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Carbohydrate Research 295 (1996) 157–177

CARBOHYDRATE RESEARCH

Disulfated oligosaccharides derived from tracheobronchial mucous glycoproteins of a patient suffering from cystic fibrosis

Deborah L. Chance, Thomas P. Mawhinney *

Departments of Biochemistry and Child Health, Room 4 Agriculture Building, University of Missouri – Columbia, Columbia, MO 65211, USA

Received 28 June 1996; accepted 29 August 1996

Abstract

Twenty novel disulfated oligosaccharides were purified in nanomolar quantities from tracheobronchial mucous glycoproteins from a patient with cystic fibrosis via cleavage by alkaline borohydride treatment, followed by anion-exchange chromatography, size-exclusion chromatography, and high-performance liquid chromatography (HPLC). In addition to positive ion fast-atom-bombardment mass spectrometry (FABMS), proposed structures for the resulting purified disulfated oligosaccharides were also based on carbohydrate permethylation analyses, periodate oxidation, complete sequential exoglycosidase digestion, and parallel analysis of desulfated products. Sulfate esters were found to reside on C-3 or C-6 of terminal D-galactose and on C-6 of internal D-galactose or 2-acetamido-2-deoxy-D-glucose residues. For this group of oligosaccharides, ranging in size from tri- to undeca-saccharides and possessing linear, di- and tri-antennary forms, it was also observed that sulfate esters could be located on the same or on different branches and that branched oligosaccharides can possess sulfate esters on C-3 and C-6 of different terminal galactose residues within the same structure. © 1996 Elsevier Science Ltd.

Keywords: Sulfated oligosaccharides; Mucous glycoproteins; Cystic fibrosis

1. Introduction

The isolation and characterization of sulfated oligosaccharides from the tracheobronchial mucous glycoproteins of patients with cystic fibrosis is an ongoing research

* Corresponding author.

PII S0008-6215(96)00231-5

focus that is an integral part of our program to understand primary and secondary molecular defense mechanisms of the human respiratory tree [1–5]. In the normal airway, the anionic makeup of respiratory mucous glycoproteins is comprised mostly of sialic acid with very low sulfate ester content. In the disease state, it is apparent that increases occur in the degree of sulfation [3–12] of respiratory mucins and/or the production of new sulfated mucins in chronic lung infection, chronic bronchitis, and in particular, cystic fibrosis, and may represent an increasingly important molecular defense mechanism in long-term disease. Furthermore, the sites, locations and number of sulfate esters on oligosaccharides may potentially play significant roles as ligands for selectins [13–18], a family of cell-adhesion proteins that are involved in the recruitment of neutrophils to inflammatory regions.

Monosulfated oligosaccharides of tracheobronchial mucous glycoproteins have been shown to possess sulfate esters predominantly on C-3 [5,19,20] and C-6 [1–5,21] of galactose and on C-6 [1–5,19,21] of *N*-acetylglucosamine residues. To date, though, very little information [5] is available on mucous glycoprotein oligosaccharides possessing more than one sulfate ester. In this study, emphasis was placed on the isolation and purification of the smaller fraction of mucous glycoprotein-derived oligosaccharides possessing two sulfate esters [2] in order to determine sulfation sites and patterns seen in cystic fibrosis mucins. Following the isolation and purification of crude disulfated oligosaccharides from tracheobronchial glycoproteins by anion-exchange and size-exclusion chromatography, the structures of twenty HPLC-purified disulfated oligosaccharides were elucidated via an unambiguous sequencing strategy [1–5]. The disulfated oligosaccharides and their desulfated analogs were subjected to permethylation analysis, coupled sequential exoglycosidase digestion and permethylation analysis, and FABMS of the disulfated oligosaccharides.

2. Results

Isolation and purification of disulfated oligosaccharides.—A heterogenous mixture of disulfated borohydride-reduced oligosaccharides, obtained from a crude mucous glycoprotein oligosaccharide preparation from which sialic acid containing oligosaccharides were previously removed [2], was isolated by anion-exchange HPLC chromatography. Four incompletely resolved fractions, coeluting with maltooligosaccharide standards 3–4, 5–6, 7 and 8–11 were obtained by Bio-Gel P-2 chromatography. Following amino acid analysis of each Bio-Gel P-2 fraction, which demonstrated no detectable glycopeptides to be present, each fraction was then subjected to Hypersil 120 Å APS-2 anion-exchange chromatography, the elution profiles of which are presented in a composite form in Fig. 1. Disulfated oligosaccharide fractions of relatively high purity ($> 85\%$) were selected for further purification. Minor fractions, or very heterogenous peaks, were excluded from this study. Repeated fractionation of each selected disulfated oligosaccharide by Bio-Gel P-2 and Hypersil 120 Å APS-2 chromatography resulted in the purification of twenty disulfated oligosaccharides, SS-VI_a through SS-CXI, in ≤ 40 nM amounts. Relative to 2-acetamido-2-deoxy-D-galactitol, each oligosaccharide, determined by carbohydrate (not shown) and permethylation analysis (Table 1), demonstrated

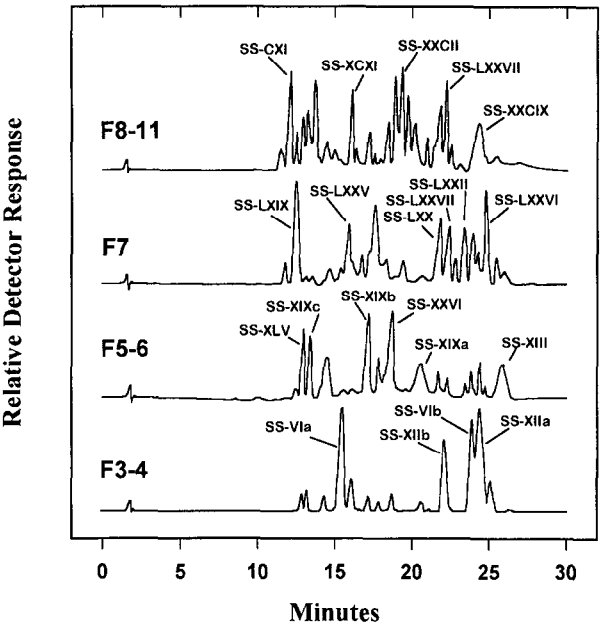


Fig. 1. HPLC anion-exchange elution profile of each pooled Bio-Gel P-2 fraction on a Hypersil 120 Å APS-2, 4.6×150 mm, metal-free column, 3 μm particle, employing a linear gradient of 90:10 to 60:40 (v/v) acetonitrile–water containing 3.8 mM NH_3CO_3 at a flow rate of 0.75 mL/min for 30 min with detection at 209 nm. Indicated disulfated oligosaccharides were individually pooled and, if required, further purified by sequential Bio-Gel P-2 and Hypersil 120 Å APS-2 chromatography.

near integral molar ratios of sulfate and, depending on which oligosaccharide being discussed, of 2-acetamido-2-deoxy-D-glucose, D-galactose, and L-fucose. In addition, based upon these molar ratios, it was concluded that all purified SS-oligosaccharides were disulfated.

Permethylation analysis of SS-VI$_a$–SS-CXII and desulfated analogs.—Further structural details are provided upon examination of the permethylation analyses of disulfated oligosaccharides SS-VI$_a$–SS-CXI and their parallel desulfated analogs DSS-VI$_a$–DSS-CXI, respectively (Tables 1 and 2). Initial analyses confirmed the carbohydrate and Bio-Gel P-2 findings for sugar composition, ratios and oligosaccharide size. As shown in Table 1, 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol was identified for SS-VI$_a$, SS-VI$_b$, SS-XXVI and SS-XLV, and 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol was found for the remaining oligosaccharides indicating one and two glycosidic linkages, respectively, on the 2-acetamido-2-deoxy-D-galactose involved in the original glycopeptide linkage of the mucous glycoprotein. Similarly, the finding of 2,4-di-*O*-methyl-D-galactose for SS-XXVI, SS-XLV, SS-XXCII, SS-XXCIX, SSXCXI and SS-CXI oligosaccharides, and their respective desulfated analogues, suggests that each oligosaccharide possesses a D-galactose residue that forms a branching point with glycosidic linkages on both C-3 and C-6. Based upon this branching data, it is apparent that SS-VI$_a$ and SS-VI$_b$ are unbranched linear oligo-

Table 1
Methylation analysis of disulfated oligosaccharides before and after treatment with α-L-fucosidase [a,b]

