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FAB CID-MS/MS characterization of tetrasaccharide tri- and tetrasulfate derived from the antigenic determinant recognized by the anti-chondroitin sulfate monoclonal antibody MO-225

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The fast atom bombardment (FAB) collision induced dissociation (CID)-mass spectrometry/mass spectrometry (MS/MS) technique was successfully applied to characterize and identify the structures of the immunoreactive trisulfated and tetrasulfated tetrasaccharides that were obtained from the chondroitin sulfate in a shark fin using a treatment with chondroitinase ABC.

Keywords: FABMS, CID, MS/MS, trisulfated tetrasaccharide, tetrasulfated tetrasaccharide, chondroitin sulfate, shark fin, monoclonal antibody, MO-225, glycosaminoglycan

Abbreviations: FABMS, fast atom bombardment mass spectrometry; CID collision induced dissociation; MS/MS, mass spectrometry/mass spectrometry; ΔUA2S-GalNAc6S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔUA-GalNAc4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔUA-GalNAcDiS, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose.

Introduction

Recently, the structure-function relationships associated with glycosaminoglycans have attracted considerable interest. In 1987, Yamagata *et al.* demonstrated that tetrasaccharide trisulfate and tetrasaccharide tetrasulfate isolated from shark fin are the smallest chondroitin sulfate fragments that can inhibit the binding of the anti-chondroitin sulfate monoclonal antibody MO-225 to various types of chondroitin sulfate proteoglycan derived from avian and mammalian tissues [1]. The structures of these compounds were deduced on the basis of the unsaturated disaccharide structures obtained by chondroitinase ABC digestion [1]. One of the disaccharide products was identical to 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔUA2S-GalNAc6S), called the D unit, that was previously characterized by chemical and enzymatic methods in the chondroitinase digests of shark cartilages [2, 3]. Because chondroitinase AC does not attack the D unit, these particular polysulfated tetrasaccharides, which contain the D unit, were isolated from the reaction mixture of chondroitinase AC (*Flavobacterium heparinum*) digestion. However, direct evidence to support the tetrasaccharide structures is still required.

Recently, we showed that fast atom bombardment ionization followed by collision induced dissociation and tandem mass spectrometry (FAB CID-MS/MS) of the molecule related ions were useful to characterize the structures of twenty sulfated unsaturated disaccharides obtained from the enzymatic digestion of chondroitin sulfate, dermatan sulfate, hyaluronan, heparin and heparan sulfate [4, 5]. Herein, we report that FAB CID-MS/MS of the molecule related ions and fragment ions has been successful in the confirmation of the proposed structure.

Materials and methods

Materials

Tetrasaccharides trisulfate (**1**) and tetrasulfate (**2**) were prepared according to the literature [1] (for structures, see

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Scheme 1). Although the isolation procedures for **1** and **2** were the same, **1** was predominantly obtained in the potassium salt forms and **2** in the sodium salt forms. The reason for this different behaviour between the two compounds is not yet clear and is under investigation.

Standard samples of 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔUA-GalNAc4S) and 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose (ΔUA-GalNAcDiS) (sodium salt) were supplied by Seikagaku Kogyo Co. (Tokyo, Japan). The potassium salt of this compound for the FAB CID-MS/MS measurements was prepared on the emitter by mixing the sample in the matrix with a KCl aqueous solution.

Mass spectrometry

The mass spectra were measured with a Finnigan MAT TSQ 700 triple stage quadrupole mass spectrometer equipped with an Ion Tech FAB gun. A xenon beam with an energy of 8 keV was used. Thioglycerol and triethanolamine were used as the matrices in the positive- and negative-ion modes, respectively. The CID-MS/MS were recorded using argon as the collision gas typically at 0.133 Pa to reduce the parent ion beam by approximately 30%. The collision energy was 5 eV or 30 eV. At least 10 scans were averaged to obtain each CID-MS/MS spectrum.

Results and discussion

To describe the fragment ions drawn in each scheme, we use the nomenclature proposed by Domon and Costello [6], and throughout this article M represents the fully protonated molecule.

*Tetrasaccharide trisulfate (**1**)*

The negative and positive ion FABMS are shown in Figs 1a and b, respectively. In Fig. 1a, the predominant molecule related ions are observed at *m/z* 1149, 1111, 1073 and 1031, corresponding to the $[M + 4K - 5H]^-$, $[M + 3K - 4H]^-$, $[M + 2K - 3H]^-$ and $[(M + 4K - 5H) - KSO_3 + H]^-$ ions, respectively. The following abundant ions, derived by the cleavages of the glycosidic bonds, were characteristically observed as shown in Scheme 1. The peaks at *m/z* 953 $[Y_3 + 3K - 3H]^-$, 828 $[C_3 + 3K - 3H]^-$, 632 $[Y_2 + 2K - 2H]^-$ and 496 $[C_2 + K - H]^-$ were examined using CID-MS/MS. The other small peaks were assigned as follows: *m/z* 790 $[C_3 + 2K - 2H]^-$, 722 $[B_3 + 2K - 4H]^-$ and 538 $[^{2,4}A_3 + K - 2H]^-$. Interestingly, compound in predominantly contains potassium ions along with a small amount of sodium ions. In the molecular ion region, the peaks at *m/z* 1133 and 1095 correspond to the ions $[M + 3K + Na - 5H]^-$ and $[M + 2K + Na - 4H]^-$, respectively. Few diagnostic ions indicating the location of the sulfate groups were observed in the FAB spectrum (Scheme 1).

