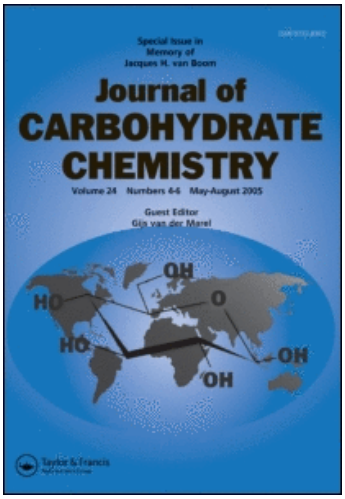

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Structural Characterization of Sulfated Glycosaminoglycans by Fast Atom Bombardment Mass Spectrometry: Application to Chondroitin Sulfate

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STRUCTURAL CHARACTERIZATION OF SULFATED GLYCOSAMINOGLYCANS BY FAST ATOM BOMBARDMENT MASS SPECTROMETRY: APPLICATION TO CHONDROITIN SULFATE

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ABSTRACT

Polysulfated oligosaccharides obtained from enzymatic digests of chondroitin sulfate have been successfully analyzed by positive and negative ion fast atom bombardment mass spectrometry. The spectra show intense cationized molecular ions and fragment ions from which sugar sequence and sites of sulfation can be readily determined. In addition, polymers of 4-O-sulfate and 6-O-sulfate positional isomers may be distinguished by fast atom bombardment mass spectrometry. The mass spectra also indicate the presence of structural homologs of chondroitin sulfate that contain more than one covalently bound sulfate moiety per two sugar residues. The effects of salt concentration, liquid matrix composition and ionization mode (negative versus positive ion abstraction) on the quality and information content of the mass spectra are discussed in detail.

INTRODUCTION

The sulfated glycosaminoglycans constitute a broad class of acidic polysaccharides that includes the chondroitin, dermatan and keratan sulfates which are widely distributed in connective tissues such as skin, collagen and bone, and heparin which is predominantly found in lung, spleen, liver and muscle tissue.[1-3] Speculation concerning the role of sulfate esters has ranged from

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simple augmentation of the anionic character of these macromolecules, (which conveys emolliency, lubricity and water-retaining capability), to more subtle structural effects such as changes in charge distribution and conformation that alter the cell surface and thus may affect cell-cell adhesion and interaction of the cell with constituents of the extracellular matrix.[1,2]

Detailed knowledge of the sequence, linkage and sites of sulfation of the glycosaminoglycans is fundamental to understanding their physiological, chemical and physical properties. One recent finding which underscores this point is the demonstration that a heparin fraction, when administered together with cortisone, causes regression of large tumor masses, inhibits angiogenesis and prevents metastases.[4] Preparations of heparin obtained from different sources exhibited a wide range of biological potency. The physiological basis for this activity and the reasons for its variability are unclear, but may well be associated with specific structural elements or domains in heparin which are as yet uncharacterized.

Unfortunately, elucidation of the molecular structures of the sulfated glycosaminoglycans is a remarkably difficult undertaking, due in large part to the lability of the sulfate residues which prevents straightforward application of conventional sequencing methods.[5] Adding further difficulty is the fact that glycosaminoglycans are structurally polydisperse and degradation by chemical or enzymatic methods introduces size heterogeneity. These subtle structural differences, together with the dominant anionic character of these oligomers, hinders chromatographic separation and purification. Sites of sulfation have previously been determined by comparing the results of compositional analyses and periodate oxidation studies obtained before and after alkaline desulfation.[5,6] These reactions lack sensitivity and invariably produce mixtures of products making the results difficult to interpret. Consequently, few structures of sulfated oligosaccharides larger than disaccharides have been unambiguously

determined, and those larger structures which have been presented are often composite or average structures rather than unique molecular entities.

In view of the chemical lability and heterogeneity of the sulfated glycosaminoglycans, physiochemical methods that would permit detailed structural information to be obtained on these materials without the need for prior chemical degradation or derivatization would be most beneficial. Recently, a number of new mass spectrometric techniques have been developed that enable direct analysis of polar and thermally labile molecules.[7] In particular, fast atom bombardment mass spectrometry (FAB MS) has been shown to be of value for structure analysis of neutral and acidic oligosaccharides and glycosphingolipids[8-12] (for a review, see Reference 8). For these reasons we have now applied FAB MS to analyze underivatized sulfated oligosaccharides obtained by enzymatic digestion of chondroitin sulfate. The results of these analyses indicate that FAB MS will be a valuable tool for structural characterization of sulfated glycosaminoglycans.