Sulfated oligosaccharide	GlcN(Me)Ac	Gal	Fuc	GalN(Me)Ac-ol	Sulfate [e]
SS-VI$_a$	4 (0.9)	2,3,4 (0.9) [c]		1,4,5,6 (1) [c]	(1.88)
SS-VI$_b$	3 (1.0)	2,4,6 (0.9)		1,4,5,6 (1) [c]	(1.91)
SS-XII$_a$	3,6 (1.0) 3,4 (0.9) [c]	2,3,4 (0.9) [c]		1,4,5 (1) [c]	(1.89)
SS-XII$_b$	3,6 (1.0) 3,4 (1.0) [c]	2,4,6 (0.9)		1,4,5 (1) [c]	(1.81)
SS-XIII	3,6 (2.1)	2,3,4 (1.9) [c]		1,4,5 (1) [c]	(2.04)
SS-XIX$_a$	3,6 (1.6) 4,6 (0.5)	2,3,4 (0.9) [c] 2,4,6 (1.9)		1,4,5 (1) [c]	(1.87)
SS-XIX$_b$	3,6 (1.4) 4,6 (0.7)	2,4 (0.9) 2,4,6 (0.9) 2,3,4,6 (1.) [c]		1,4,5 (1) [c]	(1.94)
SS-XIX$_c$	3,6 (1.1) 4,6 (1.0)	2,4,6 (2.9)		1,4,5 (1) [c]	(1.80)
SS-XXVI	3,6 (2.1)	2,4 (1.0) 2,4,6 (1.9)		1,4,5,6 (1) [c]	(1.86)
SS-XLV	6 (1.0) [d1] 3,6 (1.0)	2,4 (1.0) 2,4,6 (1.9)	2,3,4 (0.9) [c,d]	1,4,5,6 (1) [c]	(1.87)
SS-LXIX	6 (1.1) [d2] 3,6 (1.0)	2,4,6 (2.8)	2,3,4 (1.1) [c,d]	1,4,5 (1) [c]	(1.94)
SS-LXX	4 (1.1) 3,6 (1.0)	3,4,6 (1.1) [c,d3] 2,4,6 (1.9)	2,3,4 (1.0) [c,d]	1,4,5 (1) [c]	(1.92)
SS-LXXII	3,6 (1.0) 4,6 (1.0)	2,4 (0.9) 2,4,6 (0.9) 3,4,6 (1.1) [c,d3]	2,3,4 (1.1) [c,d]	1,4,5 (1) [c]	(1.88)
SS-LXXV	3,6 (1.1) 4,6 (1.0)	4,6 (1.0) [d4] 2,3,4 (1.0) [c] 2,4,6 (1.1)	2,3,4 (1.0) [c,d]	1,4,5 (1) [c]	(1.92)
SS-LXXVI	4 (0.9) 3,6 (2.1)	2,3,4 (0.9) [c] 2,4,6 (1.1) 2,3,4,6 (1.0) [c]		1,4,5 (1) [c]	(1.94)
SS-LXXVII	3,6 (2.1) 4,6 (1.0)	2,4,6 (0.9) 2,3,4 (2.0) [c]		1,4,5 (1) [c]	(1.94)
SS-XXCII	3,6 (3.1)	2,4 (0.9) 2,3,4 (1.9) [c] 2,3,4,6 (1.0) [c]		1,4,5 (1) [c]	(1.92)
SS-XXCIX	3,6 (4.0)	2,4 (0.9) 2,3,4 (1.9) [c] 2,3,4,6 (1.1) [c]		1,4,5 (1) [c]	(1.93)
SS-XCXI	3,6 (3.1) 4 (1.0)	2,4 (0.9) 2,4,6 (1.9) 2,3,4,6 (2.0) [c]		1,4,5 (1) [c]	(1.88)
SS-CXI	3,6 (3.1) 4,6 (0.9)	2,4 (0.9) 2,3,4 (0.9) [c] 2,4,6 (1.9) 3,4,6 (1.0) [c,d3]	2,3,4 (1.0) [c]	1,4,5 (1) [c]	(1.88)

[a] Data expressed as the molar ratio of the identified partially methylated alditol acetates relative to the respective permethylated D-GalN(Me)Ac-ol found for each disulfated oligosaccharide.

[b] Purified disulfated reduced oligosaccharides, isolated by Bio-Gel P-2 size-exclusion chromatography and Hypersil 120 Å APS-2 (NH_2) anion-exchange, were per-*O*-methylated, subjected to acid hydrolysis, derivatized to their respective alditol acetates, and analyzed by GLC.

[c] Residue destroyed when the respective oligosaccharide was pretreated with sodium metaperiodate.

[d] Following treatment of each native oligosaccharide with α-L-fucosidase and methylation of the product, all L-fucose and indicated residues were lost with the concomitant appearance of (d1), 3,6-di-*O*-methyl-GlcN(Me)Ac; (d2), 4,6-di-*O*-methyl-GlcN(Me)Ac; (d3), 2,3,4,6-tetra-*O*-methyl-Gal or (d4), 2,4,6-tri-*O*-methyl-Gal.

[e] Sulfate was determined both colorimetrically [3] and by GLC [22].

Table 2
Methylation analysis of the desulfated oligosaccharides produced from the respective disulfated oligosaccharide from human tracheobronchial glycoproteins [a,b]

Desulfated oligosaccharide	GlcN(Me)Ac	Gal	Fuc	GalN(Me)Ac-ol
DSS-VI$_a$	4,**6** (1.0)	2,3,4,**6** (1.1) [c]		1,4,5,6 (1) [c]
DSS-VI$_b$	3,**6** (1.0)	2,**3**,4,6 (1.0) [c]		1,4,5,6 (1) [c]
DSS-XII$_a$	3,6 (0.9) 3,4,**6** (0.9) [c]	2,3,4,**6** (1.0) [c]		1,4,5 (1) [c]
DSS-XII$_b$	3,6 (0.9) 3,4,**6** (1.0) [c]	2,**3**,4,6 (0.9) [c]		1,4,5 (1) [c]
DSS-XIII	3,6 (2.0)	2,3,4,**6** (2.1) [c]		1,4,5 (1) [c]
DSS-XIX$_a$	3,6 (1.5) 4,6 (0.5)	2,4,6 (0.9) 2,**3**,4,**6** (1.9) [c]		1,4,5 (1) [c]
DSS-XIX$_b$	3,6 (1.3) 4,6 (0.7)	2,4,**6** (1.0) 2,**3**,4,6 (0.9) [c] 2,3,4,6 (1.0) [c]		1,4,5 (1) [c]
DSS-XIX$_c$	3,6 (1.0) 4,6 (0.9)	2,4,6 (1.0) 2,**3**,4,6 (2.1) [c]		1,4,5 (1) [c]
DSS-XXVI	3,6 (2.1)	2,4 (1.0) 2,**3**,4,6 (1.9) [c]		1,4,5,6 (1) [c]
DSS-XLV	6 (1.0) 3,6 (1.0)	2,4 (1.0) 2,**3**,4,6 (1.9) [c]	2,3,4 (0.9) [c]	1,4,5,6 (1) [c]
DSS-LXIX	6 (1.0) 3,6 (1.0)	2,4,6 (1.1) 2,**3**,4,6 (2.0) [c]	2,3,4 (1.0) [c]	1,4,5 (1) [c]
DSS-LXX	4,**6** (1.0) 3,6 (1.0)	3,4,6 (1.0) [c] 2,4,6 (1.0) 2,**3**,4,6 (0.9) [c]	2,3,4 (0.9) [c]	1,4,5 (1) [c]
DSS-LXXII	3,6 (1.0) 4,6 (1.0)	2,4,**6** (1.0) 3,4,6 (1.0) [c] 2,**3**,4,6 (0.9) [c]	2,3,4 (1.0) [c]	1,4,5 (1) [c]
DSS-LXXV	3,6 (1.0) 4,6 (1.0)	4,6 (1.0) 2,**3**,4,**6** (1.9) [c]	2,3,4 (1.0) [c]	1,4,5 (1) [c]
DSS-LXXVI	4,**6** (1.0) 3,6 (2.0)	2,4,6 (1.0) 2,3,4,**6** (2.1) [c]		1,4,5 (1) [c]
DSS-LXXVII	3,6 (1.9) 4,6 (0.9)	2,4,6 (1.0) 2,3,4,**6** (1.9) [c]		1,4,5 (1) [c]
DSS-XXCII	3,6 (3.1)	2,4 (1.0) 2,3,4,**6** (3.1) [c]		1,4,5 (1) [c]
DSS-XXCIX	3,6 (4.1)	2,4 (0.9) 2,3,4,**6** (3.1) [c]		1,4,5 (1) [c]
DSS-XCXI	3,6 (3.0) 4,**6** (1.0)	2,4 (1.0) 2,4,6 (1.1) 2,**3**,4,6 (3.0) [c]		1,4,5 (1) [c]
DSS-CXI	3,6 (3.0) 4,6 (1.0)	2,4 (1.0) 2,4,6 (1.1) 3,4,6 (1.1) [c] 2,**3**,4,**6** (1.9) [c]	2,3,4 (1.0) [c]	1,4,5 (1) [c]

[a] Data expressed as the molar ratio of the identified partially methylated alditol acetates relative to the respective permethylated D-GalN(Me)Ac-ol found for each desulfated oligosaccharide analogue. Underscored boldtyped positions emphasize the new methyl ethers that appeared following the desulfation of disulfated oligosaccharides presented in Table 1.

