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Structural analysis of monosulfated side-chain oligosaccharides isolated from human tracheobronchial mucous glycoproteins

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ABSTRACT

To determine the location of some sulfate esters on respiratory mucins, an unambiguous sequencing strategy was developed for a crude, monosulfated oligosaccharide fraction derived from tracheobronchial mucous glycoproteins, isolated from sputum from a patient with cystic fibrosis, and which possessed *Ricinus communis*-I lectin affinity. Employing fractionation by Bio-Gel P-2 chromatography and high-voltage paper electrophoresis of the pool, eighteen branched and four straight-chained monosulfated oligosaccharides, each possessing at least one neutral D-galactose residue at a nonreducing terminus, were purified. Desulfated analogs of each sulfated oligosaccharide were then produced. Elucidation of their structures and sulfate ester locations was accomplished through a parallel comparative sequencing approach for the sulfated oligosaccharide and its desulfated analog. The method was based on their carbohydrate composition and parallel analysis by sequential exoglycosidase degradations, endoglycosidase digestion, permethylation analyses, and specific lectin affinities. Key to this approach was the inability for specific exoglycosidases and lectins to cleave or bind to, respectively, carbohydrates of their specificity which occupied nonreducing termini and possessed a sulfate ester. Herein we report the structures of twenty-two novel sulfated oligosaccharides. Oligosaccharides ranged from trisaccharides to heptasaccharides, were branched and unbranched, and each possessed a single sulfate ester on either C-6 of a terminal or an internal D-galactose residue or on C-6 of an internal residue of 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine).

INTRODUCTION

Tracheobronchial mucous glycoproteins refer to a heterogenous class of highly glycosylated, high molecular weight proteins which serve as a primary defense element within the respiratory tree. These respiratory glycoproteins have been shown to possess an anionic nature attributable to the presence of *N*-acetylneuraminic acid and sulfate esters[1,2]. Several reports indicate that in certain pathological states, such as chronic bronchitis, chronic lung infection and irritation, and cystic fibrosis, these tracheobronchial mucous glycoproteins are hypersecreted and exhibit an increased anionic characteristic due mainly to a marked increase in their sulfate ester content[3–9]. Although there have been several structural studies focused on the elucidation of the neutral and sialylated side-chained oligosaccharides from tracheobronchial origin[10–15], there has

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been only one report of structural investigation of the sulfated oligosaccharides that exist in these mucins[2]. In this latter study, five small, unbranched oligosaccharides, each possessing a D-galactose-6-sulfate residue at their nonreducing terminus, were characterized. An earlier study, based upon alkaline hydrolysis and the formation of 3,6-anhydro-D-galactose, suggested that the location of mucin sulfate esters resides on C-6 of D-galactose residues which possess unprotected C-3 hydroxyl groups[4].

Because of the sulfate ester's potentially important role(s) in the structure and function of tracheobronchial mucous glycoproteins, its possible involvement in the pathogenic state, and the limited amount of information pertaining to the positions of these sulfate esters, we separated a large select pool of monosulfated oligosaccharides from highly sulfated tracheobronchial mucous glycoproteins of a patient with cystic fibrosis with the intent to better define sites of sulfation on these molecules. This monosulfated oligosaccharide mixture was isolated with the use of the lectin *Ricinus communis*-I (specificity: β-D-galactose). In this way small unbranched oligosaccharides possessing D-galactose-6-sulfate residues at their nonreducing termini were removed leaving a large group of oligosaccharides that possessed at least one nonreducing terminus occupied with a neutral β-D-galactose residue. The rationale for this experimental design was to yield a mixture of monosulfated oligosaccharides that consisted of both unbranched molecules with internal sulfate esters and branched oligosaccharides that possess a sulfated ester on one nonreducing terminus or within one branch. In order to study these structures, we developed an unambiguous sequencing strategy based upon carbohydrate composition, sequential exoglycosidase degradations, endoglycosidase digestion, permethylation analyses, periodate oxidation, and lectin affinities of both the sulfated oligosaccharide and the respective chemically desulfated analog which provides complete structural information. This method takes advantage of the fact that the glycosidases employed, *i.e.*, *exo*-(1→3)-β-D-galactosidase, *exo*-(1→4)-β-D-galactosidase, exo-β-*2-acetamido-2-deoxy*-β-D-glucosidase, as well as specific lectins, *i.e.*, *Ricinus communis*-I, *Erythrina cristagalli*, and *Bandeiraea simplicifolia*-II, are unable to enzymatically cleave or bind to, respectively, carbohydrates of their specificity which both occupy nonreducing termini and possess a sulfate ester. Desulfated analogs, identical to the parent sulfated oligosaccharides, but without the sulfate esters, do not exhibit any inhibition to either exoglycosidase digestion or lectin binding and thus provide informative comparative data. *Via* this comparative analytical approach, we presently report, for the first time, the structural characterization of twenty-two monosulfated oligosaccharides displaying sulfate esters on either C-6 of D-galactose residues occupying nonreducing termini of branched oligosaccharides or on C-6 of internal D-galactose or 2-acetamido-2-deoxy-D-glucose residues of both unbranched and branched oligosaccharides.