RESULTS AND DISCUSSION

In fast atom bombardment mass spectrometry (FAB MS, also known as liquid SIMS, secondary ion mass spectrometry) sample molecules are dissolved in a viscous liquid such as glycerol (G) or thioglycerol (SG, 3-mercapto-1,2-propanediol), and ionized from this liquid/sample matrix by an impinging beam of atoms (FAB MS) or ions (liquid SIMS).[7,8,14] The distinguishing feature of this method is that sample-related ions are formed directly without prior vaporization of the sample. Thus, highly polar and thermally labile organic compounds and salts may be analyzed using microgram amounts of material. There are three principal sources for the ions observed in a FAB mass spectrum. The liquid support produces a constant and reproducible background on which molecular and fragment ions derived from the sample are superimposed. In addition, adventitious inorganic

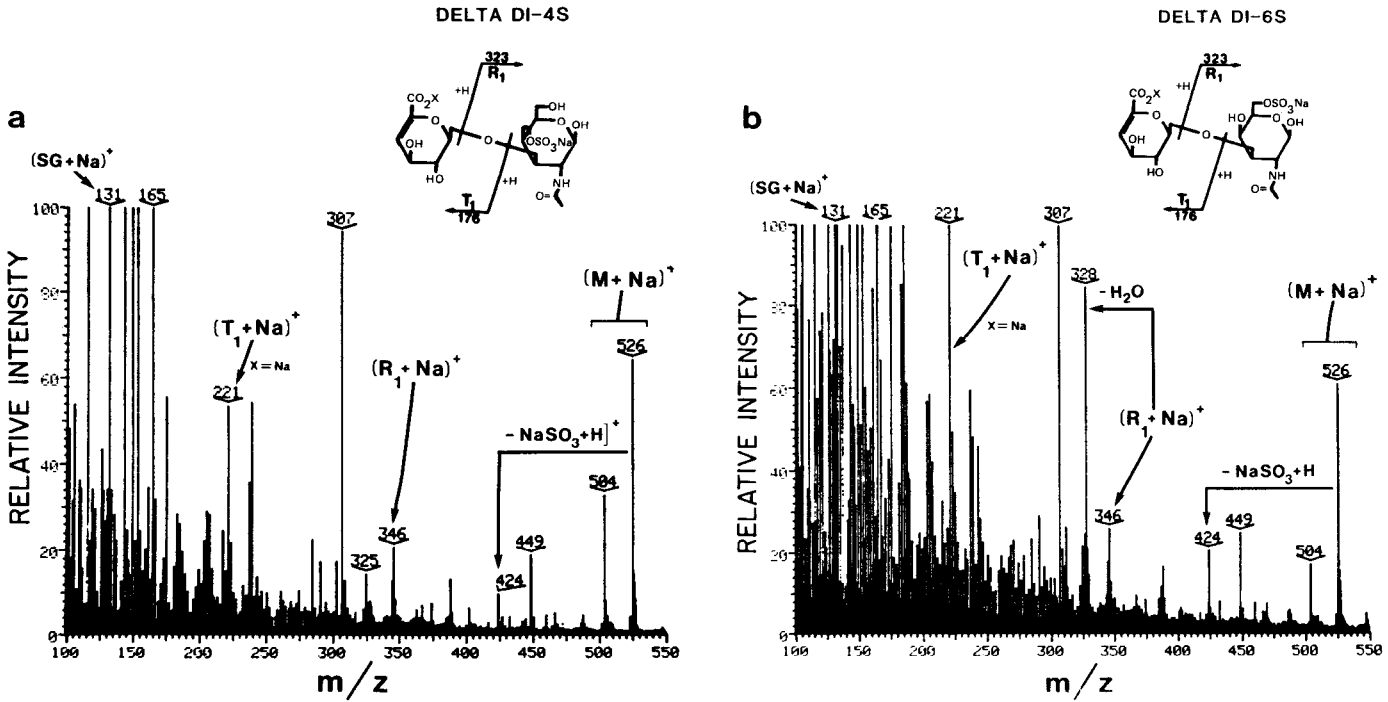


Figure 1. Positive ion FAB mass spectra of a) Δ-Di-4S, and b) Δ-Di-6S in thioglycerol (SG). Ions at m/z 165, 307 and 449 correspond to $^{-}[(Na_2SO_4)_n + Na]^{+}$ where n = 1-3.

impurities, if present at sufficient concentration, may produce a characteristic series of ions or form adducts under FAB conditions with the sample.

