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High-energy Collision-induced Fragmentation of Complex Oligosaccharides Ionized by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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The high-energy CID spectra of the MNa^+ ions from 17 underivatized oligosaccharides of the type found attached to asparagine in glycoproteins were examined with a double-focusing mass spectrometer fitted with a tandem orthogonal time-of-flight analyser. Fragment ions were observed throughout the mass range from all compounds and provided considerable structural information in the low-picomole range. The three types of fragmentation that were observed were glycosidic cleavages, cross-ring cleavages and the formation of internal cleavage ions. The major glycosidic fragmentations were B- and Y-type cleavages (Domon and Costello nomenclature). B-cleavages were particularly abundant at GlcNAc residues. Z-ions were absent when glycosidic linkages occurred at the 6-position. Cross-ring cleavages were predominantly of the $^{1,5}X$-type, which provided much sequence and branching information. $^{3,5}A$ cleavages of the core branching mannose residue were often prominent and provided information on the composition of each of the main antennae. Antenna composition was also reflected by a major internal fragment ion formed by elimination of the two GlcNAc residues of the chitobiose core together with the entire antenna at the 3-position of the core branching mannose residue. A further loss of GlcNAc as 221 mass units from this ion in the spectra of the hybrid and complex carbohydrates was indicative of the presence of a 'bisecting' (4-linked) GlcNAc substituent. Another prominent internal fragment ion containing a mannose residue and only one GlcNAc, with its substituents, was present in the spectra of the complex sugars when branching of the 3-antenna occurred. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Complex oligosaccharides attached to an asparagine residue in glycoproteins, termed *N*-linked oligosaccharides, have a common pentasaccharide core structure (structure **I**, Table 1) and one or more antennae attached to each of the mannose residues at the non-reducing terminus.[1] In mammalian systems, these antennae consist either of mannose (Man) chains (termed 'high-mannose' sugars) or chains containing *N*-acetyl-2-amino-2-deoxyglucose (GlcNAc) with or without galactose (Gal). These latter compounds are termed 'complex' if both antennae contain GlcNAc or 'hybrid' if mixed mannose- and GlcNAc-containing chains exist. Commonly, a fucose (Fuc) residue is found attached to the GlcNAc residue at the reducing terminus (termed 'core fucose') and an additional GlcNAc may be attached to the 4-position of the branching mannose (termed 'bisecting' GlcNAc). In addition, complex chains frequently terminate with a sialic acid residue.

Structural identification of these oligosaccharides requires determination of the constituent monosaccharides, their sequence, the branching pattern and the hydroxyl groups involved in the linkage of one residue with another. No single mass spectrometric (MS) technique is yet able to determine all of these parameters, but several techniques used in combination can be used to deduce the complete structure. Composition[2] and linkage[3] are best determined by gas chromatography/MS following methanolysis or hydrolysis, whereas sequence and branching information can be deduced from fragment ions produced by fast atom bombardment (FAB) or liquid secondary ion mass spectrometry (LSIMS).[4–6] Much linkage information is also accessible by FAB analysis following periodate oxidation.[7]

MS/MS techniques are particularly useful and can yield sequence, branching and some linkage information.[8–14] Many investigators have explored the utility of MS/MS techniques for linkage analysis, but much of

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this work has been performed on di-, tri- and tetrasaccharides[15–24] rather than on the larger oligosaccharides discussed in this paper. Although some linkage information has been shown to be carried by the relative abundance of certain glycosidic fragment ions, the most useful ions are those originating from cross-ring cleavages. These latter ions are mainly produced by pericyclic retro-aldol and retro-ene mechanisms and are particularly abundant in collision-induced dissociation (CID) spectra induced by high-energy collisions. They appear to be similar to those seen in the spectra of small oligosaccharides produced by energetic ionisation processes (see, for example, Refs 25–29) and in the fragmentation of oligosaccharides initiated by infrared laser desorption.[30,31]

Collision-induced fragmentation of larger oligosaccharides ionized by various techniques has been studied in several laboratories[9,13,14,32] and shown to be governed predominantly by glycosidic cleavages between the monosaccharide rings and by cross-ring cleavages. The former fragmentations, prominent at low energy, are the most common and reveal details on the sequence and branching of the constituent monosaccharides, while the cross-ring cleavages induced by higher energy collisions often reveal the linkage. Glycosidic cleavages also tend to predominate under the low-energy collision conditions occurring in post-source-decay (PSD) reactions seen in reflectron time-of-flight (TOF) instruments,[33,34] although many of the ions in these spectra appear to be the result of several such cleavages involving losses from both ends of the molecule (termed internal cleavage ions). Although MH^+ ions from both free and derivatized oligosaccharides appear to fragment more easily than MNa^+ ions,[35,36] fragmentation of the latter species produces a higher percentage of cross-ring cleavage reactions which provide linkage information on the oligosaccharide.

Matrix-assisted laser desorption/ionization (MALDI) MS has recently been found to give prominent MNa^+ ions from underivatized oligosaccharides[37–39] whose abundance bears a quantitative relationship to the amount of sample ionized.[40] Although these ions have been found to decompose spontaneously in the ion sources of magnetic sector instruments,[39] and in TOF instruments fitted with time-delay-focusing (unpublished observations from this laboratory), and to produce PSD ions in TOF mass spectrometers,[34,41] there is no information on the high-energy CID reactions of these ions formed by MALDI. Because of the pulsed nature of the MALDI ion source, it is not usually practicable to acquire CID spectra with a conventional, scanned magnetic sector mass spectrometer. However, the combination of a sector instrument with an orthogonal acceleration tandem TOF analyser provides a satisfactory solution[42] and enables product ions to be acquired with high efficiency. This paper describes the use of such an instrument to record high-energy CID spectra of underivatized oligosaccharides ionized by MALDI. This MS/MS technique provides a convenient and rapid method for ionizing underivatized oligosaccharides and for providing sequence, branching and linkage information. The spectra of 17 *N*-linked oligosaccharides are examined and the data are interpreted in terms of which ions are most useful for the identification of specific structural features such as the presence or absence of a 'bisecting' GlcNAc residue.

EXPERIMENTAL

Materials

Oligosaccharides were obtained from Oxford GlycoSciences (Abingdon, Oxfordshire, UK) and were used without further purification. 2,5-Dihydroxybenzoic acid was obtained from Aldrich Chemical (Gillingham, Dorset, UK).

Mass spectrometer

Spectra were recorded with a Micromass AutoSpec mass spectrometer fitted with an array detector[43] and an orthogonal acceleration TOF (OA-TOF) mass analyser.[44] The ion source was a standard FAB source with the addition of a nitrogen laser (337 nm), which fired through a sapphire window mounted over the ion source housing.

Sample preparation

The oligosaccharides (10–100 pmol) in aqueous solution (1 μl) were mixed on the side-inlet probe with the matrix (2,5-DHB, 3 μl of a saturated solution in acetonitrile) and allowed to dry in air. A small amount of ethanol was then added so that the mixture redissolved. This solution was again allowed to crystallize in air at ambient temperature to give a crystalline layer of about $5 \times 5\ mm^2$.

Spectral acquisition

The laser was operated at 10 Hz. During data acquisition, the laser spot was moved vertically over the target surface and the probe was slowly moved inwards in order to accommodate depletion of sample under the laser spot. Molecular (MNa^+) ions were selected with MS-1 and transmitted to the collision cell which used xenon and was operated to give a centre-of-mass collision energy of 800 eV. The push-out pulse for the OA-TOF was synchronized with the laser pulse to give a delay equivalent to the flight-time from the ion source. Fine tuning of the push-out pulse was achieved by observing the absence of the signal on the normal scanning (point) detector situated after the OA-TOF when the push-out pulse was used. Ions were accumulated by the OA-TOF detector until a satisfactory signal-to-noise ratio was obtained. This process usually took about 20–30 min.

RESULTS AND DISCUSSION

In order to specify the individual sugar residues comprising the oligosaccharides, the following scheme will be used (Fig. 1). The structure is drawn such that the

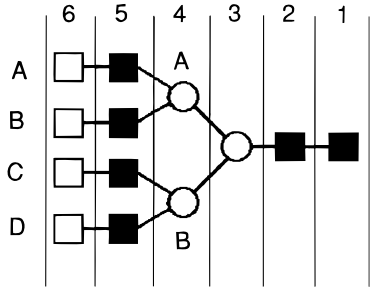


Figure 1. Scheme used to define the positions of the constituent monosaccharide rings in the oligosaccharide structure. Symbols used for the structural formulae in this and other figures: ○ = mannose, □ = galactose, ■ = *N*-acetyl-2-deoxy-2-aminoglucose and ▽ = fucose.

monosaccharide residues form columns. The columns are numbered, starting from the reducing terminus. In columns containing more than one monosaccharide residue, the units are labelled from A starting at the top. In addition, the fragmentation scheme used is based on that described by Domon and Costello[45] where ions retaining the charge at the reducing terminus are termed X (cross-ring cleavages), Y and Z (glycosidic cleavages), whereas those ions retaining the charge at the non-reducing terminus are termed A (cross-ring), B and C (glycosidic). Cross-ring cleavage ions are further designated by superscript numbers indicating the two bonds that are cleaved.

The oligosaccharides used in this study are listed in Table 1. All compounds gave abundant MNa^+ ions, as reported previously, and the monoisotopic ^{12}C peak was selected for study in each case.

General features

Targets were loaded with about 100 pmol of sugar and up to half was usually consumed in acquiring the spectra. However, a 10 pmol loading (compound **XI**) still gave a good signal-to-noise ratio. The lowest detectable amount was not determined.

The study by Lemoine *et al.*[13] showed that the high-energy CID spectra of the MNa^+ ion from permethylated oligosaccharides fragmented both by glycosidic (B, C, Y and Z ions) and by cross-ring (A and X ions) cleavages with the latter reactions becoming more prominent as the collision energy rose. The ions in the MALDI spectra discussed in this paper appeared to be similar, although with differences in the relative abundance of the various types of ions which probably reflected, in part, the presence of the labile hydroxylic hydrogen atoms. Most fragmentations involved loss of neutral species rather than radicals and were probably the result of remote-site fragmentations.

All compounds gave an ion at $[MNa - 18]^+$ representing loss of water. This fragmentation has been studied using ^{18}O and deuterium labelling by Hofmeister *et al.*[21] in the spectra generated by FAB and shown to represent a specific loss of the OH group attached to C-1 of the reducing terminal monosaccharide (GlcNAc-1, Fig. 1) together with a hydrogen atom from a hydroxy group. It is probable that the same reaction is occurring in the spectra reported in this paper. All spectra additionally contained a prominent Na^+ ion.

High-mannose oligosaccharides (I–VII)

The spectrum of a representative high-mannose sugar, $(Man)_9(GlcNAc)_2$ (**VII**), is shown in Fig. 2 and the fragmentations producing the major ions in its spectrum are shown in Fig. 3(a) and (b). Glycosidic cleavages (Figs 3(a) and 4) were prominent. The resulting B and Y ions were generally the most abundant products and were observed for all glycosidic bonds in the molecule. B-type cleavages (B_5) adjacent to the central GlcNAc residue were particularly abundant (*m/z* 1684.7, Fig. 2), an observation also common to FAB spectra. A hydrogen atom was transferred from the ionic fragment to the eliminated GlcNAc residue in the formation of this B fragment (Fig. 4) and from the eliminated neutral fragment to the ion in the formation of the Y ions (Fig. 4). Additional work by Hofmeister *et al.*[21] using deuterium labelling has shown that, in the CID spectra of native oligosaccharides ionized by FAB, this hydrogen atom originates from a hydroxyl group and not from C-2, which is the apparent source in the fragmentation of permethyl derivatives.[9] We have shown by use of deuterium labelling, that hydrogen atoms from hydroxyl groups are involved in B and Y cleavages during spontaneous in-source decay (ISD) of MALDI-produced MNa^+ ions from these compounds (to be published) suggesting that a similar mechanism occurs in the high-energy CID spectra reported here. This transfer of hydroxylic hydrogen atoms probably accounts for the greater relative abundance of B and Y ions in these spectra compared with the spectra of permethylated oligosaccharides reported by Lemoine *et al.*[13]

The ions labelled as being formed by Z-cleavages in Fig. 2 may, in fact, be due to loss of water from the Y ions; permethylation would be needed to distinguish these alternative routes, as emphasized by Reinhold *et al.*[32] Again, if a glycosidic cleavage is involved, a hydrogen transfer from the ionic fragment to the neutral particle would be involved. Z-type ions were observed from each glycosidic bond except that to the 6-position of the branching mannose (mannose 3, Fig. 1). The absence of Z ions from 6-linked sugar residues appeared to be a general phenomenon; e.g. Z ions were not observed from 6-linked fucose residues (compounds **II**, **VIII**, **IX**, **XI** and **XIII**) and may serve to define linkage at this position. It was not possible to determine if a $Z_{4\gamma}$ cleavage occurred [see Fig. 3(a)] because the resulting fragment ion (*m/z* 1563.5) was isobaric with the $Z_{4\alpha}$ and $Z_{4\beta}$ ions. The lack of Z ions from 6-linked sugars is consistent with the generally low relative abundance of ions produced by loss of water or alcohols from hydroxy or substituted hydroxy groups attached to primary carbon atoms observed under electron impact conditions, compared with similar losses of these residues when attached to alicyclic rings. C ions, on the other hand were prominent from this linkage.

