn-Xaa-Ser(Thr) in glycoproteins) has been shown to produce much more diagnostic spectra than fragmentation of positive ions as the result of loss of specific hydroxylic protons in the initial fragmentation event [1–4]. Many samples of released *N*-glycans contain both neutral and acidic compounds (carrying sialic acid, sulfate or phosphate). When examined in negative ion mode with a small amount of a suitable ammonium salt in the ESI spray to stabilize the resulting ions, the neutral glycans ionize as $[M+anion]^-$ species whereas the anionic glycans form $[M-H]^-$ and/or $[M-nH]^{n-}$ ions. Fragmentation of these $[M-nH]^{n-}$ ions from anionic glycans produces less diagnostic fragmentation patterns than fragmentation of the $[M+anion]^-$ species because the proton loss tends to be predominantly from the acidic group thus inhibiting formation of many of the ions seen in the spectra of the neutral compounds. Nevertheless, much structural information can still be obtained as demonstrated by the study by Sagi et al. [5] on large multiantennary glycans from erythropoietin, ionized mainly as triply charged ions. However, fragmentation of the smaller glycans in various charge sites, particularly with reference to the occurrence of the diagnostic ions reported earlier [1–4] has received comparatively little attention. A possible reason is the generally unstable nature of these compounds, particularly when analysed by MALDI-TOF MS where loss of the sialic acid moieties is often extensive [6,7]. Stabilization can be achieved by salt formation or preparation of derivatives such as methyl esters [8–13] (as well as permethylation), amides [12,14,15], methylamides [16] or pyridylethylamido derivatives [17]. The resulting neutralization of the acidic group enables the compounds to be examined in positive ion mode or in negative mode if adducted with an anion or further derivatized appropriately.

Abbreviations: CI, chemical ionization; CID, collision-induced decomposition; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride; ESI, electrospray; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; MALDI, matrix-assisted laser desorption/ionization; Man, mannose; MS, mass spectrometry; Neu5Ac, *N*-acetylneuraminic acid (sialic acid); PAGE, polyacrylamide gel electrophoresis; PSD, post-source decay; Q, quadrupole; SDS, sodium dodecyl sulfate; TOF, time-of-flight.

* Corresponding author. Tel.: +44 0 1865 275750; fax: +44 0 1865 275216.
E-mail address: david.harvey@bioch.ox.ac.uk (David.J. Harvey).

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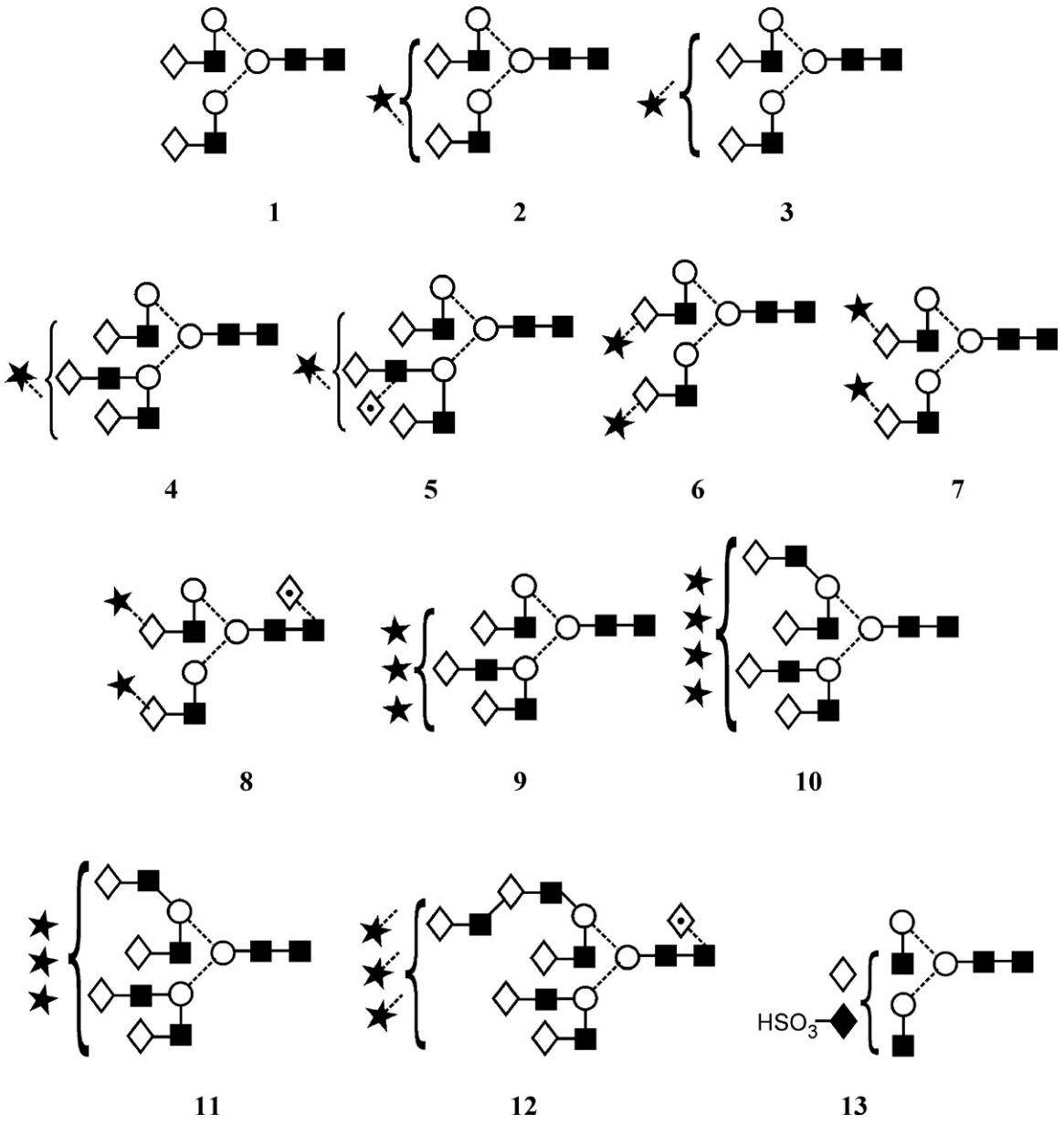

Scheme 1. Structures of the glycans used in this paper. Key to symbols: ■ = GlcNAc; ◯ = mannose; ◊ = galactose; ⋆ = *N*-acetylneuraminic acid (sialic acid). The angle of the bonds linking the symbols define the linkage with full lines indicating β-bonds and broken lines showing α-bonds [33].

Most of the work on negative ion fragmentation of sialylated *N*-linked glycans has been performed with sialylated versions of the biantennary compound (**1**, Scheme 1). The negative ion fragmentation of the 2-pyridylamidated di-sialylated biantennary glycan and a corresponding glycopeptide derived from ovalbumin, were reported by Ito et al. [18] in 2006. Both compounds yielded a prominent B_6 fragment (ions are named according to the convention devised by Domon and Costello [19]) resulting from cleavage between the core GlcNAc residues but, in addition, the glycopeptide exhibited a prominent $^{2,4}A_7$ cross-ring fragment because of the closed reducing-terminal GlcNAc residue. The B_3 ion (Neu5Ac-Gal-GlcNAc) was prominent in the spectrum of the derivatized glycan and both spectra contained abundant B_1 ions at *m*/*z* 290 representing sialic acid. Seymour et al. [9] also reported the CID fragmentation of the free and methyl esterified biantennary glycan. $^{0,2}A_7$, $^{0,2}A_6$, B_3 and particularly the B_1 ion were prominent. An ion at *m*/*z* 424 represented a cross-ring cleavage (probably $^{1,3}A_4$) together with loss of sialic acid. In these respects, the spectra were similar to those reported in this paper for the free glycans. Wheeler and Harvey reported an extensive study of the negative ion fragmentation of several milk-derived and biantennary *N*-glycans in 2000. Sialic acids were linked in both α2 → 3 and α2 → 6 configurations and several ions were noted that allowed the linkage of the sialic acids to be determined. Details are discussed later in this paper.