[b] Desulfation was accomplished by treating the respective disulfated oligosaccharide with 0.06 M anhydrous methanolic hydrogen chloride and isolating and purifying the desulfated product by Bio-Gel P-2 chromatography. Aliquots were then per-*O*-methylated, subjected to acid hydrolysis, derivatized to their respective alditol acetates, and analyzed by GLC–MS.

[c] Residue destroyed when the respective desulfated oligosaccharide was pretreated with sodium metaperiodate.

saccharides, that SS-XII_a, SS-XII_b, SS-XIII, SS-XIX_a, SS-XIX_b, SS-XIX_c, SS-XXVI, SS-XLV, SS-LXIX, SS-LXX, SS-LXXII, SS-LXXV, SS-LXXVI, SS-LXXVII are biantennary, and that SS-XXCII, SS-XXCIX, SS-XCXI and SS-CXI are triantennary structures.

In addition to branching information, a further comparison of the methylation of the data of the disulfated oligosaccharides in Table 1 with that of the parallel desulfated analogs in Table 2 yields important glycosidic and precise sulfate ester linkage information. First, for SS-VI_a, SS-LXX, and SS-LXXVI, and SS-XCXI, the elimination of 2-deoxy-4-*O*-methyl-2-(*N*-methylacetamido)-D-glucose (Table 1) and the concomitant molar increase in 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose (Table 2), as a result of desulfation, indicates the site of sulfation to be C-6 of internal *N*-acetyl-D-glucosamine residues also possessing C-3 glycosidic linkages. Analogously, the loss of 2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)-D-glucose (Table 1) from SS-VI_b, upon desulfation, and concomitant molar increase in 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose (Table 2) would suggest the site of sulfation to be on C-6 of an internal *N*-acetyl-D-glucosamine residue which also possesses a C-4 glycosidic linkage. Second, the two disulfated oligosaccharides SS-XIX_b and SS-LXXII demonstrated the presence of a C-6 sulfate ester on an internal D-galactose residue possessing a C-3 glycosidic linkage by the elimination of 2,4-di-*O*-methyl-D-galactose and the parallel increase in 2,4,6-tri-*O*-methyl-D-galactose upon desulfation. Third, all other sulfations were localized on nonreducing termini occupied by D-galactose residues. Notably, SS-VI_a, SS-XII_b, SS-XIX_a, SS-LXXV, SS-LXXVI, and SS-CXI were shown to possess a single C-6 sulfate-containing D-galactose residue on a nonreducing terminus by the loss of one 2,3,4-tri-*O*-methyl-D-galactose from each disulfated oligosaccharide (Table 1) to a parallel molar increase in 2,3,4,6-tetra-*O*-methyl-D-galactose of the respective desulfated analogue (Table 2). Similarly, SS-VI_b, SS-XII_b, SS-XIX_a, SS-XIX_b, SS-LXX, SS-LXXII, SS-LXXV, SS-XCXI and SS-CXI displayed a single C-3 sulfate-containing D-galactose residue occupying a nonreducing terminus by the loss of one 2,4,6-tri-*O*-methyl-D-galactose unit from disulfated oligosaccharide to a parallel molar increase in 2,3,4,6-tetra-*O*-methyl-D-galactose of the respective desulfated analogue. SS-XII_a, SS-XIII, SS-LXXVII, SS-XXCII and SS-XXCIX, upon desulfation, revealed the loss of two 2,3,4-tri-*O*-methyl-D-galactose residues to a concomitant increase in 2,3,4,6-tetra-*O*-methyl-D-galactose, indicating that each of these disulfated oligosaccharides possessed two nonreducing terminal galactose-6-sulfate residues. The elimination of two 2,4,6-tri-*O*-methyl-D-galactose residues to 2,3,4,6-tetra-*O*-methyl-D-galactose, for SS-XIX_c, SS-XXVI, SS-SLV and SS-LXIX, also indicated that each possessed two nonreducing terminal galactose-3-sulfate residues.

For those disulfated oligosaccharides possessing L-fucose, permethylation analysis before and after treatment of the oligosaccharides with α-L-fucosidase (Table 1) clearly demonstrated their glycosidic linkages and the sugar moieties involved with them. For SS-XLV and SS-LXIX, α-L-fucose was determined to occupy C-3 and C-4, respectively, of a single internal *N*-acetyl-D-glucosamine residue, which also possessed other glycosidic bonds on C-4 and C-3, respectively. SS-LXX, SS-LXXII, and CXI were demonstrated to possess a single α-L-fucose residue on C-2 of D-galactose residues occupying nonreducing termini as evidenced by the loss of 3,4,6-tri-*O*-methyl-galactose

(Table 1) to 2,3,4,6-tetra-*O*-methyl-galactose. Lastly, the appearance of 2,4,6-tri-*O*-methyl-galactose and the loss of 4,6-di-*O*-methyl-galactose showed the α-L-fucose residue of LXXV to reside on C-2 of an internal galactose residue which also possessed an additional C-3 glycosidic linkage.

Periodate oxidation analysis of SS-VI$_a$–SS-CXII and desulfated analogs.—Carbohydrate residues that were destroyed when each respective oligosaccharide was subjected to periodate oxidation are also indicated in Tables 1 and 2. Under these conditions, all carbohydrate residues possessing unsubstituted vicinal hydroxyl groups within the respective SS- or DSS-oligosaccharide molecule were destroyed. Notably, all nonreducing terminus galactose residues that possessed C-3 sulfate esters survived the treatment, whereas, all similar residues possessing C-6 sulfate esters were destroyed, thus confirming the above permethylation results for sulfate ester localization on nonreducing termini. All 2-acetamido-2-deoxy-D-galactitol residues produced a 2-acetamido(methylamino)-2-deoxy-1,4-di-*O*-methyl-D-threitol product, following periodate treatment and sodium borohydride reduction, confirming a C-3 linkage for each oligosaccharide on this residue. Lastly, these results also confirm the permethylation results by verifying identified, unsubstituted vicinal hydroxyl groups.

Sequential exoglycosidase analysis.—The results for the exoglycosidase analyses for SS-VIa–SS-CXI and the desulfated DSS-VIa–DSS-CXI analogues are presented in Table 3. For ease of discussion, and based upon these findings, the above analytical results and the mass spectral data, presented later, the proposed structures for SS-VIa–SS-CXI are presented in Table 4.

As shown in Table 3, the disulfated oligosaccharides SS-VIa, SS-VIb, SS-XIIa, SS-XIIb, SS-XIXa, SS-XIXc, SS-XXVI and SS-LXXVII were resistant to *exo*-(1 → 3)-β-D-galactosidase, *exo*-(1 → 4)-β-D-galactosidase, *exo*-2-acetamido-2-deoxy-β-D-glucosidase, and α-L-fucosidase digestion. Following desulfation, each of these oligosaccharides was readily sequentially digested, indicating that each nonreducing terminus was occupied with a sulfated sugar, confirming the above permethylation and periodate oxidation results. Additionally, in conjunction with the permethylation findings, these sequential digestions provided both the anomeric configurations and the primary glycosidic sequences of their linear or biantennary structures.

