Glycoconjugate Journal (1995) **12**: 162–172

Negative-ion fast atom bombardment tandem mass spectrometry for characterization of sulfated unsaturated disaccharides from heparin and heparan sulfate

TADASHI II[1], MASAYUKI KUBOTA[2], SATOSHI OKUDA[3], TAKASHI HIRANO[2] and MAMORU OHASHI[2]*

[1] *Research and Development Laboratories, Soda Aromatic Co., Ltd., Noda-shi, Chiba 270-02, Japan*
[2] *Department of Applied Physics and Chemistry, The University of Electro-Communications, Chofu, Tokyo 182, Japan*
[3] *School of Agricultural Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan*

Received 5 August 1994, revised 20 September 1994

Negative-ion fast atom bombardment tandem mass spectrometry has been used in the characterization of non-, mono-, di- and trisulfated disaccharides from heparin and heparan sulfate. The positional isomers of the sulfate group of monosulfated disaccharides were distinguished from each other by negative-ion fast atom bombardment tandem mass spectra, which provide an easy way of identifying the positional isomers. This fast atom bombardment collision induced dissociation mass spectrometry/mass spectrometry technique was also applied successfully to the characterization of di- and trisulfated disaccharides.

Keywords: unsaturated disaccharide, glycosaminoglycan, heparin, heparan sulfate, FABMS, CID, MS/MS

Abbreviations: FABMS, fast atom bombardment mass spectrometry; CID, collision induced dissociation; MIKE, mass analysed ion kinetic energy; MS/MS, mass spectrometry/mass spectrometry; HPLC, high performance liquid chromatography; ΔUA, D-gluco-4-enepyranosyluronic acid; CS, chondroitin sulfate; DS, dermatan sulfate; HA, hyaluronan; Hep, heparin; HS, heparan sulfate; ΔUA(1→4) GlcNAc, 2-acetamido-2-deoxy-4-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ΔUA(1→4)GlcNAc6S, 2-acetamido-2-deoxy-4-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔUA2S(1→4)GlcNAc, 2-acetamido-2-deoxy-4-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ΔUA2S(1→4)GlcNAc6S, 2-acetamido-2-deoxy-4-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔUA(1→4)GlcN6S, 2-amino-2-deoxy-4-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔUA2S(1→4)GlcN, 2-amino-2-deoxy-4-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ΔUA2S(1→4)GlcN6S, 2-amino-2-deoxy-4-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔUA(1→4)GlcNS, 2-deoxy-2-sulfamino-4-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ΔUA(1→4)GlcNS6S, 2-deoxy-2-sulfamino-4-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔUA2S(1→4)GlcNS, 2-deoxy-2-sulfamino-4-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ΔUA2S(1→4)GlcNS6S, 2-deoxy-2-sulfamino-4-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔUA(1→3)GalNAc, 2-acetamido-2-deoxy-3-O-(β-D-Gluco-4-enepyranosyluronic acid)-D-galatose; ΔUA(1→3)GalNAc4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔUA(1→3)GalNAc6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔUA2S(1→3)GalNAc, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔUA2S(1→3)GalNAc4S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔUA2S(1→3)GalNAc6S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔUA(1→3)GalNAcDiS, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose; ΔUA(1→3)GlcNAc, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose.

* To whom correspondence should be addressed.

Introduction

In vertebrates, sulfated glycosaminoglycans are covalently linked to core proteins to form proteoglycans, which are distributed in a variety of tissues and cells and play important roles in a wide range of biological processes [1, 2].

Sulfated glycosaminoglycans are anionic, polydisperse, microheterogeneous, linear polysaccharides that are composed of alternating glycosidically linked hexosamine and hexuronic acid residues. On the basis of differences in types of saccharide constituents, sulfation and linkage configuration and position, sulfated glycosaminoglycans can be divided into two families, *i.e.* the chondroitin sulfate (CS)/dermatan sulfate (DS) family and the heparin (Hep)/heparan sulfate (HS) family [3, 4]. CS and DS are galactosaminoglycans composed of repeating–hexuronic acid-(1→3)-*N*-acetyl-D-galactosamine (GalNAc)-(β1→4) disaccharide units, in which the hexuronic acids are either β-D-glucuronic acid or α-L-iduronic acid [3, 4]. Moreover, the disaccharide units are *O*-sulfated to various extents at C-6 and/or C-4 of the GalNAc residues and at C-2 of hexuronic acid residues [3, 4]. Hep and HS are glucosaminoglycans composed of repeating -hexuronic acid-(1→4)-D-glucosamine (GlcN)-(α1→4) disaccharide units. The hexuronic acid residues are either β-D-glucuronic acid or α-L-iduronic acid and the GlcN residues are either *N*-acetylated or *N*-sulfated. In addition, the disaccharide units are *O*-sulfated to various extents at C-6 and/or C-3 of the GlcN residues and at C-2 of hexuronic acid residues [3, 4].

An exhaustive treatment of CS or DS with chondroitinase ABC affords a mixture of eight unsaturated disaccharides which have the common disaccharide structure, ΔUA-(1→3)-GalNAc, where ΔUA represents β-D-gluco-4-enepyranosyluronic acid residue [5–7]. On the other hand, an exhaustive treatment of Hep or HS with a mixture of heparinase, heparitinase I and II leads to the formation of eight unsaturated disaccharides which have the common disaccharide structure, ΔUA-(1→4)-GlcNR, where GlcNR represents either *N*-acetylated (GlcNAc) or *N*-sulfated GlcN (GlcNS) residue (for structures, see Table 1) [7–9]. Both sets of eight unsaturated disaccharides include one nonsulfated, three monosulfated, three disulfated and one trisulfated component(s) [5–9].

Fast atom bombardment mass spectrometry (FABMS) is one of the best methods for obtaining structural information on *O*-sulfated sugar compounds even with small amounts of samples. Previously, several papers dealing with the analysis of sulfated oligosaccharides on FABMS have been published [10–14]. Recently, Lamb *et al.* demonstrated that collision induced dissociation (CID) -mass analysed ion kinetic energy (MIKE) spectra on the negative-ion FABMS were useful for distinguishing isomeric monosulfated disaccharides from glycosaminoglycans [14]. We have also shown that CID-mass spectrometry/mass spectrometry (MS/MS) on both positive- and negative-ion modes provides a new easy method for the characterization of all of the eight unsaturated disaccharides, especially the respective three positional isomers of mono- and disulfated disaccharides from CS and DS [15].

Here we report the characterization of eight unsaturated disaccharides from Hep and HS and three GlcN-containing disaccharides derived from *N*-sulfo-GlcN-containing unsaturated disaccharides from Hep and HS by *N*-desulfation (for structures, see Table 1) based on the negative-ion FAB CID-MS/MS.

Materials and methods

Materials The unsaturated disaccharides investigated are listed in Table 1. All compounds (sodium salts) were obtained from Sigma Chemical Co. (USA) and were used without further purification.

Mass spectrometry Mass spectra were recorded on 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. About a 1 μl aliquot of an aqueous sample solution (*ca.* 1.0 mg in 50 μl) was mixed with 1 μl of triethanolamine or diethanolamine as the matrix (discussed below) and then placed on the FAB target.

CID MS/MS spectra were taken using argon as the collision gas at typically 0.133 Pa to reduce the beam of parent ions by approximately 30%. Collision energies were used at 30 eV. At least 10 scans were averaged to obtain each MS/MS spectrum.