C ions were formed by hydrogen transfer from the neutral to the ionic fragment. However, the C ion resulting from loss of the chitobiose core was found to be accompanied by another abundant ion containing two fewer hydrogen atoms and its formation presumably involved a hydrogen transfer in the other direction, namely away from the ionic fragment (Fig. 4). The

Table 1. Structures of the oligosaccharides

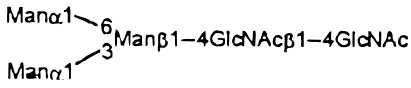
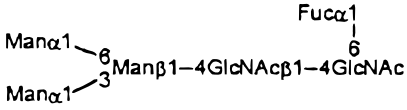
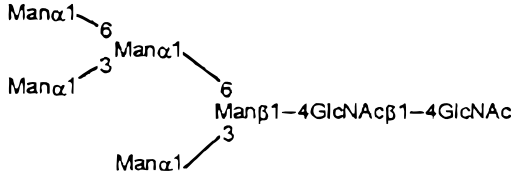
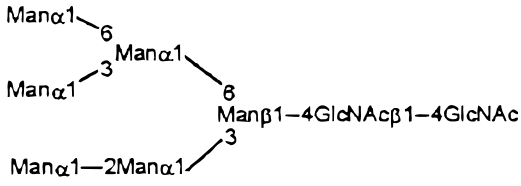
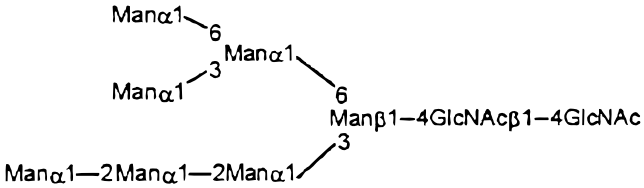
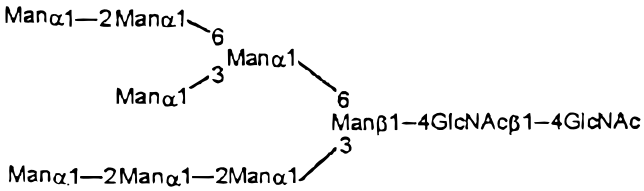
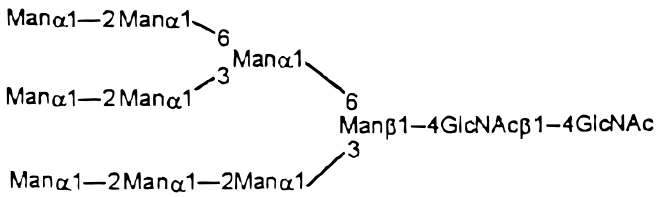
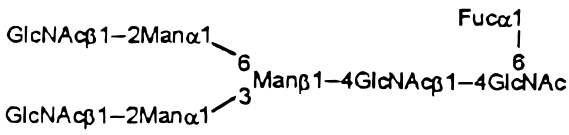
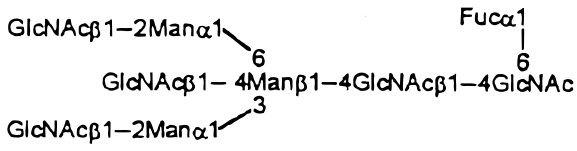
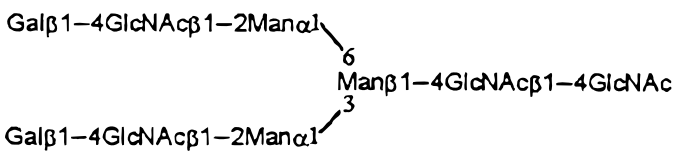
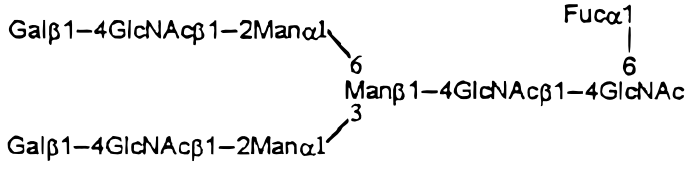
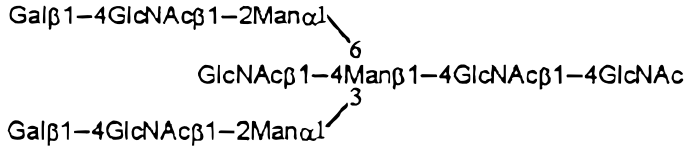
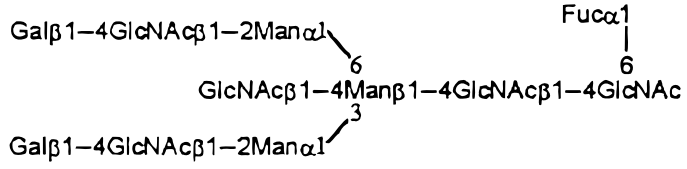
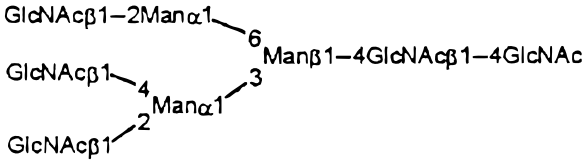
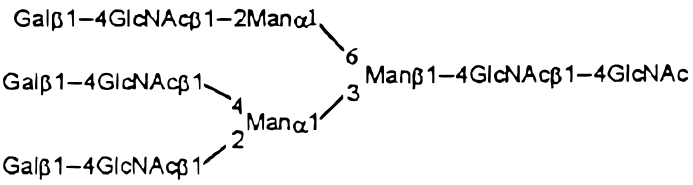
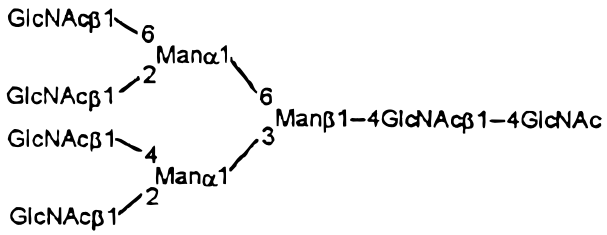
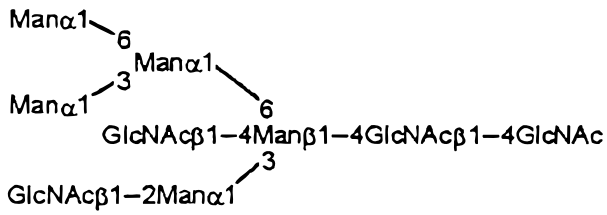
Compound No.	Structure[a]	Mass [MNa+] (Da)[b]
I		933.3
II		1079.4
III		1257.4
IV		1419.5
V		1581.5
VI		1743.6
VII		1905.6
VIII		1485.5
IX		1688.6
X		1663.6
XI		1809.7

Table 1. *Continued*

Compound No.	Structure[a]	Mass [MNa^+] (Da)
XII		1866.7
XIII		2012.7
XIV		1542.6
XV		2028.7
XVI		1745.6
XVII		1663.6

[a] Gal = D-galactose; Man = D-mannose; Fuc = L-fucose (6-deoxy-β-L-galactopyranose); GlcNAc = 2-acetamido-2-deoxy-D-glucose.
[b] Monoisotopic mass.

initial structure shown in Fig. 4 is unstable and probably rearranges to a hydroxy ketone or hydroxy epoxide. Similar abundant C and C-2H ions were also found from fragmentation of this glycosidic bond in the other spectra and weak C-2H ions were frequently observed to accompany other C ions.

The major ion which could be used to differentiate the substitution on the two antenna was an internal cleavage ion, termed ion *a* produced by elimination of the two GlcNAc residues from the chitobiose core, together with the sugar residues comprising the antenna attached to C-3 of the core-mannose residue (mannose 3, Fig. 1) (*m/z* 995.4, Fig. 2). Its abundance, relative to the base peak (discounting the molecular ion and Na^+), is listed in Table 2 for the high-mannose sugars. Its composition was confirmed by comparing its mass in the spectra of all 17 sugars. Two mechanisms can be proposed for its formation, namely a B-type cleavage between the GlcNAc residue at position 2 (Fig. 1) and the core branching mannose (mannose-3, Fig. 1) together with elimination of the antenna linked to C-3 of this mannose residue with retention of its linking oxygen (Fig. 5, path a), or a C-cleavage with elimination of both the 3-antenna and its linking oxygen (Fig. 5, path b). The latter mechanism is favoured because inspection of molecular models shows that the 3-mannose (Fig. 1) can abstract a proton from the hydroxy group at C-2 of the core branching mannose,

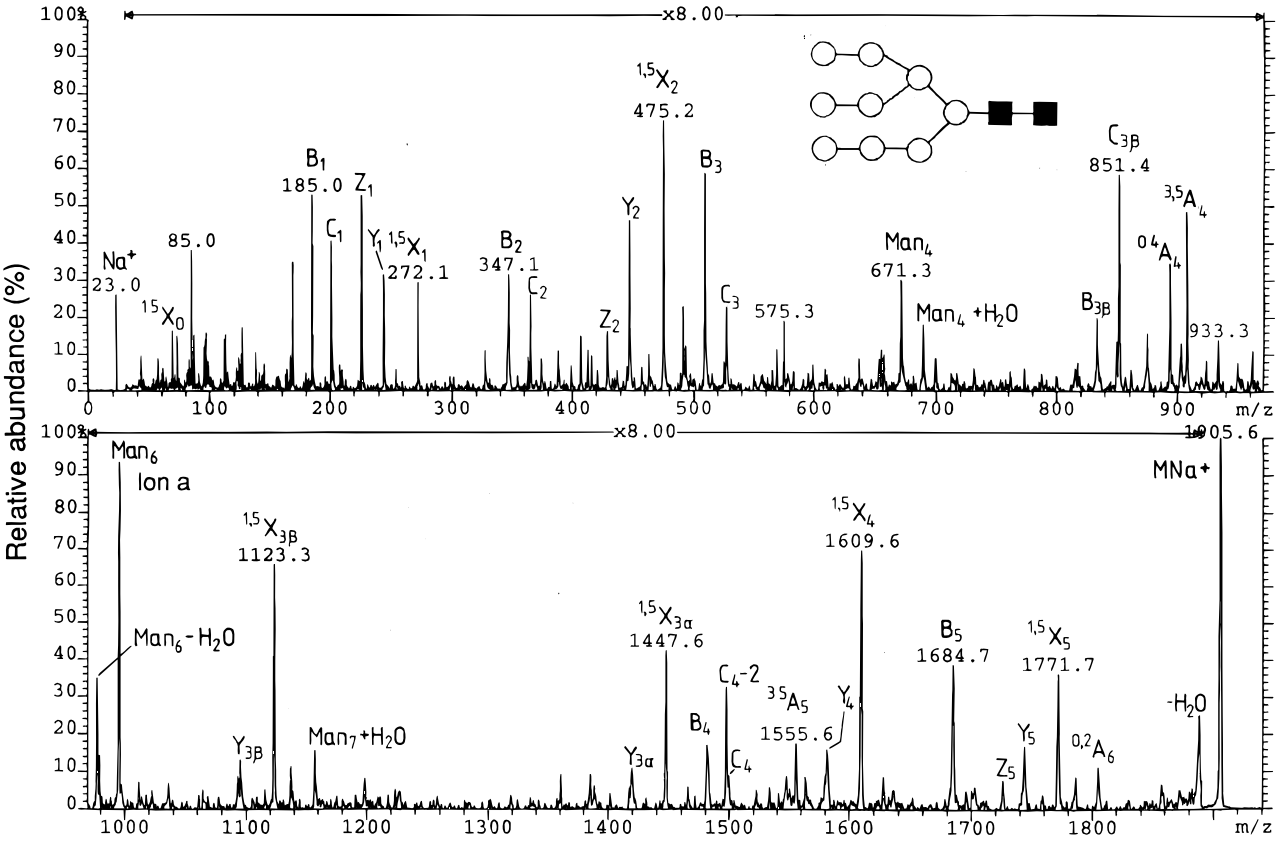


Figure 2. CID spectrum of the high-mannose sugar $(Man)_9(GlcNAc)_2$ (**VII**). Ions are labelled with the ion structures as defined in Fig. 3 with the addition that H = hexose and N = GlcNAc. The ion labelled Man_4 (*m/z* 671.3) is an internal fragment with a composition of $(Man)_4$ but of undetermined origin. Masses are monoisotopic.