In the positive ion spectrum (Fig. 1b), the molecule related ions were also observed at *m/z* 1189 $[M - 4H + 5K]^+$, 1173 $[M - 4H + 4K + Na]^+$, 1151 $[M - 3H + 4K]^+$ and 1135 $[M - 3H + 3K + Na]^+$, which clearly indicates that the molecular mass of a tetrasaccharide trisulfate is 998 mass units. The other peaks are ions derived by the loss of potassium sulfite with hydrogen transfer i.e. 118 U ($- KSO_3 + H$) from the molecule related ions.

The CID-MS/MS of the negative ion at *m/z* 1149 is shown in Fig. 2a, in which the abundant peak at *m/z* 828 corresponds to $[C_3 + 3K - 3H]^-$. The diagnostic peaks at *m/z* 650, 576 and 538 correspond to $[^{0,2}A_3 + 2K - 3H]^-$, $[^{2,4}A_3 + 2K - 3H]^-$ and $[^{2,4}A_3 + K - 2H]^-$, respectively. These peaks clearly indicate that a sulfate group is present at the C-2 position of the glucuronic acid moiety on the reducing end site (Scheme 2a). The assignment of the other daughter ions are illustrated in Scheme 2a.

The CID/MS/MS of the fragment ion at *m/z* 953, which corresponds to $[Y_3 + 3K - 3H]^-$, is shown in Fig. 2b. The base peak at *m/z* 632 corresponds to $[C_{2'} + 2K - 2H]^-$, where $C_{2'}$ is the fragment ion that corresponds to the C_2 fragment of the trisaccharide parent ion (*m/z* 953) and whose structure is shown in Scheme 2b. The CID-MS/MS of the trisaccharide ion at *m/z* 828 corresponds to $[C_3 + 3K - 3H]^-$ and is shown in Fig. 3a. The major peaks at *m/z* 650, 576 and 538 correspond to $[^{0,2}A_{3'} + 2K - 3H]^-$, $[^{2,4}A_{3'} + 2K - 3H]^-$ and $[^{2,4}A_{3'} + K - 2H]^-$, respectively (Scheme 3a). This fragmentation also confirms that the sulfate group is located at the C-2 position in the glucuronic acid moiety on the reducing end site of **1**.

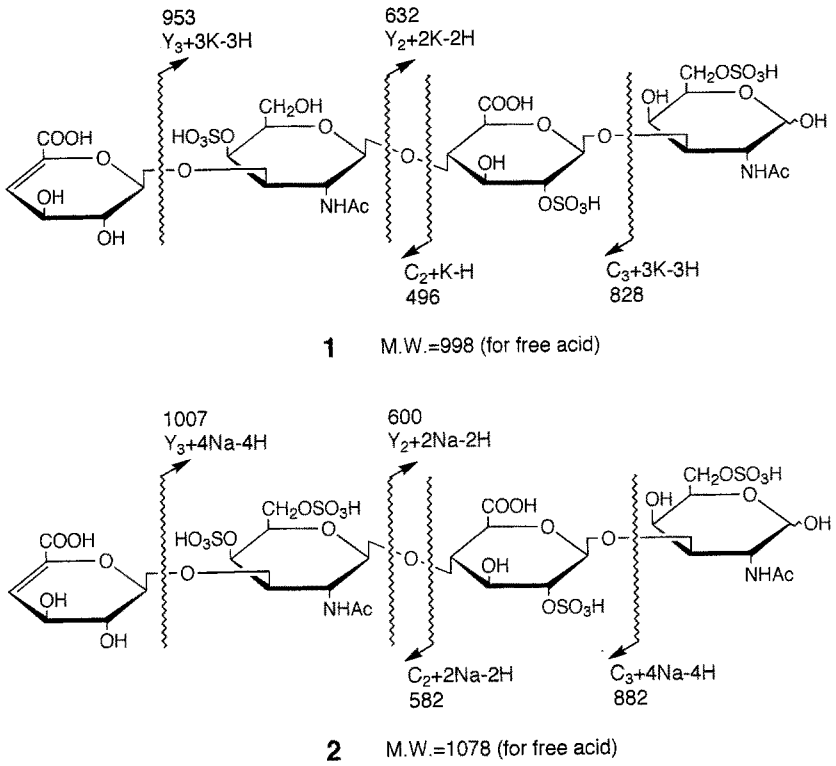
The CID-MS/MS of the disaccharide ion at *m/z* 632 $[Y_2 + 2K - 2H]^-$ is shown in Fig. 3b. The major ions are at *m/z* 342 $[^{0,2}X_{1'} - HSO_3]^-$, 311 $[C_{1'} + K - H]^-$, 282 $[Z_{1'} - 2H]^-$ and 139 $[O_3SOH_2CCHO]^-$, which are consistent with the structure shown in Scheme 3b. The presence of the peak at *m/z* 139 confirms the C-6 sulfate group in the galactosamine moiety on the reducing end site of **1**.

The CID-MS/MS (Fig. 4a) of the disaccharide ion at *m/z* 496 which corresponds to $[C_2 + K - H]^-$ is almost superimposable on the FAB CID-MS/MS (Fig. 4b) of the molecule related ion $[M + K - 2H]^-$ of the potassium salt of 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔUA-GalNAc4S). This confirms the location of the sulfate group at C-4 in the galactosamine moiety on the non-reducing end site of **1**. A similar fragmentation pattern was observed in the spectrum of the sodium salt of the same compound [4].