Results and discussion

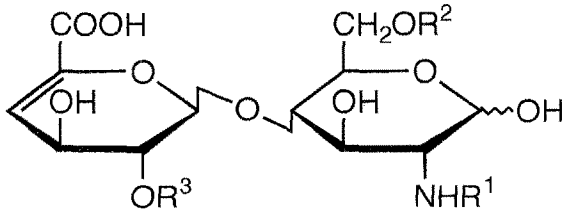
Negative-ion FAB Mass Spectra of Non-, Mono-, Di- and Trisulfated Unsaturated Disaccharides

As summarized in Table 2, the negative-ion FAB spectra of non-(ΔUA(1→4)GlcNAc), mono-(ΔUA(1→4)GlcNAc6S, ΔUA2S(1→4)GlcNAc, ΔUA(1→4)GlcNS, ΔUA(1→4)GlcN6S and ΔUA2S(1→4)GlcN), di-(ΔUA2S(1→4)GlcNAc6S, ΔUA(1→4)GlcNS6S, ΔUA2S(1→4)GlcNS and ΔUA2S(1→4)GlcN6S) and trisulfated (ΔUA2S(1→4)-GlcNS6S) unsaturated disaccharides exhibited unambiguously the peaks of molecule related ions $[M + n\mathrm{Na}-(n + 1)\mathrm{H}]^-$ (where $n = 0$–4), as reported earlier [10–15]. M represents the fully protonated molecule throughout this article. As a typical example, the spectra of ΔUA(1→4)GlcNAc6S, ΔUA(1→4)GlcNS6S and ΔUA(1→4)GlcN6S are shown in Fig. 1a, b and c, respectively. The spectra of the GlcN-containing disaccharides such as ΔUA(1→4)GlcN6S show the sodium adduct ions less remarkably than those of other disaccharides, ΔUA(1→4)GlcNAc6S and ΔUA(1→4)GlcNS6S. This may be caused by an amphoteric electrolyte character of the GlcN-containing disaccharides.

Triethanolamine has been usually used as the matrix in the negative-ion FABMS for sulfated oligosaccharides [12–15], because it gives abundant molecule related ions and structurally significant fragment ions. However, when triethanolamine was used as the matrix for disaccharides having a primary amino group such as ΔUA(1→4)GlcN6S, ΔUA2S(1→4)GlcN and ΔUA2S(1→4)GlcN6S, in addition to $[M + n\mathrm{Na}-(n + 1)\mathrm{H}]^-$

Table 1. Compounds investigated.

Compound	*RMM*[a]	R^1	R^2	R^3
Unsaturated disaccharides from heparin/heparan sulfate				
a) GlcNAc-containing disaccharide				
ΔUA(1→4)GlcNAc	379	Ac	H	H
ΔUA(1→4)GlcNAc6S	459	Ac	SO_3H	H
ΔUA2S(1→4)GlcNAc	459	Ac	H	SO_3H
ΔUA2S(1→4)GlcNAc6S	539	Ac	SO_3H	SO_3H
b) GlcNS-containing disaccharide				
ΔUA(1→4)GlcNS	417	SO_3H	H	H
ΔUA(1→4)GlcNS6S	497	SO_3H	SO_3H	H
ΔUA2S(1→4)GlcNS	497	SO_3H	H	SO_3H
ΔUA2S(1→4)GlcNS6S	577	SO_3H	SO_3H	SO_3H
c) GlcN-containing disaccharide derivative				
ΔUA(1→4)GlcN6S	417	H	SO_3H	H
ΔUA2S(1→4)GlcN	417	H	H	SO_3H
ΔUA2S(1→4)GlcN6S	497	H	SO_3H	SO_3H



[a] RMM represents the relative molecular mass of the free acid.

Table 2. Molecule related and major fragment ions (*m/z*) and relative peak intensities (%) in the negative ion FABMS of non-, mono-, di- and trisulfated unsaturated disaccharides from heparin / heparan sulfate.

Compound[a]	*Molecule related ions*[b, c]					*Major fragment ions*					
	$[M-H]^-$	$[M + Na-2H]^-$	$[M + 2Na-3H]^-$	$[M + 3Na-4H]^-$	$[M + 4Na-5H]^-$						
ΔUA(1→4)GlcNAc	378 (100)	400 (8)						277 (26)	$[(M-H)-101]^-$		
ΔUA(1→4)GlcNAc6S	458 (61)	480 (100)	502 (7)			401 (13)	$[(M + 2Na-3H)-101]^-$	379 (35)	$[(M + Na-2H)-101]^-$		
ΔUA2S(1→4)GlcNAc	458 (100)	480 (55)	502 (2)			401 (12)	$[(M + 2Na-3H)-101]^-$	379 (40)	$[(M + Na-2H)-101]^-$		
ΔUA2S(1→4)GlCNAc6S		560 (100)	582 (50)	604 (4)		503 (9)	$[(M + 3Na-4H)-101]^-$	481 (52)	$[(M + 2Na-3H)-101]^-$	458 (16)	$[(M + Na-2H)-NaSO_3+H]^-$
ΔUA(1→4)GlcNS	416 (82)	438 (100)									
ΔUA(1→4)GlcNS6S	496 (13)	518 (84)	540 (100)	562 (5)				416 (21)	$[(M + Na-2H)-NaSO_3 + H]^-$	438 (38)	$[(M + 2Na-3H)-NaSO_3+H]^-$
ΔUA2S(1→4)GlcNS	496 (8)	518 (100)	540 (87)	562 (4)				416 (23)	$[(M + Na-2H)-NaSO_3 + H]^-$	438 (9)	$[(M + 2Na-3H)-NaSO_3+H]^-$
ΔUA2S(1→4)GlcNS6S		598 (4)	620 (100)	642 (70)	664 (9)			518 (24)	$[(M + 2Na-3H)-NaSO_3 + H]^-$	540 (10)	$[(M + 3Na-4H)-NaSO_3+H]^-$
ΔUA(1→4)GlcN6S	416 (100)	438 (38)									
ΔUA2S(1→4)GlcN	416 (100)	438 (12)									
ΔUA2S(1→4)GlcN6S	496 (29)	518 (100)	540 (29)					416 (35)	$[(M + Na-2H)-NaSO_3 + H]^-$		

[a] See Table 1 for structures.

[b] M represents the fully protonated acid.

[c] Peaks are normalized to the base peak.