The glycosaminoglycan disaccharide samples considered in the present study were obtained by chondroitinase digestion of chondroitin sulfate which releases two major components, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose and the corresponding 6-O-sulfogalactose isomer, abbreviated Δ-Di-4S and Δ-Di-6S, respectively. The positive and negative ion FAB mass spectra of these isomeric sulfated disaccharides (Figs. 1 and 2, respectively) are dominated by molecular and structurally significant fragment ions. The molecular weights of these polyanionic materials as determined by FAB MS includes the sum of all countercations, a variable factor which depends on the acidity of the anionic groups and the microenvironment of the sample. Thus, molecular weight related ions in the FAB mass spectra of the sulfated oligosaccharides appear as multiplets separated by 22 daltons because sodium cations (present as a result of sample isolation or purification) replace protons as the countercations to the sulfate and carboxylate anions (Tables I-III). In order to simplify structural representation of these molecules we have drawn sodium as the counterion for each sulfate residue and "X" as the counterion for each carboxylate group, where X may be either a proton or a sodium cation. For example, the molecular weight of the monosodium salt of either Δ-Di-4S or Δ-Di-6S is 481 daltons (X=H). Thus, the ions at m/z 504 and 526 in the positive ion FAB mass spectra (Fig. 1, Table I) correspond to $(M + Na)^+$ for X=H and X=Na, respectively. Similarly, the ions at m/z 480 and 502 in the negative ion spectra correspond to $(M-H)^-$ for X=H and X=Na, respectively (Fig. 2, Table I). Losses of sodium sulfite with hydrogen replacement (i.e., $-NaSO_3$, $+H$) are commonly observed from sulfate containing parent and fragment ions resulting in satellite peaks 102 daltons lower in mass (e.g., Fig. 1, Table I). Ions at m/z 165, 307, 449... in the

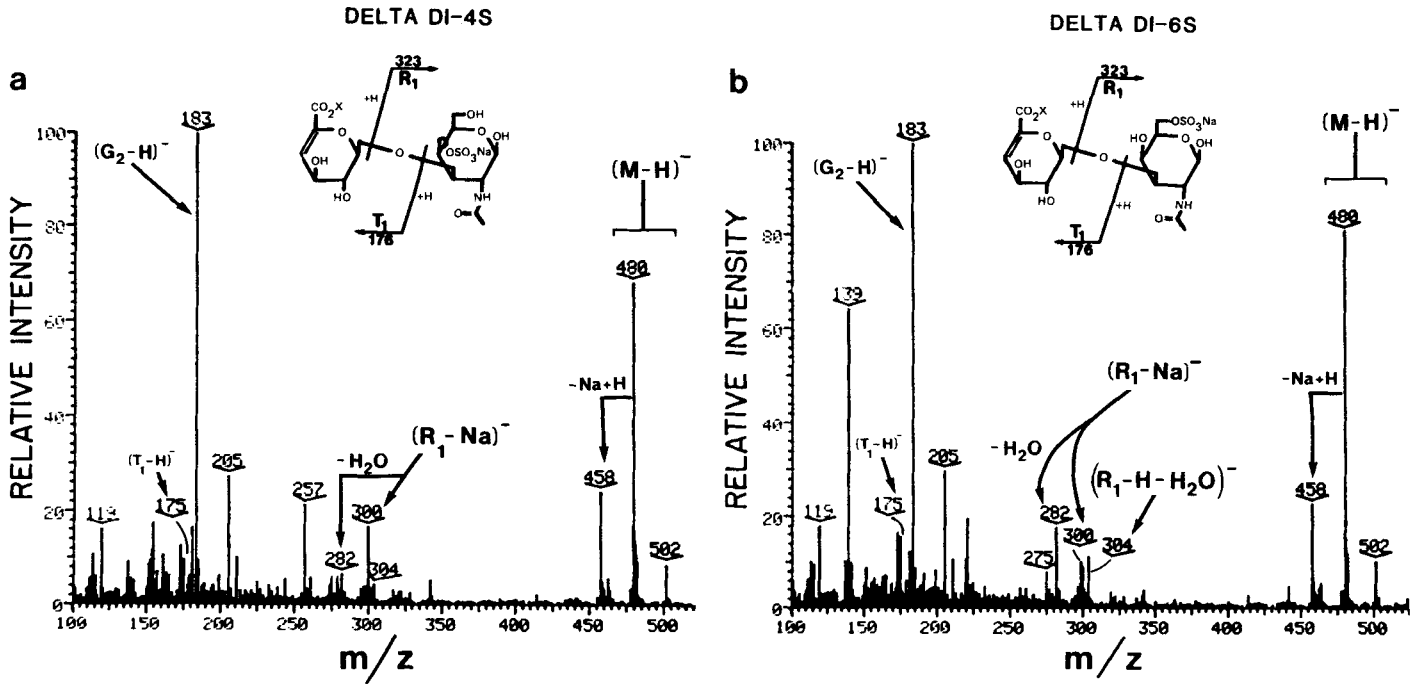


Figure 2. Negative ion FAB mass spectra of a) Δ-Di-4S, and b) Δ-Di-6S in glycerol (G).