It was clear from the permethylation analyses that SS-XIIb, SS-XIXa, SS-LXXV and SS-CXI possess both a galactose-3-sulfate and a galactose-6-sulfate residue on the nonreducing termini of two different branches. Discernment of which branch contained the respective sulfated residue was accomplished by a sequence of periodate oxidation, desulfation, enzymatic digestion and subsequent permethylation analysis of the final product. Since periodate oxidation and subsequent reduction with $NaBH_4$ destroys nonreducing terminal galactose-6-sulfate residues, resulting in a glycosidically linked remnant on the nonreducing terminus that interferes with subsequent enzymatic degradation of the respective branch, only those branches that possess terminal galactose-3-sulfate residues, after subsequent desulfation, will then be digested. Permethylation analysis of the final enzymatic product, therefore, definitively provides the location of the galactose-3-sulfate possessing branch on 2-acetamido-2-deoxy-D-galactitol, i.e., the finding of 2-deoxy-1,3,4,5-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol for SS-XIX$_a$ and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol for SS-XII$_b$ indi-

Table 3
Sequential exoglycosidase digestion of disulfated oligosaccharides and their respective desulfated product

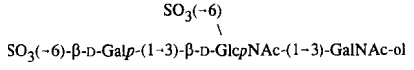
Oligosaccharide	Sequence of exoglycosidase released carbohydrates (molar ratio) [a]
SS-VIa	*no digestion observed*
Desulfated product VIa	Galβ1 → 3(0.96)GlcNAcβ(1.04) + GalNAc-ol(1)
SS-VIb	*no digestion observed*
Desulfated product VIb	Galβ1 → 4(1.01)GlcNAcβ(1.05) + GalNAc-ol(1)
SS-XIIa	*no digestion observed*
Desulfated product XIIa	Galβ1 → 4(0.97)GlcNAcβ(2.11) + GalNAc-ol(1)
SS-XIIb	*no digestion observed*
Desulfated product XIIb	Galβ1 → 4(1.03)GlcNAcβ(2.07) + GalNAc-ol(1)
SS-XIII	*no digestion observed*
Desulfated product XIII	Galβ1 → 4(2.10)GlcNAcβ(2.03) + GalNAc-ol(1)
SS-XIXa	*no digestion observed*
Desulfated product XIXa	Galβ1 → 3(1.47)Galβ1 → 4(0.56)GlcNAc β(1.04)Galβ1 → 4(0.94)GlcNAcβ(0.97) + GalNAc-ol(1)
SS-XIXb	Galβ1 → 3(1.04)
Desulfated product XIXb	Galβ1 → 3(0.72)Galβ1 → 4(0.35)GlcNAc β(0.96)Galβ1 → 4(0.96)GlcNAcβ(0.94) + GalNAc-ol(1)
SS-XIXc	*no digestion observed*
Desulfated product XIXc	Galβ1 → 3(2.08)GlcNAcβ(1.03)Gal β1 → 4(0.94)GlcNAcβ(1.06) + GalNAc-ol(1)
SS-XXVI	*no digestion observed*
Desulfated product XXVI	Galβ1 → 4(1.89)GlcNAcβ(2.10)Gal β1 → 3(1.03) + GalNAc-ol(1)
SS-XLV	*no digestion observed*
Desulfated product XLV	Galβ1 → 4(1.91)GlcNAcβ(1.06)Fuc α(1.04)GlcNAcβ(0.97)Galβ1 → 3(1.03) + GalNAc-ol(1)
SS-LXIX	*no digestion observed*
Desulfated product LXIX	Galβ1 → 3(1.90)Fucα(0.99)GlcNAc β(0.93)Galβ1 → 4(0.93)GlcNAcβ(0.94) + GalNAc-ol(1)

SS-LXX	Fucα(1.06)Galβ1 → 3(0.94)
Desulfated product LXX	Galβ1 → 3(1.06)GlcNAcβ(1.01)Galβ1 → 4(0.96)GlcNAcβ(0.96) + GalNAc-ol(1)
SS-LXXII	Fucα(0.98)Galβ1 → 3(1.01)GlcNAcβ(1.04)
Desulfated product LXXII	Galβ1 → 3(0.96)Galβ1 → 4(1.03)GlcNAcβ(0.97) + GalNAc-ol(1)
SS-LXXV	*no digestion observed*
Desulfated product LXXV	Galβ1 → 3(1.91)GlcNAcβ(0.93)Fucα(0.97)Galβ1 → 4(0.91)GlcNAcβ(0.95) + GalNAc-ol(1)
SS-LXXVI	Galβ1 → 3(0.93)
Desulfated product LXXVI	Galβ1 → 4(0.94)GlcNAcβ(1.97)Galβ1 → 4(0.95)GlcNAcβ(0.93) + GalNAc-ol(1)
SS-LXXVII	*no digestion observed*
Desulfated product LXXVII	Galβ1 → 3(1.03)Galβ1 → 4(0.94)GlcNAcβ(1.96)Galβ1 → 4(0.93)GlcNAcβ(0.93) + GalNAc-ol(1)
SS-XXCII	Galβ1 → 4(0.99)GlcNAcβ(1.00)
Desulfated product XXCII	Galβ1 → 4(1.93)GlcNAcβ(1.97)Galβ1 → 3(0.91) + GalNAc-ol(1)
SS-XXCIX	Galβ1 → 4(1.02)GlcNAcβ(0.98)
Desulfated product XXCIX	Galβ1 → 4(1.94)GlcNAcβ(1.97)Galβ1 → 4(0.94)GlcNAcβ(0.93) + GalNAc-ol(1)
SS-XCXI	Galβ1 → 3(0.99)Galβ1 → 4(1.02)GlcNAcβ(1.07)
Desulfated product XCXI	Galβ1 → 4(0.97)GlcNAcβ(2.10)Galβ1 → 3(0.88)Galβ1 → 4(0.94)GlcNAcβ(0.94) + GalNAc-ol(1)
SS-CXI	Fucα(1.04)Galβ1 → 4(1.01)GlcNAcβ(0.98)
Desulfated product CXI	Galβ1 → 3(0.95)Galβ1 → 4(0.93)GlcNAcβ(1.98)Galβ1 → 3(0.93)Galβ1 → 4(0.91)GlcNAcβ(0.95) + GalNAc-ol(1)

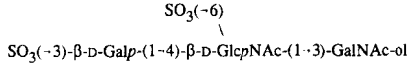
[a] Each reduced disulfated oligosaccharide was subjected to sequential exoglycosidase digestions with *exo*-(1,3)-β-D-galactosidase, *exo*-(1,4)-β-D-galactosidase, and *exo*-2-acetamido-2-deoxy-β-D-glucosidase. When no further digestions were observed, each oligosaccharide possessing a L-fucose residue on a nonreducing terminus was then treated with α-L-fucosidase and then resubjected to the sequential digestions above. Disulfated oligosaccharides possessing an internal L-fucose residue were treated with α-L-fucosidase only after initial sequential exoglycosidase digestion, desulfation, and when no other subsequent exoglycosidase liberations were observed. Data expressed, in order from left to right, as the sequentially identified enzymatically liberated carbohydrate (molar ratio of the liberated sugar relative to 2-acetamido-2-deoxy-β-D-galactitol of the respective oligosaccharide). At each enzymatic step the liberated carbohydrate was quantitated by GLC–MS as the alditol acetate. When no further liberations were observed, an aliquot of each respective disulfated oligosaccharide product was then subjected to permethylation analysis. The remaining disulfated product was then desulfated, as described in Table 2, and resubjected to complete sequential exoglycosidase digestion.

Table 4
Proposed structures for disulfated oligosaccharides from human tracheobronchial glycoproteins