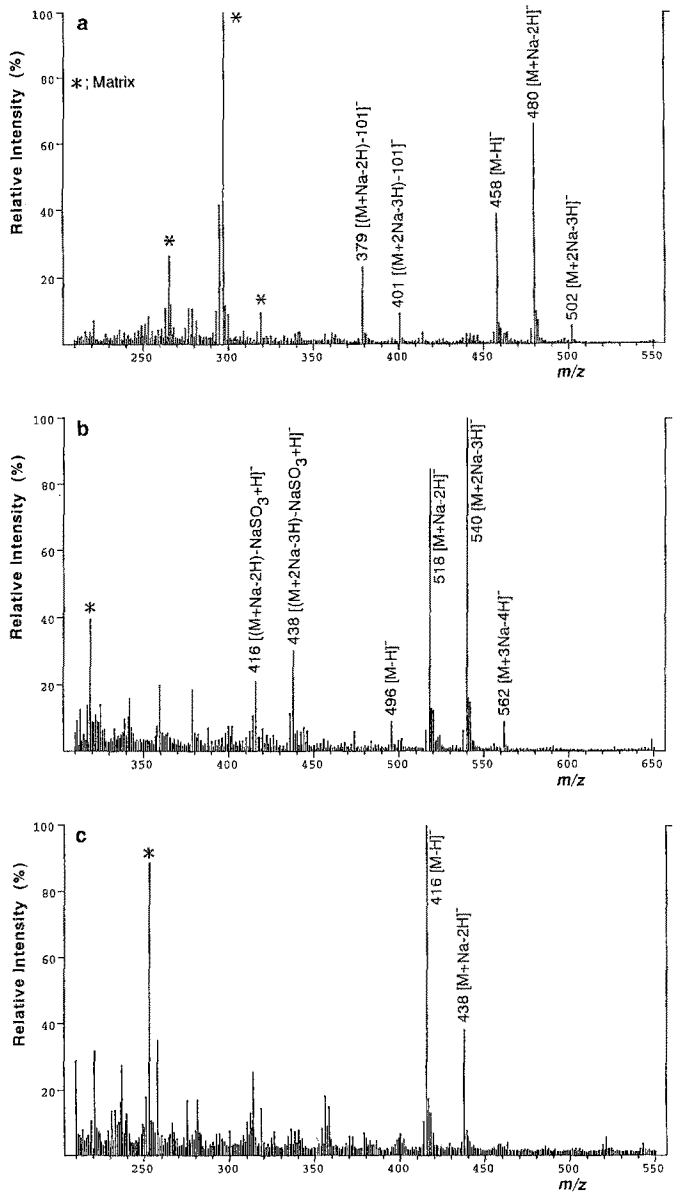


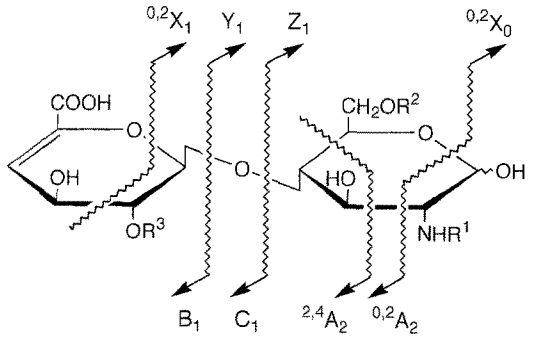
Figure 1. Negative-ion FAB mass spectra of sulfated unsaturated disaccharides. (a) ΔUA(1→4)GlcNAc6S; (b) ΔUA(1→4)GlcNS6S; (c) ΔUA(1→4)GlcN6S.

ions, unexpected molecule related ions of 26 and 42 units higher in mass appeared [16, 17]. Although the mass of $[M-H+42]^-$ ion (*m/z* 458) of ΔUA(1→4)GlcN6S coincided with that of $[M-H]^-$ ion of ΔUA(1→4)GlcNAc6S, the CID-MS/MS spectra of those ions were not identical (data shown below). Thus the structure of this adduct ion cannot be identical to that of the *N*-acetyl derivative. Since these adduct ions would be mistaken as the molecular ion of the *N*-acetyl derivative, diethanolamine was used as the matrix for disaccharides having a primary amino group such as ΔUA(1→4)GlcN6S, ΔUA2S(1→4)GlcN and ΔUA2S(1→4)GlcN6S.

The loss of sodium sulfite with hydrogen transfer (i.e. 102 units, $-NaSO_3+H$) from the molecule related ions was also commonly observed in the spectra of di- and trisulfated unsaturated disaccharides (Table 2, Fig. 1) [10–13, 15]. This elimination was more remarkable for GlcNS-containing disaccharides than for GlcNAc- and GlcN-containing disaccharides. It is noted that the spectra of disaccharides having a GlcNAc residue such as ΔUA(1→4)GlcNAc, ΔUA(1→4)GlcNAc6S, ΔUA2S(1→4)GlcNAc and ΔUA2S(1→4)GlcNAc6S, show the loss of 101 units from the molecule related ions (Table 2, Fig. 1a). These ions are produced by the elimination of AcNHCHCHOH due to the cleavage of the GlcNAc residue. This result shows that it is possible to distinguish the GlcNAc-containing disaccharides from not only the GlcN- and GlcNS-containing disaccharides, but also the GalNAc-containing disaccharides from CS and DS having a (1→3)-glycosidic linkage as reported in the previous report [15]. However, it is impossible to distinguish the mono- or disulfated positional isomers from these negative-ion FABMS alone.

CID-MS/MS Spectra of the Negative-ion FAB of GlcNAc-Containing Unsaturated Disaccharides

(1) Nonsulfated disaccharide The CID-MS/MS spectra of $[M-H]^-$ ion (*m/z* 378) and $[M+Na-2H]^-$ ion (*m/z* 400) of ΔUA (1→4)GlcNAc are shown in Fig. 2a and b, respectively. To describe the fragment ion drawn in each Figure, we use the nomenclature shown in Scheme 1, proposed by Domon and Costello [18]. The spectrum of $[M-H]^-$ ion (Fig. 2a) exhibits an intense and a weak peak at *m/z* 175 and 157 corresponding to $[C_1]^-$ and $[B_1-2H]^-$ ions, respectively, both of which are produced by the cleavage of the glycosidic bond, and also characteristic peaks at *m/z* 277 and 259 corresponding to $[^{0,2}A_2-H]^-$ and $[^{0,2}A_2-H-H_2O]^-$ ions, respectively, which are derived by the cleavage of the GlcNAc residue at the reducing end. These ions produced by the cleavage of the sugar ring are characteristic for polysaccharides having (1→4)-glycosidic linkages [19], and are not observed in the spectra of unsaturated disaccharides from CS, DS and hyaluronan (HA) having (1→3)-glycosidic linkages as we previously reported [15]. The spectrum of $[M+Na-2H]^-$ ion (Fig. 2b) shows peaks at *m/z* 239, 299 and 340



Scheme 1. The nomenclature for disaccharide fragmentation proposed by Domon and Costello [18].

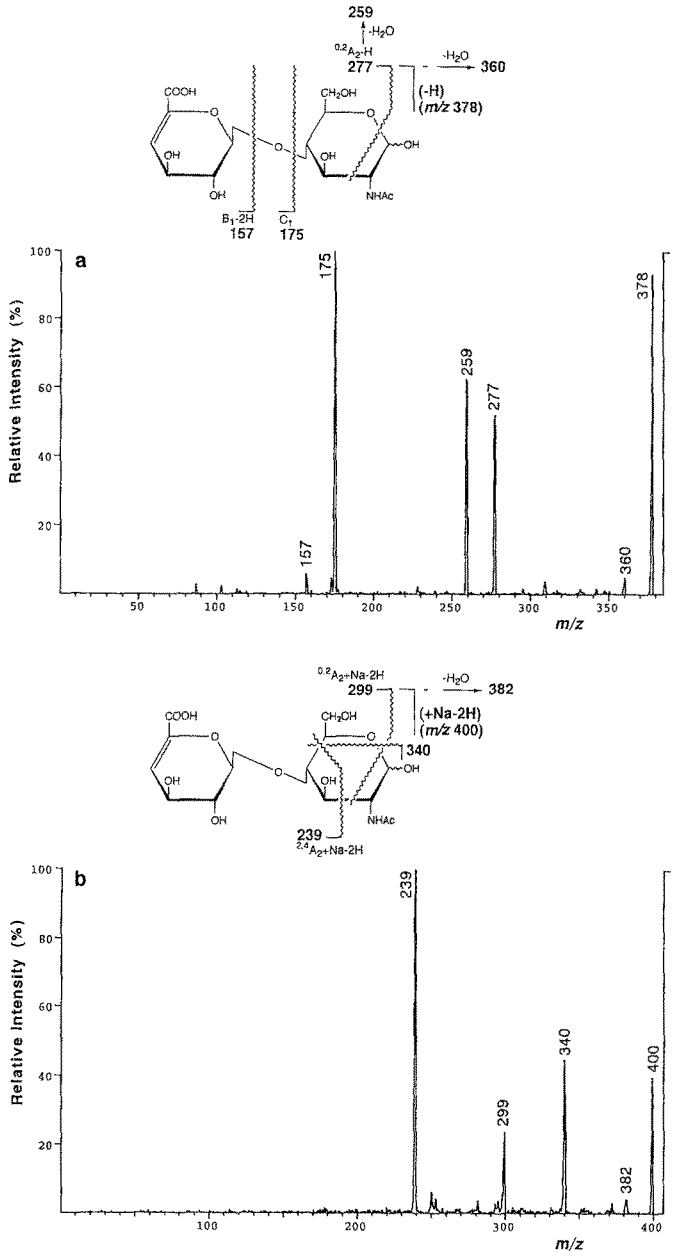


Figure 2. MS/MS spectra of nonsulfated unsaturated disaccharides ΔUA(1→4)GlcNAc. (a) the $[M–H]^-$ ion (*m/z* 378) as the parent ion; (b) the $[M + Na–2H]^-$ ion (*m/z* 400) as the parent ion.

corresponding to $[^{2,4}A_2 + Na–2H]^-$, $[^{0,2}A_2 + Na–2H]^-$ and $[^{0,4}A_2 + Na–2H]^-$ ions, respectively, derived from the cleavage of the sugar ring. On the basis of the presence of these characteristic ions, nonsulfated unsaturated disaccharide from Hep and HS, ΔUA(1→4)GlcNAc, can be easily distinguished from ΔUA(1→3)GalNAc and ΔUA(1→3)GlcNAc obtained from CS, DS and HA, respectively [15].