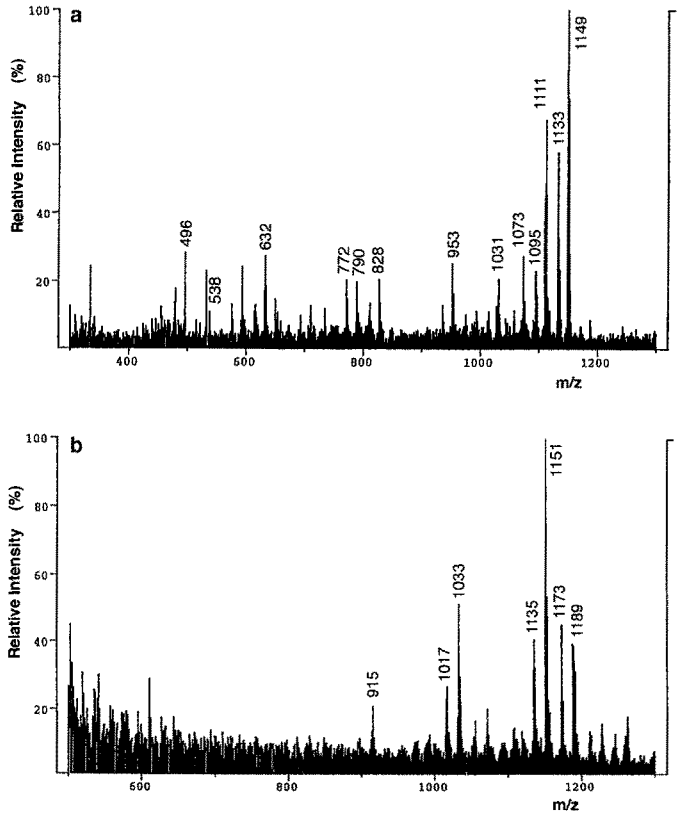
On the basis of these results, combined with the previous report which shows that this compound gives equimolar amounts of ΔUA-GalNAc4S and ΔUA2S-GalNAc6S upon chondroitinase ABC digestion [1], the complete structure of the tetrasaccharide trisulfate(**1**) is now confirmed.

Tetrasaccharide tetrasulfate (2)

The negative and positive ion FABMS are shown in Figs 5a and 5b, respectively. In the negative ion spectrum the following significant peaks were observed as shown in Scheme 1: *m/z* 1187 $[M + 5Na - 6H]^-$, 1165 $[M + 4Na - 5H]^-$,



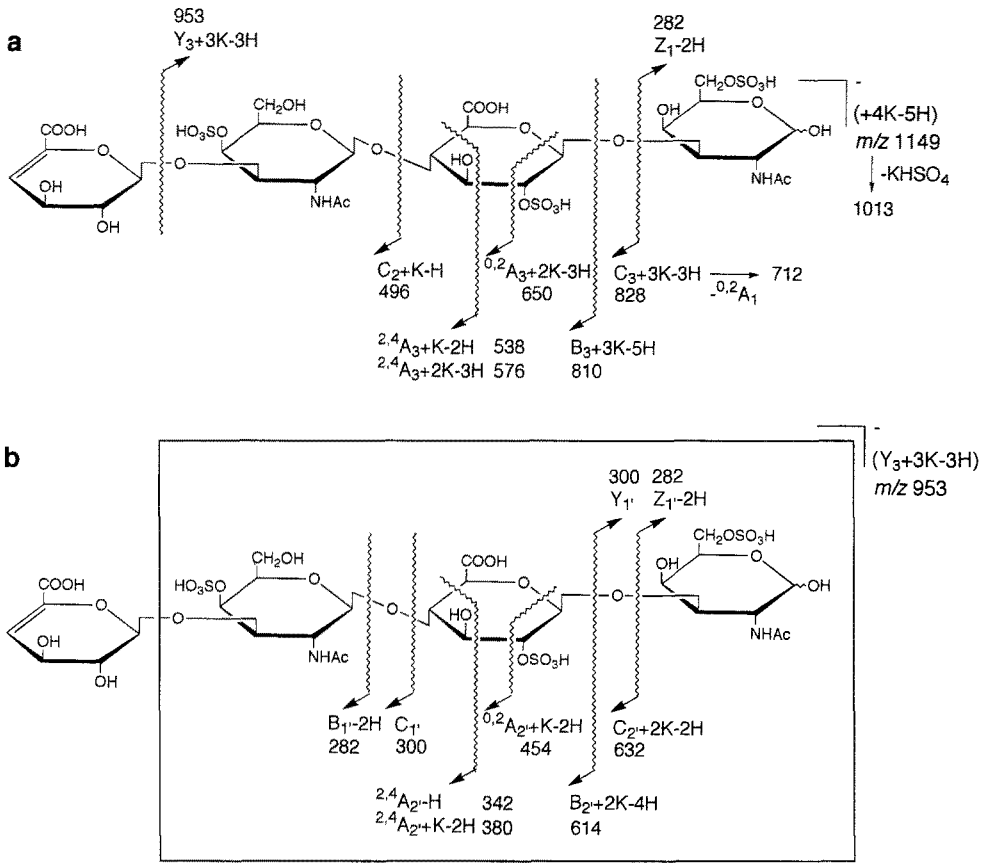
Scheme 1. The structures and fragmentations of the negative ion FABMS of tetrasaccharide trisulfate (**1**) and tetrasulfate (**2**).