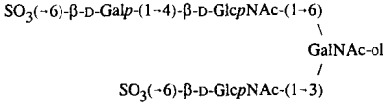
SS-VI$_a$



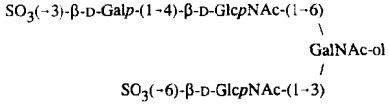
SS-VI$_b$



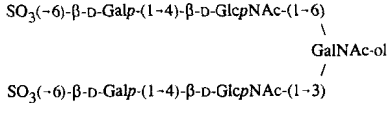
SS-XII$_a$



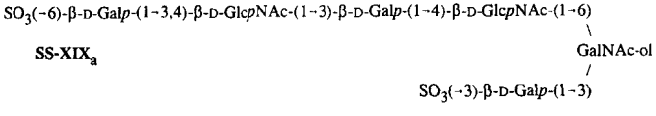
SS-XII$_b$



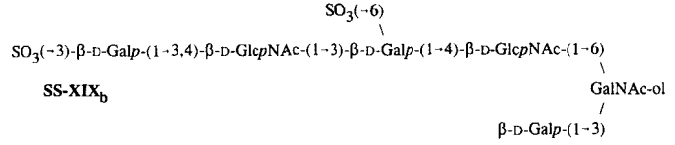
SS-XIII



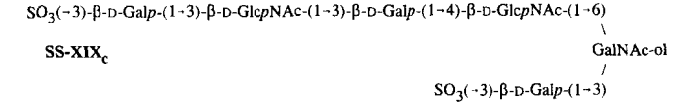
SS-XIX$_a$



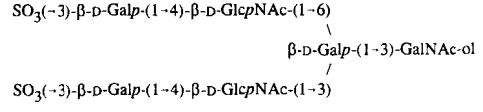
SS-XIX$_b$



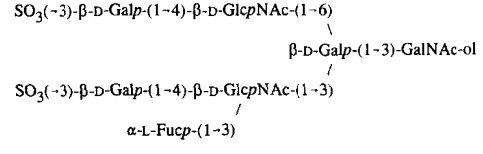
SS-XIX$_c$



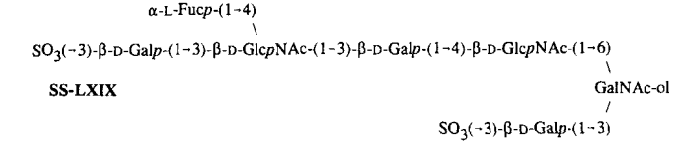
SS-XXVI

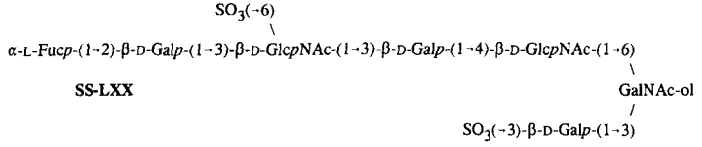


SS-XLV

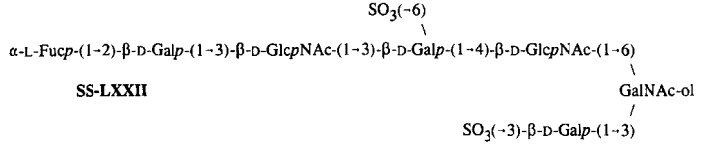


SS-LXIX

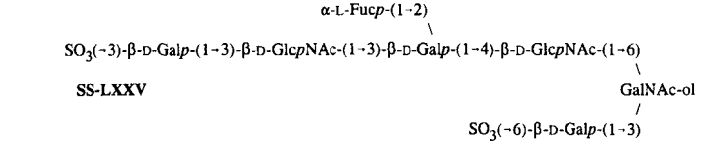




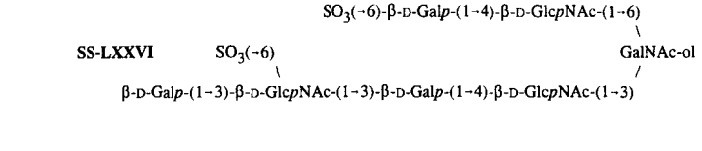
SS-LXX

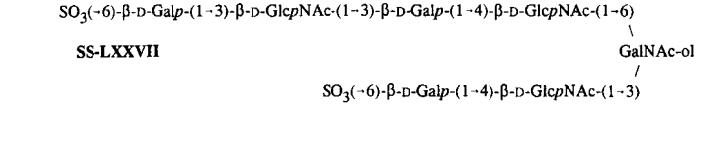


SS-LXXII

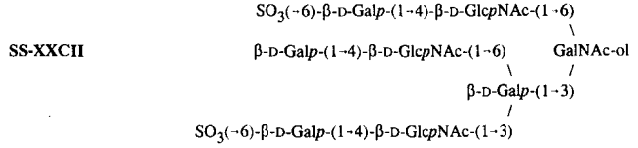


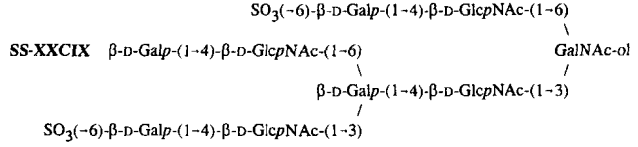
SS-LXXV

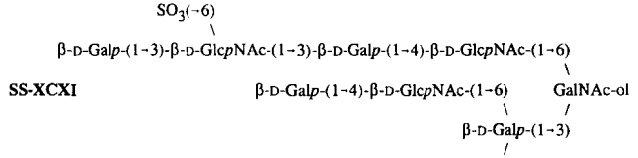


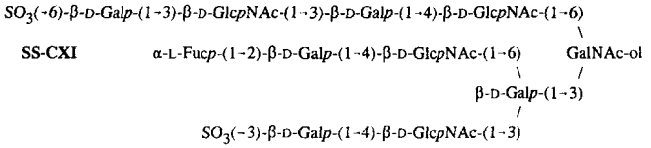


SS-LXXVII









cated that a galactose-3-sulfate containing branch existed on C-3 and C-6 of 2-acetamido-2-deoxy-D-galactitol, respectively. This same approach when applied to SS-LXXV, after α-L-fucosidase treatment of the disulfated oligosaccharide, resulted in the production of 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol following permethylation of the product and clearly demonstrated that galactose-3-sulfate resided on the C-6 branch of 2-acetamido-2-deoxy-D-galactitol. The finding of 2,3,4-tri-*O*-methyl-D-galactose when the triantennary SS-CXI was similarly analyzed, but without α-L-fucosidase treatment, determined that the galactose-3-sulfate bearing branch was located on C-3 of a galactose residue and that the α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc branch (Table 3) resided on C-6 of the same residue.

For all triantennary structures, i.e., SS-XXCII, SS-XXCIX, SS-XCXI and SS-CXI, the nonsulfated branch was sequentially digested providing its carbohydrate sequence, anomeric configuration, and branching location. In each case, this nonsulfated branch occurred on C-6 of a galactose residue, which also possessed another C-3 glycosidic linkage.

For SS-XIIa, SS-XIIb, SS-XIXa, SS-XIXb, SS-XIXc, SS-LXIX, SS-LXX, SS-LXXII, and SS-LXXV, the identification of the branch on C-3 of 2-acetamido-2-deoxy-D-galactitol, following desulfation, was simplified by the fact that the branch was comprised of a single 2-acetamido-2-deoxy-D-glucose or D-galactose sugar residue. Once enzymatically cleaved, permethylation of the product demonstrated the loss of 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol and the appearance of 2-deoxy-1,3,4,5-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol, indicating a C-3 linkage.

Branch localization via sequential exoglycosidase digestion and permethylation analysis was also simplified due to the singular and repetitive occurrence of the lactosamine disaccharide units within the structures. For example, when the desulfated SS-XIII oligosaccharide underwent exoglycosidase treatment, it exhibited the sequential loss of two (1 → 4)-β-D-galactose and then two β-linked 2-acetamido-2-deoxy-D-glucose residues with the appearance of one residue of 2-acetamido-2-deoxy-D-galactitol (Table 3). Parallel permethylation analysis of the starting material and enzymatic products demonstrated the sequential loss of two 2,3,4,6-tetra-*O*-methyl-D-galactose and two 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose residues with the appearance of two 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucose residues. The concomitant loss of 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol and the appearance of 2-deoxy-1,3,4,5,6-penta-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol upon the last sequential enzymatic step with *exo*-2-acetamido-2-deoxy-β-D-glucosidase demonstrated C-3 and C-6 glycosidic linkages to this sugar. From these data it is apparent that SS-XIII is symmetrical in its biantennary structure, possessing single lactosamine residues on C-3 and C-6 of 2-acetamido-2-deoxy-D-galactitol. Other permethylation and FABMS data also confirm this arrangement.

The inability to chromatographically resolve some structurally similar disulfated oligosaccharides is reflected in the permethylation and sequential exoglycosidase data in Tables 1–3 for SS-XIXa and SS-XIXb. Taking SS-XIXa, for example, it was apparent that this disulfated oligosaccharide and its respective desulfated analogue possessed two 2-acetamido-2-deoxy-D-glucose residues in its structure (Tables 1 and 2), though the nonintegral relationship of 1.55:0.46 of 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methyl-

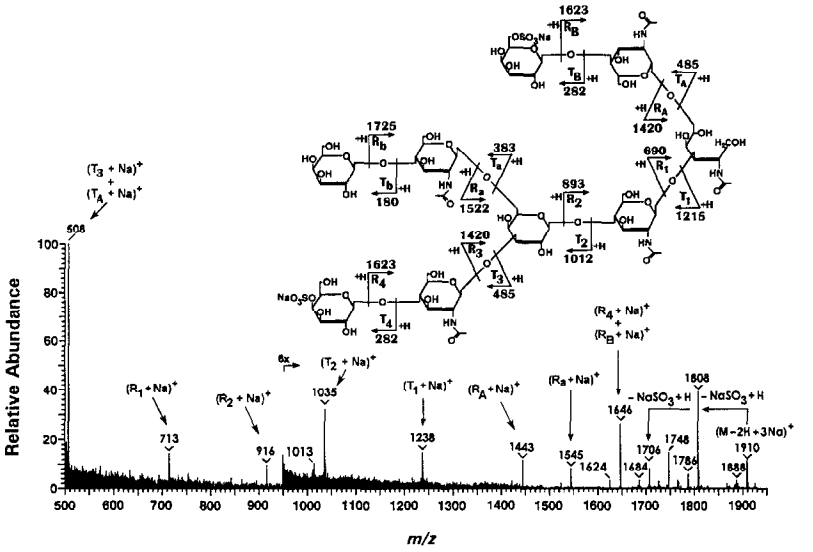


Fig. 2. FABMS, positive-ion mode, of the disulfated nonasaccharide SS-XXCIX, using 1-thioglycerol as a liquid matrix. The proposed structure for SS-XXCIX is based upon carbohydrate, sequential exoglycosidase digestion, permethylation analysis and FABMS data.