Table 2. Selected fragment ionic masses and relative abundances for ions in the CID spectra of the high-mannose sugars

	Compound						
Ion	I	II	III	IV	V	VI	VII
MNa^+	933.3[a]	1079.8	1257.4	1419.9	1581.9	1743.4	1905.6
Base	475.2	347.2	475.2	475.3	671.5	689.8	995.4
a	347.2	347.2	671.2	671.2	671.5	833.0	995.4
	(73)[b]	(100)	(62)	(96)	(100)	(82)	(100)
$^{3,5}A$	583.2	583.2	583.4	583.4	583.4	705.2	907.2
	(61)	(45)	(10)	(19)	(19)	(25)	(53)
$^{1,5}X_2$	475.2	621.3	475.2	475.3	475.3	475.4	475.2
	(100)	(49)	(100)	(100)	(64)	(76)	(79)
$^{1,5}X_{3\alpha}$	799.0	945.1	1123.3	1123.4	1123.4	1286.2	1447.6
	(56)	(31)	(75)	(37)	(73)	(40)	(70)
$^{1,5}X_{3\beta}$	799.0	945.1	799.3	961.5	1123.4	1123.1	1123.3
	(56)	(31)	(50)	(37)	(73)	(40)	(70)
$^{1,5}X_{4\alpha}$	—	—	—	1285.6	1285.6	1447.6	1609.6
				(42)	(36)	(82)	(75)
$^{1,5}X_{4\beta}$	—	—	1123.3	1285.6	1447.7	1608.9	1609.6
			(75)	(42)	(39)	(71)	(75)
$^{1,5}X_{4\gamma}$	—	—	1123.3	1285.6	1447.7	1447.6	1609.6
			(75)	(42)	(39)	(82)	(75)
$^{1,5}X_{5\alpha}$	—	—	—	—	1447.7	1608.9	1771.7
					(39)	(71)	(39)
$^{1,5}X_{5\beta}$	—	—	—	—	—	—	1771.7
							(39)
$^{1,5}X_{5\gamma}$	—	—	—	—	—	1608.9	1771.0
						(71)	(39)

[a] Measured mass (*m/z*).
[b] Abundance (%) relative to that of the largest fragment ion.

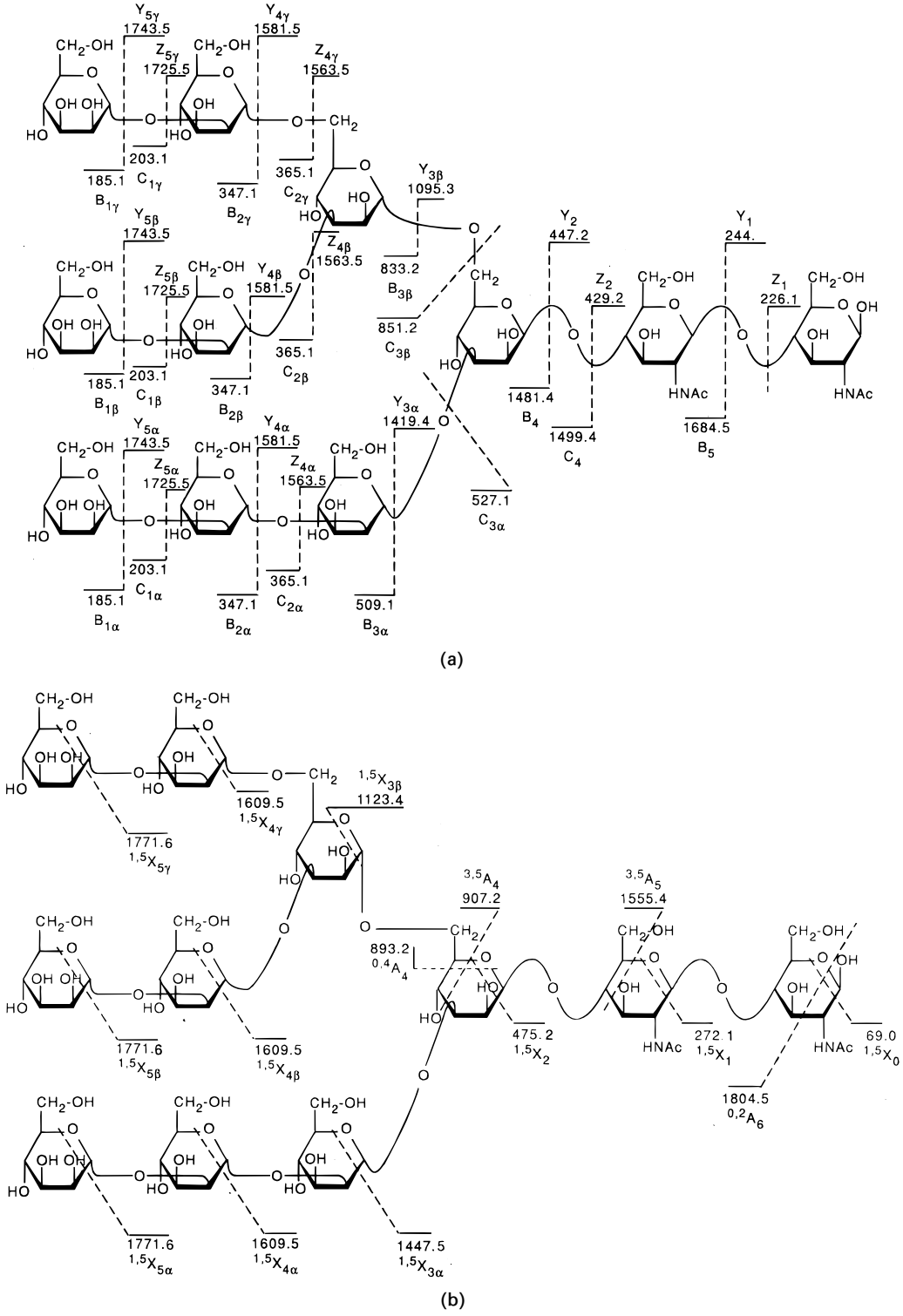


Figure 3. (a) Glycosidic cleavages found in the CID spectrum of $(Man)_9(GlcNAc)_2$ (**VII**). The fragmentation scheme is based on that proposed by Domon and Costello.[45] Hydrogen transfers have been omitted for clarity. The masses shown for each fragmentation are the calculated monoisotopic masses. (b) Cross-ring cleavages found in the CID spectrum of $(Man)_9(GlcNAc)_2$ (**VII**).

allowing an epoxide ring to form from the oxygen atom to C-3. It has often been reported that there is a preferential loss of the group attached to the 3-position of sugars (water, methanol or a sugar residue, depending on the compound and derivative),[4,6,10,41,46,47] probably either for the same reason or, in the case of B-type oxonium ion formation, by forming a conjugated system.[4]

It is not clear if ion *a* is formed by consecutive reactions or whether it involves a concerted internal fragmentation. Kovacik *et al.*[14] have proposed the occurrence of concerted internal cleavages to account for ions appearing at 16 mass units lower than Y-cleavage ions in the spectra of certain linear oligosaccharides. However, in the present case, the observed ion represents *loss* of the terminal sugar residues whereas

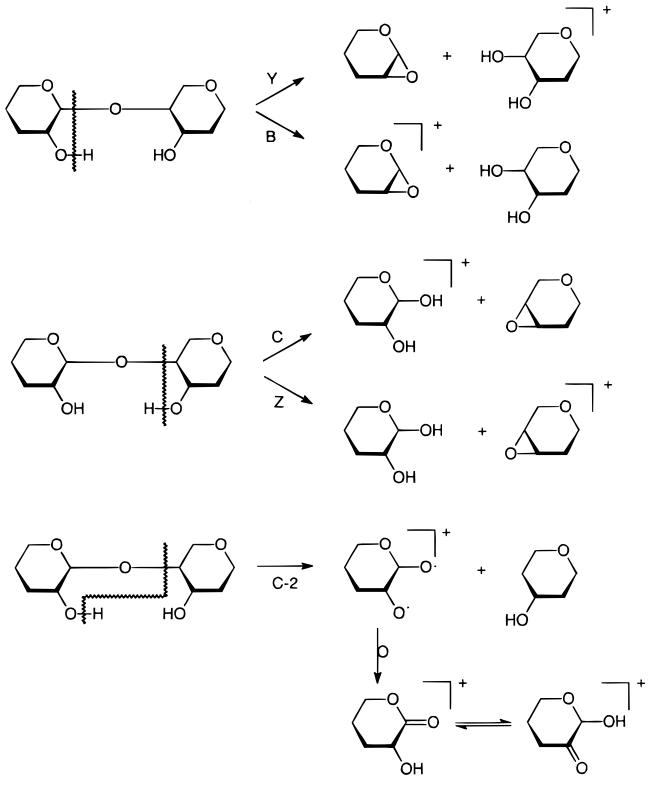


Figure 4. Scheme showing formation of the glycosidic cleavage ions. The origin of the migrating hydroxylic hydrogen atoms is unknown and is only shown for illustrative purposes. Remaining sugar residues and hydroxy groups have been removed for clarity.

the ions reported by Kovacik *et al.*[14] *contained* the terminal residues and were formed by expulsion of an internal fragment. Nevertheless, the proposal supports the occurrence of interactions between sugar rings during fragmentation and the possibility of complex internal rearrangement reactions. Similar internal cleavages have also been observed under Cl conditions[48] and in PSD spectra.[34] Inspection of molecular models shows that, with the sugar rings in their normal, chair, configuration, no suitable interactions are possible between the 2-GlcNAc and 4B-mannose residues, suggesting the absence of a concerted mechanism. However, this lack of interaction with the sugar rings in their normal chair conformation may not rule out a concerted reaction for the formation of ion *a* if the energy is sufficient to enable the rings to adopt a boat conformation. Such a mechanism has, in fact, been proposed to account for the relative abundance of the rearrangement ion at *m/z* 147 in the electron impact mass spectra of the trimethylsilyl derivatives of steroids.[49] Although the ionization mode was different in this work, the results demonstrate that ground-state conformations are not necessarily involved in rearrangement reactions in the mass spectrometer. If the energy imparted to these oligosaccharides were sufficient to achieve such a conformational change, then the 4-mannose ring could come to within bonding distance of the internal GlcNAc residue and possibly initiate a concerted reaction. However, another argument against a concerted mechanism is that many other ions produced by internal cleavages are present in these spectra whose formation appears to be the result of secondary, tertiary or quaternary fragmentations. The latter two fragmentations, which involve the rupture of three and four bonds, respectively, are unlikely to be formed through concerted mechanisms.

Ion *a* was usually accompanied by a triplet of ions at 18, 16 and 14 mass units lower in mass. The loss of 18 mass units probably corresponded to loss of water from ion *a* whereas the ion appearing 14 mass units lower

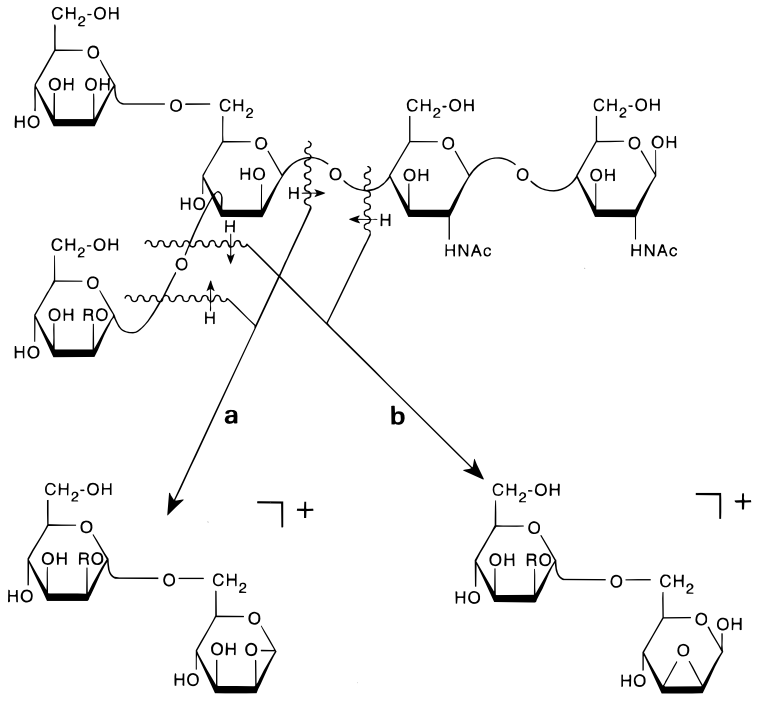


Figure 5. Scheme showing the proposed alternative routes for the formation of ion *a*. R = remainder of glycan.

Table 3. Masses of the ions formed by $^{1,5}X$ cleavages in the spectra of oligosaccharides with structures $(Man)_{1-9}(GlcNAc)_2$

			Number of mannose or partial mannose residues lost								
Man[a]	Compound	MW[b]	1	2	3	4	5	6	7	8	9
1	—	609.2	475.2	—	—	—	—	—	—	—	—
2(A)[c]	—	771.3	637.2	475.2	—	—	—	—	—	—	—
2(B)	—	771.3	637.2	475.2	—	—	—	—	—	—	—
3	I	933.3	799.3	—	475.2	—	—	—	—	—	—
4(A)	—	1095.4	961.3	799.3	—	475.2	—	—	—	—	—
4(B)	—	1095.4	961.3	799.3	—	475.2	—	—	—	—	—
4(C)	—	1095.4	961.3	799.3	—	475.2	—	—	—	—	—
5	III	1257.4	1123.4	—	799.3	—	475.2	—	—	—	—
6(A)	—	1419.5	1285.4	1123.4	—	799.3	—	475.2	—	—	—
6(B)	—	1419.5	1285.4	1123.4	—	799.3	—	475.2	—	—	—
6(C)	IV	1419.5	1285.4	1123.4	961.3	—	—	475.2	—	—	—
7(A)	—	1581.5	1447.5	1285.4	—	961.3	—	—	475.2	—	—
7(B)	—	1581.5	1447.5	1285.4	—	961.3	—	—	475.2	—	—
7(C)	V	1581.5	1447.5	1285.4	1123.4	—	—	—	475.2	—	—
8(AB)	—	1743.6	1609.5	1447.5	—	—	961.3	—	—	475.2	—
8(AC)	VI	1743.6	1609.5	1447.5	1285.4	1123.4	—	—	—	475.2	—
8(BC)	–	1743.6	1609.5	1447.5	1285.4	1123.4	—	—	—	475.2	—
9	VII	1905.6	1771.6	1609.5	1447.5	—	1123.4	—	—	—	475.2

[a] Number of mannose residues in the structure.
[b] Molecular weight (monoisotopic).
[c] Letters in parentheses denote the isomer (with reference to Fig. 1). The letters represent the presence of mannose on the respective arms lettering from the top.

may represent a W-type cleavage as discussed below. The genesis of the other ion is not clear.