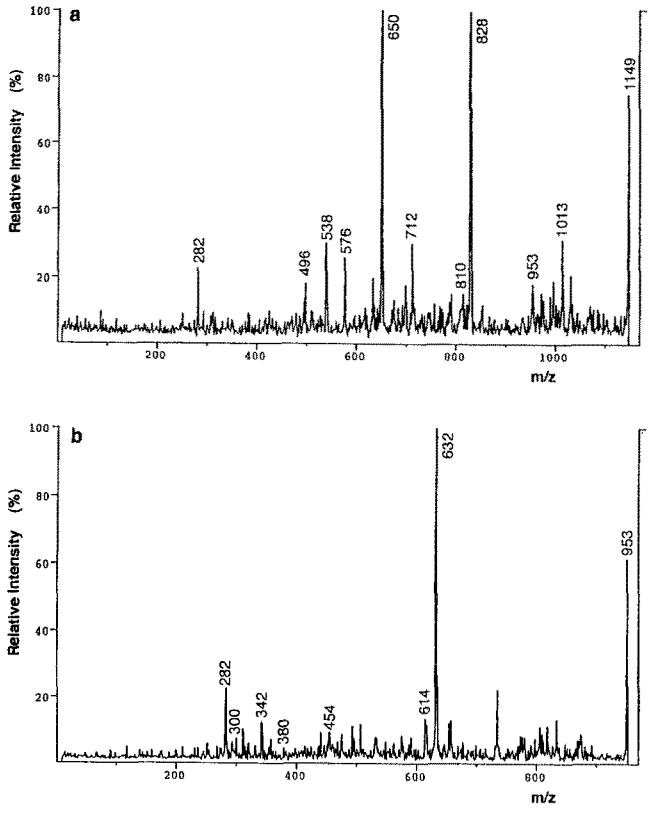
1143 [M + 3Na – 4H]$^-$, 1085 [1187 – $NaSO_3$ + H]$^-$, 1063 [1165 – $NaSO_3$ + H]$^-$, 1007 [Y_3 + 4Na – 4H]$^-$, 882 [C_3 + 4Na – 4H]$^-$, 600 [Y_2 + 2Na – 2H]$^-$ and 582 [C_2 + 2Na – 2H]$^-$. These ions were analysed using CID-MS/MS. As previously discussed, the spectral features were similar to those of compound **1**. The other small fragment ions were assigned as follows: *m/z* 860 [C_3 + 3Na – 3H]$^-$, 842 [B_3 + 3Na – 5H]$^-$ and 624 [$^{2,4}A_3$ + 2Na – 3H]$^-$. The structure elucidation of **2** was impossible using only the FAB spectrum. In the positive ion spectrum, the abundant peaks in the molecular region are at *m/z* 1211, 1189 and 1167 and correspond to the [M – 5H + 6Na]$^+$, [M – 4H + 5Na]$^+$ and [M – 3H + 4Na]$^+$ ions, respectively, which indicate the molecular mass of tetrasaccharide tetrasulfate (**2**).

The CID-MS/MS of the molecule related ion at *m/z* 1187 [M + 5Na – 6H]$^-$ is shown in Fig. 6a and exhibits peaks at *m/z* 1007 [Y_3 + 4Na – 4H]$^-$, 882 [C_3 + 4Na – 4H]$^-$, 864 [B_3 + 4Na – 6H]$^-$, 720 [$^{0,2}A_3$ + 3Na – 4H]$^-$, 624 [$^{2,4}A_3$ + 2Na - 3H]$^-$ and 582 [C_2 + 2Na – 2H]$^-$. The detection of ring cleavage ions, $^{0,2}A_3$ and $^{2,4}A_3$, indicates that the sulfate group is present at the C-2 position in the glucuronic acid moiety on the reducing end site. The fragmentation of **2** is shown in Scheme 4a.

Figure 1. The FAB mass spectra of tetrasaccharide trisulfate (**1**). (a) negative ion mode; (b) positive ion mode.



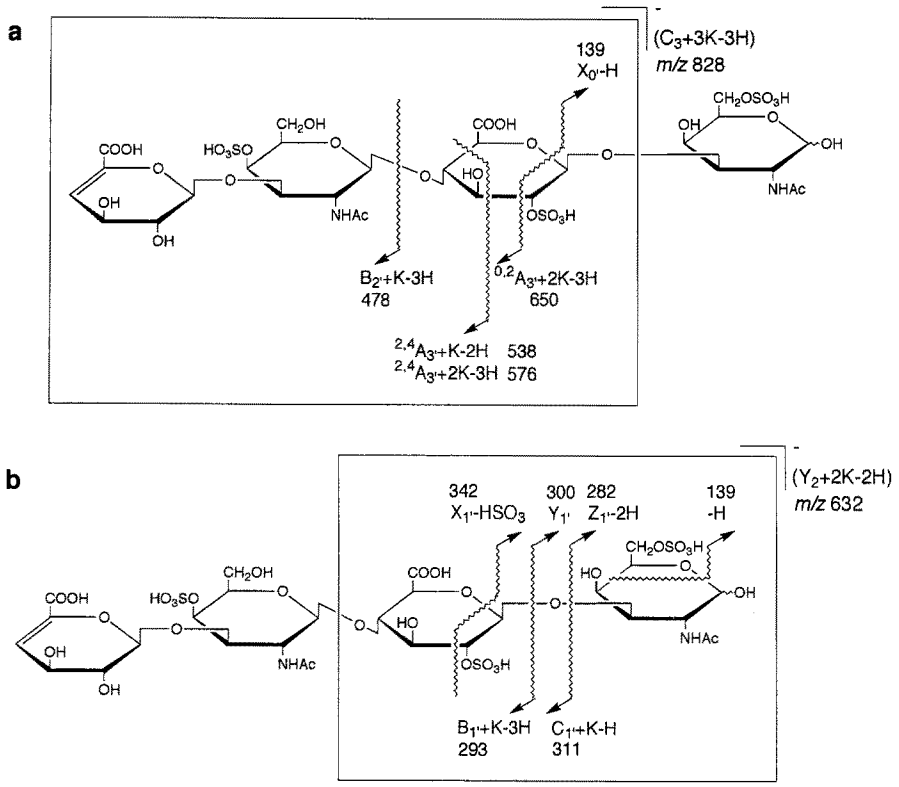
Scheme 2. The fragmentation of the negative ion CID-MS/MS of tetrasaccharide trisulfate (**1**). (a) the $[M + 4K - 5H]^-$ ion (*m/z* 1149) as the parent ion: (b) the fragment ion that corresponds to $[Y_3 + 3K - 3H]^-$ (*m/z* 953) as the parent ion.