TABLE I

Molecular Weight Related Ions for $(GlcU(4\text{-ene})\text{—}GlcNAcSO_4)^{-2}(H_m, Na_n)^{+2}$

Countercations	Molecular Weight	$(M-H)^-$	$(M + Na)^+$
$2H^+$	459	<u>458</u>[a]	482
H^+, Na^+	481	<u>480</u>	<u>504</u>
$2Na^+$	503	<u>502</u>	<u>526</u>

$$R\text{-}O\text{-}SO_3Na \xrightarrow[-SO_3Na,\ +H]{102} R\text{-}OH$$

[a]Underlines indicate ions observed in the mass spectra.

positive ion spectra of these commercially obtained disaccharides are due to contamination with Na_2SO_4, (see Fig. 1 and discussion below).

The sequence of the disaccharide samples is provided by intense fragment ions arising by simple cleavage on either side of the glycosidic oxygen accompanied by hydrogen attachment to the fragment which retains the charge. Nonreducing terminal end fragments are referred to as T_n ions (e.g., m/z 221 = $(T_1 + Na)^+$ for X=Na, Fig. 1), while charge retention on fragments arising from the reducing end are labeled R_n (e.g., m/z 300 = $(R_1 - Na)^-$, Fig. 2).

The 4-O-sulfate and 6-O-sulfate isomers may be clearly distinguished by comparing their positive and negative ion FAB mass spectra due to the ease with which the 6-O-sulfate isomer loses H_2O from the reducing end fragment R_1 (Scheme 1). For example, in the positive ion FAB spectra of Δ-Di-6S (Fig. 1b) a major peak is present at m/z 328 (corresponding to $[(R_1 + Na) -$

TABLE II

Molecular Weight Related Ions for $(GlcU\text{-}GlcNAcSO_4)_2^{-4}(H_m, Na_n)^{+4}$

Countercations	Molecular Weight	$(M - H)^-$	$(M + Na)^+$
$4H^+$	936	935[a]	959
$3H^+, Na^+$	958	957	981
$2H^+, 2Na^+$	980	979	1003
$H^+, 3Na^+$	1002	1001	1025
$4Na^+$	1024	1023	1047

Ions 102u above $(M - H)^-$ or $(M + Na)^+$ correspond to molecules with an additional $NaSO_3$ covalently bound ($-H$, $+NaSO_3$ = +102u).

Ions 142u above $(M - H)^-$ or $(M + Na)^+$ correspond to noncovalent adducts with Na_2SO_4.

[a]Underlines indicate ions observed in the mass spectra; dashed underlines indicates weak intensity.

TABLE III

Molecular Weight Related Ions for $(GlcU\text{-}GlcNAcSO_4)_3^{-6}(H_m, Na_n)^{+6}$

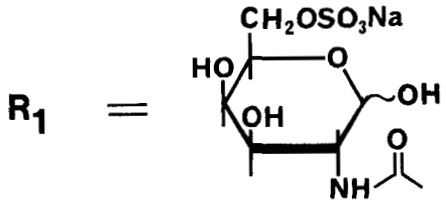
Countercations	Molecular Weight	$(M - H)^-$	$(M + Na)^+$
$6H^+$	1395	1394	1418
$5H^+, Na^+$	1417	1416	1440
$4H^+, 2Na^+$	1439	1438[a]	1462
$3H^+, 3Na^+$	1461	1460	1484
$2H^+, 4Na^+$	1483	1482	1506
$H^+, 5Na^+$	1505	1504	1528
$6Na^+$	1527	1526	1550

Ions 102u above $(M - H)^-$ or $(M + Na)^+$ correspond to molecules with an additional $NaSO_3$ covalently bound ($-H$, $+NaSO_3$ = +102u).

Ions 142u above $(M - H)^-$ or $(M + Na)^+$ correspond to noncovalent adducts with Na_2SO_4.

[a]Underlines indicate ions observed in the mass spectra; dashed underlines indicates weak intensity.

SCHEME 1



Negative Ion Mode	m/z	Positive Ion Mode	m/z
$(R_1 - H)^-$	= 322	$(R_1 + Na)^+$	= 346
$[(R_1 - H) - H_2O]^-$	= 304		
$(R_1 - Na)^-$	= 300	$[(R_1 + Na) - H_2O]^+$	= 328
$[(R_1 - Na) - H_2O]^-$	= 282		

$H_2O)^+$] which is virtually absent in the mass spectrum of the 4S isomer (Fig. 1a). This selective dehydration is also observed under negative ion conditions by the ratio of $[(R_1 - Na) - H_2O]^-$ to $(R_1 - Na)^-$ (Fig. 2, m/z 282 and 300, respectively). Tetrasaccharides and larger homopolymers of 4-O-sulfate and 6-O-sulfate oligomers may also be distinguished on this basis (see below). The data suggest dehydration involves the axial hydroxyl at C-4 of the $GlcNAcSO_4$ residue, but this has not yet been conclusively shown.