The ions in the spectrum of compound **VII** (Fig. 2) labelled as Man-4 and Man-7 are undoubtedly also secondary internal cleavage products. The ion labelled Man-4 probably arises from loss of the chitobiose core together with the C-6 chain from the branching mannose, whereas that labelled Man-7 can be envisaged

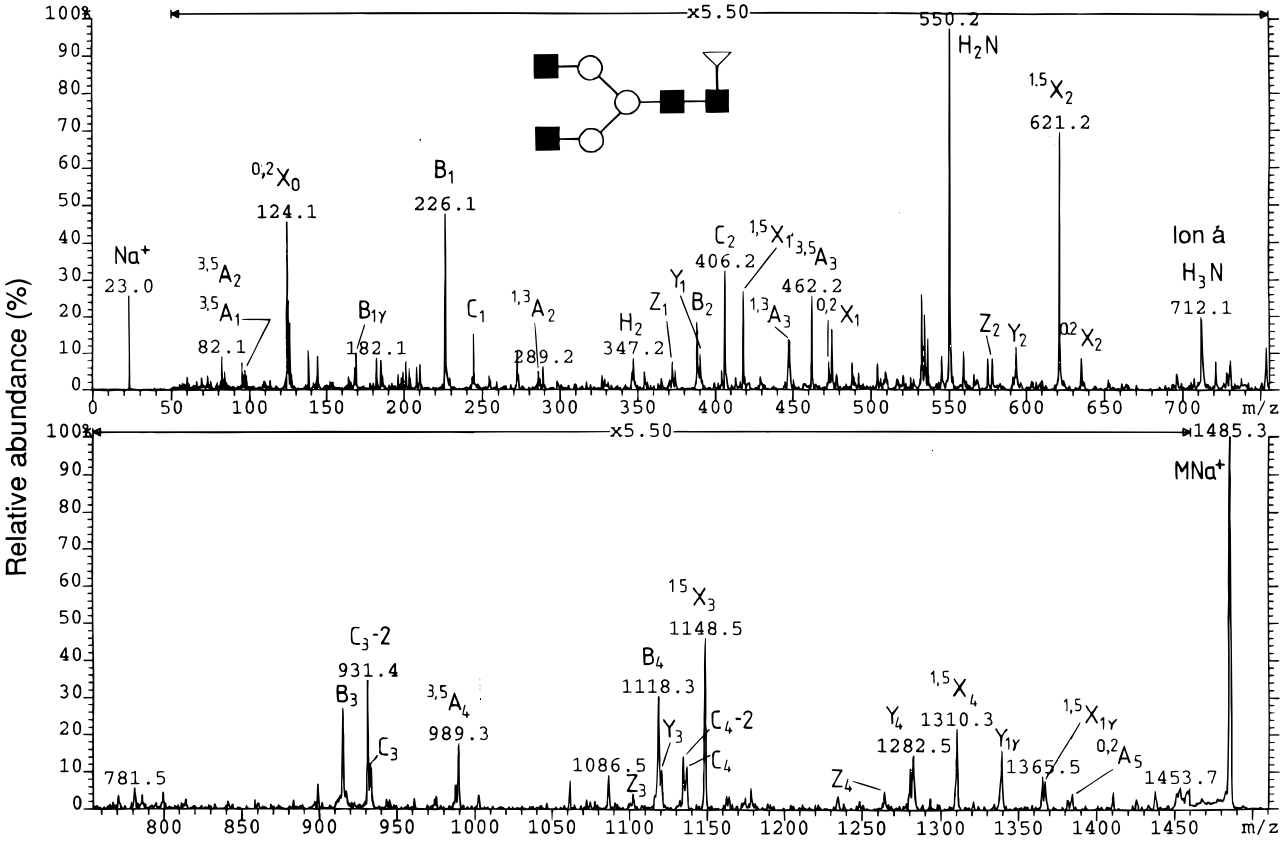


Figure 6. CID spectrum of the complex-type sugar $(Man)_3(GlcNAc)_4Fuc$ (**VIII**). Ion structures are as defined in Fig. 7 and the note to Fig. 2. The ion labelled H_2N (*m/z* 550.2) is formed by loss of the 'bisecting' GlcNAc from ion *a* (*m/z* 712.1).

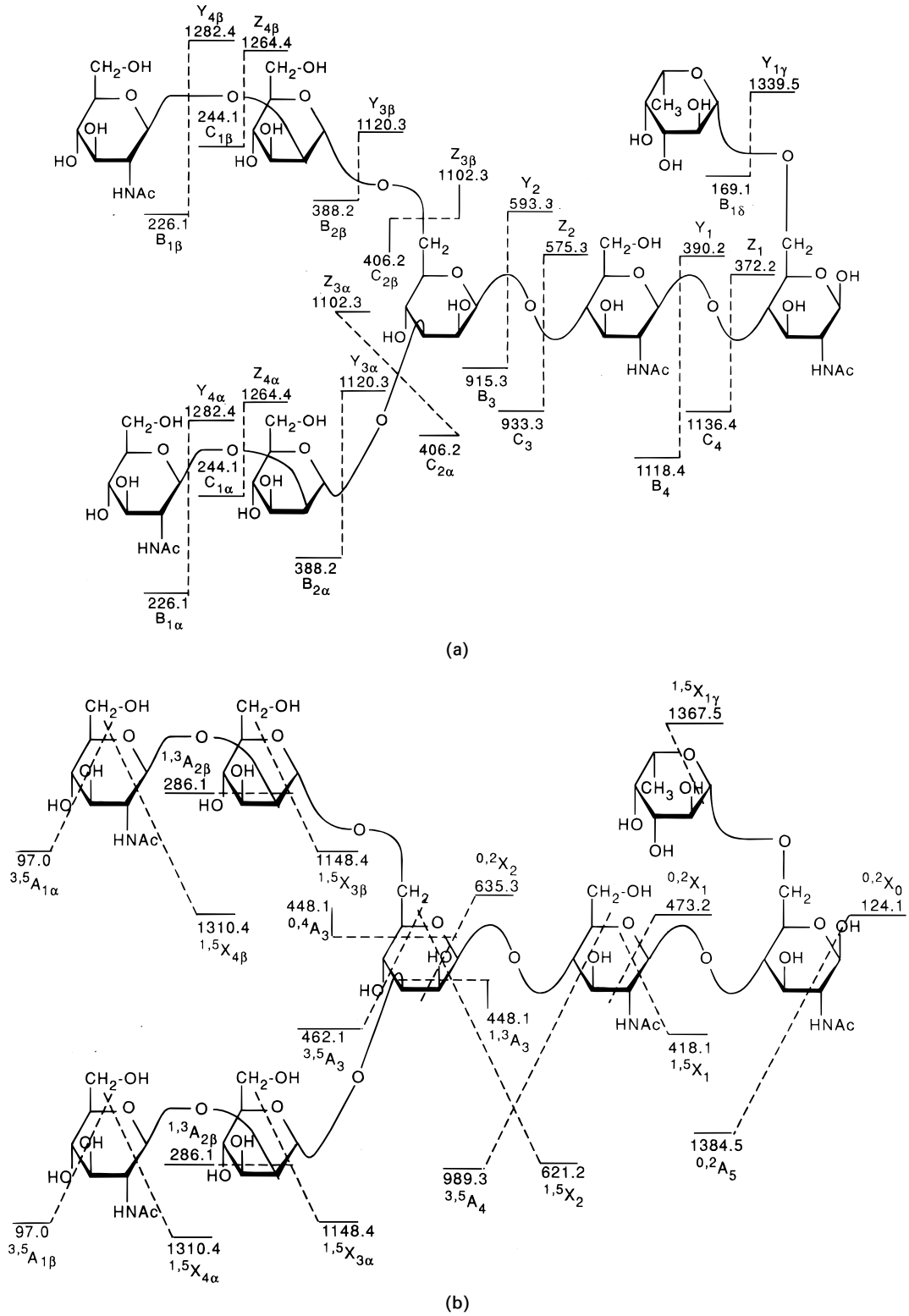


Figure 7. (a) Glycosidic cleavages found in the CID spectrum $(Man)_3(GlcNAc)_4Fuc$ (**VIII**). (b) Cross-ring cleavages found in the CID spectrum of $(Man)_3(GlcNAc)_4Fuc$ (**VIII**).

as having lost the chitobiose core and a $(Man)_2$ fragment.

$^{1,5}X$-type cross-ring cleavages, which are usually weak or non-existent in PSD[34] and ISD[39,50] spectra, were among the most abundant ions in the spectra of these oligosaccharides (Table 2). They were observed to occur from all sugar residues and revealed the branching pattern of the antennae as shown in Table 3. For example, the Man-6C structure could be distinguished from the Man-6A and -6B structures by the presence of the ion at *m/z* 961.3 and the absence of that at *m/z* 799.3. The other two structures contained *m/z* 700.3 but not *m/z* 961.3. Some isomeric structures containing chains of equivalent length, such as the Man-6A and -B

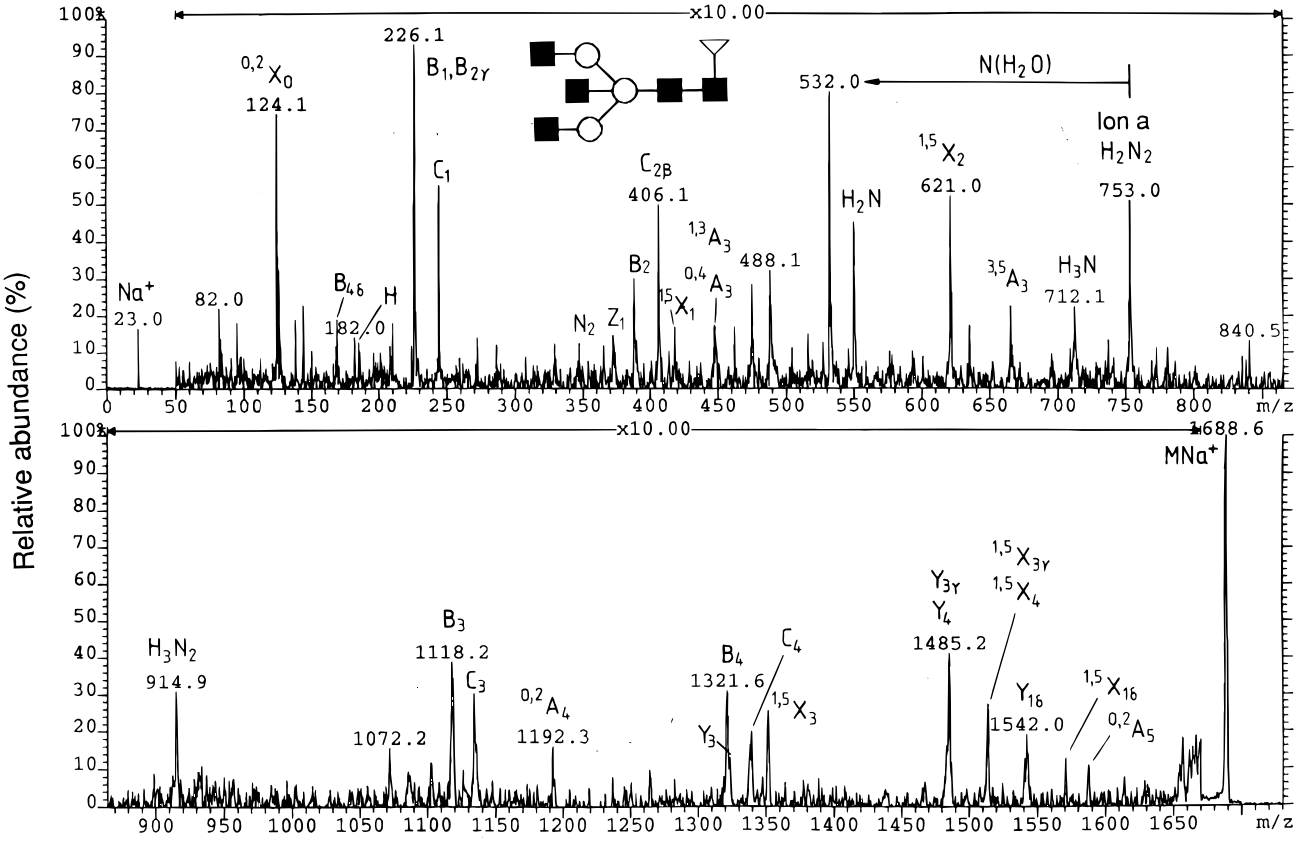


Figure 8. CID spectrum of the 'bisected' complex-type sugar $(Man)_3(GlcNAc)_5Fuc$ (**IX**). Ion structures are as defined in Fig. 9 and the note to Fig. 2. The ion labelled H_2N is formed by loss of the 'bisecting' GlcNAc from ion *a* (*m/z* 753.0). The ion labelled H_3N_2 (*m/z* 914.9) is an internal fragment with the composition $(Man)_3(GlcNAc)_2$ but of undetermined origin.

isomers (Table 3), however, could not be differentiated in this manner.