EXPERIMENTAL

Materials. — A sodium borohydride-reduced monosulfated oligosaccharide fraction (8.53 g) from a tracheobronchial mucous glycoprotein pool, from which sialic

acid- and L-fucose-containing oligosaccharides were removed, for a singular 14-year-old male patient with cystic fibrosis was obtained as previously described[2]. Immobilized lectins, *Ricinus communis*-I (RCA-I; specificity: β-D-galactose), *Erythrina cristagalli* (ECA; specificity: β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc) and *Bandeiraea simplicifolia*-II (BSA-II; specificity: 2-acetamido-2-deoxy-β-D-glucose), on Sepharose 4B gel were purchased from E-Y Laboratories (San Mateo, CA 94401). All solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI 53233) and distilled prior to use.

Analytical procedures. — Carbohydrate and permethylation analysis of $NaBH_4$-reduced monosulfated oligosaccharides and their respective desulfated products were performed as previously described[2,16–18]. For these studies, gas–liquid chromatographic (g.l.c.) separation of the alditol and 2-amino-2-deoxy-hexitol acetates were performed on a Varian model 3700 gas-liquid chromatograph equipped with a Quadrex 25 m × 0.25 mm I.D. fused silica capillary column with a bonded 0.25 μm OV-17 stationary phase (New Haven, CT 06525), and with a split–splitless injector and dual flame ionization detector. With a split ratio of 1:30, a 1 μL aliquot of alditol acetate sample in $CHCl_3$ was injected at 170°, and after an initial hold of 4 min, it was then programmed at 6°/min to 250° at a He flow rate of 1.5 mL/min. Separate aliquots of reduced monosulfated oligosaccharides and their desulfated analogs (1.5 mg) were subjected to periodate oxidation (Smith degradation) by treatment with sodium metaperiodate, reduction of the products with $NaBH_4$, followed by carbohydrate and permethylation analysis as previously described[2]. Sulfate was determined both colorimetrically by the benzidine procedure of Antonopoulos[19] and by g.l.c. as its *O-tert*-butyldimethylsilyl derivative[20]. The latter procedure was also employed to detect the presence of phosphate or other inorganic oxyanions. Sialic acid was analyzed as its per-*O*-trimethylsilyl-*O*-trimethylsilyl oxime by g.l.c.[17]. Amino acids were determined on a Beckman 6300 amino acid analyzer employing post-column ninhydrin colorimetry[21]. Bio-Gel P-2 column effluents were monitored by a Gilson model 131 refractive index detector (Middleton, WI 53562) and initially analyzed for total carbohydrate content *via* the ferricyanide colorimetric method[22].

Mass spectrometry. — Mass spectra for all carbohydrate derivatives 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°, and a transfer temperature of 218°.

Isolation of monosulfated oligosaccharides possessing a free β-D-galactose residue at a nonreducing terminus. — Individual 50-mg aliquots of the heterogenous mixture of monosulfated oligosaccharides were applied to a 1.0 × 10.0 cm column of immobilized *Ricinus communis*-I (RCA-I) lectin on Sepharose 4B (pre-equilibrated with 5mM NaCl). Following the initial elution of unbound oligosaccharides (13.4 mg, 22.8%) with three bed-volumes of distilled water, the retained oligosaccharides (36.1 mg, 72.2%) were eluted from the column with a calcium-free buffer containing M NaCl in 0.05M Tris–HCl, pH 8.5 (ref. 23). Oligosaccharides exhibiting RCA-I affinity were then applied (20-mg aliquots) to a Bio-Gel P-2 (− 400 mesh), 1.0 × 100 cm, column and eluted with