In other studies, Casal et al. [20] examined the fragmentation of sialylated *O*-glycans by ion trap mass spectrometry and noted an ion at *m*/*z* 597 in the spectra of α2 → 6-linked sialic acid isomers that was not present in the spectra recorded from beam instruments. The explanation appeared to be the formation of an internal hydrogen bond that was specific to the α2 → 6-isomer. Other major fragments (negative ion mode) were mainly B, Y and X-type ions. Anomeric fragmentation of α- and β-isomers of 1-*O*-octadecyl-3-*O*-(*N*-acetyl)neuraminyl-*sn*glycerol sodium salt has allowed anomeric differentiation [21]. The chemical ionization (CI)

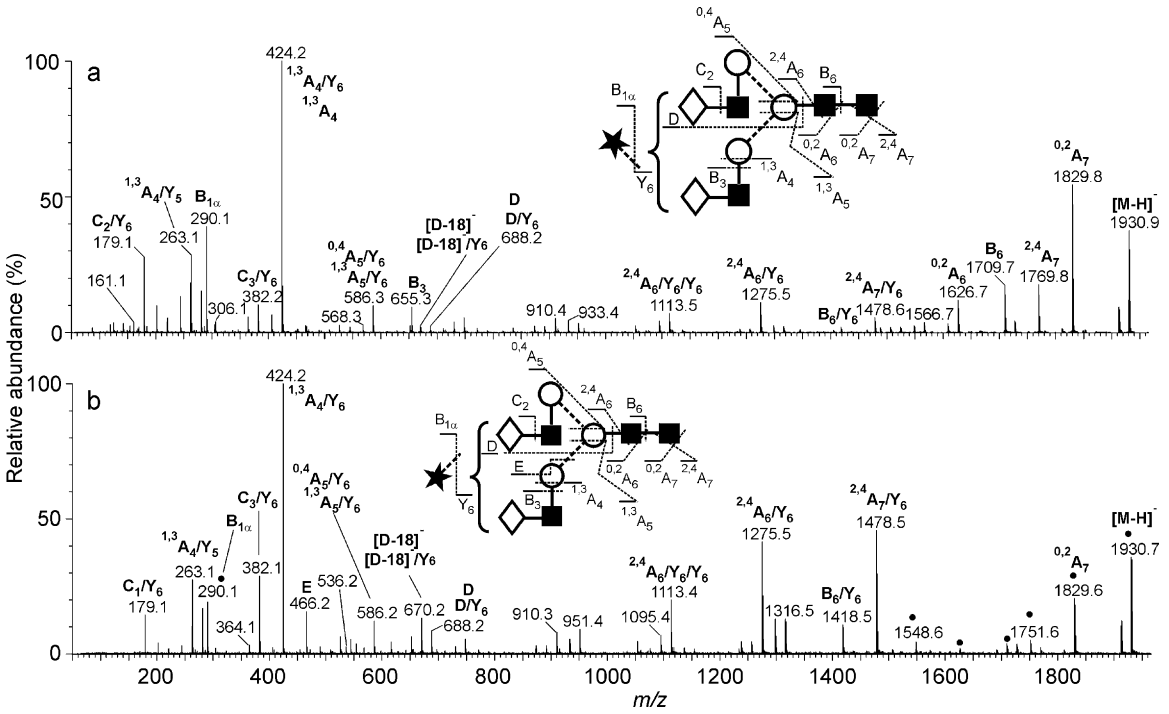

Fig. 1. (a) Negative ion MS/MS spectrum of the α2 → 6-linked monosialylated biantennary glycan **2**; (b) Negative ion MS/MS spectrum of the α2 → 3-linked monosialylated biantennary glycan **3**. Ions that shift in the spectrum of the [$^{13}C_1$]-labelled analogue are indicated with a filled circle.

fragmentation of several sialic acids themselves was reported by Shukla et al. [22].

This paper examines the fragmentation of singly, doubly and some triply charged bi-, tri- and tetra-antennary glycans with from one to four sialic acid groups and a carbohydrate with a sulfated GalNAc residue. Emphasis is placed on ions that allow the linkage of the acid groups and the general topology of the glycans to be determined.

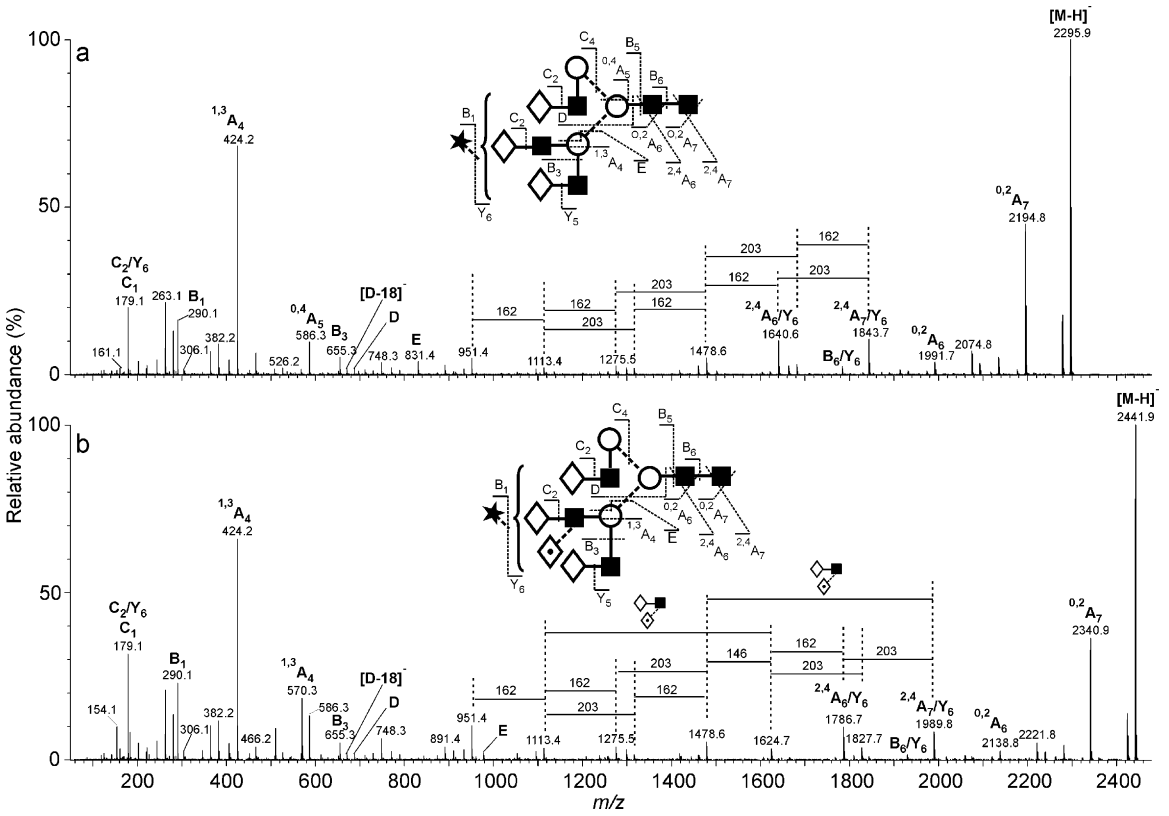

Fig. 2. (a) Negative ion MS/MS spectrum of the α2 → 6-linked monosialylated triantennary glycan **4**; (b) Negative ion MS/MS spectrum of the α2 → 6-linked monosialylated triantennary glycan **5** containing a fucose residue in the 3-antenna.