A prominent cross-ring cleavage $^{3,5}A$ ion from the core-mannose residue was present in most of the spectra (*m/z* 907.2, Fig. 2 and Table 4 for its mass in the other spectra). This ion, by effectively bisecting the core branching mannose, allowed the antennae compositions to be determined. $^{0,4}A$ Ions were also present in some spectra and again served to define the antennae composition. The equivalent ($^{3,5}A$ and $^{0,4}A$) ions appeared to be present, although weak, from the other branched mannose residue (the 4A-mannose) in the spectrum of $(Man)_9(GlcNAc)_2$ (**VII**) (*m/z* 421 and 407), but their equivalents were not seen in the spectra of the other compounds containing branch points other than at the core mannose. The $^{1,3}A$ ions observed by Lemoine *et al.*[13] appeared to be absent. Cross-ring fragmentation of the reducing-terminal GlcNAc residue (GlcNAc-1, Fig. 1) to give the $^{0,2}A$ and $^{2,4}A$ fragments was much less pronounced than in the corresponding ISD[39,50] and PSD[34,50] spectra of these compounds. It should be noted, however, that all cross-ring fragments were equivalent to those observed in PSD-MALDI spectra.

It appears to be easier to detect linkage in the CID spectra of permethylated oligosaccharides than in these spectra of native sugars, probably because the competing glycosidic cleavage reactions are less abundant in the spectra of the methylated compounds as the result of the lack of hydroxylic hydrogen atoms. Thus, the linkage-specific $^{0,4}A$, $^{3,5}A$ and $^{1,3}A$ cleavages employed by Reinhold *et al.*[32] in the spectra of the permethylated sugars were weak in the MALDI-CID spectra of native sugars reported in this paper, except at the core-mannose residue as discussed above.

Complex biantennary oligosaccharides (VIII–XIII)

The CID spectra of $(Man)_3(GlcNAc)_4Fuc$ (**VIII**) and $(Man)_3(GlcNAc)_5Fuc$ (**IX**) which differ by the presence of a bisecting GlcNAc residue are shown in Figs 6 and 8. Fragmentations responsible for the major ions are shown in Figs 7 and 9. Figures 10 and 11 show the spectra and major fragmentation pattern for the galactosylated derivative (**XI**) of compound **VIII**. Y-type glycosidic cleavages yielded relatively weak ions but B cleavages adjacent to GlcNAc residues gave very prominent ions paralleling the behaviour of these oligosaccharides in FAB spectra.[4] In the spectra of the sugars containing outer-arm galactose (e.g. compound **XI**, Fig. 10), the B_2 cleavage invariably gave the base peak (Table 4). C-ions were also prominent but those originating from the chitobiose core were accompanied by ions of higher abundance that appeared two mass units lower, as observed for the high-mannose sugars. Z ions were missing for cleavages at the 6-position to the branching mannose (6-antenna and fucose in the spectra of compounds **VIII**, **IX**, **XI** and **XIII**).

The internal cleavage ion *a* was prominent in all spectra (Table 4), but it should be noted that ions of the

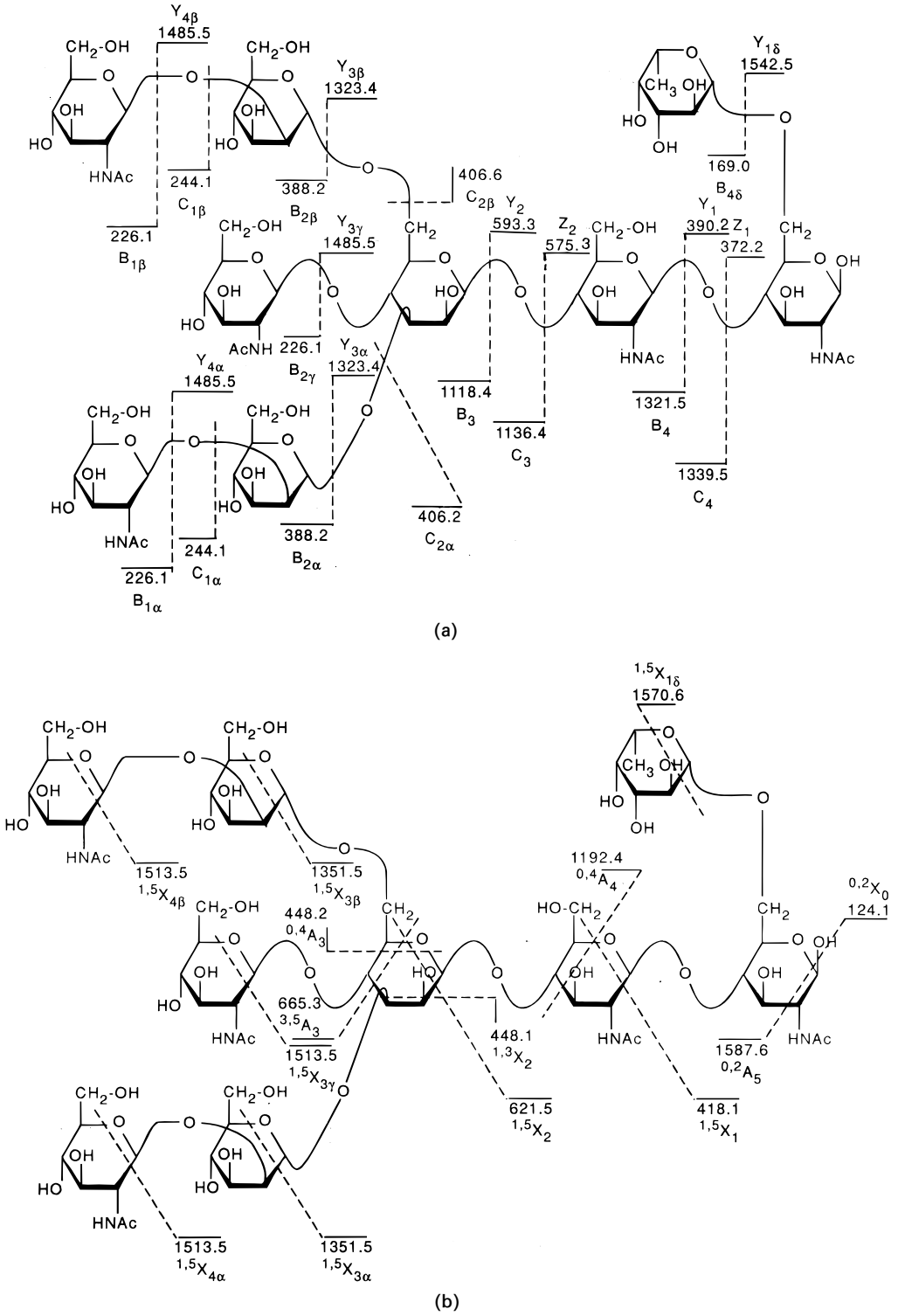


Figure 9. (a) Glycosidic cleavages found in the CID spectrum $(Man)_3(GlcNAc)_5Fuc$ (**IX**). (b) Cross-ring cleavages found in the CID spectrum of $(Man)_3(GlcNAc)_5Fuc$ (**IX**).

same mass could be formed by other internal cleavages although, in general, these other ions appeared to be formed in relatively low abundance. The presence of a bisecting GlcNAc residue was clearly indicated by a prominent loss of the GlcNAc residue as an intact molecule (221 mass units) from ion *a* (Fig. 12), as illustrated by the pair of ions at *m/z* 753.3 and 532.2 in Fig. 8 (see also Table 4). In the fragmentation of compounds lacking the bisecting GlcNAc residue, ion *a* lost water, probably from C-4 of the core-mannose residue, to give an ion of the same mass as that formed by loss of GlcNAc (221 mass units) when the bisecting GlcNAc was present (e.g. *m/z* 532 in Fig. 8).

In common with the spectra of the high-mannose oligosaccharides, the cross-ring $^{1,5}X$ ions were of high relative abundance (Table 4). Several of the other cross-ring cleavages reported by Reinhold *et al.*[32] as defining linkage were found; these were particularly abundant

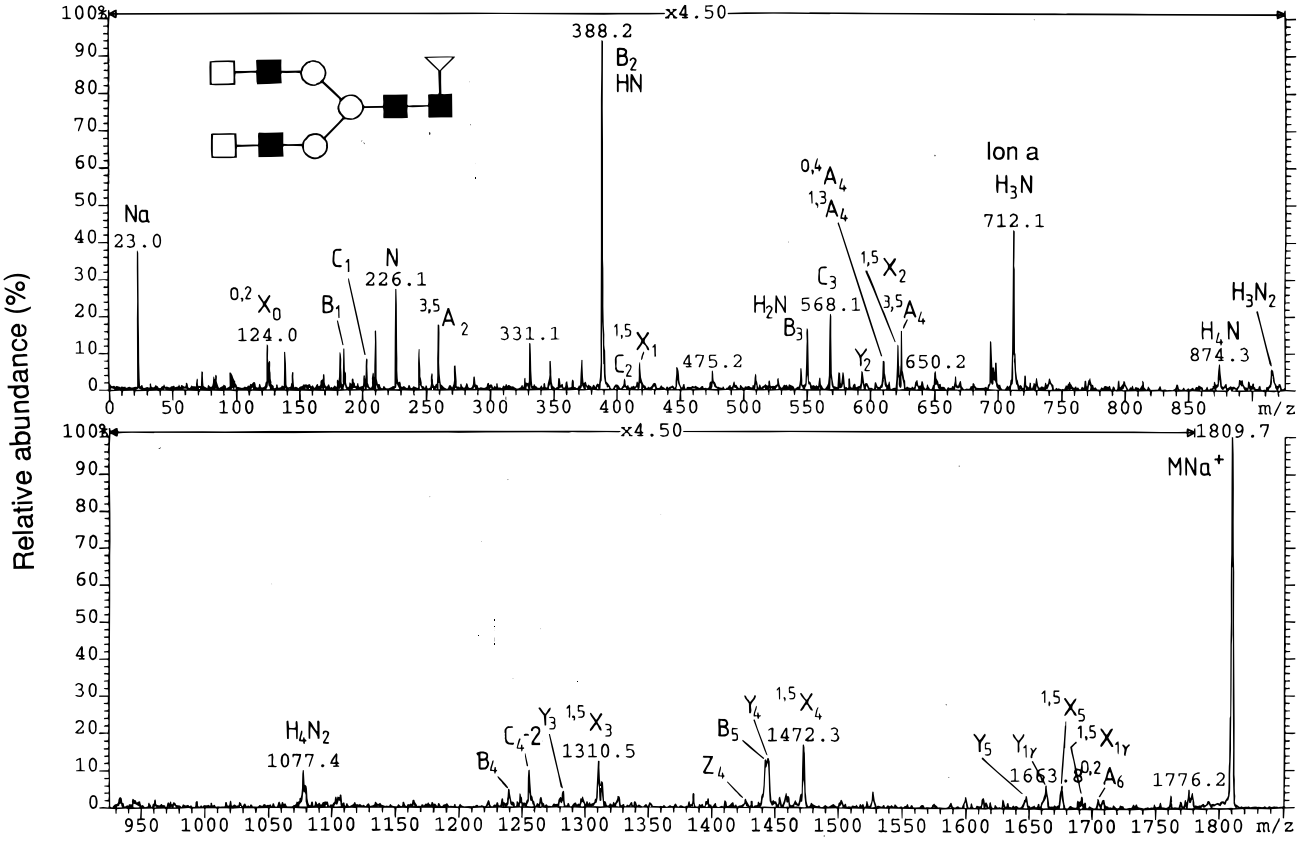


Figure 10. CID spectrum of the complex-type sugar $(Gal)_2(Man)_3(HexNAc)_4Fuc$ (**XI**). Ion structures are as defined in Fig. 11 and the note to Fig. 2. The ions labelled N (*m/z* 226.1), HN (*m/z* 388.3, isobaric with B_2), H_2N (*m/z* 550.3, isobaric with B_3), H_3N (*m/z* 712.1, mainly ion *a*), H_4N (*m/z* 874.3, $(Hex)_4GlcNAc$), H_3N_2 (*m/z* 915.3, $(Hex)_3(GlcNAc)_2$) and H_4N_2 (*m/z* 1077.4, $(Hex)_4(GlcNAc)_2$) are internal cleavage ions of undetermined origin.

for the 3-(branching)mannose residue. Thus the $^{1,3}A$ cleavage defined linkage at the 2- or 3-positions and, in the case of the core mannose, these linkage positions could sometimes be differentiated by the $^{0,2}X$ ion. The composition of the antennae was best indicated by the mass of the $^{3,5}A$ ion from the core mannose (Table 4) and sometimes also by the mass of the $^{0,4}A$ ion. However this latter ion was isobaric with the $^{1,3}A$ ion in the spectra of compounds with two equivalent antennae and would need permethylation in order to isolate it.