The CID-MS/MS of the trisaccharide ion $[Y_3 + 4Na - 4H]^-$ at *m/z* 1007 exhibits diagnostic ions at *m/z* 702 $[C_{2'} + 3Na - 3H]^-$, 540 $[^{0,2}A_{2'} + 2Na - 3H]^-$, 488 $[^{2,4}A_{2'} + 3Na - 4H]^-$, 466 $[^{2,4}A_{2'} + 2Na - 3H]^-$, 444 $[^{2,4}A_{2'} + Na - 2H]^-$ and 402 $[C_{1-} + Na - H]^-$ as shown in Fig. 6b and Scheme 4b. The CID-MS/MS of the trisaccharide ion at *m/z* 882 that corresponds to $[C_3 + 4Na - 4H]^-$ is shown in Fig. 7a. The abundant peaks are characteristically observed at *m/z* 720, 668, 646 and 624 and correspond to $[^{0,2}A_{3'} + 3Na - 4H]^-$, $[^{2,4}A_{3'} + 4Na - 5H]^-$, $[^{2,4}A_{3'} + 3Na - 4H]^-$ and $[^{2,4}A_{3'} + 2Na - 3H]^-$, respectively (Scheme 5a). These fragmentations also confirm that the C-2 position in the glucuronic acid moiety on the reducing end site of **2** is sulfated.

The CID-MS/MS of the disaccharide ion of **2** at *m/z* 600 $[Y_2 + 2Na - 2H]^-$, as shown in Fig. 7b, produced the dominant ions at *m/z 300* $[Y_{1'}]^-$, 304 $[Z_{1'} + Na - 3H]^-$, 295 $[C_{1'} + Na - H]^-$, 282 $[Z_{1'} - 2H]^-$ and 277 $[B_{1'} + Na - 3H]^-$, which were derived by the cleavage of the glycosidic bond. The presence of the peak at *m/z*139 in the low mass region indicates that the C-6 position in the galactosamine moiety on the reducing end site is sulfated (Scheme 5b).

Figure 2. The CID-MS/MS spectra of tetrasaccharide trisulfate (**1**). (a) the $[M + 4K - 5H]^-$ ion (*m/z* 1149) as the parent ion; (b) the fragment that corresponds to $[Y_3 + 3K - 3H]^-$ (*m/z* 953) as the parent ion.



Scheme 3. The fragmentation of the negative ion CID-MS/MS of tetrasaccharide trisulfate (**1**). (a) the fragment ion that corresponds to $[C_3 + 3K - 3H]^-$ (*m/z* 828) as the parent ion; (b) the fragment ion that corresponds to $[Y_2 + 2K - 2H]^-$ (*m/z* 632) as the parent ion.

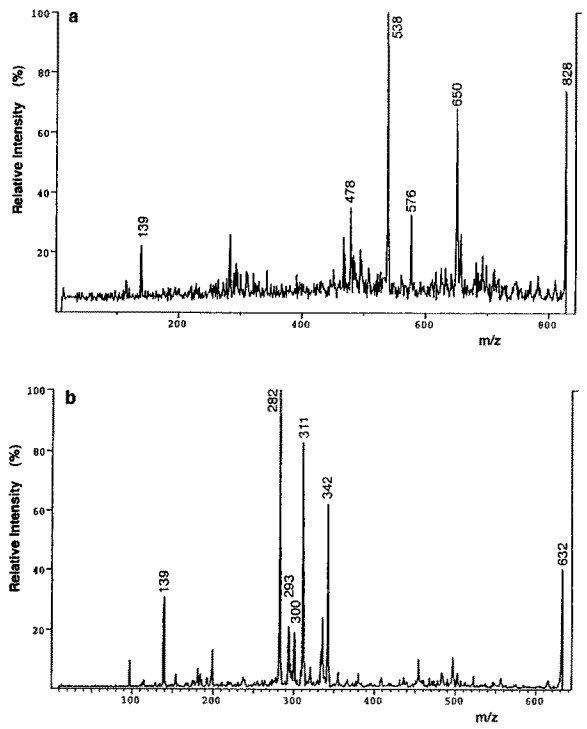


Figure 3. The CID-MS/MS spectra of tetrasaccharide trisulfate (**1**). (a) the fragment ion that corresponds to [C3 + 3K – 3H]– (m/z 828) as the parent ion; (b) the fragment ion that corresponds to $[Y2 + 2K - 2H]^-$ (m/z 632) as the parent ion.