0.01% NaCl at a flow rate of 0.4 mL/min and collected in 3.0-mL fractions. Five defined fractions corresponding to tri- through hepta-saccharides (d.p. 3, 6.4%; d.p. 4, 19.5%; d.p. 5, 7.2%; d.p. 6, 14.5%; d.p. 7, 16.1%) were individually pooled and lyophilized. Oligosaccharides larger than heptasaccharides and most of the contaminating glycopeptides eluted earlier and were poorly resolved. Each Bio-Gel P-2 collected fraction was then resubjected to Bio-Gel P-2 chromatography until no overlap with other fractions was found. High-voltage paper electrophoresis of 2.5–3.5 mg of each fraction was then performed on Whatman 3M paper for 95 min at 50 V/cm using a 0.1 M pyridine acetate buffer at pH 4.6, as described[2,24]. Detection was with periodate–benzidine[25]. Separated oligosaccharides S-VI–S-XXVII were individually pooled from a number of parallel runs, and then each pooled fraction was repeatedly subjected to Bio-Gel P-2 chromatography and paper electrophoresis until both carbohydrate and permethylation analyses demonstrated that both constituent sugars and glycosidic linkages existed in near integral molar ratios (purity >96%).

Oligosaccharide analysis via *sequential exoglycosidase degradation.* — For the purpose of comparative analytical data, aliquots (5–25 μM) of each monosulfated oligosaccharide and monosaccharide standards were solvolytically desulfated in 0.06M anhydrous methanolic hydrogen chloride with constant shaking for 48 h at 5°[26] and subjected to Bio-Gel P-2 column chromatography, as previously described[2]. Each monosulfated oligosaccharide (S) and its respective desulfated oligosaccharide analog (DS) were then subjected to carbohydrate and permethylation analysis, followed by sequential exoglycosidase digestion employing the following enzymes: (a) *exo*-(1→3)-β-D-galactosidase (EC 3.2.1.23, 0.01 U) from bovine testes (Boehringer Mannheim Corp., Indianapolis, IN 46250), in 5mM citrate buffer, pH 4.6, for 48 h at 37°; (b) *exo*-(1→4)-β-D-galactosidase (0.01 U) from *D. pneumoniae* (Boehringer Mannheim), in 5mM citrate buffer, pH 6.0, for 48 h at 37°; and (c) 2-acetamido-2-deoxy-β-D-glucosidase (0.1 U) from bovine kidney and *D. pneumoniae* (Boehringer Mannheim), in 5mM citrate buffer, pH 4.6, for 48 h at 37°. All digestions were performed in 0.5 mL which contained 20 μL toluene and 5 μg of the internal standard, D-*glycero*-D-*galo*-heptitol. Each enzymatic procedure utilized 5 μM of the respective oligosaccharide with the reactions being terminated by heating for 5 min at 100°. Enzymatically liberated monosaccharides were then analyzed as their alditol acetates, as above, by treatment of 10 μL of each digestion mixture with 100 μL of 1.0% $NaBH_4$ for 40 min at 4°, termination of the reaction with 1% acetic acid at 0°, removal of boric acid with repeated methanol evaporations, acetylation of the alditols with 1:2 (v/v) pyridine–acetic anhydride (0.5 mL). The sulfated oligosaccharide product in the remaining enzymatic digest was then isolated from salts, monosaccharides, and enzyme by gel filtration on Sephadex G-25 (0.9 × 100 cm column, superfine, dry bead diameter 10–40 μm) and lyophilized. The oligosaccharide product was further purified and characterized by Bio-Gel P-2 chromatography, and it was then subjected to permethylation and sulfate analysis and the next enzymatic step, as described above. Sequential glycosidase treatment of the desulfated analogs, in all cases, went to completion. Sequential glycosidase treatment of each sulfated oligosaccharide was terminated when no further enzymatic digestion was

observed. Following permethylation analysis, each resulting sulfated glycosidase-resistant product was then desulfated with methanolic hydrogen chloride, and the product was then subjected to complete sequential exoglycosidase and lectin analysis as outlined.