Tetra-, hexa- and octasaccharides from homopolymers of chondroitin 4- and 6-O-sulfate were also successfully analyzed by FAB MS, (see Figs. 3-4, Tables II and III). These materials were prepared by hyaluronidase digestion and separated according to their degree of polymerization by gel permeation chromatography.[13] As described above for the unsaturated sulfated disaccharides, sequence specific fragments for these saturated

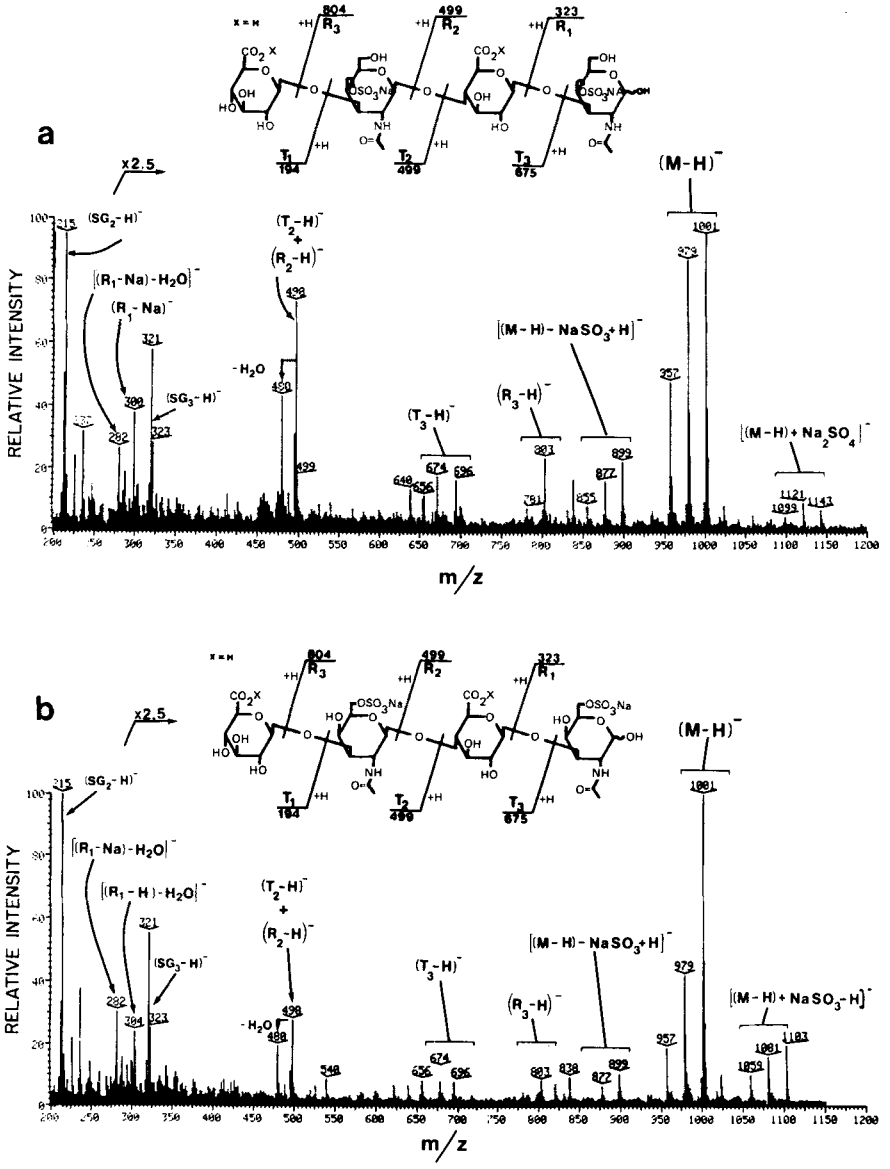


Figure 3. Negative ion FAB mass spectra of a) chondroitin 4-O-sulfate tetrasaccharide, and b) chondroitin 6-O-sulfate tetrasaccharide in thioglycerol (SG).