(2) Monosulfated disaccharides The results of the CID-MS/MS spectra of $[M–H]^-$ and $[M–2H + Na]^-$ ions of ΔUA(1→4)GlcNAc6S and ΔUA2S(1→4)GlcNAc are summarized in Table 3, where pronounced differences between the two isomers are observed. In the spectra of $[M–H]^-$ ions, the diagnostic peaks of ΔUA(1→4)GlcNAc6S were found at *m/z* 357 ($[^{0,2}A_2–H]^-$), 342($[^{0,2}X_1–H]^-$), 300 ($[Y_1]^-$), 282 ($[Z_1–2H]^-$) and 175 ($[C_1]^-$), while those of ΔUA2S(1→4)GlcNAc were found at *m/z* 342 ($[^{0,2}X_1–H]^-$, 237 ($[B_1–2H]^-$), 157 ($[(B_1–2H)–SO_3]^-$), and 97 ($[HSO_4]^-$). In the spectra of $[M + Na–2H]^-$ ions, the diagnostic peaks of ΔUA(1→4)GlcNAc6S were observed at *m/z* 379 ($[^{0,2}A_2 + Na–2H]^-$) and 139 ($[OHCCH_2OSO_3]^-$, while those of ΔUA2S(1→4)GlcNAc were observed at *m/z* 379 ($[^{0,2}A_2 + Na–2H]^-$), 361 ($[(^{0,2}A_2 + Na –2H)–H_2O]^-$), 319 ($[^{2,4}A_2 + Na–2H]^-$), 277 ($[C_1 + Na–H]^-$) and 259 ($[B_1 + Na–3H]^-$).

On the basis of this fragmentation behaviour of $[M–H]^-$ and $[M–2H + Na]^-$ ions, the positional isomers, ΔUA(1→4)GlcNAc6S and ΔUA2S(1→4)GlcNAc can be easily distinguished from each other and from the other three isomers, ΔUA(1→3)GalNAc4S, ΔUA(1→3)GalNAc6S and ΔUA2S(1→3)GalNAc derived from CS and DS [15].

The spectral features obtained here at low collision energy (30 eV), do not resemble those of the CID-MIKE spectra of $[M–H]^-$ and $[M + Na–2H]^-$ ions at high collision energy (8 ke V) reported by Lamb *et al.* [14], but are similar to those of the CID-MS/MS spectra of $[M–H]^-$ ions at low collision energy in the electrospray ionization (ESI) MS, recently reported by Chai *et al.* [20].

(3) Disulfated disaccharides As shown in Fig. 3a and b, the CID-MS/MS spectra of $[M + Na–2H]^-$ (*m/z* 560) and $[M + 2Na–3H]^-$ (*m/z* 582) ions of ΔUA2S(1→4)GlcNAc6S give characteristically the B, C, Y, Z, A and X-type fragment ions. The assignment of the fragment ions are illustrated in the Figure. These observations indicate that the CID-MS/MS is also useful to identify and distinguish this disulfate from the isomers, ΔUA2S(1→3)GalNAc4S, ΔUA2S(1→3)GalNAc6S and ΔUA(1→3)GalNAcDiS from CS and DS [15].

CID-MS/MS Spectra of the Negative-ion FAB of GlcNS-Containing Unsaturated Disaccharides

(1) Monosulfated disaccharide The CID-MS/MS spectrum of $[M–H]^-$ ion (*m/z* 416) of ΔUA(1→4)GlcNS, as shown in Fig. 4a, shows the predominant peak at *m/z* 138 corresponding to the $[^{0,2}X_0–H]^-$ ion characteristic of GlcNS-containing unsaturated disaccharides in the low mass region. Other ions are produced by the cleavage of the glycosidic bond (*i.e.* $[B_1–2H]^-$ (*m/z* 157), $[C_1]^-$ (*m/z* 175), $[Z_1–2H]^-$ (*m/z* 240) and $[Y_1]^-$ (*m/z* 258)), and of the ΔUA residue of the non-reducing end (*i.e.* $[^{0,2}X_1–H]^-$ (*m/z* 300)). Interestingly, an ion derived by the loss of H_2O from the $[Z_1–2H]^-$ (*m/z* 240) ion was observed at *m/z* 222.

(2) Disulfated disaccharides The CID-MS/MS spectrum of the $[M–H]^-$ ion (*m/z* 496) of ΔUA(1→4)GlcNS6S is shown in Fig. 4b. The result is similar to that of ΔUA(1→4)GlcNS, except for the observation of $[^{0,2}A_2–H]^-$ at *m/z* 357 and

Table 3. Major ions in MS/MS of $[M–H]^-$ and $[M + Na–2H]^-$ ions of ΔUA(1→4)GlcNAc6S and ΔUA2S(1→4)GlcNAc (*m/z*, relative peak intensities).

$[M–H]^-$		$[M + Na–2H]^-$		
ΔUA(1→4)GlcNAc6S	*ΔUA2S(1→4)GlcNAc*	*ΔUA(1→4)GlcNAc6S*	*ΔUA2S(1→4)GlcNAc*	*Ion structure*
458	458	480	480	Parent ion
440 (13)		462 (2)		Parent ion – H_2O
		379 (77)	379 (5)	$^{0,2}A_2$ + Na – 2H
		361 (8)	361 (14)	$^{0,2}A_2$ + Na – 2H – H_2O
357 (43)				$^{0,2}A_2$ – H
342 (51)	342 (11)			$^{0,2}X_1$ – H
339 (9)				$^{0,2}A_2$ – H – H_2O
			319 (100)	$^{2,4}A_2$ + Na – 2H
300 (45)		300 (3)		Y_1
282 (17)				Z_1 – 2H
			277 (9)	C_1 + Na – H
		264 (5)		Z_1 – 2H – H_2O
259 (21)				$^{0,2}A_2$ – H – H_2SO_4
			259 (5)	B_1 + Na – 3H
		245 (8)		$^{0,2}A_2$ + Na – 2H – H_2O – $^{0,2}A_1$
241 (19)				$^{0,2}X_1$ – H – $^{0,2}X_0$
	237 (5)			B_1 – 2H
217 (15)				$^{2,4}A_2$ – H
199 (55)				Y_1 – $^{0,2}X_0$
175 (100)				C_1
			161 (12)	B_1 + Na – 3H – H_2SO_4
		161 (10)		$OHCCH_2OSO_3$ + Na – H
	157 (91)			B_1 – 2H – SO_3
139 (54)		139 (100)		$OHCCH_2OSO_3$
			139 (7)	B_1 + Na – 3H – $NaHSO_4$
97 (79)	97 (100)	97 (7)		HSO_4
85 (13)	85 (26)			$^{0,3}A_1$–H

$[(M–H)–SO_3]^-$ (*m/z* 416) ions. These observations indicate that major fragmentations are caused by the elimination of SO_3 from the ions produced by the cleavage of the glycosidic bonds and the sugar rings.