As with the high-mannose oligosaccharides, cross-ring cleavages of the reducing-terminal GlcNAc (GlcNAc-1) residue were weak. $^{0,2}A$-Cleavages (loss of 101 mass units) were generally the most abundant. The $^{3,5}A$ cleavages could be used to locate a core fucose residue to C-6.

Triantennary and tetraantennary oligosaccharides (XIV–XVI)

Most of the glycosidic and cross-ring cleavages found in the spectra of the biantennary oligosaccharides were also found in the spectra of these sugars (see, for example, Figs 13 and 14). However, there were several ions that appeared to have no strict parallel in the spectra of the biantennary oligosaccharides. The most prominent of these was the abundant ion at *m/z* 404 (termed ion *b*) in the spectra of the sugars not possessing a galactose residue at the reducing terminus of the antennae (compounds **XIV** and **XVI**). This ion shifted by the equivalent of one galactose residue in the spectrum of the compound in which the galactose was present $[(Gal)_3(Man)_3(HexNAc)_5$ **XV**]. The ion's even mass indicated the presence of one nitrogen atom (assuming that the ion retained the sodium atom), which was consistent with the presence of a GlcNAc and a mannose residue together with the oxygen linking it to the core mannose residue (position 3, Fig. 1). The presence of the ion in the spectra of both the tri- and tetra-antennary sugars, both of which contained a branched 3-antenna, but not in that of the biantennary oligosaccharides indicated that it originated from the 3-antenna by elimination of a GlcNAc residue (Fig. 15). Hence this ion appears to represent an internal cleavage fragment from the 3-antenna. However, it is not possible to determine if its formation was a concerted or a two-step process without the spectra of additional compounds. The presence of this ion, however, was a clear indication of a branched antenna but insufficient compounds were available to determine if its formation was linkage specific. Also, it was unfortunate that the other common triantennary oligosaccharide, having the additional antenna at C-6 of the 4A-mannose, was not available for checking if this ion was specific to the 3-antenna.

The prominent ions at *m/z* 739 in the spectra of the tri- and tetra-antennary sugars not containing galactose

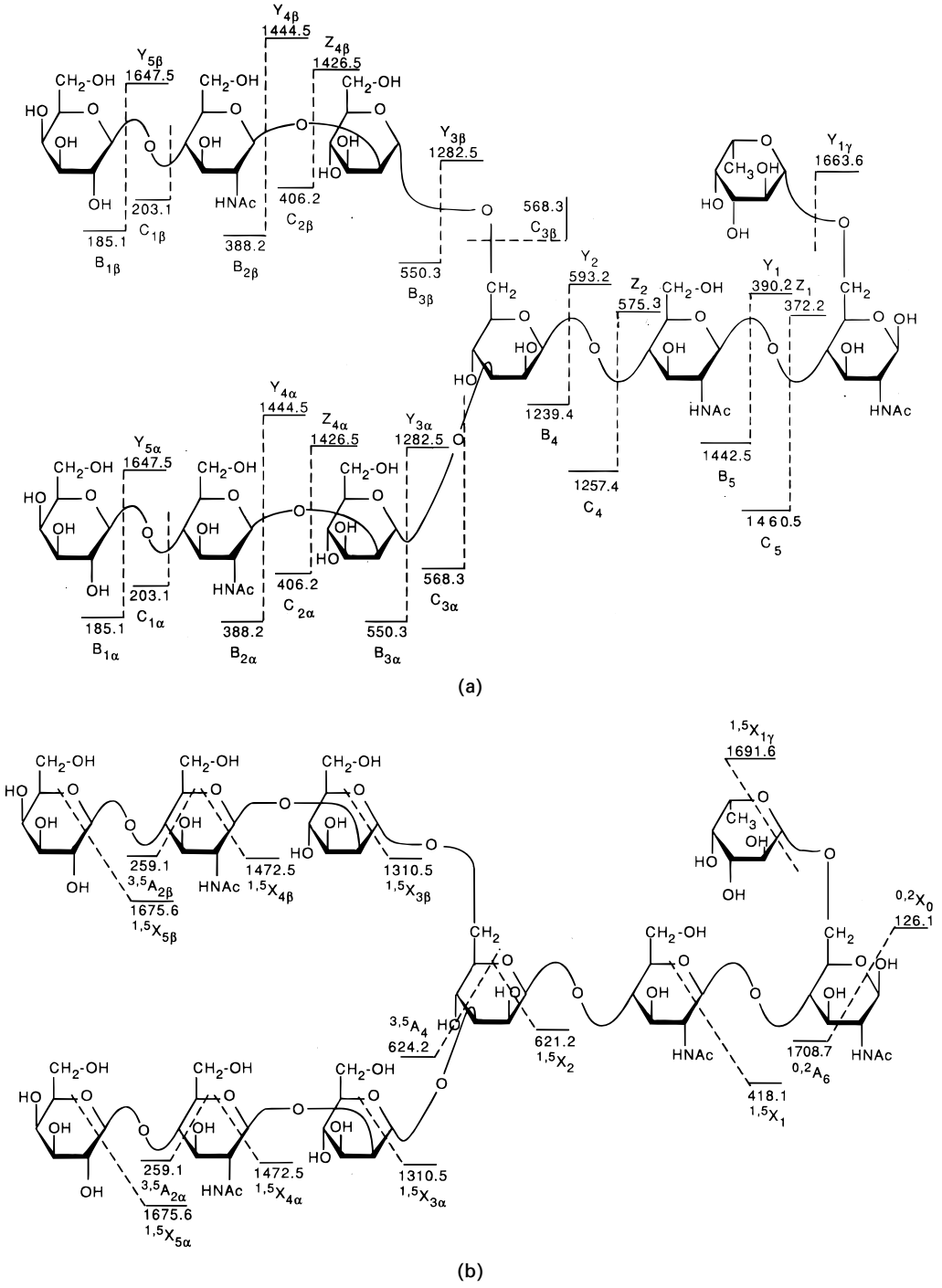


Figure 11. (a) Glycosidic cleavages found in the CID spectrum $(Gal)_2(Man)_3(HexNAc)_4Fuc$ (**XI**). (b) Cross-ring cleavages found in the CID spectrum of $(Gal)_2(Man)_3(HexNAc)_4Fuc$ (**XI**).

(see Fig. 13 and Table 4) and at *m/z* 1063 when galactose was present appeared to be examples of another type of internal cleavage ion (termed in *c*) involving loss of the chitobiose core and the 6-antenna possibly by a W-type cleavage[13] (Fig. 16). These ions could, therefore, also be used to define the antennae structure and showed that the triantennary sugars **XIV** and **XV** clearly contained two GlcNAc residues on the lower (3) arm. The presence of ion *c* in the spectra of the biantennary sugars discussed above possibly accounted for the ion appearing at 14 mass units below ion *a*.

Several other ions served to define the triantennary structure of compounds **XIV** and **XV**. Thus, for example, the difference in mass between the $^{1,5}X_{3\alpha}$ and $^{1,5}X_{3\beta}$ ions (Table 4) indicated asymmetric substitution on the antennae. Ion *a* was present at *m/z* 550 in the spectrum of compound **XIV** and at *m/z* 712 in that of compound **XV**, showing the absence of branching on the 6-antenna. The $^{3,5}A$ and $^{3,5}X$ ions from the branching core mannose confirmed that the additional GlcNAc residue was situated on the 3-antenna. The structure was also reflected in the higher abundance of

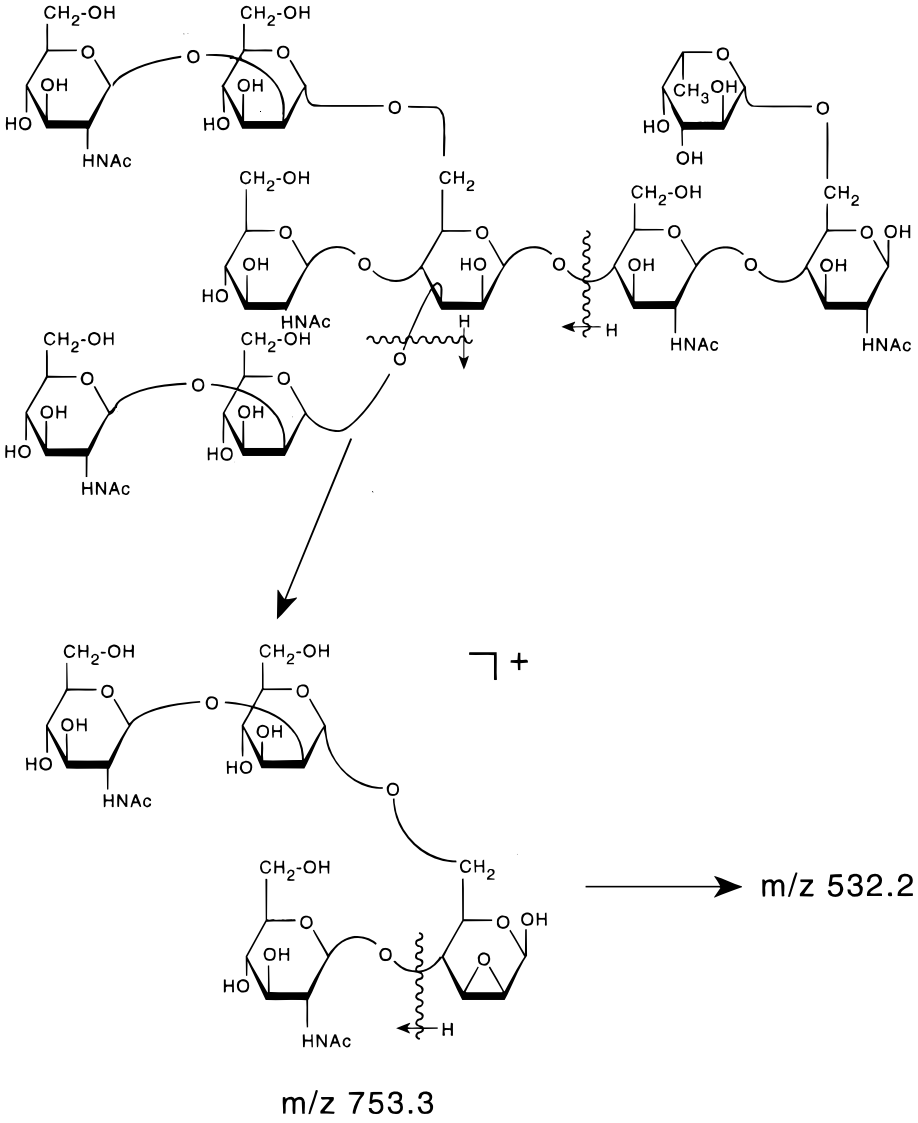


Figure 12. Scheme showing the loss of a bisecting (4-linked) GlcNAc residue from ion *a* in the MALDI mass spectrum of compound **XII**.

the $Y_{3\alpha}$ ions compared with that of the $Y_{3\beta}$ ions. The absence of a bisecting GlcNAc residue was reflected by the absence of a 221 Da (GlcNAc) loss from ion *a*. A weak ion corresponding to loss of water from ion *a*, probably from the 4-position, was, however, present.

Hybrid oligosaccharide $(Man)_5(GlcNAc)_4$(XVII)

Figure 17 shows the spectrum of this compound and the proposed fragmentation is shown in Fig. 18. Features common to both the biantennary and high-mannose structures were present. Ion *a* at *m/z* 874 indicated the presence of the one GlcNAc and three mannose residues bound to the core mannose and the loss of an intact GlcNAc sugar from ion *a* to give the ion at *m/z* 653 indicated the presence of the bisecting GlcNAc residue at C-4 of the core mannose. Absence of the $Z_{3\gamma}$ ion at *m/z* 1159.4 was consistent with the presence of the three mannose residues being attached to C-6 of the core-mannose residue. The possible presence of $Z_{4\alpha}$ and $Z_{3\beta}$ ions was masked by the isobaric B_4 ion. The $Y_{3\gamma}$ and $^{1,5}X_{3\gamma}$ ions at *m/z* 1177.5 and 1205.5, respectively, defined the three mannose residues which were linked at their central (4) mannose residue and the $^{3,5}A_3$ and $^{0,4}A_3$ ions at *m/z* 786.2 and 569.3 defined the linkage as being at C-6 of the 3-mannose. Although no ion was found that defined the linkage of the 3-antenna directly, the presence of the $^{0,2}X_2$ ion at *m/z* 489.2 showed that the 2-hydroxy group of the 3-mannose residue was free, thus leaving the 3-position as the only remaining substitution site. Most of the other ions appeared to be produced by conventional mechanisms (see Fig. 18).