2. Materials and methods

2.1. Materials

Most *N*-glycans were released with protein *N*-glycosidase F from human serum glycoproteins from within SDS-PAGE gels as described earlier [23,24]. Briefly, the glycoproteins were run in a low density SDS-PAGE gel, the gel bands containing the glycoproteins were excised and the glycoproteins were reduced and alkylated. The gel pieces were then incubated with protein *N*-glycosidase F (PNGase F) overnight at 37 °C to remove the glycans which were extracted with water and acetonitrile. Full experimental details have been published [25]. Biantennary glycans containing ^{13}C-labelled sialic acids (label in the COOH group) were synthesised by the action of α2 → 3- and α2 → 6-sialyltransferases on the isolated biantennary glycan (Oxford GlycoSciences, Abingdon, UK) as described earlier [26]. The sialic acid linkage in these and other glycans was checked by MALDI-TOF MS using the linkage-specific derivatization method described by Wheeler et al. [13], employing derivatization with methanol catalysed with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM) [27]. The structures of the glycans used in this study are shown in Scheme 1.

2.2. Electrospray mass spectrometry

Negative ion electrospray mass spectrometry was performed with a Waters quadrupole-time-of-flight (Q-TOF) Ultima Global instrument (Waters MS Technologies, Manchester, UK). Samples in 1:1 (v:v) methanol:water were infused through Proxeon nanospray capillaries (Proxeon Biosystems, Odense, Denmark, now part of Thermo Fisher Scientific). The ion source conditions were: temperature, 120 °C; infusion needle potential, 1.2 kV; cone voltage 100 V; RF-1 voltage 180 V. Spectra (2 s scans) were acquired with a digitization rate of 4 GHz and accumulated until a satisfactory signal:noise ratio had been obtained. Mass accuracy was about 20 ppm. For MS/MS data acquisition, the parent ion was selected at low resolution (about 4 *m*/*z* mass window) to allow transmission of isotope peaks and fragmented with argon at a pressure (recorded on the instrument's pressure gauge) of 0.5 mBar. The voltage on the collision cell was adjusted with mass and charge to give an even distribution of fragment ions across the mass scale. Typical values were 80–120 V. Other voltages were as recommended by the manufacturer. Instrument control, data acquisition and processing were performed with MassLynx software Version 4.0.

3. Results and discussion

3.1. Sialylated glycans

3.1.1. Mono-sialylated glycans (**2, 3**)

Fig. 1a and b shows the MS/MS spectrum of biantennary glycan containing single α2 → 6- (**2**, Scheme 1) and α2 → 3-linked sialic acids (**3**), respectively. Ions that shift in the spectrum of the [$^{13}C_1$]-labelled analogue of the α2 → 3-linked isomer (**3**) are indicated with a dot. The specific antenna to which the sialic acid was linked is not known but both isomers are probably present.

These mono-sialylated compounds produced $[M-H]^-$ ions mainly as the result of loss of the labile acidic proton from the sialic acid moiety and, consequently, many of the diagnostic ions that were present in the spectra of the corresponding neutral glycans were of low abundance or absent. Rather than forming the abundant $^{2,4}A_7$, B_6 and $^{2,4}A_6$ ions that characterise spectra of the neutral glycans, the α2 → 6-linked sialylated glycans produced, as the most prominent ion in the high-mass region, a $^{0,2}A_7$ ion (*m*/*z* 1829 in Fig. 1a). This ion was accompanied by other less abundant cross-ring and glycosidic fragments of the reducing terminal GlcNAc, namely $^{2,4}A_7$, $^{0,2}A_6$ and B_6. Loss of sialic acid (Y_6 cleavage) left what is essentially the $[M-H]^-$ ion of the corresponding neutral glycan and this ion fragmented in the conventional manner producing $^{2,4}A_7/Y_6$, B_7/Y_6 and $^{2,4}A_7/Y_6$ ions at *m*/*z* 1478, 1418 and 1275, respectively. These ions were much more abundant in the spectrum of the α2 → 3-linked isomer (Fig. 1b) and their proposed compositions were supported by the absence of the ^{13}C label from the labelled analogues.

Ions in the lower half of the spectrum reflected the structure of the antennae. The ion at *m*/*z* 290 is the B_1 (sialic acid) ion. Yamagaki and Nakanishi [28] have reported that α2 → 3-linked sialic acids are eliminated much more readily than α2 → 6-linked acids in the post-source decay (PSD) spectra of sialyllactoses and have proposed that this difference could be used diagnostically. In these collision-induced decomposition (CID) spectra of *N*-linked glycans, the situation appears more complicated. The relative abundance of the B_1 fragment (sialic acid) is lower in the spectra of the compounds that contain an α2 → 6-linked sialic acid than in the spectra of the isomeric compounds, suggesting otherwise. However, ions that involve additional cleavages, such as the $^{2,4}A_7/Y_6$ and $^{2,4}A_6/Y_6$ are much more prominent in the spectra of the compounds containing α2 → 3-linked sialic acids, in agreement with this proposal. The α2 → 6-linked sialic acid also gave rise to the weak, but diagnostic $^{0,4}A_2$–CO_2 fragment at *m*/*z* 306 [26,29–31]. This ion was absent from the spectra of sialylated glycans containing only α2 → 3-linked sialic acids (Fig. 1b) [26]. Another ion that appeared to reflect the linkage of the sialic acid was *m*/*z* 536 (low abundance), present in the spectra of the α2 → 3-linked isomer (both mono- and di-sialylated glycans). This ion retained the ^{13}C label and appeared to be an internal $B_3/^{0,4}X_1$ fragment, the X-cleavage producing loss of the $C_4O_4H_8$ fragment from the sialic acid. However, the relative abundance of this ion was usually too low for it to be a reliable indicator of linkage. The B_2 ion (*m*/*z* 452) was insignificant in these spectra but the B_3 ion at *m*/*z* 655 was prominent and diagnostic in the spectrum of the α2 → 6-linked-isomer reflecting the previously reported tendency for abundant B-type cleavage ions to be produced adjacent to GlcNAc. The reason for its lower relative abundance in the spectrum of the α2 → 3-linked-isomer is unclear.

Many of the other singly charged ions in this region of the spectrum were internal fragments having lost the sialic acid in a Y_6 cleavage. Thus, *m*/*z* 179 was the C_2/Y_6 fragment from the antenna containing the sialic acid or C_1 where no sialic acid was present, accompanied by a very weak B_2/Y_6 or B_1 ion (no sialic acid) at *m*/*z* 161 in the spectrum of the α2–6-isomer. The major ion at *m*/*z* 424 was a $^{1,3}A_4/Y_6$ fragment and the ion at *m*/*z* 586 appeared to be formed by cross-ring cleavage of the branching mannose residue ($^{0,4}A_5$ or $^{1,3}A_5$ cleavage). The D and $[D-18]^-$ ions [4] that result from formal loss of the chitobiose core and the 3-antenna (exact mechanism not determined) are among the most useful ions in the spectra of the neutral glycans because they give the composition of the 6-antenna. Although these ions were of low relative abundance in these spectra, the corresponding D/Y_6 and $[D-18]^-/Y_6$ ions were present at *m*/*z* 688 and 670, respectively. Other ions are annotated in Fig. 1.