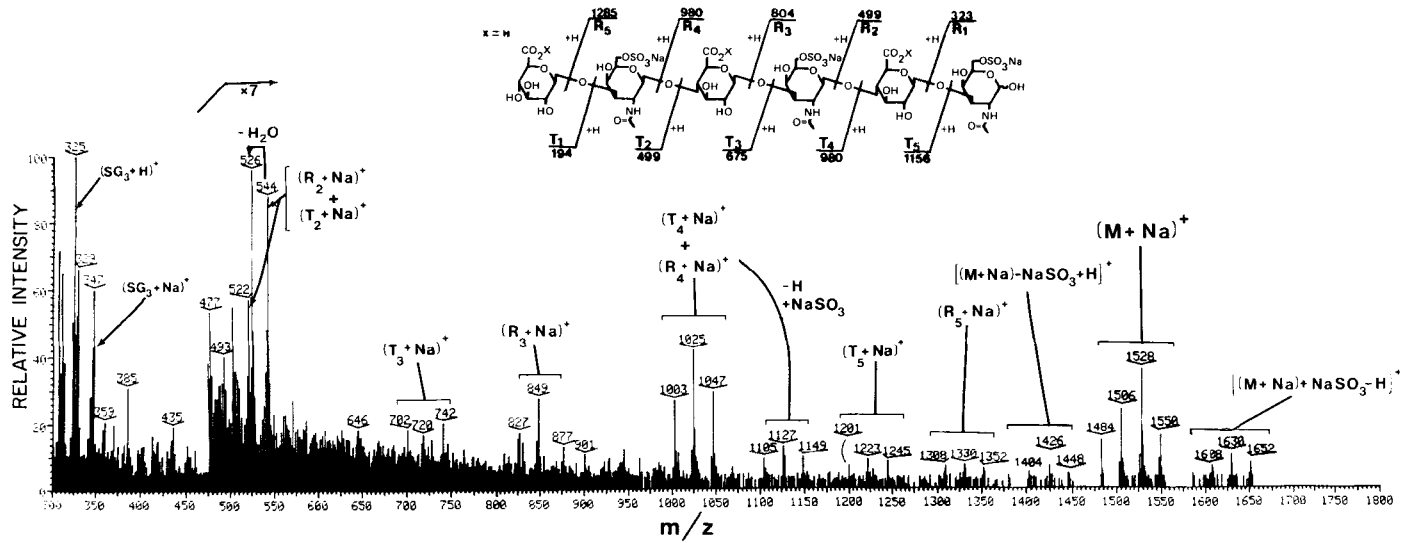


Figure 4. Positive ion FAB mass spectrum of chondroitin 6-O-sulfate hexasaccharide in thioglycerol.

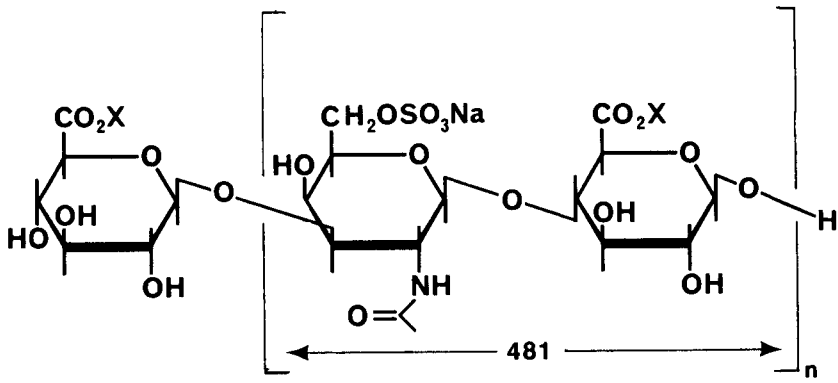
oligomers arise by cleavage and hydrogen attachment to the glycosidic oxygen with charge retained on either the terminal or reducing end of the molecule. Ions due to loss of H_2O from sequence specific fragments are also commonly observed, (e.g., Fig. 3a, m/z 480 = $[(R_2 - H) - H_2O]^-$ and Fig. 4, m/z 702 = $[(T_3 + Na) - H_2O]^+$). Scheme 2 illustrates the general fragmentation behavior of the oligomers in both positive and negative ion FAB MS. Since these oligomers are composed of repeating disaccharides, terminal or reducing end fragments with an odd number of sugar residues (T_z and R_z series, where z = 3,5,7..., Scheme 2) have unique masses, whereas fragments with an even number of sugar residues are isobaric (T_e and R_e series, Scheme 2). Reduction with $NaBD_4$ increments all ions containing the reducing end by 3 daltons, thereby eliminating this mass redundancy. Structures assigned to fragment ions have been corroborated by comparison of the FAB mass spectra obtained on samples prior to and after reduction.

Homopolymers of the 4-O-sulfate and 6-O-sulfate positional isomers of chondroitin sulfate with four or more sugar residues may be distinguished by the same criteria described above for the unsaturated disaccharides. Fragments corresponding to $[(R_1 - H) - H_2O]^-$ and $[(R_1 - Na) - H_2O]^-$, (m/z 304 and 282, respectively), are prominent in the spectra of the 6-O-sulfate tetra- and hexasaccharides whereas $(R_1 - Na)^-$ is of negligible abundance (e.g., Fig. 3b, m/z 300). In contrast, $(R_1 - Na)^-$ is the most significant peak in this region in the spectra of the 4-O-sulfate polymers (e.g., Fig. 3a). The tetra- and hexasaccharide chondroitin sulfate oligomers described in this report have been shown by other methods to be reasonably pure homopolymers of either the 4- or 6-O-sulfate disaccharide units, with less than 5% of the 4,6-O-sulfate copolymer present.[13]

Structural homologs of chondroitin sulfate that contain more than one covalently bound sulfate moiety per two sugar units were observed in the FAB mass spectra of the 6-O-sulfate tetra-, hexa- and octasaccharides, but not in the spectra of