CONCLUSIONS

High-energy collisional activation of the MNa^+ ions from these oligosaccharides ionized by MALDI produced informative spectra with fragment ions distributed evenly across the mass range. All ions appeared to contain sodium and were probably formed by charge-

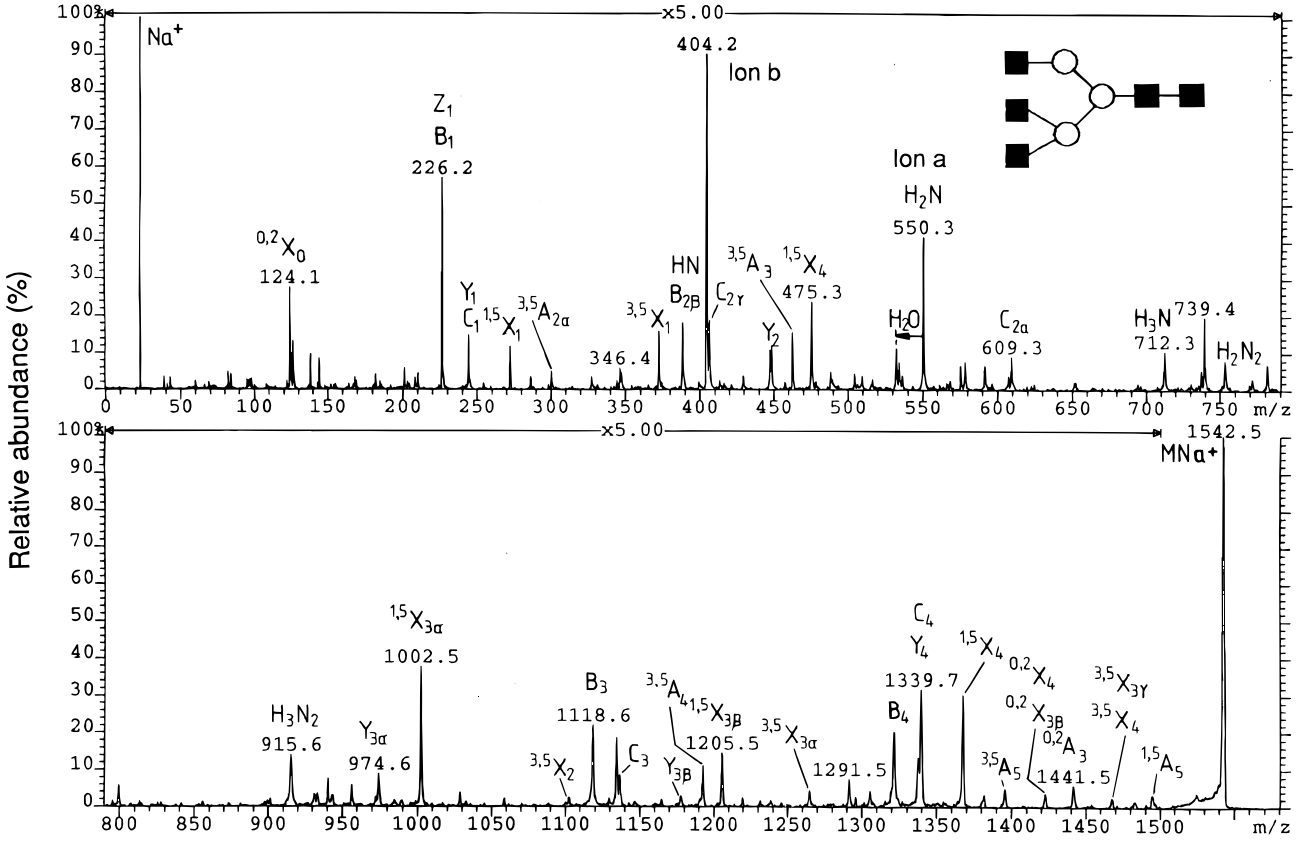


Figure 13. CID spectrum of the complex-type sugar $(Man)_3(HexNAc)_5$ (**XIV**). Ion structures are as defined in Fig. 14 and the note to Fig. 2. The ions labelled HN (*m/z* 388.3, isobaric with $B_{2\beta}$), H_2N (*m/z* 550.3, isobaric with B_3), H_3N (*m/z* 712.3, mainly ion *a*), H_2N_2 (*m/z* 753.3, $(Hex)_2(GlcNAc)_2$) and H_3N_2 (*m/z* 915.3, $(Hex)_3(GlcNAc)_2$) are internal cleavage ions of undetermined origin.

remote processes. Three types of ion were found: those formed by glycosidic cleavage, those formed by cross-ring cleavage and those formed by internal glycosidic cleavages. The relative abundance of the cross-ring cleavage ions was very much higher than in the spectra obtained by either PSD or ISD. B, C or C-2 and Y ions were all produced in abundance from most glycosidic linkages and the related internal fragments were often the most abundant ions in the spectra. B ions were particularly abundant when cleavage occurred adjacent to GlcNAc. The profusion of glycosidic cleavage ions was probably the result of the free hydroxy groups in these native oligosaccharides as it appears to be the hydroxylic hydrogen atoms that migrate during the glycosidic cleavages.[21] An adverse consequence of the abundant glycosidic cleavage ions was that several of the cross-ring cleavages defining linkage, as reported by others,[13,32] appeared to be absent.

Some cross-ring cleavages were, however, abundant. In particular, $^{1,5}X$ cleavages were found for all constituent monosaccharide residues and usually defined the branching pattern. Other cleavages were particularly abundant around the branching 3-mannose and enabled the linkage pattern to be ascertained. In particular, the $^{3,5}A$ ion clearly distinguished between substituents on the 3- or 6-arm of this mannose residue.

The following ions were found to be particularly useful in defining the structure of these compounds.

1. The branching pattern around the core mannose was defined by:
   ion *a* which contained the 6-arm and bisecting antenna at C-4;
   the $^{3,5}A$ ion which again contained these two groups;
   the $^{0,4}A$ ion which contained only the 6-antenna.
   In some cases the $Y_{3\alpha}$ ion was more abundant than the $Y_{3\beta}$ fragment.
2. The presence of a core fucose residue (usually at C-6) on the reducing-terminal GlcNAc residue was best identified by the mass of the abundant B ion formed by cleavage between the 1- and 2-GlcNAc residues (loss of 367.2 Da). Its position at either C-6 or C-3 would be reflected by the mass of the $^{3,5}A$ or $^{2,4}A$ ions formed by cleavage through the reducing terminal GlcNAc residue.
3. A bisecting GlcNAc residue at C-4 of the 3-mannose residue was revealed by the prominent loss of an intact GlcNAc sugar (221 Da) from ion *a*.
4. The composition of the antenna was revealed by the mass of the prominent B ions produced by cleavage adjacent to the constituent GlcNAc residues.
5. Branching of the 3-antenna (and possibly also of the 6-arm) of the complex-type sugars gave a prominent internal cleavage ion as the result of a B-type loss of the chitobiose core together with a W-type elimination of the 6-antenna.
6. The remainder of the branching pattern was mostly revealed by the masses of the $^{1,5}X$ ions.
7. Linkage at the 6-position resulted in the absence of the corresponding Z-type glycosidic cleavage ion.

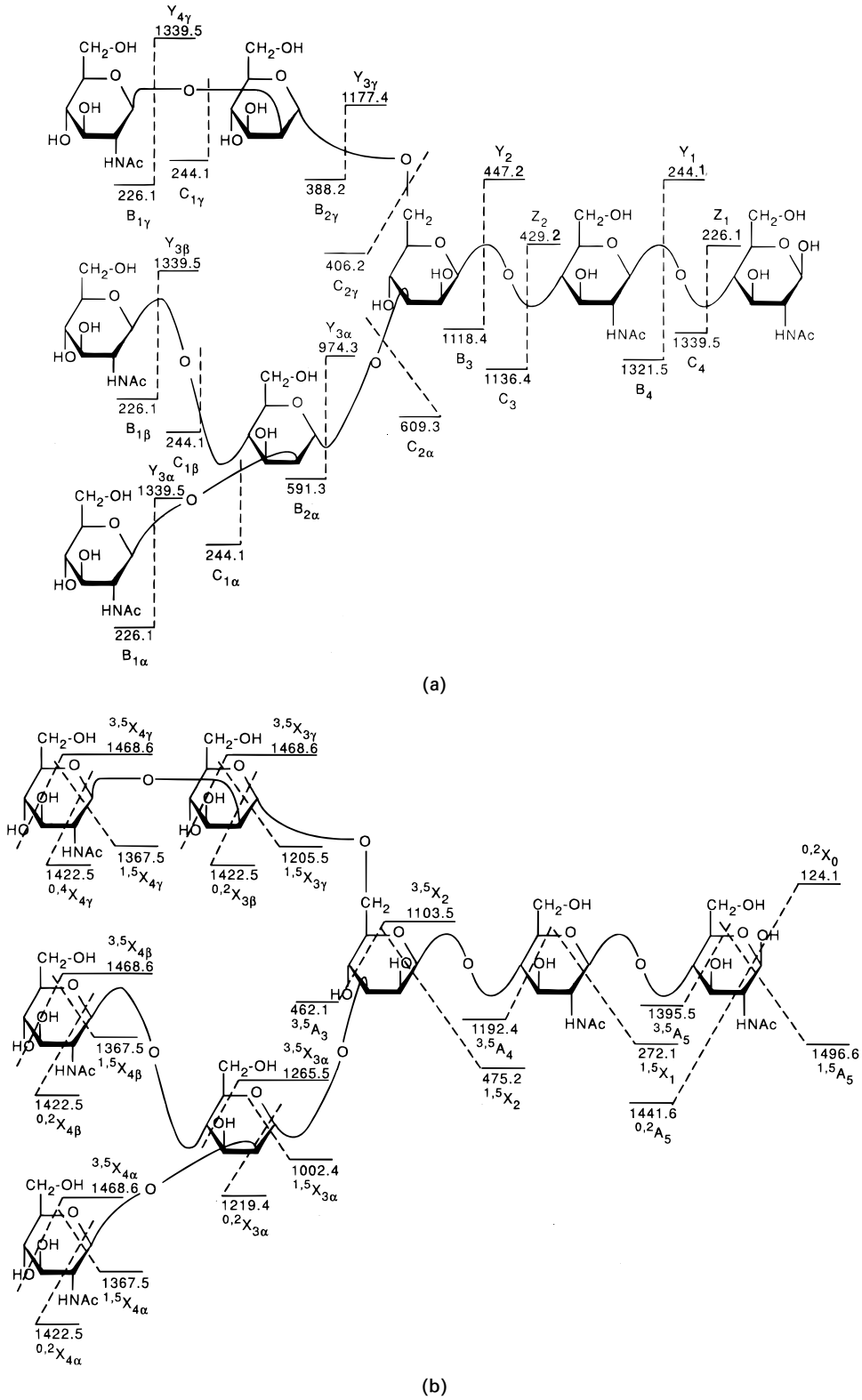


Figure 14. (a) Glycosidic cleavages found in the CID spectrum of $(Man)_3(HexNAc)_5$ (**XIV**). (b) Cross-ring cleavages found in the CID spectrum of $(Man)_3(HexNAc)_5$ (**XIV**).

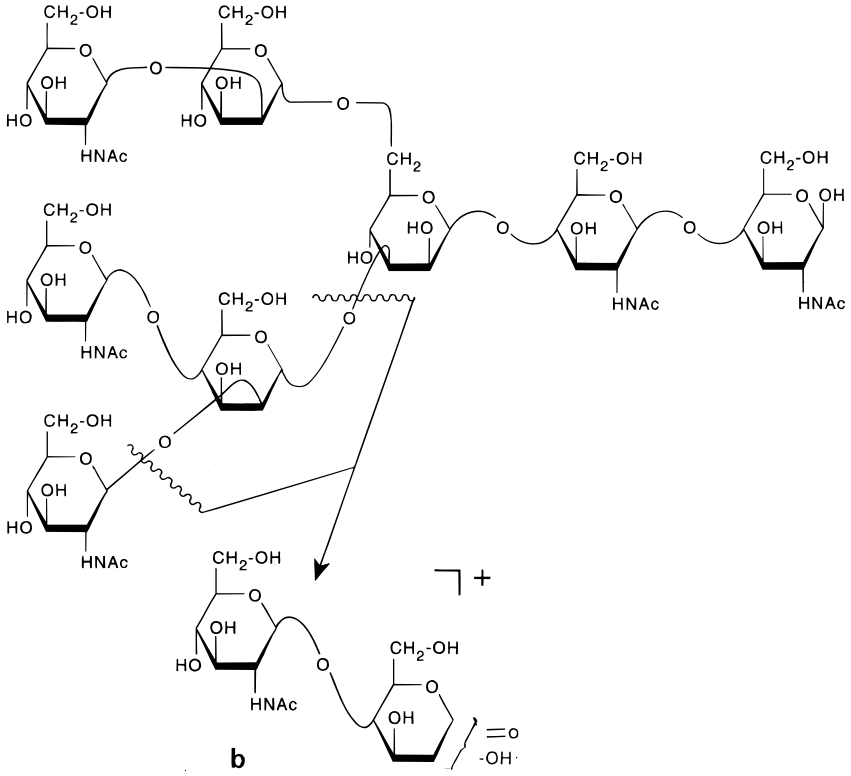


Figure 15. Scheme showing the formation of ion *b*.