Additionally, aliquots of each sulfated oligosaccharide and its desulfated analog (≈ 1.5 mg) was treated with *endo*-(1→4)-β-D-galactosidase (EC 3.2.1.103) from *Bacteriodes fragilis* (Boehringer Mannheim Corp.), in 5mM citrate buffer, pH 5.8, for 16 h at 37°. Products derived from oligosaccharides susceptible to digestion by this endoglycosidase were separated by Bio-Gel P-2 chromatography and subjected to carbohydrate and permethylation analysis.

Lectin affinity analysis. — Lectin affinity studies were initially performed before enzymatic hydrolysis and then at each sequential enzymatic step of each sulfated oligosaccharide and desulfated analog. Oligosaccharide products were individually applied to 0.5 × 2.0 cm columns, each containing immobilized RCA-I, ECA, or BSA-II lectin on Sepharose 4B. Columns were then washed with 10 bed-volumes of 5mM NaCl, and retained oligosaccharides were eluted with a calcium-free buffer containing M NaCl in 0.05M Tris–HCl, pH 8.5, as described above.

RESULTS AND DISCUSSION

Isolation and purification of monosulfated oligosaccharides possessing a free β-D-galactose residue at a nonreducing terminus. — A representative elution profile showing the Bio-Gel P-2 fractionation of a crude mixture of the RCA-I lectin binding monosulfated oligosaccharides displaying five distinctive peaks corresponding to tri- through hepta-saccharides (F3–F7, respectively) is presented in Fig. 1A. Larger oligosaccharides and most glycopeptides eluted earlier and were not resolved by this column. A scan composite showing the initial individual high-voltage paper electrophoretic profiles of the Bio-Gel P-2 fractions F3–F7 is given in Fig. 2. With repeated fractionation by Bio-Gel P-2 chromatography and high-voltage paper electrophoresis, the twenty-two indicated monosulfated oligosaccharides in Fig. 2, S-VI–S-XXVII, were purified. Subsequent analyses demonstrated that 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, and D-galactose were the only sugars detected. Near-integral molar ratios of 2-acetamido-2-deoxy-D-glucose, D-galactose, and sulfate and their glycosidic linkages relative to 2-acetamido-2-deoxy-D-galactitol, determined by carbohydrate analysis (not shown) and by permethylation analysis (Table I), respectively, were found for each oligosaccharide. In all cases, the total constitutive monosaccharide composition (*i.e.*, total residues per oligosaccharide) were in excellent agreement with the respective Bio-Gel P-2 indication of monosulfated oligosaccharide size in Fig. 1A. None of the purified oligosaccharides were found to possess sialic acid, L-fucose, phosphate esters, or amino acids.

Desulfated analogs of S-VI–S-XXVII. — Desulfation of an aliquot of each monosulfated oligosaccharide with cold methanolic hydrogen chloride, followed by Bio-Gel P-2 chromatographic purification of the product, resulted in the recovery