The spectra of the singly charged ion from monosialylated triantennary glycans without (**4**) and with (**5**) a fucose residue are shown in Fig. 2a and b. The fucosylated compound, from human α1-acid glycoprotein, is unusual in that the fucose residue is attached to the β1 → 4-branch of the 3-antenna rather than in the more common 6-position of the reducing-terminal (core) GlcNAc. Retention of the fucose by the B_6/Y_6, $^{2,4}A_7/Y_6$ and $^{2,4}A_6/Y_6$ ions reflected its absence from the chitobiose core and the $^{1,3}A_4/Y_6$ ion at *m*/*z* 570 (Gal-(Fuc)GlcNAc-CH=CH–O^-) confirmed its location on an antenna. Absence of a C_1 ion at *m*/*z* 325 (Fuc-Gal) ion was consistent

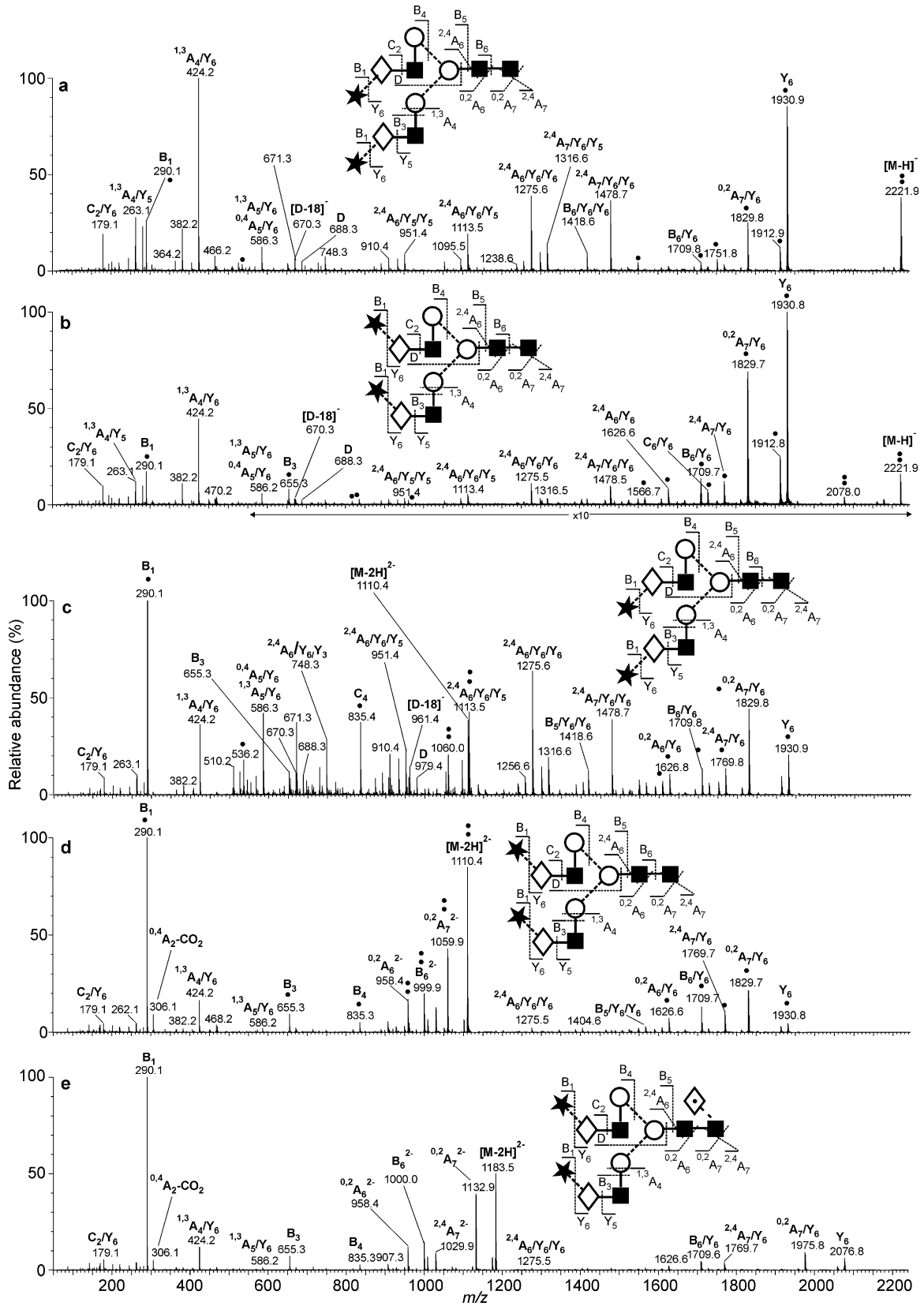

Fig. 3. (a) Singly charged negative ion MS/MS spectrum of the α2 → 3-linked di-sialylated biantennary glycan **6**, (b) Singly charged negative ion MS/MS spectrum of the α2 → 6-linked di-sialylated biantennary glycan **7**. (c) doubly charged negative ion MS/MS spectrum of the α2 → 3-linked di-sialylated biantennary glycan **6**, (d) the corresponding spectrum of the α2 → 6-linked di-sialylated biantennary glycan **7**. Ions that shift in the spectra of the $^{13}C_1$-labelled compounds are indicated by filled circles (one filled circle for one mass unit shift and two filled circles for two). (e) Doubly charged negative ion MS/MS spectrum of the α2 → 6-linked di-sialylated biantennary glycan containing a core fucose residue (**8**).