SCHEME 2

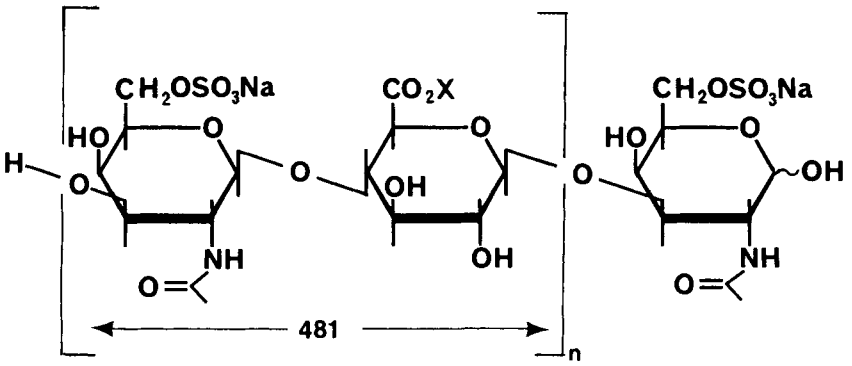
T_Z Series: T_3, T_5, T_7...=194 + n(481)



Negative Ion Mode	n	all X=H	1X=Na	2X=Na
$(T_3 - H)^-$	1	674	696	718
$(T_5 - H)^-$	2	1155	1177	1199
$(T_7 - H)^-$	3	1636	1658	1680
Positive Ion Mode				
$(T_3 + Na)^+$	1	698	720	742
$(T_5 + Na)^+$	2	1179	1201	1223
$(T_7 + Na)^+$	3	1660	1682	1704

SCHEME 2, cont.

R_Z Series: R_3, R_5, R_7...=323 + n(481)

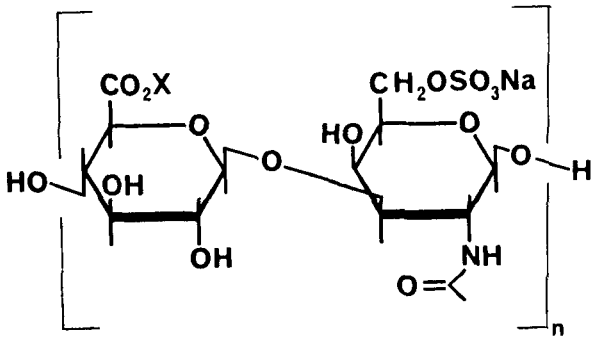


Negative Ion Mode	n	all X=H	1X=Na	2X=Na
$(R_3 - H)^-$	1	803	825	847
$(R_5 - H)^-$	2	1284	1306	1328
$(R_7 - H)^-$	3	1765	1787	1809
Positive Ion Mode				
$(R_3 + Na)^+$	1	827	849	871
$(R_5 + Na)^+$	2	1308	1330	1352
$(R_7 + Na)^+$	3	1789	1811	1833

(continued)

SCHEME 2, cont.

$T_e=R_e$ Series: T_2/R_2, T_4/R_4, T_6/R_6...=n(481)+18



Negative Ion Mode	n	all X=H	1X=Na	2X=Na
$(T_2-H)^-$/ $(R_2-H)^-$	1	498	520	542
$(T_4-H)^-$/ $(R_4-H)^-$	2	979	1001	1023
$(T_6-H)^-$/ $(R_6-H)^-$	3	1460	1482	1504
Positive Ion Mode				
$(T_2+Na)^+$ / $(R_2+Na)^+$	1	522	544	566
$(T_4+Na)^+$ / $(R_4+Na)^+$	2	1003	1025	1047
$(T_6+Na)^+$ / $(R_6+Na)^+$	3	1484	1506	1528

the 4-O-sulfate isomers. These "oversulfated" chondroitins are indicated by the cluster of ions 102 daltons above the protonated parent ions (Figs. 3b and 4). These ions are due to covalent attachment of an additional sulfite group ($+NaSO_3$, $-H$), and should not be confused with the FAB generated adduct ions of Na_2SO_4 (142 dalton shift; e.g., Fig. 3a) which are apparent to a variable degree in the mass spectra of both isomers. Oversulfation at the disaccharide level has previously been reported in squid and shark cartilage,[15-17] and oversulfated disaccharides have been found in high concentration in pathologically altered human bone.[18] Several sulfotransferases that may catalyze oversulfation are known, one of which has recently been shown to specifically catalyze the incorporation of sulfate into the 6-position of the nonreducing terminus of chondroitin 4-O-sulfate.[19] Specifically shifted sequence fragments are not apparent in these FAB mass spectra. Although the additional sulfates are probably not randomly distributed in the intact 50,000 dalton polymer, enzymatic hydrolysis of chondroitin with hyaluronidase may generate overlapping fragments of otherwise identical sequence (assuming oversulfation does not modify enzymatic activity) which "scrambles" the apparent positions of the additional sulfates. Mass spectral analysis of polymers which have been separated by charge as well as size may enable specific sites of oversulfation to be identified.