The results of the negative-ion CID-MS/MS of $[M + Na–2H]^-$ ions (*m/z* 518) of isomeric disulfated disaccharides, ΔUA(1→4)GlcNS6S and ΔUA2S(1→4)GlcNS, indicate that it is possible to distinguish GlcNS-containing disulfated disaccharides. The CID-MS/MS spectrum of $[M + Na–2H]^-$ ion (*m/z* 518) of ΔUA(1→4)GlcNS6S, as shown in Fig. 5a, showed characteristic fragment ions similar to those observed in the case of $[M + Na–2H]^-$ (*m/z* 480) of ΔUA(1→4) GlcNAc6S (Table 3). The CID-MS/MS spectra of $[M + Na–2H]^-$ ions of ΔUA2S(1→4)GlcN (Fig. 5b) and ΔUA2S(1→4)GlcNAc (Table 3) closely resemble each other. In the spectrum of ΔUA(1→4)GlcNS6S, three intense peaks at *m/z* 438, 379 and 139 that correspond to $[(M + Na–2H) – SO_3]^-$, $[^{0,2}A_2 + Na–2H]^-$ and $[OHCCH_2OSO_3]^-$ ions, respectively, are observed. The elimination of SO_3 from $[Y_1]^-$ (*m/z* 338) and $[Z_1–2H]^-$ (*m/z* 320) also yielded weak peaks at *m/z* 258 and 240, respectively. In the spectrum of ΔUA2S(1→4)GlcNS, however, the predominant peak at *m/z* 319 corresponds to the $[^{2,4}A_2 + Na–2H]^-$ ion. The cleavage of the sugar ring also yields the weak peaks at *m/z* 357 ($[^{0,2}A_2–H]^-$ and 402 ($[^{0,2}X_1 + Na–2H]^-$. The origins of these fragment ions are rationalized as illustrated in Fig. 5. These observations show that the CID-MS/MS of $[M + Na–2H]^-$ ions of positional isomers, ΔUA(1→4)GlcNS6S and ΔUA2S(1→4)GlcNS, provides an easy way of distinguishing them.

(3) Trisulfated disaccharide As shown in Fig. 6, the CID-MS/MS spectrum of $[M + 2Na–3H]^-$ ion (m/z 620) of trisulfated disaccharide ΔUA2S(1→4)GlcNS6S exhibits dominant peaks at *m/z* 481, 459 and 319 corresponding to $[^{0,2}A_2 + 2Na–3H]^-$, $[^{0,2}A_2 + Na–2H]^-$ and $[^{2,4}A_2 + Na–2H]^-$ ions, respectively. Other characteristic peaks are assigned as illustrated in the Figure. These observation indicate that the spectrum can be used for identification of this trisulfate.

The CID-MS/MS spectra of $[M + Na–2H]^-$ ion of ΔUA(1→4)GlcNS, of $[M + 2Na–3H]^-$ ions of ΔUA(1→4) GlcNS6S and ΔUA2S (1→4)GlcNS, and of the [M +

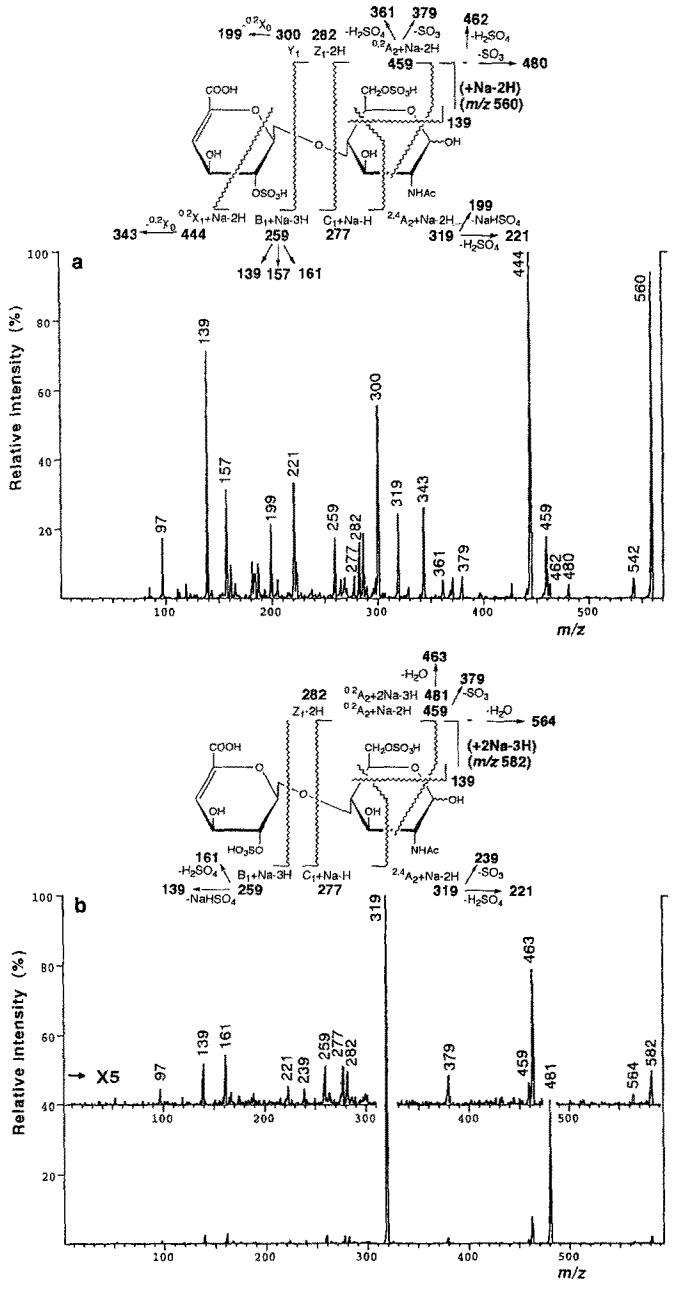


Figure 3. MS/MS spectra of disulfated unsaturated disaccharides ΔUA2S(1→4)GlcNAc6S. (a) the $[M + Na–2H]^-$ ion (*m/z* 560) as the parent ion; (b) the $[M + 2Na–3H]^-$ ion (*m/z* 582) as the parent ion.

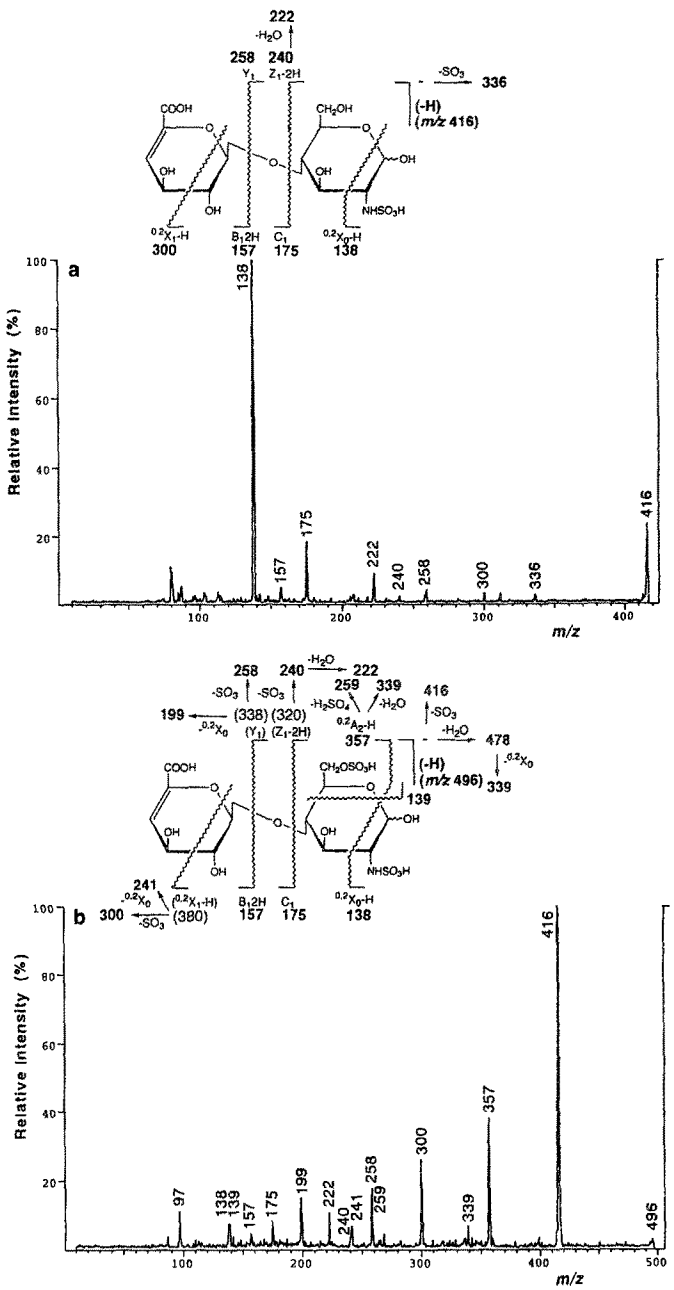


Figure 4. MS/MS spectra of GlcNS-containing unsaturated disaccharides having $[M–H]^-$ ions as the parent ion. (a) ΔUA(1→4)GlcNS (*m/z* 416); (b) ΔUA(1→4)GlcNS6S (*m/z* 496).