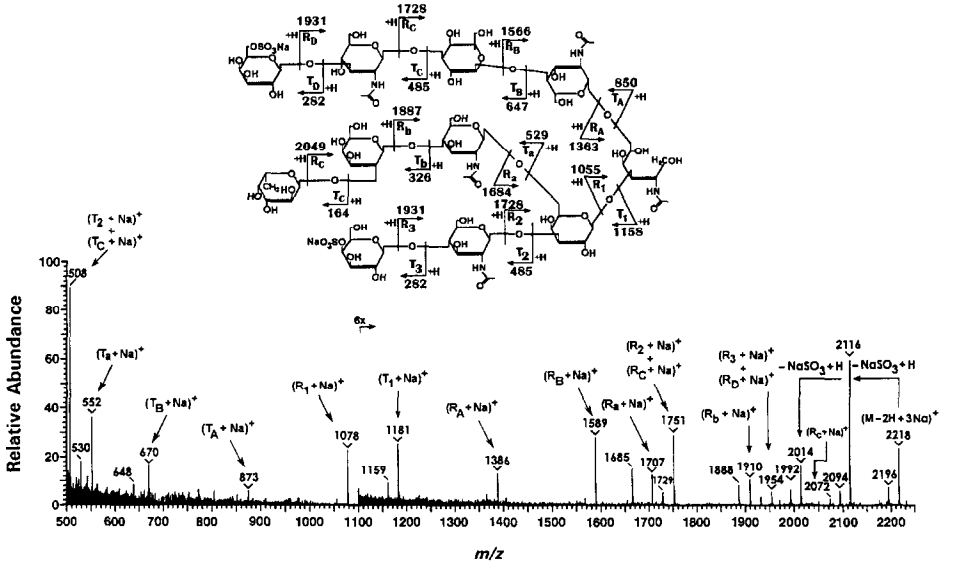
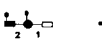
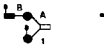
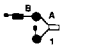
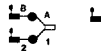
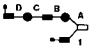
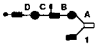


Fig. 3. FABMS, positive-ion mode, of the disulfated undecasaccharide SS-CXI, using 1-thioglycerol as a liquid matrix. The proposed structure for SS-CXI is based upon carbohydrate, sequential exoglycosidase digestion, permethylation analysis and FABMS data.

acetamido)-D-glucose and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose, respectively, clearly indicated that a single mixed glycosidic linkage was present on one of these residues. Sequential exoglycosidase digestion (Table 3) and permethylation analysis of the products indicated that an initial mole ratio of 1.47 of (1 → 3)-β-D-galactose, relative to 2-acetamido-2-deoxy-D-galactitol, was liberated when treated with *exo*-(1 → 3)-β-D-galactosidase. As a result of this digestion, permethylation of the product showed that 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol was completely lost with the concomitant appearance of 2-deoxy-1,3,4,5-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol, indicating that one mole ratio of (1 → 3)-β-D-galactose resided on C-3 of this residue and that the remaining mole ratio of 0.47 of (1 → 3)-β-D-galactose must exist on the other branch. Also completely lost was the 0.46 molar ratio of 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose (Table 1) with the concomitant gain of an equivalent molar ratio of 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucose (not shown). Upon further digestion of the desulfated oligosaccharide with *exo*-(1 → 4)-β-D-galactosidase, which liberated a 0.56 mole ratio of (1 → 3)-β-D-galactose, permethylation of the product resulted in the appearance of a

Table 5
Prominent fragment ions (*m/z*) in the fast-atom-bombardment mass spectra of disulfated oligosaccharides [a]

Prominent fragment ions (*m/z*) in the fast-atom-bombardment mass spectra of disulfated oligosaccharides [a]

Fragment ion	SS-VI$_a$	SS-VI$_b$	SS-XII$_a$	SS-XII$_b$	SS-XIII	SS-XIX$_a$	SS-XIX$_b$
							
$(M + Na)^+$ [b]	815 , 793	815 , 793	1018 , 996	1018 , 996	1180 , 1158	1342 , 1320	1342 , 1320
$(M + Na)^+$ -$NaSO_3$ + H[c]	713 , 691	713 , 691	916 , 894	916 , 894	1078 , 1056	1240 , 1218	1240 , 1218
$(M + Na)^+$ -$2NaSO_3$ + 2H[c]	611 , 589	611 , 589	814 , 792	814 , 792	976 , 954	1138 , 1116	1138 , 1116
T_1 / R_1[d]	610 / 246	610 / 246	346 / 713	346 / 713	508 / 713	305 / 1078	203 / 1180
T_2 / R_2	305 / 551	305 / 551			305 / 916		
T_A / R_A			508 / 551	508 / 551	508 / 713	873 / 510	975 / 408
T_B / R_B			305 / 754	305 / 754	305 / 916	670 / 713	772 / 611
T_C / R_C						508 / 875	508 / 875
T_D / R_D						305 / 1078	305 / 1078

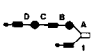
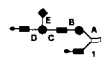
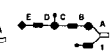
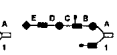
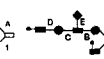
Fragment ion	SS-XIX$_c$	SS-XXVI	SS-XLV	SS-LXIX	SS-LXX	SS-LXXII	SS-LXXV
							
$(M + Na)^+$ [b]	1342 , 1320	1342 ,1320	1488 , 1466	1488 , 1466	1488 , 1466	1488 , 1466	1488 , 1466
$(M + Na)^+$ -$NaSO_3$ + H[c]	1240 , 1218	1240 ,1218	1386 , 1364	1386 , 1364	1386 , 1364	1386 , 1364	1386 , 1364
$(M + Na)^+$ -$2NaSO_3$ + 2H[c]	1138 , 1116	1138 , 1116	1284 , 1262	1284 , 1262	1284 , 1262	1284 , 1262	1284 , 1262
T_1 / R_1[d]	305 / 1078	1137 / 246	1283 / 246	305 /1224	305 /1224	305 /1224	305 /1224
T_2 / R_2		508 / 875	654 / 875				
T_3 / R_3		305 / 1078	305 / 1224				
T_4 / R_4			187 / 1342				
T_A / R_A	873 / 510	508 / 875	508 / 1021	1019 / 510	1019 / 510	1019 / 510	1019 / 510
T_B / R_B	670 / 713	305 / 1078	305 /1224	816 / 713	816 / 713	816 / 713	816 / 713
T_C / R_C	508 / 875			654 / 875	654 / 875	552 / 977	508 / 1021
T_D / R_D	305 / 1078			305 /1224	349 / 1180	349 / 1180	305 /1224
T_E / R_E				187 / 1342	187 / 1342	187 / 1342	187 / 1342

Table 5 (continued)

Fragment ion	SS-LXXVI	SS-LXXVII	SS-XXCII	SS-XXCIX	SS-XCXI	SS-CXI
						
$(M + Na)^+$ [b]	1545 , 1523	1545 , 1523	1707 , 1685	1910 , 1888	2072 , 2050	2218 , 2196
$(M + Na)^+ - NaSO_3 + H$ [c]	1443 , 1421	1443 , 1421	1605 , 1583	1808 , 1786	1970 , 1948	2116 , 2094
$(M + Na)^+ - 2NaSO_3 + 2H$ [c]	1341 , 1319	1341 , 1319	1503 , 1481	1706 , 1684	1868 , 1846	2014 , 1992
T_1 / R_1 [d]	873 / 713	508 / 1078	1035 / 713	1238 / 713	1035 , 1078	1181 , 1078
T_2 / R_2	670 / 916	305 / 1281	508 / 1240	1035 / 916	508 / 1605	508 / 1751
T_3 / R_3	508 / 1078		305 / 1443	508 / 1443	305 / 1808	305 / 1954
T_4 / R_4	203 / 1383			305 / 1646		
T_A / R_A	508 / 1078	873 / 713	508 / 1240	508 / 1443	873 / 1240	873 / 1386
T_B / R_B	305 / 1281	670 / 916	305 / 1443	305 / 1646	670 / 1443	670 / 1589
T_C / R_C		508 / 1078			508 / 1605	508 / 1751
T_D / R_D		305 / 1281			203 / 1910	305 / 1954
T_a / R_a			406 / 1342	406 / 1545	406 / 1707	552 / 1707
T_b / R_b			203 / 1545	203 / 1748	203 / 1910	349 / 1910
T_c / R_c						187 / 2072

[a] FAB mass spectral fragment ions were obtained in the positive-ion mode for each underivatized disulfated. Spectra were acquired at +8 keV with the respective oligosaccharide in a matrix of 1-thioglycerol. The proposed structures are based upon carbohydrate, sequential exoglycosidase digestion, permethylation analysis and FABMS data. Symbols: ▬, D-galactose; ▙, D-galactose-6-sulfate; ▬▪, D-galactose-3-sulfate; ●, 2-i acetamido-2-deoxy-D-glucose; ◭, 2-acetamido-2-deoxy-D-glucose-6-sulfate; ◆, L-fucose; ▭, 2-acetamido-2-deoxy-d-galactitol.