Table 4. Selected fragment ionic masses and relative abundances for ions in the CID spectra of the complex and hybrid sugars

	Compound									
Ion	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII
MNa^+	1485.3[a]	1888.6	1663.7	1809.7	1867.1	2013.3	1542.5	2029.6	1746.2	1663.5
Base	550.2	226.1	388.2	388.2	388.3	388.2	404.2	388.3	404.3	475.3
a	550.2	753.0	712.3	712.1	915.4	915.5	550.3	712.4	753.2	874.4
	(100)[b]	(56)	(63)	(46)	(16)	(21)	(46)	(15)	(69)	(80)
a − N[c]	532.1	532.0	694.1	694.4	694.4	694.2	532.0	694.4	735.3	653.3
a − H_2O	(14)	(87)	(6)	(21)	(25)	(22)	(3)	(3)	(20)	(83)
b	—	—	—	—	—	—	404.0	566.4	404.3	
							(100)	(31)	(100)	—
c	536.2	—	698.4	698.3	—	—	739.4	1063.6	739.4	—
	(14)		(15)	(8)			(27)	(10)	(12)	
$^{3,5}A$	462.2	665.2	624.3	624.3	827.2	827.2	462.2	624.3	665.3	786.4
	(26)	(24)	(32)	(16)	(16)	(5)	(17)	(8)	(26)	(28)
$^{1,5}X_1$	418.2	418.2	272.1	418.1	272.1	418.1	272.1	272.1	272.1	272.1
	(28)	(19)	(12)	(8)	(9)	(5)	(13)	(7)	(6)	(17)
$^{1,5}X_2$	621.2	621.2	475.3	621.2	475.4	621.3	475.2	475.4	475.3	475.3
	(73)	(56)	(17)	(13)	(19)	(10)	(26)	(8)	(16)	(100)
$^{1,5}X_{3\alpha}$	1148.5	1351.5	1164.6	1310.5	1368.1	1514.1	1002.5	1164.7	1205.3	1326.5
	(49)	(27)	(38)	(14)	(19)	(11)	(48)	(14)	(70)	(24)
$^{1,5}X_{3\beta}$	1148.5	1351.5	1164.6	1310.5	1368.1	1514.1	1205.5	1530.2	1205.3	1326.5
	(49)	(27)	(38)	(14)	(19)	(11)	(15)	(9)	(70)	(24)
$^{1,5}X_{4\alpha}$	1310.3	1513.6	1326.6	1472.5	1530.1	1676.5	1387.5	1692.4	1570.3	1488.5
	(22)	(29)	(50)	(19)	(71)	(14)	(33)	(28)	(72)	(41)
$^{1,5}X_{4\beta}$	1310.3	1513.6	1326.6	1472.5	1530.1	1676.5	1387.5	1692.4	1570.3	1529.6
	(22)	(29)	(50)	(19)	(71)	(14)	(33)	(28)	(72)	(36)
$^{1,5}X_5$	—	—	1529.8	1676.3	1733.2	1878.7	—	1895.3	—	—
			(22)	(29)	(18)	(7)	—	(4)		

[a] Measured mass (*m/z*).
[b] Abundance (%) relative to that of the largest fragment ion.
[c] The ion formed by loss of GlcNAc from ion *a*.

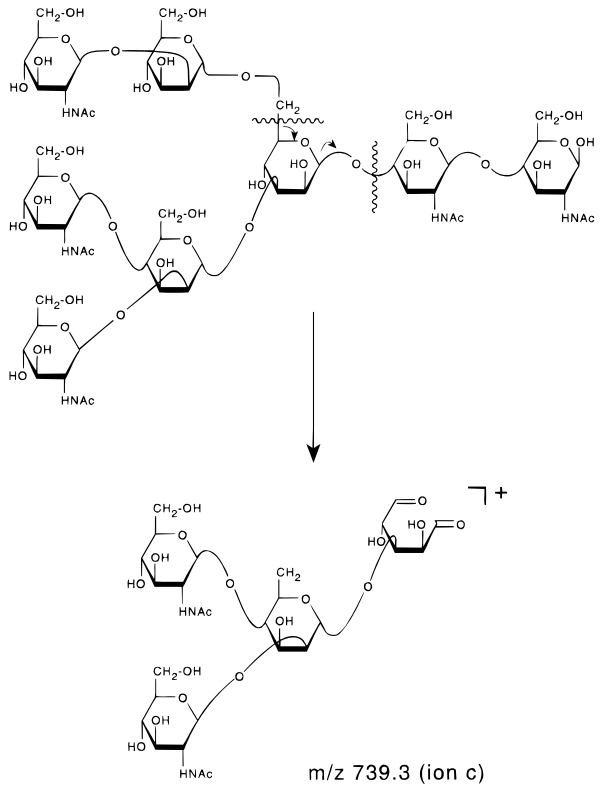


Figure 16. Scheme showing the formation of ion *c*.

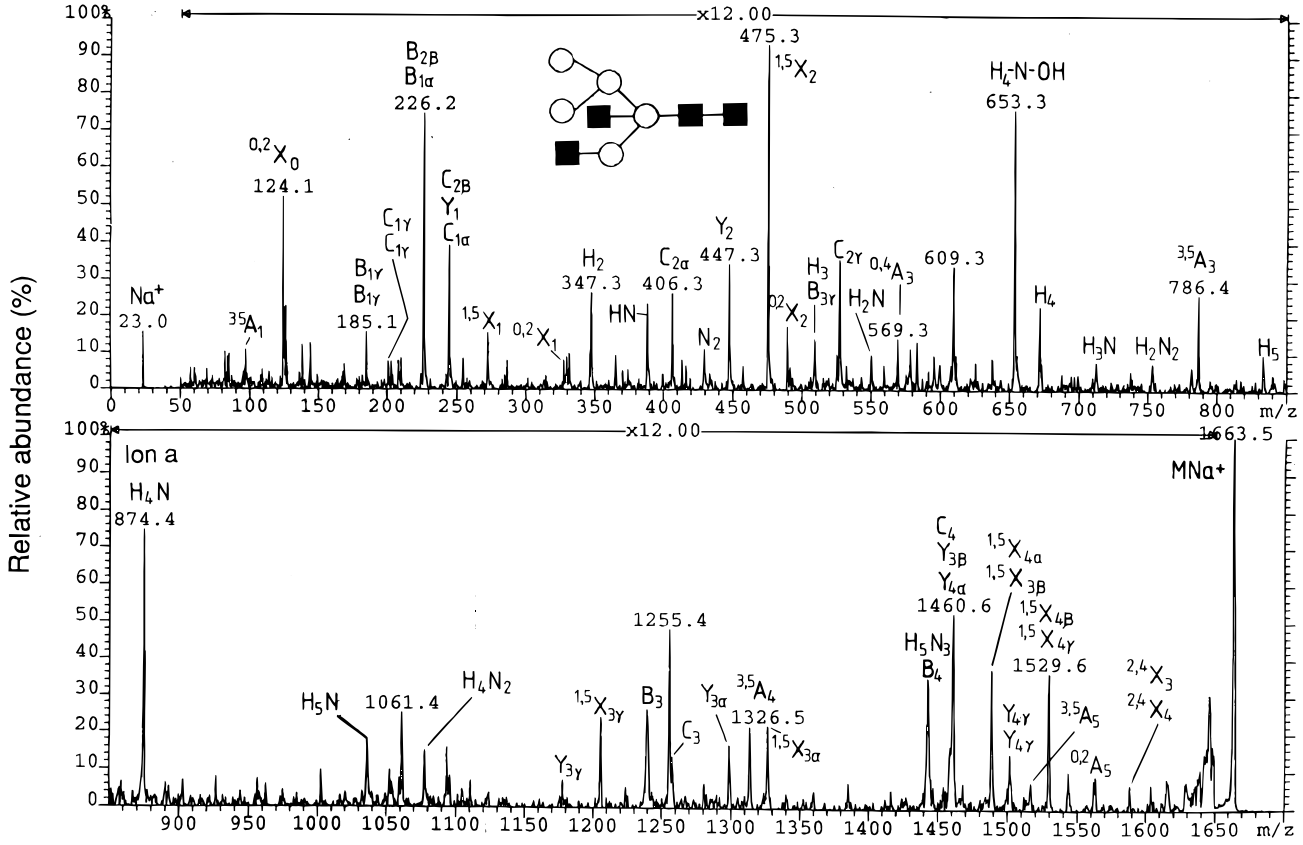


Figure 17. CID spectrum of the hybrid sugar $(Man)_5(GlcNAc)_4$ (**XVII**). Ion structures are as defined in Fig. 18 and the note to Fig. 2. The ions labelled HN (*m/z* 388.3, isobaric with B_2), N_2 (*m/z* 429.3 $(GlcNAc)_2$), H_2N (*m/z* 550.3, isobaric with B_3), H_4 (*m/z* 671.4, $(Hex)_4$), H_3N (*m/z* 712.1, ion *a*), H_2N_2 (*m/z* 753.4, $(Hex)_2(HexNAc)_2$), H_5 (*m/z* 833.5, $(Hex)_5$), H_4N (*m/z* 874.4, $(Hex)_4GlcNAc$, mainly ion *a*), H_5N (*m/z* 1036.6, $(Hex)_5GlcNAc$), H_4N_2 (*m/z* 1077.8, $(Hex)_4(GlcNAc)_2$) and H_5N_3 (*m/z* 1442.5, $(Hex)_5(GlcNAc)_3$, isobaric with B_4) are internal cleavage ions of undetermined origin.

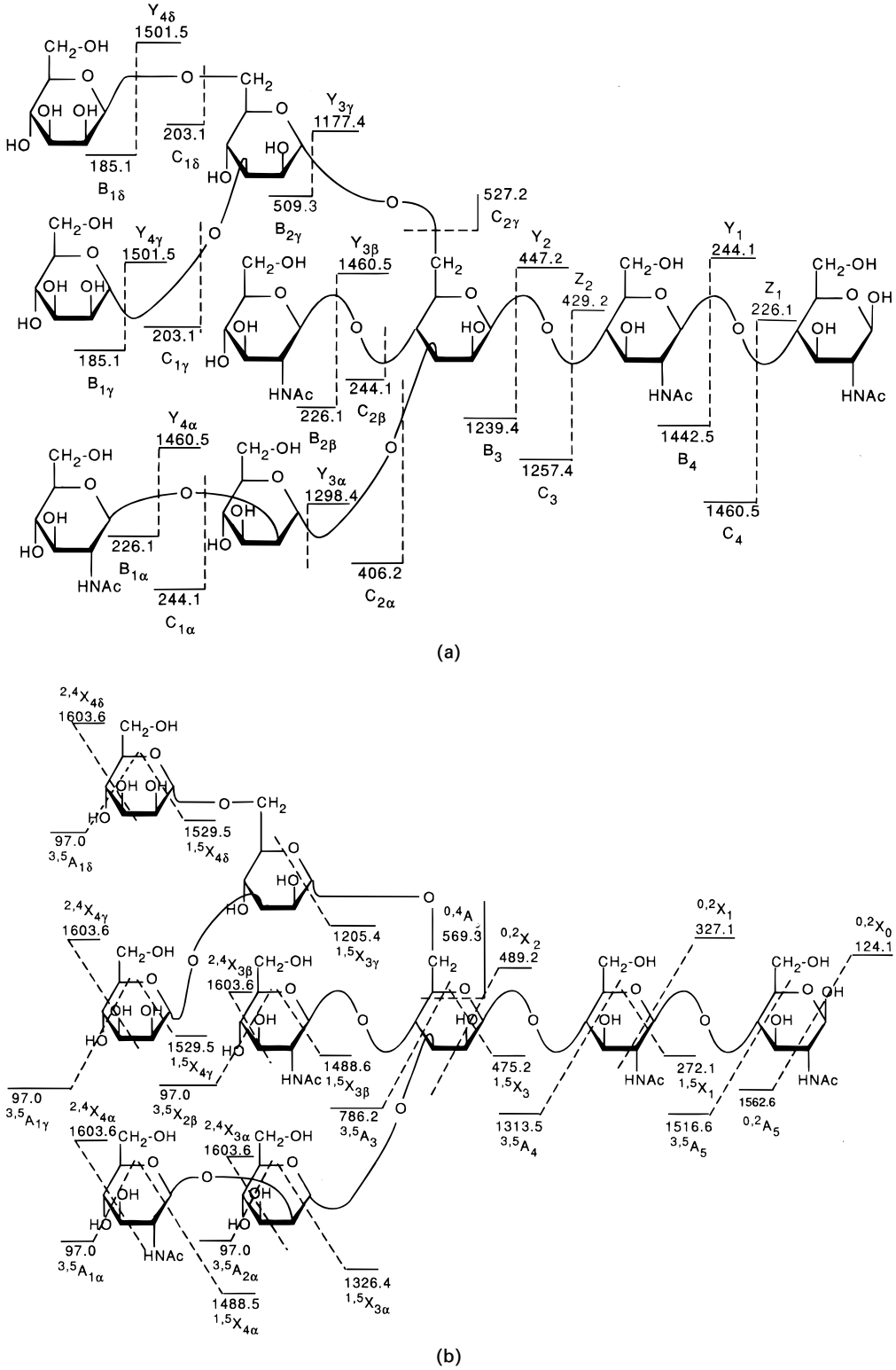


Figure 18. (a) Glycosidic cleavages found in the CID spectrum of $(Man)_5(GlcNAc)_4$ (**XVII**). (b) Cross-ring cleavages found in the CID spectrum of $(Man)_5(GlcNAc)_4$ (**XVII**).

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