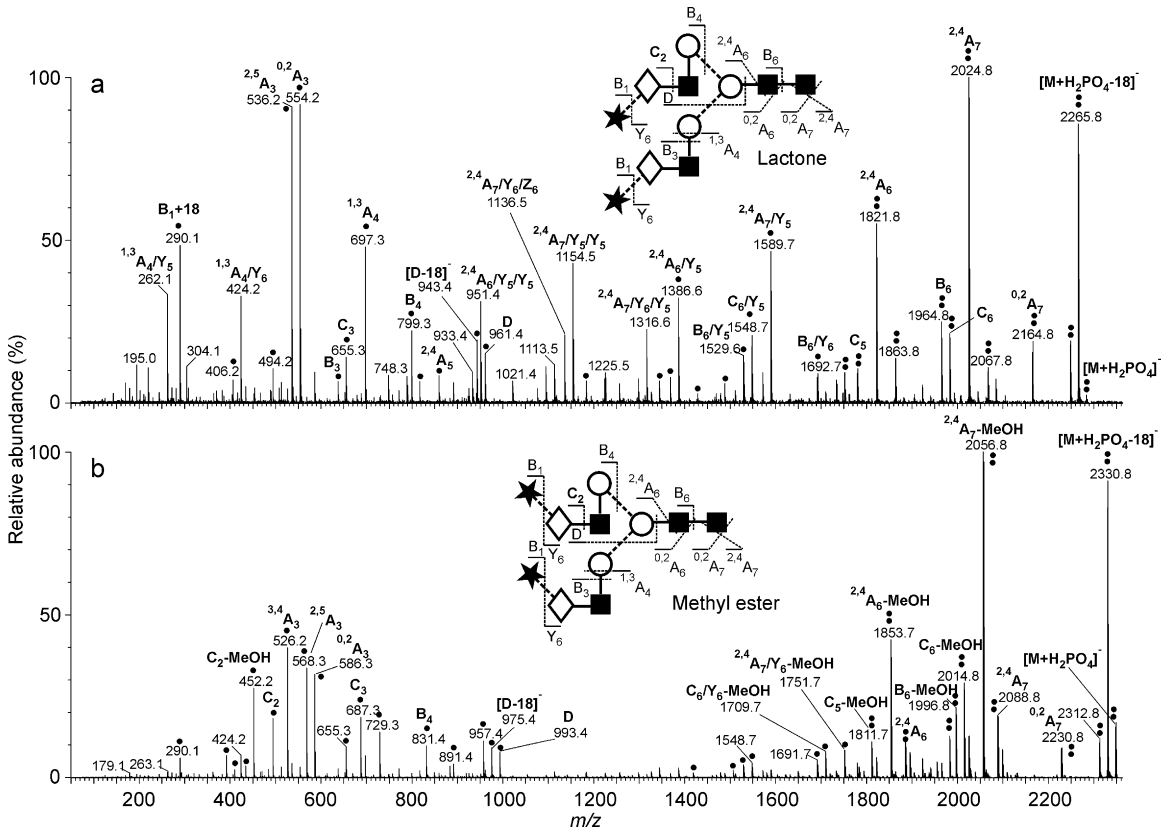

Fig. 4. Singly charged negative ion MS/MS spectrum of the phosphate adducts of (a) the α2 → 3-linked di-sialylated biantennary glycan **6** as its di-lactone with (b) the α2 → 6-linked di-sialylated biantennary glycan **7** as its di-methyl ester. Ions that shift in the spectra of the $^{13}C_1$-labelled compounds are indicated by filled circles.

with its location on a GlcNAc residue. The ion at *m/z* 831 (termed ion E) in the spectrum of the unfucosylated compound showed that the triantennary glycan was the isomer with a branched 3-antenna and the shift of this ion to *m/z* 977 in the spectrum of the fucosylated glycan (Fig. 2b) showed that the fucose was located on this antenna [32]. The presence of the ion at *m/z* 306 indicated an α2 → 6-linked sialic acid. Other fragments were largely similar to those from the biantennary glycans. Thus, even though some of these diagnostic ions were of lower abundance than in the spectra of unsialylated compounds, they were still abundant enough to define the structure of the glycan.

3.1.2. Di-sialylated glycans (**6**, **7**)

3.1.2.1. Singly charged ions. Fig. 3a and b shows the MS/MS spectra of the singly charged α2 → 3- (**6**) and α2 → 6-linked (**7**) disialylated biantennary glycans. Both spectra showed an abundant initial loss of sialic acid to give *m/z* 1930 and then, the remainder of the ions were similar to those of the monosialylated glycans. Thus, the ions at *m/z* 1829, 1478, 1418 and 1275 were the $^{0,2}A_7/Y_6$, $^{2,4}A_7/Y_6/Y_6$, $B_6/Y_6/Y_6$ and $^{2,4}A_6/Y_6/Y_6$ ions, respectively. In general, these spectra showed the same general differences between the α2 → 3- and α2 → 6-isomers as the spectra of the singly sialylated glycans, Thus, the $^{0,2}A_7/Y_6$, $^{2,4}A_7/Y_6/Y_6$, $B_6/Y_6/Y_6$ and $^{2,4}A_6/Y_6/Y_6$ ions were much more abundant in the spectra of the α2 → 3- than in the spectra of the α2 → 6-linked isomers.

3.1.2.2. Doubly charged ions. The spectra of the doubly charged ions from the α2 → 3- and α2 → 6-linked di-sialylated biantennary glycans (**6** and **7**) are shown in Fig. 3c and 3d, respectively. The $[M-2H]^{2-}$ molecular ion at *m/z* 1110.4 from the α2 → 6-linked-compound displayed the same type of fragmentation as was seen with the singly charged monosialylated analogue (Fig. 1) namely production of an abundant $^{0,2}A_7$ doubly charged ion and a series of doubly charged cross-ring fragments from the reducing-terminal GlcNAc residue. Most other ions were singly charged. Thus, loss of one of the sialic acid residues (Y_6 cleavage) yielded *m/z* 1930.9 and this ion fragmented in an analogous manner to the singly charged ion from the mono-sialylated glycans. Ions at the low mass end of the spectrum were singly charged and mirrored those seen with the mono-sialylated analogue.

Fragmentation of the α2 → 3-linked-isomer (**6**) was somewhat different in that the $^{0,2}A_7$ and $^{0,2}A_7/Y_6$ and most other cross-ring fragments from the reducing-terminal GlcNAc were of lower relative abundance than in the spectrum of the other isomer whereas the $^{2,4}A_7/Y_6/Y_6$, $B_6/Y_6/Y_6$ and $^{2,4}A_6/Y_6/Y_6$ singly charged ions were much more abundant. At the lower end of the spectrum, the B_3 ion (*m/z* 655, $[Neu5Ac\text{-}Gal\text{-}GlcNAc\text{-}H]^-$) was of much lower relative abundance than in the spectrum of the α2 → 6-linked-isomer (**7**) but more prominent was the ion at *m/z* 671. This latter ion appeared to have the composition of $[Neu5Ac\text{-}Gal\text{-}GlcNAc\text{-}CH{=}CH - O - CO_2]^-$ i.e., the product of a $^{1,3}A_4$ cross-ring cleavage with loss of the COOH group from the sialic acid. This structure was supported by the absence of a ^{13}C label from the labelled compounds and by the odd ionic mass which was consistent with the presence of two nitrogen atoms. In general, the diagnostic ions that were present in the spectra of the neutral, un-sialylated, glycans were of higher relative abundance in the spectra of the α2 → 3-linked sialylated biantennary glycans than in those of the other isomer and this was reflected by the reasonably prominent D and $[D-18]^-$ ions at *m/z* 979 and 961 that were diagnostic of the composition of the 6-antenna [4]. The corresponding internal fragments namely the D/Y_6 and $[D-18]^-/Y_6$ ions were present at *m/z* 688 and 670, respectively having lost the capping sialic acid residue. The $^{0,4}A_2/-CO_2$ ion at *m/z* 306 was missing, as reported earlier [26].