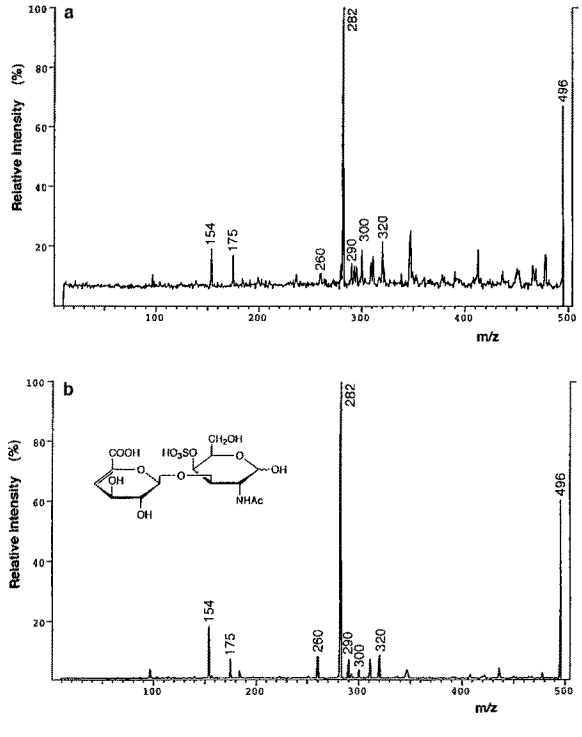


Figure 4. The CID-MS/MS spectra. (a) the fragment ion of tetrasaccharide trisulfate (1), that corresponds to $[C2 + K - H]^-$ (m/z 496) as the parent ion; (b) the $[M + K - 2H]^-$ ion of DUA-GalNAc4S (m/z 496) as the parent ion.

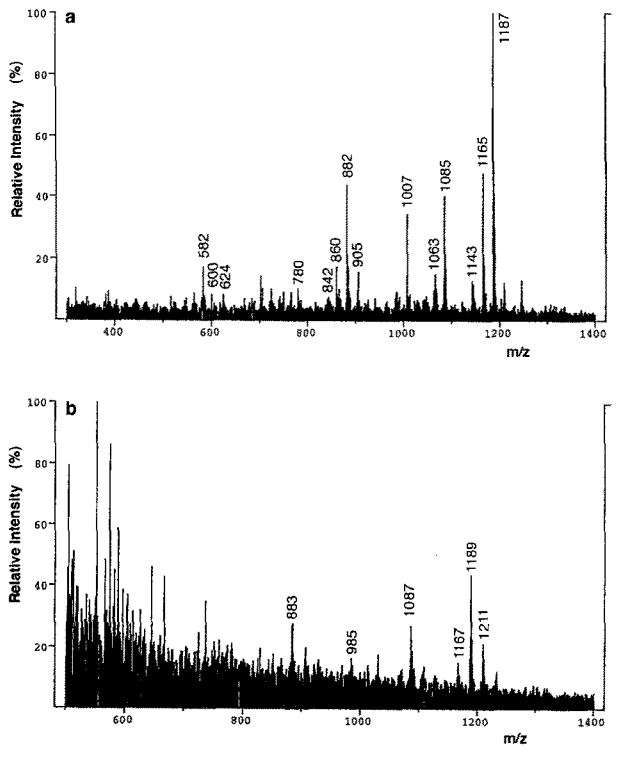


Figure 5. The FAB mass spectra of tetrasaccharide tetrasulfate (**2**). (a). negative ion mode; (b) positive ion mode.

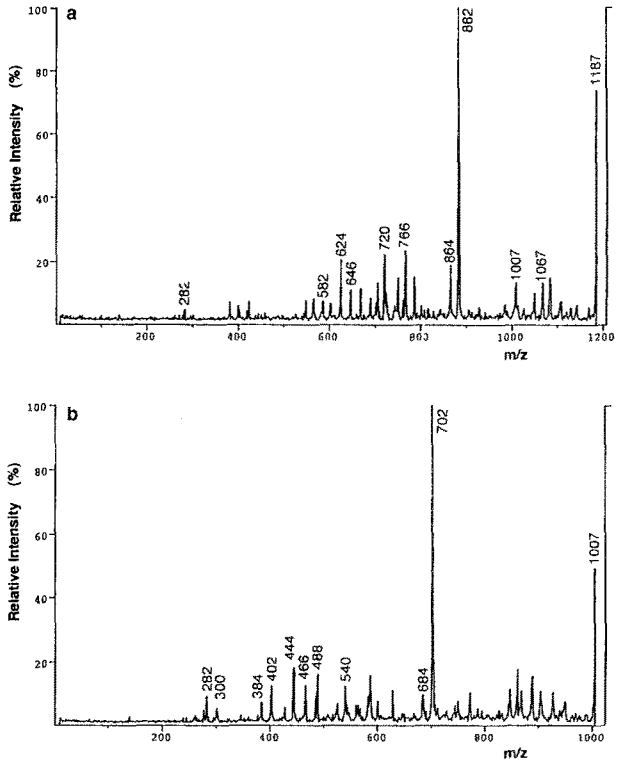
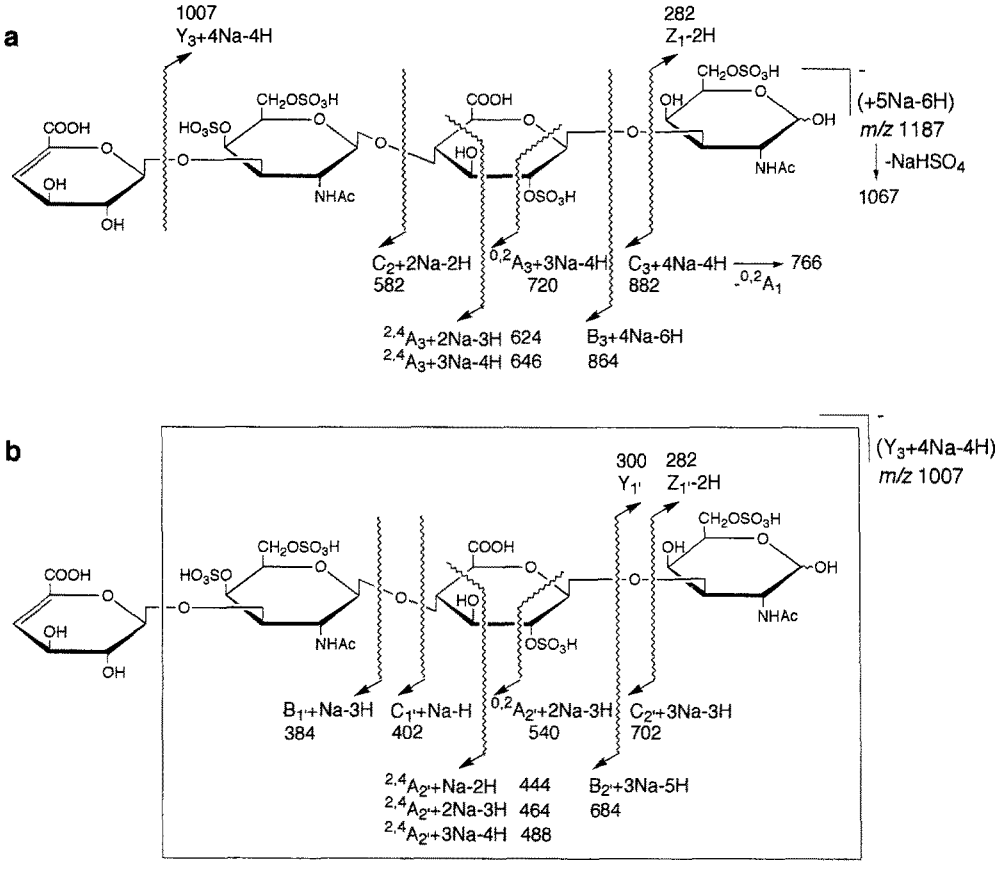