$3Na–4H]^-$ ion of ΔUA2S(1→4)GlcNS6S showed complicated and less informative fragment ions (data not shown).

CID-MS/MS Spectra of the Negative-Ion FAB of GlcN-Containing Unsaturated Disaccharides

(1) Monosulfated disaccharides The results of the negative-ion CID-MS/MS of $[M–H]^-$ ions (*m/z* 416) of isomeric monosulfated disaccharides, ΔUA(1→4)GlcN6S and ΔUA2S(1→4)GlcN, indicate that it is possible to distinguish monosulfated GlcN-containing disaccharides. The CID-MS/MS spectrum of the $[M–H]^-$ ion (*m/z* 416) of ΔUA(1→4)GlcN6S, as shown in Fig. 7a, gave characteristic fragment ions similar to those observed in the case of $[M–H]^-$ (*m/z* 458) of ΔUA(1→4)GlcNAc6S (Table 3). The CID-MS/MS spectra of $[M–H]^-$ ions of ΔUA2S(1→4)GlcN (Fig. 7b) and ΔUA2S(1→4)GlcNAc (Table 3) closely resemble each other. In the spectrum of ΔUA(1→4)GlcN6S, predominant peaks at *m/z* 357, 300 and 258 corresponding to $[^{0,2}A_2–H]^-$, $[^{0,2}X_1–H]^-$ and $[Y_1]^-$ ions, respectively, produced by the cleavage of the *O*-sulfated GlcN, ΔUA and the glyco-

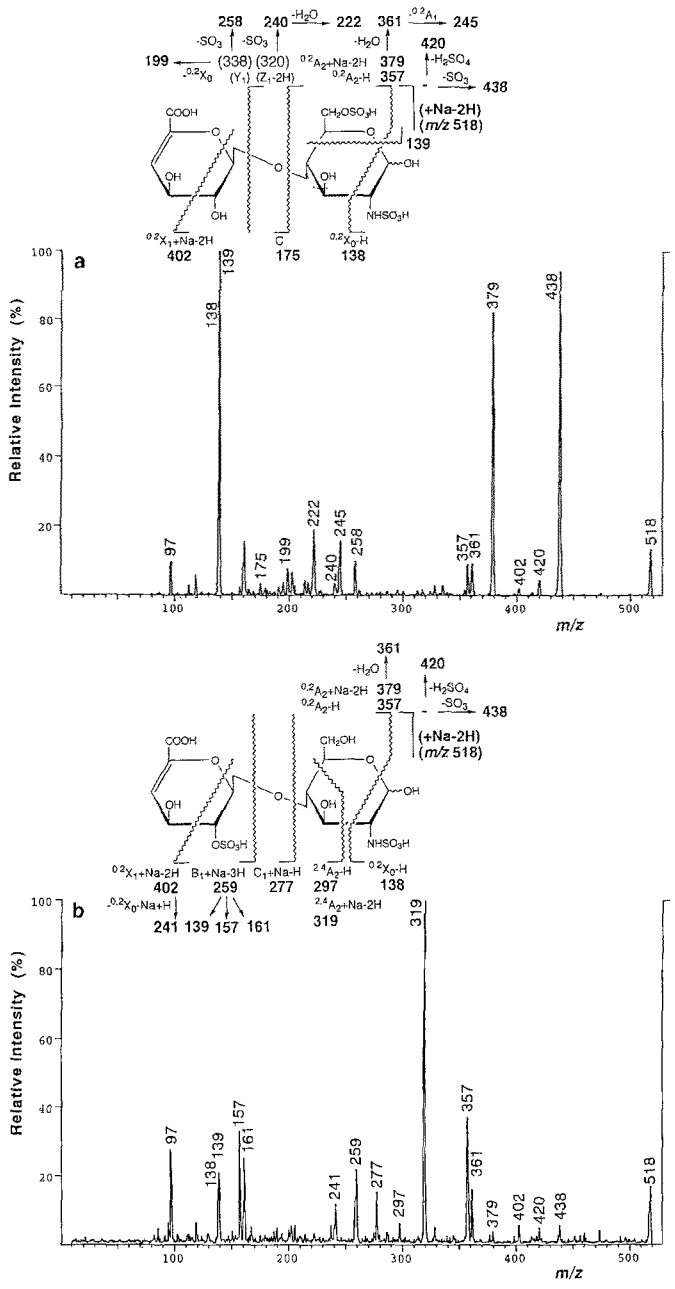


Figure 5. MS/MS spectra of disulfated unsaturated disaccharides having $[M + Na–2H]^-$ ions (*m/z* 518), as the parent ion. (a) ΔUA(1→4)GlcNS6S; (b) ΔUA2S(1→4)GlcNS.

sidic bond are observed. The elimination of the $^{0,2}X_1$ unit from $[(M–H)–H_2O]^-$ (*m/z* 398), $[^{0,2}X_1–H]^-$ (*m/z* 300) and $[Y_1]^-$ (*m/z* 258) also yielded three intense peaks at *m/z* 339, 241 and 199, respectively. In the spectrum of ΔUA2S(1→4)GlcN, two intense peaks at *m/z* 157 and 97 correspond to the $[(B_1–2H)–SO_3]^-$ and $[HSO_4]^-$ ions, respectively. The cleavage of the sugar ring also yielded weak peaks at *m/z* 357 ($[^{0,2}A_2–H]^-$) and 300 ($[^{0,2}X_1–H]^-$).

The CID-MS/MS spectra of $[M + Na–2H]^-$ ions (*m/z* 438) of ΔUA(1→4)GlcN6S and ΔUA2S(1→4)GlcN are shown in

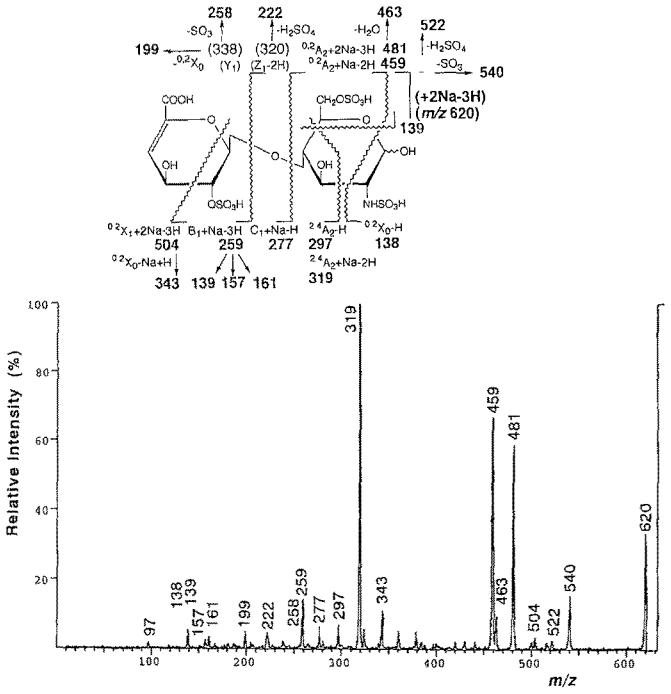


Figure 6. MS/MS spectrum of the trisulfated unsaturated disaccharide ΔUA2S(1→4)GlcNS6S having $[M + 2Na–3H]^-$ ions (*m/z* 620), as the parent ion.