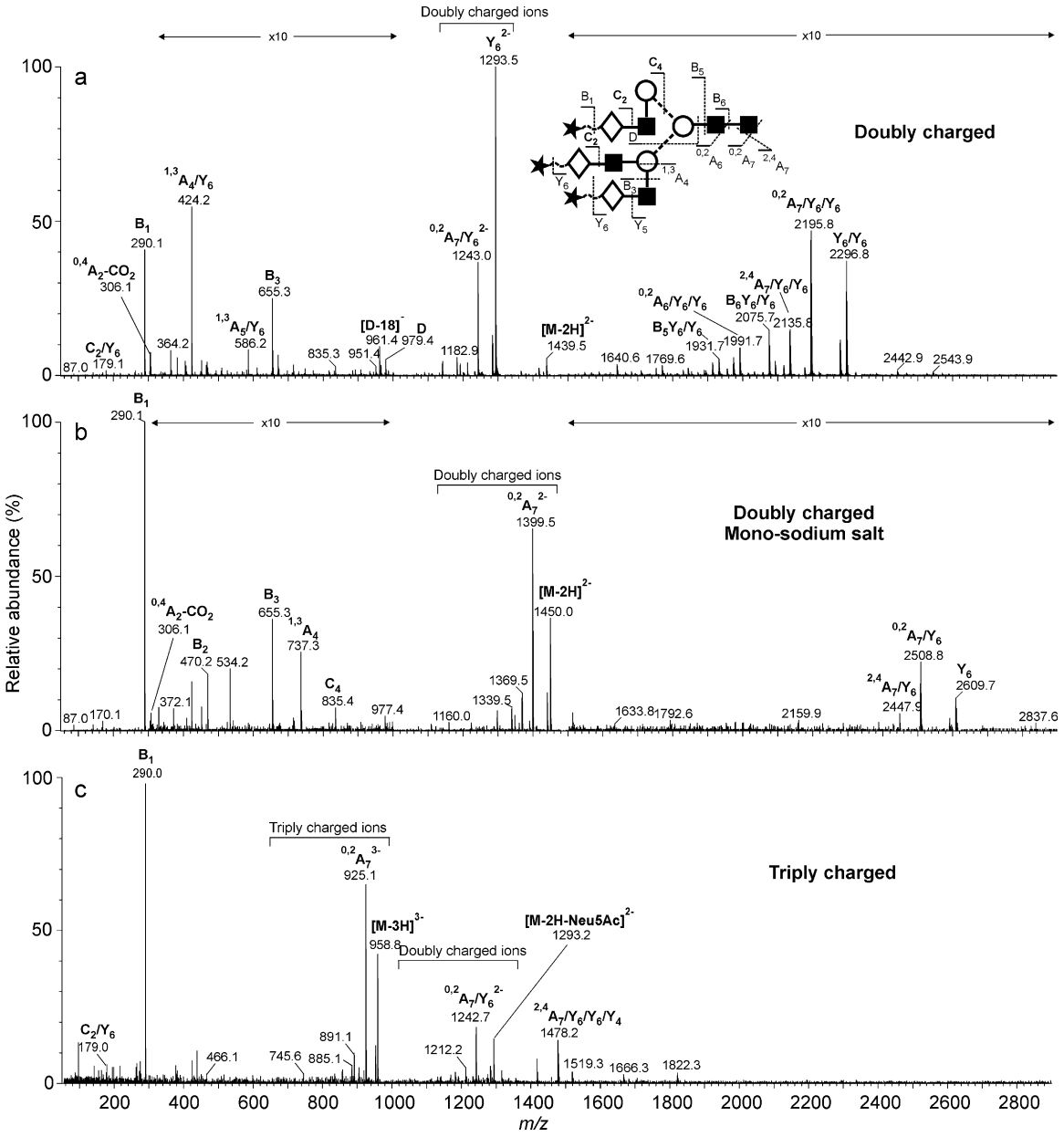

Fig. 5. Doubly charged negative ion MS/MS spectrum of (a) the tri-sialylated triantennary glycan **9**, (b) the MS/MS spectrum of its mono-sodium salt, (c) triply charged ion from the triantennary glycan.

The presence of a fucose residue on the reducing terminal (core) GlcNAc residue (**8**) could be detected by its loss in the formation of the $^{2,4}A_7/Y_6$ and B_6/Y_6 ions (*m*/*z* 1769.7 and 1709.6, respectively) and the doubly charged $^{2,4}A_7$ and B_6 ions (*m*/*z* 1029.9 and 1000.0, respectively, Fig. 3e). These fragments were, thus, at the same masses as in the spectrum of the compound without core fucose.

3.1.3. Derivative formation from the sialic acids

Neutralization of the sialic acids by derivatization was performed to prevent ionization by loss of the carboxyl proton in an attempt to boost the relative abundance of the diagnostic fragment ions that are formed by loss of hydroxylic protons. When these glycans were reacted with methanol in the presence of DMT-MM, the glycans with two α2 → 6-linked sialic acids (**7**) formed methyl esters whereas those with two α2 → 3-linked sialic acids (**6**) formed lactones [13]. The spectra of these compounds are shown in Fig. 4a and b, respectively. All compounds are as their phosphate adducts; phosphate is the adduct normally seen when compounds of this type are recovered from biological matrices. Fragmentation of these neutral glycans was similar to that of the corresponding neutral compounds lacking sialic acid. Thus, abstraction of a hydroxylic proton and further cleavage resulted in formation of the $^{2,4}A_7$ ion at *m*/*z* 2024 in Spectrum 4a and *m*/*z* 2088 in Spectrum 4b. These ions eliminated methanol in the case of the compounds with a methyl ester group to give the major ions at *m*/*z* 2056 (Spectrum 4b). Losses of methanol proved to be problematic in the interpretation of these spectra. In glycans with three or more methylated sialic acids (data not shown) the spectra were dominated by such ions with several losses of methanol occurring from most fragments. Retention of both ^{13}C-labels indicated retention of both sialic acid moieties in the $^{2,4}A_7$ fragments. Other ions in this region of the spectra were the B_6 (*m*/*z* 1964 (Spectrum 4a) and *m*/*z* 1996 (Spectrum 4b) and $^{2,4}A_6$ ions at *m*/*z* 1821 and 1853 in the two spectra, respectively.

Fragmentation of the compounds with α2 → 3-linked sialic acids (lactones) produced more abundant diagnostic ions than the