Figure 6. The CID-MS/MS spectra of tetrasaccharide tetrasulfate (**2**). (a) the $[M + 5Na - 6H]^-$ ion (*m/z* 1187) as the parent ion; (b) the fragment ion that corresponds to $[Y_3 + 4Na - 4H]^-$ (*m/z* 1007) as the parent ion.



Scheme 4. The fragmentation of the negative ion CID-MS/MS of tetrasaccharide tetrasulfate (**2**). (a) the $[M + 5Na - 6H]^-$ ion (*m/z* 1187) as the parent ion; (b) the fragment ion that corresponds to $[Y_3 + 4Na - 4H]^-$ (*m/z* 1007) as the parent ion.

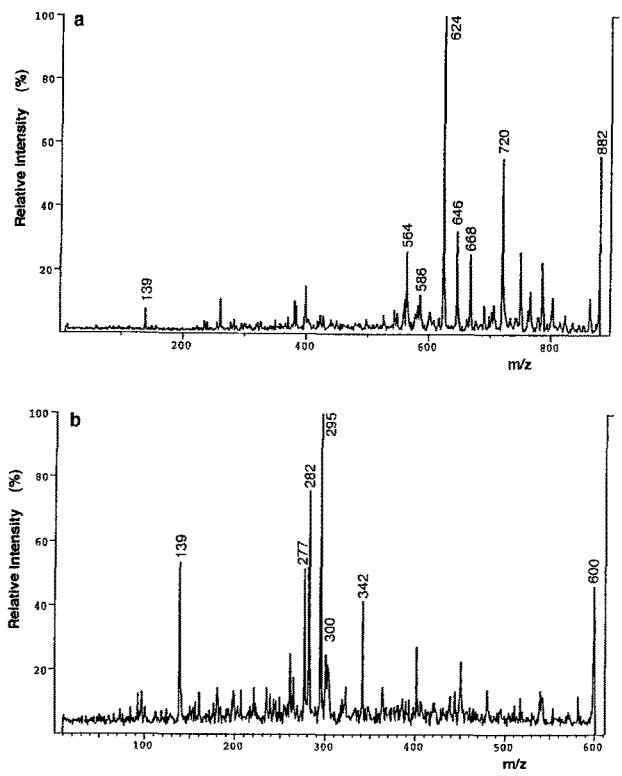


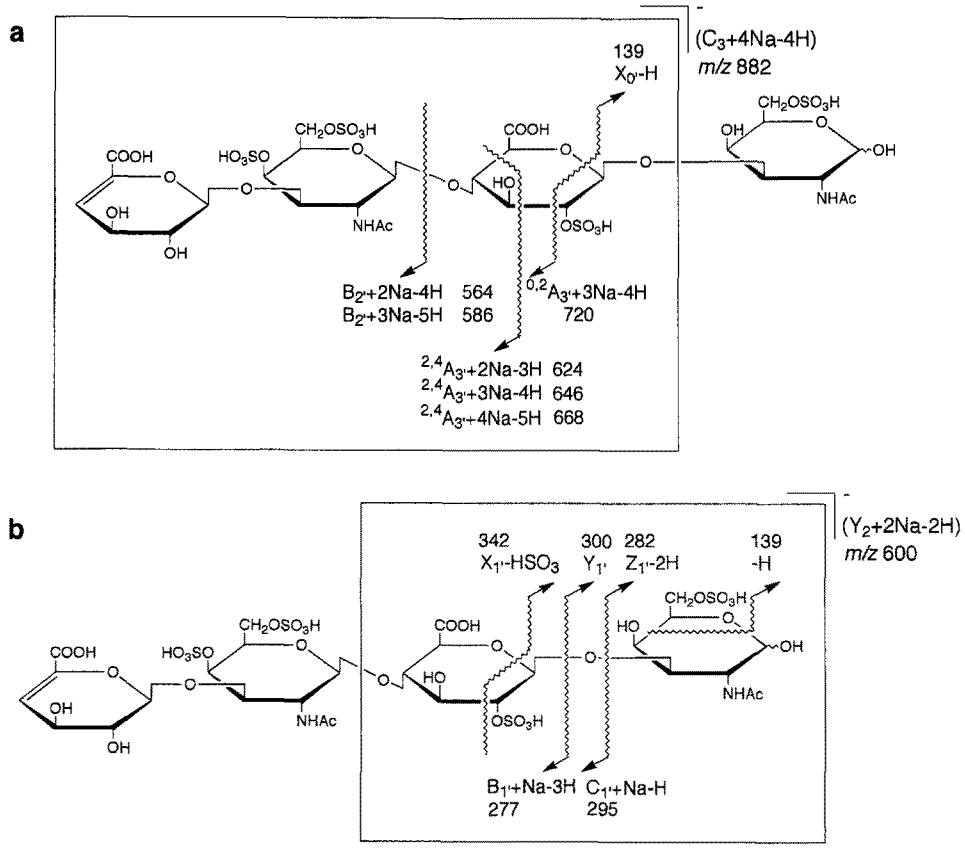
Figure 7. The CID-MS/MS spectra of tetrasaccharide tetrasulfate (**2**). (a) the fragment ion that corresponds to $[C_3 + 4Na - 4H]^-$ (*m/z* 882) as the parent ion; (b) the fragment ion that corresponds to $[Y_2 + 2Na - 2H]^-$ (*m/z* 600) as the parent ion.