Fig. 8, where more pronounced differences between these positional isomers are observed. Similar differences in the spectra of $[M + Na–2H]^-$ ions are also seen in the case of ΔUA(1→4)GlcNS6S and ΔUA2S(1→4)GlcNS. The similarity of those spectral features suggests that the CID-MS/MS of ΔUA(1→4)GlcNS6S and ΔUA2S(1→4)GlcNS demonstrates the loss of the SO_3 group from the *N*-sulfo groups giving the ions of ΔUA(1→4)GlcN6S and ΔUA2S(1→4)GlcN, respectively, as intermediates. The origins of these fragment ions are rationalized as illustrated in Fig. 8.

These observations show that the CID-MS/MS of $[M–H]^-$ and $[M + Na–2H]^-$ ions of positional isomers, ΔUA(1→4)GlcN6S and ΔUA2S(1→4)GlcN, provides an easy way of distinguishing them.

(2) Disulfated disaccharide As shown in Fig. 9a, the CID-MS/MS spectrum of the $[M + Na–2H]^-$ (*m/z* 518) ion of disulfated disaccharide ΔUA2S(1→4)GlcN6S exhibits characteristic dominant peaks at *m/z* 459, 402, 343, 319 and 258 corresponding to $[^{0,2}A_2 + Na–2H]^-$, $[^{0,2}X_1 + Na–2H]^-$, $[(^{0,2}X_1 + Na–2H)–^{0,2}X_0]^-$, $[^{2,4}A_2 + Na–2H]^-$ and $[Y_1]^-$, respectively. In the low mass region abundant ions arising from the sulfate group are observed at *m/z* 97 ($[HSO_4]^-$) and 139 ($[OHCCH_2OSO_3]^-$). The assignments of other fragment ions are illustrated in this Figure. In contrast to the CID-MS/MS spectrum of $[M + Na–2H]^-$ ion, that of the $[M + 2Na–3H]^-$ (*m/z* 540) ion of ΔUA2S(1→4)GlcN6S, as shown in Fig. 9b, gave a simple spectrum, which showed a predominant peak at

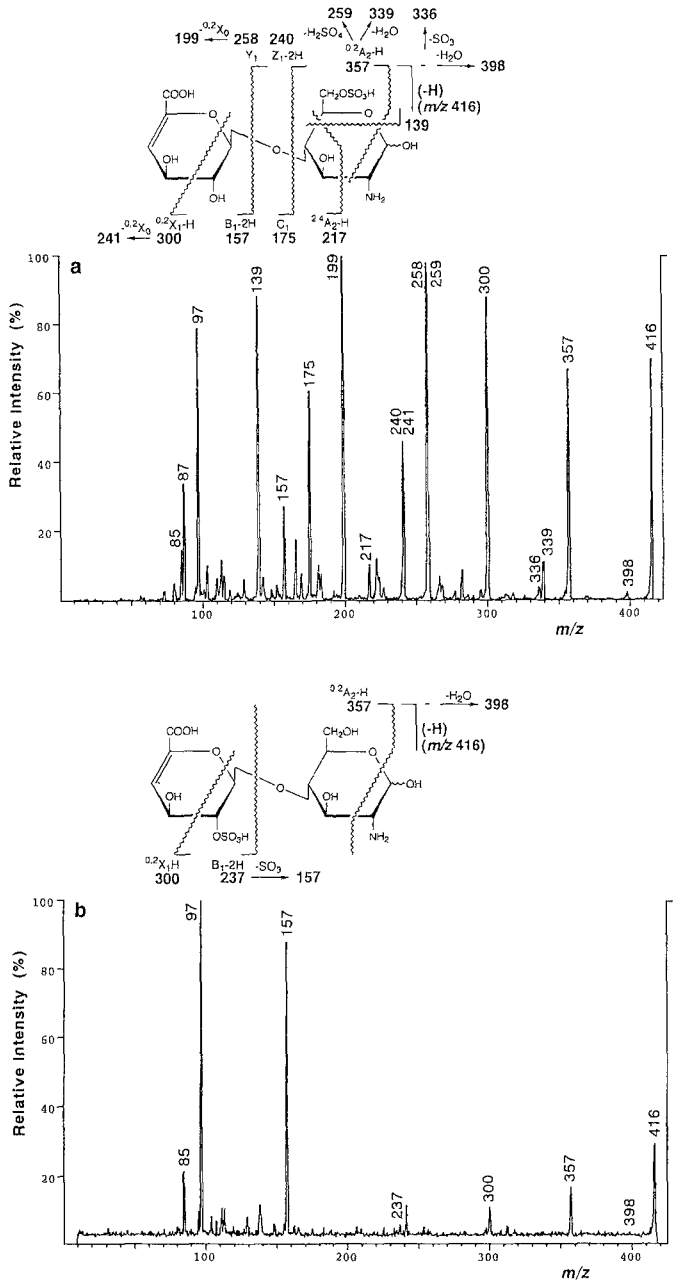


Figure 7. MS/MS spectra of monosulfated unsaturated disaccharides having $[M–H]^-$ ions (*m/z* 416), as the parent ion. (a) ΔUA(1→4)GlcN6S, (b) ΔUA(1→4)GlcN.

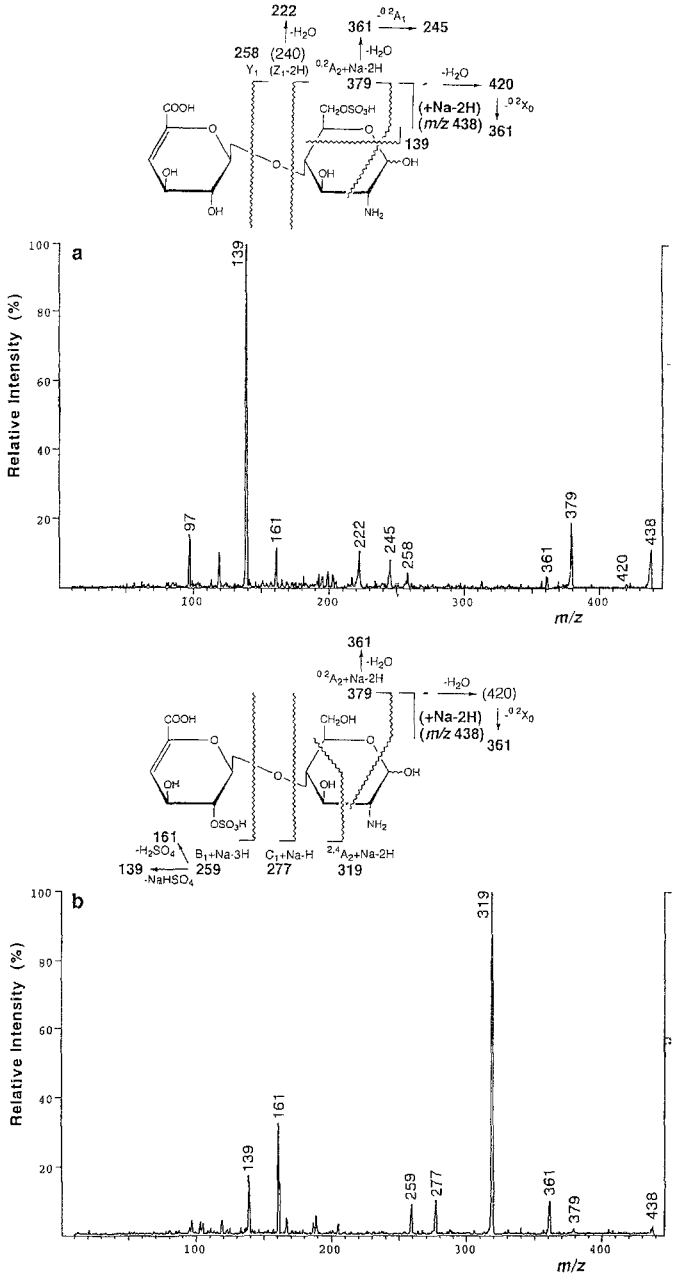


Figure 8. MS/MS spectra of monosulfated unsaturated disaccharides having $[M + Na–2H]^-$ ions (*m/z* 438), as the parent ion. (a) ΔUA(1→4)GlcN6S; (b) ΔUA2S(1→4)GlcN.

m/z 319 ($[^{2,4}A_2 + Na–2H]^-$) and a few weak peaks, as illustrated in the Figure. These results indicate that the CID-MS/MS can be used for identification of ΔUA2S(1→4)GlcN6S, and for differentiation of this compound from the isomer, ΔUA2S(1→4)GlcNS.