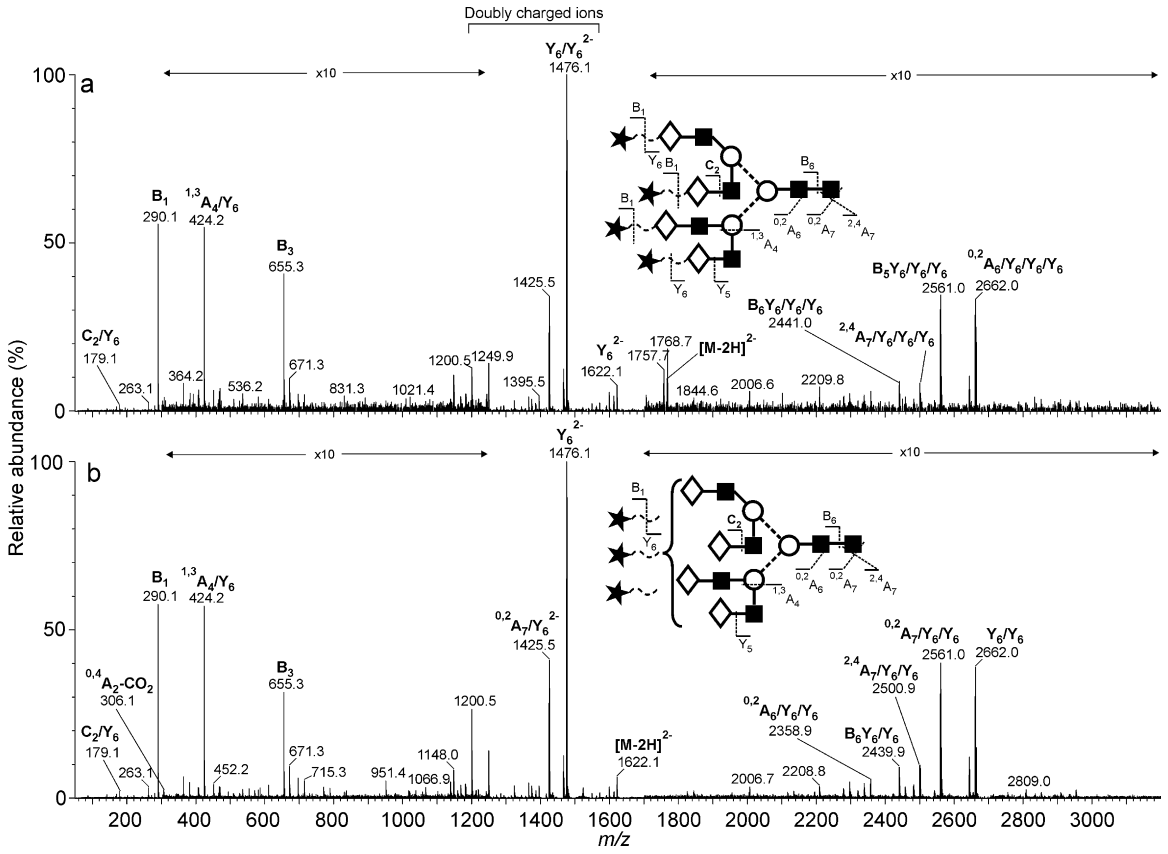

Fig. 6. Doubly charged negative ion MS/MS spectrum of (a) the tetra-sialylated tetra-antennary glycan **10**, (b) the corresponding spectrum of the tri-sialylated analogue (**11**).

compound with two α2 → 6-linked sialic acids (methyl esters). Ions at *m*/*z* 1589 and 1386 (Fig. 4a) contained only one sialic acid as shown by the ^{13}C-labelling and corresponded to further losses of Neu5Ac-Gal and Neu5Ac-Gal-GlcNAc (B cleavages) from the $^{2,4}A_7$ ion (*m*/*z* 2024). In the case of the compounds with sialic acids in mixed linkages, the loss of methanol from the $^{2,4}A_7$ ion to give *m*/*z* 2024 effectively generated a lactone and produced a spectrum (not shown) almost identical to that shown in Fig. 4a from the α2 → 3-linked glycan. Thus determination of the linkage site of each sialic acid was not possible. However, Amano et al. [15] have recorded the negative ion spectrum of a sialylated biantennary glycan (pyrene derivative) with the acid group derivatized as its amide and noted the appearance of an abundant D-type ion containing the intact derivatized sialic acid. This type of derivative, therefore, offers the possibility of determining the antenna to which α2 → 6-linked sialic acids are attached.

D and $[D-18]^-$ ions were present at *m*/*z* 961 and 943 in the case of the compounds with lactonized sialic acids and at *m*/*z* 993 and 975, respectively where the sialic acid was as its methyl ester. In the latter case, these ions were accompanied by a further $[M-36]^-$ ion (*m*/*z* 957) as frequently seen in the spectra of some of the larger *N*-glycans [4]. The pair of ions at *m*/*z* 554/536 (lactones) and 586,568 (methyl esters) contained one sialic acid and could be accounted for by $^{0,2}A_3$ and $^{2,5}A_3$ cleavages, respectively. Other significant ions are labelled in Fig. 4.

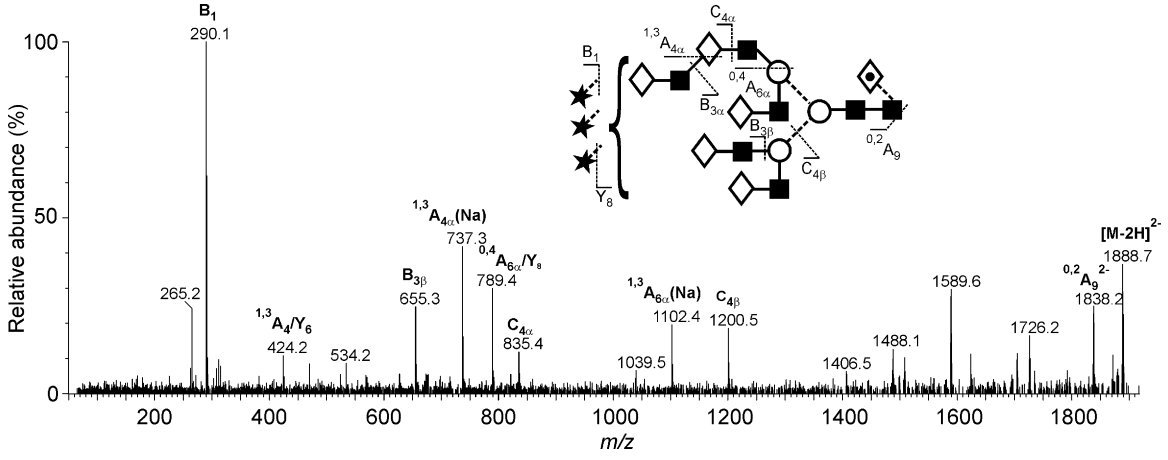

Fig. 7. Low mass region of the negative ion CID spectrum of a core-fucosylated tetra-antennary *N*-glycan containing an antenna extended with a *N*-acetyllactosamine group (**12**). The structure is of the major constituent but other isomers are probably present.

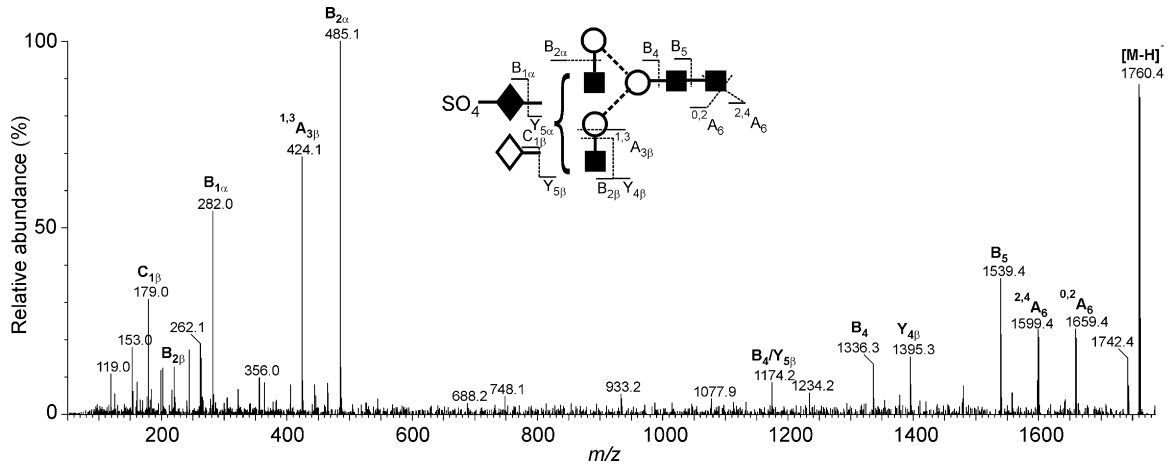

Fig. 8. Singly charged negative ion MS/MS spectrum of the monosulfated glycan (**13**). The spectrum is probably from a mixture of isomers where the sulfated GalNAc can be on either antenna. Isomer-specific ions were not found. Fragmentation is shown for the isomer with the sulfated GalNAc residue on the 6-antenna.