A characteristic feature of FAB mass spectra is that sample related ions appear superimposed on a relatively constant and predictable background spectrum due to the liquid support. We have found that thioglycerol is the preferred matrix for FAB analysis of the sulfated sugars.[8] The yield of sample-related ions is 2-3 fold greater for oligomers dissolved in thioglycerol and matrix-related ions are reduced by approximately one-half compared to glycerol (Fig. 5). The overall

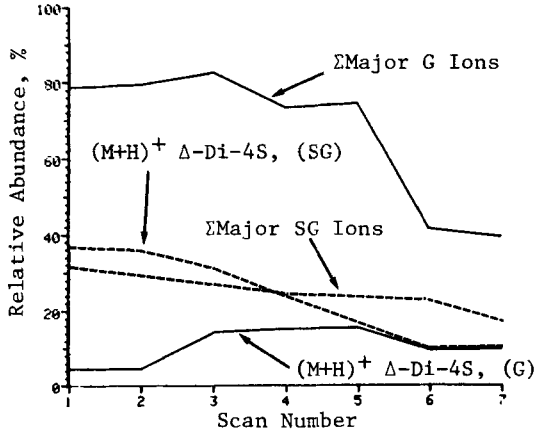


Figure 5. Comparison of the yields of the protonated molecular ion $(M+H)^+$ of Δ-Di-4S disaccharide for equivalent amounts of sample dissolved in glycerol (G, ——) versus thioglycerol (SG, ----). The relative abundance of matrix related ions are plotted as the sum of the three most intense glycerol (G) related (——) versus thioglycerol (SG) related (----) ions; see text for discussion.

result is a 4-6 fold increase in sensitivity when thioglycerol is used as the liquid matrix. However, thioglycerol is more volatile than glycerol which shortens observation time, and it is more susceptible to oxidation.

Molecular ion abundances are enhanced in the negative versus positive ion FAB mass spectra of the sulfated oligosaccharides. In addition, matrix-related ions and interferences from salt-cluster ions (such as $[(Na_2SO_4)_n + Na]^+$, m/z 165, 307, 339... Fig. 1) are substantially reduced in the negative ion mode, which also contributes to the apparently increased signal/noise ratio. The cation concentration also exerts a strong influence on the appearance of mass spectra. The highest sensitivity and greatest abundance of molecular and structurally informative ions are obtained when a molar equivalent of Na to carbohydrate-bound sulfate is present. Samples which have been desalted by cation

exchange show a 2-3 fold decrease in overall sensitivity (data not shown). Furthermore, ions due to loss of sulfate from molecular and fragment ions are more intense which makes spectral interpretation more difficult.

The results presented here clearly suggest that FAB MS will be very useful for structural analysis of sulfated glycosaminoglycans. The methods described may enable, for the first time, detailed structural characterization of this class of biopolymer at physiologically significant levels. We are presently extending our studies to the characterization of the clinically important degradation products of heparin. Preliminary FAB MS experiments on polysulfated oligosaccharides from heparinase digests have yielded excellent data that are entirely consistent with the findings presented here.[20]

EXPERIMENTAL

Tetra-, hexa- and octasaccharides of chondroitin 4- and 6-O-sulfate were generously supplied by H. Edward Conrad, University of Illinois, Urbana, IL. These fractions were obtained from whale and shark cartilage by hyaluronidase digestion, purified by gel filtration and characterized by Conrad, et al., as described.[13] The unsaturated disaccharides 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose and the corresponding 6-O-sulfogalactose isomer were obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan and are distributed by Miles Laboratories, Elkhart, IN.

Mass spectrometry experiments were carried out on the Finnigan-MAT 312 double focussing mass spectrometer. A Finnigan-MAT SS-200 data system controls the instrument and acquires, processes and stores the data. For fast atom bombardment (FAB) the instrument is equipped with an Ion Tech (Teddington, Middlesex, England) B-11 fine beam saddle field fast atom source; xenon was used as the reagent gas. Approximately 100 μg of the underivatized sulfated oligosaccharides were dissolved in 10 to 50 μl of distilled water and aliquots of

these solutions containing from 5-20 μg of sample were loaded by syringe onto a glycerol or thioglycerol (3-mercapto-1,2-propanediol) coated copper target attached to a direct insertion probe. The probe was then inserted into the ion source (combined electron impact/chemical ionization/FAB) of the mass spectrometer and bombarded with the xenon beam (operating parameters: source pressure 9 x 10^{-6} Torr Xe, FAB tube voltage = 8 kV, tube current = 2.0 mA).

ACKNOWLEDGEMENTS

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