[b] For each $(M+Na)^+$ is indicated two molecular-weight-related ions (m/z, m/z) which are due to variations in the resident counterions (i.e., Na^+ and H^+) and for SS-VI_a through SS-CXI correspond to $(3Na^{3+})^{3+}$ and $(H^{1+} + 2Na^{2+})^{3+}$, respectively.

[c] For each observed molecular-weight-related ion for $(M+Na)^+$ a loss of one and two 102 units was also noted and corresponded to the eliminations of sodium sulfite with hydrogen replacements.

[d] Nonreducing terminal end fragments are identified as T_n ions while reducing end fragments are referred to as R_n ions [23], where 'n' for both T and R fragment ions is either a numeral or an uppercase letter to differentiate between fragment ions arising from the $1 \rightarrow 3$ or $1 \rightarrow 6$ oligosaccharide branch, respectively, on 2-acetamido-2-deoxy-D-galactitol, or a lowercase letter to distinguish an internal branch.

1.04 molar ratio of 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucose relative to 2-deoxy-1,4,5-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol in the oligosaccharide. This clearly demonstrated the fact that this penultimate amino sugar possessed a heterogenous glycosidic linkage of either C-3 or C-4 of D-galactose with an approximate ratio of 47% and 53%, respectively, and that SS-XIXa consisted of two disulfated oligosaccharides with identical primary structures differing only by the linkage configuration of one nonreducing terminus D-galactose residue. SS-XIXa, sulfated twice on the sample branch, was also determined to be a mix of two similar oligosaccharides, though the absence of sulfation of the D-galactose residue on the C-3 of 2-acetamido-2-deoxy-D-galactitol permitted it to be removed prior to desulfation.

FABMS analysis of SS-VI_a–SS-CXI.—In addition to the detailed enzymatic and permethylation analyses performed on these disulfated oligosaccharides, additional structure confirmation was achieved by positive-ion FABMS. Representative mass

spectra are presented in Figs. 2 and 3, showing the mass spectral results for the nonasaccharide SS-XXCIX and undecasaccharide SS-CXI, respectively. A comprehensive listing of the prominent fragment ions observed for each disulfated species is presented in Table 5. All FAB mass spectra of disulfated oligosaccharides displayed (1) prominent molecular weight related ions $(M - 2H + 3Na)^+$, $(M - H + 2Na)^+$, and $(M + Na)^+$ which present themselves as multiplets, 22 amu. apart, and which are attributable to variations in the counteraction species (i.e., sodium and proton) that may reside on the sulfate esters; (2) two prominent losses of 102 amu. each from each respective disulfated oligosaccharide representing the loss of sodium sulfite with hydrogen replacement from the molecule and confirming the presence of two sulfate esters and lastly; (3) structurally significant fragment ions for each glycosidic linkage, representing cleavage around the glycosidic oxygen with hydrogen replacement, providing confirmatory sequence information. For ease of presentation, nonreducing terminal end fragments are identified as T_n ions while reducing end fragments are referred to as R_n ions [23], where '*n*' for both T and R fragment ions is either a numeral or a capital letter to differentiate between fragment ions arising from the $1 \rightarrow 3$ or $1 \rightarrow 6$ oligosaccharide branch, respectively, on 2-acetamido-2-deoxy-D-galactitol. For the triantennary structures, SS-XXCII, SS-XXCIX, SS-XCXI and SS-CXI, '*n*' is also presented as a small letter to designate an internal branch not originating from 2-acetamido-2-deoxy-D-galactitol.

These disulfated oligosaccharide FAB mass spectral characteristics can readily be observed within the mass spectrum of the nonasaccharide SS-XXCIX, shown in Fig. 2 and presented in tabular form in Table 5, which shows the molecular weight related ions *m/z* 1910 $(M - 2H + 3Na)^+$ and *m/z* 1888 $(M - H + 2Na)^+$, and, the two losses of sulfite and hydrogen replacement, *m/z* 1808 and 1706, and two structurally important fragment ions for each glycosidic linkage within the structure: T_1, *m/z* 1238; R_1, *m/z* 713; T_2, *m/z* 1035; R_2, *m/z* 916; T_3, *m/z* 508; R_3 *m/z* 1443; T_4, *m/z* 305; R_4, *m/z* 1646; T_A, *m/z* 508; R_A, *m/z* 1443; T_B, *m/z* 305; R_B, *m/z* 1646; T_a, *m/z* 406; R_a, *m/z* 1545; T_b, *m/z* 203; and R_b, *m/z* 1748. These FAB results are consistent with all carbohydrate, periodate oxidation, permethylation and sequential exoglycosidase digestion data and with the proposed structure in Table 4.

The positive-ion FAB spectrum of the undecasaccharide SS-CXI, presented in Fig. 3 and Table 5, is very similar to SS-XXCIX in that it also displays prominent molecular weight related multiplet ions $(M - 2H + 3Na)^+$ and $(M - H + 2Na)^+$, i.e., *m/z* 2218 and 2196, exhibits two separate losses of sodium sulfite and hydrogen replacement, i.e., *m/z* 2116 and 2014, and presents two highly informative structural fragment ions for each glycosidic linkage, i.e., T_1, *m/z* 1181; R_1, *m/z* 1078; T_2, *m/z* 508; R_2, *m/z* 1751; T_3, *m/z* 305; R_3, *m/z* 1954; T_A, *m/z* 873; R_A, *m/z* 1386; T_B, *m/z* 670; R_B, *m/z* 1589; T_C, *m/z* 508; R_C, *m/z* 1751; T_D, *m/z* 305; R_D, *m/z* 1954; T_a, *m/z* 552; R_a, *m/z* 1707; T_b, *m/z* 349; R_b, *m/z* 1910; T_c, *m/z* 187; and R_c, *m/z* 2072. The presence of generally weaker -22 amu fragment ions for many non-molecular-weight-related fragment ions reflect a $(H^{1+} + 2Na^{2+})^{3+}$ or a $(2H^{2+} + Na^{1+})^{3+}$ countercation composition, depending on which fragment ion is being considered. As above for SS-XXCIX, these FAB results are also consistent with all carbohydrate, periodate oxidation, permethylation and sequential exoglycosidase digestion data and with the proposed structure in Table 4.

The prominent fragment ions observed for all disulfated oligosaccharides are presented in Table 5 along with a symbolic representation of each oligosaccharide structure with labeled glycosidic linkages. It is noteworthy that each disulfated oligosaccharide produced molecular weight related ions $(M - 2H + 3Na)^+$, $(M - H + 2Na)^+$, and $(M + Na)^+$ as multiplets, demonstrated the dual losses of sodium sulfite with hydrogen replacement to confirm disulfation, and yielded both T_n and R_n fragment ions for all respective glycosidic linkages.

3. Discussion

For reasons of structural elucidation and location of anionic sites, considerable efforts have been focused on determining the structures of neutral, sialylated, sulfated, and sulfated and sialylated oligosaccharides from high molecular weight human tracheobronchial mucous glycoproteins [1–5,24–30]. The sulfated oligosaccharides are of particular importance in light of the fact that increased sulfation of mucous glycoprotein oligosaccharide side chains may play an important role as a molecular defense mechanism in chronic lung disease. While the functions of sulfation of carbohydrates in general are not fully understood, *O*-sulfation would appear to participate in the regulation of such significant biological events as blood clotting (in the case of activation of the anticoagulant heparin), cellular migration (as effecting binding via ligands for endothelial and immune cells such as glycam-1), and infection (inhibiting colonization of infectious agents such as *Helicobacter pylori* by gastric mucin) [31–33]. 3-*O*-Sulfo-galactose, 6-*O*-sulfo-galactose and 2-acetamino-2-deoxy-3-*O*-sulfo-glucose appear to be important components for attachment among the selectin binding glycoproteins, [32,34]. In chronic pulmonary disease such as cystic fibrosis, the marked increase in the amount of oligosaccharide sulfation may reflect the long-term expression of normal defense mechanisms, mechanisms whose mode of action are not yet clear and whose consequences are unknown.