The CID-MS/MS of the disaccharide ion at *m/z* 582, $[C_2 + 2Na - 2H]^-$, which is the disaccharide part of the non-reducing end of **2**, is shown in Fig. 8a. It is identical to the CID-MS/MS of the molecule related ion, $[M + 2Na - 3H]^-$, of the sodium salt of 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose (ΔUA-GalNAcDiS), as shown in Fig. 8b [4].

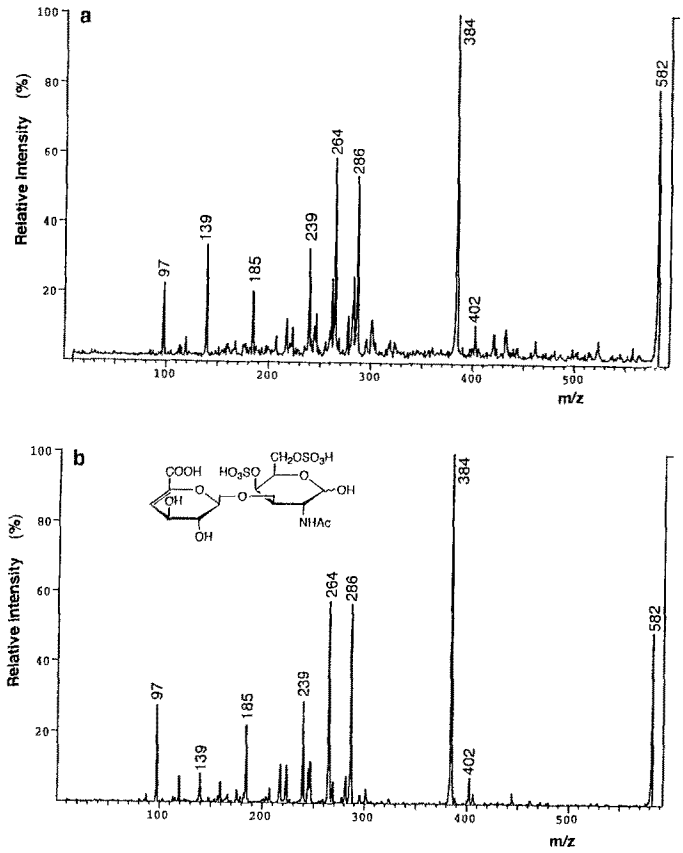
These results confirm the previously reported structure of tetrasaccharide tetrasulfate (**2**), based on the formation of equimolar amounts of ΔUA-GalNAcDiS and ΔUA2S-GalNAc6S by digestion with chondroitinase ABC [1]. The mass spectrometric studies described here provide direct evidence for the structures of the polysulfated trisaccharides, especially the position of the sulfate group in the glucuronic acid moiety on the reducing end site.

Conclusions

The FAB mass spectra of the two sulfated tetrasaccharides that were obtained from the chondroitin sulfate in a shark fin exhibit molecule related ion peaks in both the negative and positive ion modes. In the negative ion FABMS, the diagnostic fragment ions that were derived by the cleavage of the glycosidic bonds are detected. On the other hand, the negative ion CID-MS/MS of the molecule related ions, as well as the fragment ions, produce spectra that are characteristic of the structures of tetrasaccharides with regard to the sugar sequences as well as the positions of the sulfate groups.



Scheme 5. The fragmentation of the negative ion CID-MS/MS of tetrasaccharide tetrasulfate (**2**). (a) the fragment ion that corresponds to $[C_3 + 4Na - 4H]^-$ (*m/z* 882) as the parent ion; (b) the fragment ion that corresponds to $[Y_2 + 2Na - 2H]^-$ (*m/z* 600) as the parent ion.



These results confirm the structures of tetrasaccharide trisulfate and tetrasulfate as previously reported by Yamagata *et al.* [1].

Acknowledgements

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Figure 8. The CID-MS/MS spectra. (a) the fragment ion of tetrasaccharide tetrasulfate (**2**) that corresponds to $[C_2 + 2Na - 2H]^-$ (*m/z* 582) as the parent ion; (b) the $[M + 2Na - 3H]^-$ ion of ΔUA-GalNAcDiS (*m/z* 582) as the parent ion.