3.1.4. Tri-sialylated glycans

Fig. 5a shows the spectra of the doubly charged ion from the trisialylated triantennary glycan (**9**) obtained from human serum. Linkage analysis by methylation with DMT-MM showed it to contain about 60% of the isomer in which all sialic acids were $\alpha2\rightarrow$6-linked with the remainder having one $\alpha2\rightarrow$3-linked sialic acid. This predominance of $\alpha2\rightarrow$6-linked-sialic acid accounts for the presence of the singly charged $^{0,4}A_2/-CO_2$ ion at *m/z* 306. In addition, two groups of doubly charged cross-ring fragments from the reducing terminus following Y_6 loss of one of the sialic acids (*m/z* 1243.0) and singly charged after loss of both such groups (*m/z* 2195.8, and 1991.7) and were present. These doubly charged ions from a doubly charged precursor indicates the presence of three sialic acid moieties in the molecule. Ions in the lower region of both spectra (*m/z* 179 (C_2/Y_6), 290 (B_1), 424 ($^{0,4}A_4/Y_6$ or $^{1,3}A_4/Y_6$) and 655 (B_3) were as in the spectra discussed above. The diagnostic D, $[D-18]^-$ ions (containing sialic acid) appeared at *m/z* 979 and 961, respectively, consistent with the triantennary isomer containing a branched 3-antenna (**9**). However, the E ion [32] indicating branching of the 3-antenna that appeared at *m/z* 831 in the spectrum of the neutral triantennary glycan was not present, neither was its sialylated counterpart (*m/z* 1413). In the spectrum of a trisialylated triantennary glycan with branching of the other antenna (not shown), neither this ion or the D and $[D-18]^-$ ions appeared.

The spectrum of the triply charged ion from the triantennary glycan (*m/z* 958.8, Fig. 5c) was characterized by loss of a sialic acid residue (Y_6 cleavage) to give the doubly charged ion at *m/z* 1292.7 (^{13}C isotope labelled in Fig. 5c) which further formed an $[^{0,2}A_7/Y_6]^{2-}$ ion at *m/z* 1242. Few other diagnostic fragments were present. Singly charged ions in the upper region of the spectra were the result of elimination of sialic acid and Y-type cleavages of the antennae (e.g., *m/z* 1477.4 = $^{2,4}A_7/Y_6/Y_6/Y_3$). D and $[D-18]^-$ ions were not observed.

The spectrum shown in Fig. 5b is of the same trisialylated glycan as that shown in Fig. 5a but where one of the sialic acids has formed a sodium salt. This modification induced formation of the ion at *m/z* 737 which is the equivalent of the cross-ring cleavage ion at *m/z* 424 with the addition of the sodium salt of a sialic acid. This ion is typical of the spectra of these sialylated glycans when sodium salts are present. Because only two free sialic acid residues exist in this compound, the doubly charged cross-ring fragments from the reducing terminus were produced directly from the molecular ion and formation of the singly charged fragments occurred with loss of only one such acid.

3.1.5. Tetra-sialylated glycans

The fragmentation spectra of the doubly charged ion from the tetra-sialylated tetra-antennary glycan (**10**, *m/z* 1766.6) was somewhat less informative than the spectra of the smaller glycans because of the low relative abundance of many of the fragment ions (Fig. 6a). Dominant ions were as above with the singly charged fragments involving loss of three sialic acids. The doubly charged ions formed from the doubly charged molecular ion mainly involved loss of two sialic acids (Fig. 6a). Very similar spectra were observed from the tetra-antennary glycans with only three sialic acids (**11**, Fig. 6b). Ions are annotated in Fig. 6 and further information can be found in the paper by Sagi et al. [5] who have discussed the fragmentation of these tetra-antennary glycans in some detail.

3.1.6. Complex glycans carrying N-acetyllactosamine extensions to their antennae

Fig. 7 shows the low mass region of the doubly charged ion from a complex tetra-antennary glycan of composition $Gal_5GlcNAc_7Man_3Fuc_1Neu5Ac_3Na$ (**12**) released from a recombinant erythropoietin and containing at least one *N*-acetyllactosamine extension to an antenna (the exact structure was unknown). Ions at *m/z* 655, 737 and 835 correspond to B_3, $^{1,3}A_{4\alpha}/Y_6$ (with sodium) and C_4, respectively. The presence of the *N*-acetyllactosamine extended antenna was revealed by the appearance of corresponding ions containing an additional Gal-GlcNAc group at *m/z* 789.3 ($^{1,3}A_{6\alpha}/Y_8$, Gal-GlcNAc-Gal-GlcNAc-CH=CH–O^-) and 1102 ($^{1,3}A_{6\alpha}$, Neu5Ac(Na)-Gal-GlcNAc-Gal-GlcNAc-CH=CH–O^-). The prominent ion at *m/z* 1200.5 was also present in the spectrum of the triantennary glycan and was probably the $C_{4\beta}$ ion containing a single sialic acid but this was not confirmed. The presence of the fucose on the core GlcNAc was reflected by the absence of the ion at *m/z* 570 (see above).

3.2. Sulfated glycans

The MS/MS spectra of sulfated glycans showed many features in common with those of the sialylated glycans because of charge localization on the sulfate group. Fig. 8 shows the spectrum of a mixture of biantennary glycans containing one sulfated GalNAc residue on either antenna (**13**). The dominant fragments in the lower mass region were the B_2 ion, $[SO_3$-GalNAc-GlcNAc$]^-$ (*m/z* 485) and the B_1 fragment, $[SO_3$-GalNAc$]^-$ (*m/z* 282). In common with the spectra of the $[M-H]^-$ ions from sialylated glycans, fragmentation at the reducing terminus produced the $^{0,2}A_6$, $^{2,4}A_6$, B_5 and B_4 fragments

at m/z 1659, 1599, 1539 and 1336, respectively. Other diagnostic ions typical of the corresponding neutral glycan include m/z 179 (C_1) and 424 ($^{1,3}A_3$). Thus, the spectrum contained some of the diagnostic fragments found in the spectra of the neutral glycans but differed from the spectra of the sialylated glycans in producing major B_1 and B_2 cleavages from the non-reducing terminus rather than cross-ring fragments such as m/z 424.

4. Conclusions

Although these anionic glycans gave deprotonated ions with proton loss predominantly from the acid groups, their fragmentation spectra yielded a considerable amount of information. Spectra of singly-charged ions contained many of the fragments previously observed from [M+adduct]$^-$ ions generated from neutral compounds. Spectra of more highly sialylated glycans, both singly and doubly charged, were dominated by ions produced by loss of sialic acid groups to give mainly singly charged fragments. These ions yielded information on the composition of the chitobiose core but many of the antennae-specific ions that were observed in the spectra of neutral glycans were missing. Spectra of glycans with $\alpha2\rightarrow$ 6-linked-sialic acids were generally dominated by $^{0,2}A_7$ cleavage products rather than the $^{2,4}A_7$ ions that dominate the spectra of the neutral glycans, whereas glycans with $\alpha2\rightarrow$ 3-linked-sialic acids produced spectra that more closely resemble those of the neutral compounds. The most useful ion for detecting the presence of compounds with $\alpha2\rightarrow$ 6-linked-sialic acids was, as reported earlier, the $^{0,2}A_4$–CO_2 ion at m/z 306. Linkage of the sialic acids could also be determined by linkage-specific derivatization with the lactone derivatives produced from glycans with $\alpha2\rightarrow$ 3-linked-sialic acids yielding spectra resembling those of the neutral compounds. The spectra of the methyl esters of the compounds with $\alpha2\rightarrow$ 6-linked-sialic were characteristically different but complicated by the presence of ions produced by loss of methanol. Singly charged sulfated glycans fragmented in a similar manner to the sialylated compounds but the lower end of the spectra were dominated by B_1 (sulfated GalNAc) and B_2 fragments rather than the cross-ring fragments common to the corresponding spectra of the sialylated compounds.

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