Conclusion

Negative-ion FAB mass spectra of the sodium salts of eight disaccharides from Hep and HS and three derivatives show distinct molecule related ions, giving clear molecular weight information. For GlcNAc-containing disaccharides, the observations of the ions produced by the elimination of the AcNHCHCHOH ($^{0,2}X_0$ residue) indicate clearly that it is possible to distinguish these disaccharides from isomeric sulfated disaccharides from CS and DS.

The CID-MS/MS spectra of the selected molecule related ions can be used for differentiating the respective two positional isomers of mono- and disulfated disaccharides from Hep and HS, and for distinguishing GlcNS-containing mono-

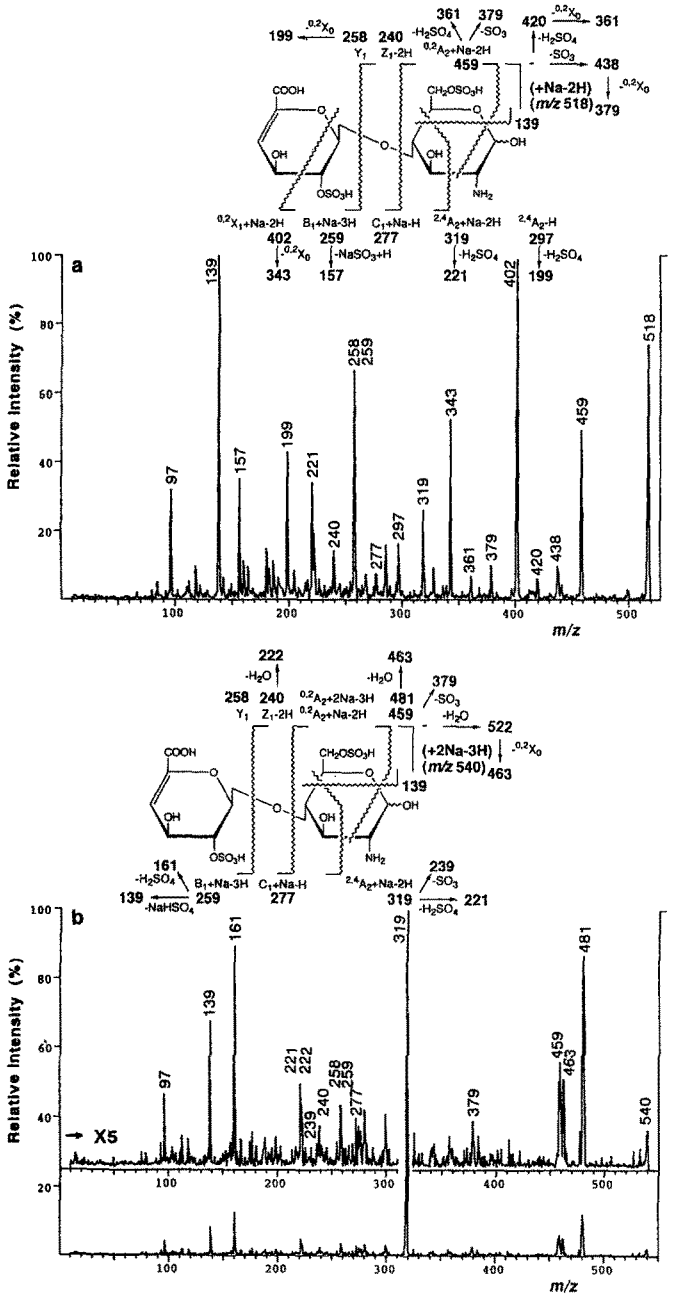


Figure 9. MS/MS spectra of disulfated unsaturated disaccharides ΔUA2S(1→4)GlcN6S. (a) the $[M + Na-2H]^-$ ion (*m/z* 518) as the parent ion; (b) the $[M + 2Na-3H]^-$ ion (*m/z* 540) as the parent ion.

and disulfated disaccharides from respective isomeric artefacts. The tandem FABMS also provides useful information on the identification of di- and trisulfated disaccharides. Our results suggest that the negative-ion tandem FABMS will provide a new rapid and convenient method for the analysis of disaccharides from Hep and HS.

Recently, Da Col *et al.* [21] showed that negative-ion ESI CID-MS/MS was useful for characterizing the structures of sulfated glycosaminoglycans after enzymatic digestion. We have previously succeeded in the systematic characterization of nine unsaturated disaccharides from CS, DS and HA [15] by FAB CID-MS/MS. Thus, negative-ion FAB as well as ESI CID-MS/MS will be very useful for disaccharide analyses of many glycosaminoglycans in various biological materials. We are currently extending our studies, based on the positive-ion FAB CID-MS/MS, to the characterizations of disaccharides from Hep and HS and the results will be published elsewhere.

Acknowledgements

This work was partially supported by the Grant-in-Aid for Scientific Research on Priority Areas No. 06240247 from the Ministry of Education, Science and Culture, Japan.

References

1. Poole AR (1986) *Biochem J* **236**:1–14.
2. Rouslahti E (1989) *J Biol Chem* **264**:13369–72.
3. Fransson LÅ (1987) *Trends Biochem Sci* **12**:406–11.
4. Suzuki S, Mizutani A, Koike Y, Kato M, Yoshida K, Kimata K (1991) *Pure Appl Chem* **63**:545–54.
5. Al-Hakim A, Linhardt RJ (1991) *Anal Biochem* **195**:68–73.
6. Yoshida K, Miyauchi S, Kikuchi H, Tawada A, Tokuyasu K (1989) *Anal Biochem* **177**:327–32.
7. Yamada S, Yoshida K, Sugiura M, Sugahara K (1992) *J Biochem (Tokyo)* **112**:440–47.
8. Ampofo SA, Wang HM, Linhardt RJ (1991) *Anal Biochem* **199**:3249–55.
9. Desai UR, Wang HM, Ampofo SA, Linhardt RJ (1993) *Anal Biochem* **213**:120–27.
10. Carr SA, Reinhold VN (1984) *Carbohydr Chem* **3**:381–401.
11. Reinhold VN, Carr SA, Green BN, Petitou M, Choay J, Sinay P (1987) *Carhohydr Res* **161**:305–13.
12. Mallis LM, Wang HM, Loganathan D, Linhardt RJ (1989) *Anal Chem* **61**:1453–58.
13. Linhardt RJ, Wang HM, Loganathan D, Lamb DJ, Mallis LM (1992) *Carbohydr Res* **225**:137–45.
14. Lamb DJ, Wang HM, Mallis LM, Linhardt RJ (1992) *J Am Soc Mass Spectrom* **3**:797–803.
15. Ii T, Okuda S, Hirano T, Ohashi M (1994) *Glycoconjugate J* **11**:123–32.
16. Nores GA, Hanai N, Levery SB, Eaton HL, Salyan MEK, Hakomori S (1988) *Carbohydr Res* **179**:393–410.
17. Morita M, Matsubara T, Hayashi A (1990) *Proc Japanese Soc Biomed Mass Spectrom (Japan)* **15**:103–6.
18. Domon B, Costello CE (1988) *Glycoconjugate J* **5**:397–409.
19. Garozzo D, Giuffrida M, Impallomeri G, Ballistreri A, Montaudo G (1990) *Anal Chem* **62**:279–86.
20. Chai W, Green BM, Lawson AM (1993) *Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics* **41**:85a–85b.
21. Da Col R, Silvestro L, Naggi A, Torri G, Baiocchi C, Moltrasio D, Cedro A, Viano I (1993) *J Chromatogr* **647**:289–300.