The sequencing strategy employed here allowed for the successful elucidation of twenty disulfated oligosaccharides derived from tracheobronchial mucous glycoproteins of a patient with cystic fibrosis. The combined use of permethylation analysis, periodate oxidation, and sequential exoglycosidase digestion of disulfated species and the parallel analysis of the desulfated products provided complete unambiguous structural characterization of the disulfated species, including glycosidic linkages and sulfate ester locations. Sequential exoglycosidase digestion/permethylation analysis, while labor intensive, proved an effective and accurate tool for structural elucidation of samples of limited quantity and for similar molecular species which were copurified. Positive-ion mode FABMS analysis of the sulfated species unequivocally confirmed these carbohydrate structures. The mucous glycoprotein derived oligosaccharides in this study include C-3 or C-6 sulfate esters of internal or terminal D-galactose residues and C-6 sulfate esters of internal 2-acetamido-2-deoxy-D-glucose residues and linear as well as di- and tri-antenary structures. Though the presence of two sulfate esters residing on the same chain of a branched structure was not uncommon, galactose sulfate was more often the terminal sugar on two separate chains on branched structures.

While the physiological effects of sulfation of mucous glycoproteins and the regulation of this modification are not clear, characterization studies such as these which define what these clinically significant modifications are such that their potential actions can be studied, will surely help elucidate the structure/function relationships of sulfated oligosaccharides of tracheobronchial and other mucous glycoproteins in health and disease.

4. Experimental

Materials.—A mixture of disulfated oligosaccharides (91 mg) were isolated from a mucous glycoprotein pool for a 16-year-old male patient (blood type A, nonsecretor status) with cystic fibrosis (Δf508 homozygote gentotype) by subjecting the glycoproteins to alkaline-borohydride treatment, lectin affinity chromatographic removal of sialic acid containing oligosaccharides, and then separating the disulfated moieties (fractions 55 through 120) via gradient anion-exchange chromatography utilizing a SynChroprep AX300 HPLC column, as previously described [2–4]. All *exo*-glycosidases were purchased from Boehringer Mannheim Corp. (Indianapolis, IN 46250), and all solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI 53233) and distilled prior to use. Bio-Gel P-2 was purchased from Bio-Rad (Richmond, CA 94804), and all neutral and amino sugars and maltooligosaccharides were purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IL 60085). Galactose-6-sulfate and glucosamine-6-sulfate were purchased from Sigma Chemical Co. (St. Louis, MO 63178). Galactose-3-sulfate and galactose-4-sulfate were synthesized as previously described [2,35].

Analytical procedures.—Each purified disulfated oligosaccharide (SS), before and after α-L-fucosidase treatment, and its respective desulfated analog (DSS), before and after α-L-fucosidase digestion, was subjected to carbohydrate and permethylation analysis, periodate oxidation and product analysis, and analyzed for inorganic oxyanions, sialic acids and amino acids, as previously described [2,3,36–38]. Aliquots of Bio-Gel P-2 column fractions were analyzed for total carbohydrate content via the ferricyanide colorimetric method [39].

Mass spectrometry.—GLC–EI-mass spectra for all monosaccharide derivatives, and sugars released during sequential exoglycosidase digestion, were obtained and verified against standards on a Kratos MS 50 S mass spectrometer interfaced with a Carlo Erba Model 4160 gas chromatograph. Mass spectra were recorded at 70 eV with an ionization current of 50 μA, a source temperature of 250 °C, and a transfer temperature 218 °C. FABMS (positive-ion mode) spectra were obtained employing a VG ZAB-HF mass spectrometer, as previously described [4,5].

Isolation of disulfated oligosaccharides.—Lyophilized disulfated oligosaccharide mixtures were first desalted on a Sephadex G-25-SF (Pharmacia, Piscataway, NJ 08854), 1.0 × 20 cm column, using distilled water, and were lyophilized. This crude mixture of disulfated oligosaccharides was then applied (10-mg aliquots) to a Bio-Gel P-2 (−400 mesh), 1.0 × 90 cm column and eluted with 0.01% NaCl and 2 mM NaN_3 at a flow rate of 0.42 mL/min and collected in 1.5-mL fractions [4]. Four fractions of indicated

oligosaccharide ranges that corresponded to the elution times of standard maltotriose through maltoundecaose (dp 3–4, 8.1%; dp 5–6, 11.2%; dp 7, 6.7%; dp 8–11, 14.3%) were collected and individually pooled, desalted, lyophilized, and aliquots of each were tested for contaminating glycopeptides via amino acid analysis as previously described [3]. HPLC anion-exchange chromatography of 0.75–1.5 mg of each Bio-Gel P-2 fraction was then performed on a Shandon Hypersil 120 Å APS-2, 4.6 × 150 mm, metal-free column (3 μm particle size, Alltech, Deerfield, IL 60015), as previously described [4]. Isolated oligosaccharides SS-VI_a through SS-CXI were then individually pooled from several chromatographic runs and each resubjected to Bio-Gel P-2 and Hypersil 120 Å APS-2 chromatography until the carbohydrate, sulfate and permethylation analyses demonstrated that constituent sugars, sulfate and glycosidic linkages, respectively, existed in near integral molar ratios.

Oligosaccharide analysis via sequential exoglycosidase degradation.—Because of the limited sample size ($\leq$ 40 nM) of each of the isolated disulfated oligosaccharides, a modified sequential exoglycosidase digestion [3,4] was employed. Each respective disulfated oligosaccharide (2–5 nM), dissolved in 1.0 mL of 5 mM citrate buffer, pH 5.2, containing 20 μL toluene and 5 μg each of the internal standards α-D-glucoheptitol and 3-*O*-methyl-D-glucose, was sequentially treated for 48 h each with *exo*-(*1* → *3*)-β-D-galactosidase (EC 3.2.1.23, 0.01 U) from bovine testes, *exo*-(*1* → *4*)-β-D-galactosidase (0.01 U) from *Diplococcus pneumoniae*, and 2-acetamido-2-deoxy-β-D-glucosidase (0.1 U) from bovine kidney and *Diplococcus pneumoniae* at 37 °C. At the end of each enzymatic digestion, the reaction was stopped by placing the digestion tube into boiling water for 5 min. After a 10–25 μL aliquot of the reaction mixture was taken for permethylation analysis, the pH was checked and the next sequential exoglycosidase was added and digestion commenced. This enzymatic digestion sequence was repeated until no further digestions were observed. Those disulfated oligosaccharides possessing L-fucose residues on nonreducing termini, as determined by prior methylation analysis of the disulfated oligosaccharides and their respective desulfated analogues, were first treated with α-L-fucosidase (EC 3.2.1.51, 0.05 U) from bovine kidney then subjected to the above enzymatic digestion sequence. For all reactions, when no further enzymatic digestions were observed, each respective disulfated product was subjected to Bio-Gel P-2 chromatography, and permethylation analysis [3,4]. The remaining exoglycosidase-resistant disulfated product was then solvolytically desulfated in 0.06 M anhydrous methanolic hydrogen chloride, as previously described [2], and the resulting neutral product was then subjected to the modified sequential exoglycosidase digestion, as described above, to complete the digestion of the oligosaccharide. For those desulfated oligosaccharides possessing an internal L-fucose residue, treatment with α-L-fucosidase was performed when no further digestions with *exo*-(*1,3*)-β-D-galactosidase, *exo*-(*1,4*)-β-D-galactosidase and 2-acetamido-2-deoxy-β-D-glucosidase produced a liberated monosaccharide. This was then followed with the normal enzymatic sequence until complete digestion of the oligosaccharide was accomplished.

For those disulfated oligosaccharides possessing sulfate esters on C-3 and C-6 of different galactose residues on nonreducing termini, as determined by permethylation analysis above, an aliquot of each oligosaccharide was subjected to periodate oxidation–sodium borohydride reduction, to destroy nonreducing terminal galactose-6-

sulfate residues, followed by desulfation and subsequent purification by Bio-Gel P-2 chromatography, as noted above. With a glycosidically linked remnant now blocking any exoglycosidase digestion of the oligosaccharide branch that possessed the galactose-6-sulfate residue, each respective disulfated oligosaccharide (2–5 nM) was then taken up in the digestion buffer and subjected to the modified sequential exoglycosidase protocol described above.

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