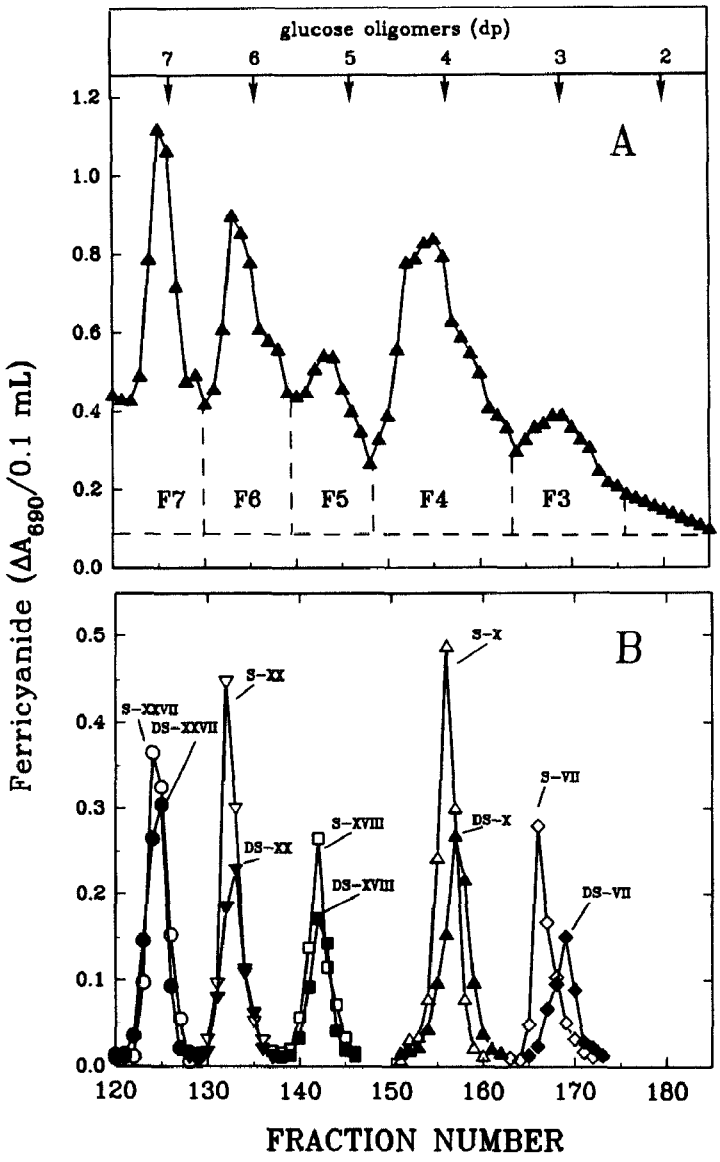


Fig. 1. Bio-Gel P-2 elution profile (A) of the crude mixture of RCA-I binding monosulfated oligosaccharides and a composite elution profile (B) on the same column showing separations of representative purified monosulfated RCA-I binding oligosaccharides (open symbols) for each oligosaccharide size that was isolated by Whatman 3M high-voltage paper electrophoresis. Also superimposed upon the composite profile (B) are the elution profiles corresponding to the purified desulfated analogs (DS, closed symbols) of each representative oligosaccharide shown. (A) Bio-Gel P-2 (~400 mesh), 1.0 × 100 cm, column was employed using 0.01% NaCl as the eluent. The sample was applied to the column and then eluted at a flow rate of 0.4 mL/min and a collected fraction size of 3.0 mL. Designated fractions F3–F7, each corresponding to the specified elution volumes of standard D-glucose oligomers, were individually pooled and lyophilized. Elution was colorimetrically monitored by determination of total reducing sugar *via* the ferricyanide method at 690 nm. (B) The column size and eluting solvent were the same as in (A). The figure presents a composite picture of 10 individual chromatographic runs. As representatives of each oligosaccharide size analyzed, sulfated oligosaccharide S-VII, S-XVIII, S-XX, and S-XXVII, corresponding to tri- to hepta-saccharides,

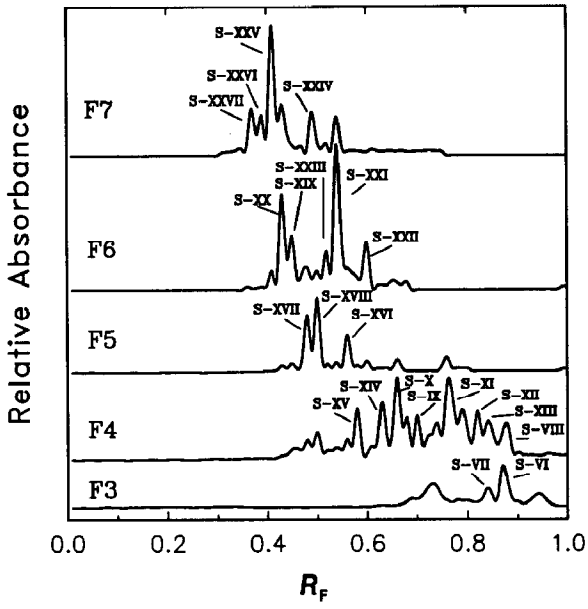


Fig. 2. High-voltage paper electrophoresis of the individually pooled fractions F3–F7 from the Bio-Gel P-2 column (Fig. 1A). Fractions were individually applied and separated on Whatman 3M paper for 95 min at 50 V/cm using a 0.1M pyridine acetate buffer at pH 4.6. A periodate–benzidine reagent was used to visualize oligosaccharides and carbohydrates. Stained sample lanes were then immediately scanned at 535 nm. Designated monosulfated oligosaccharides S-VI–S-XXVII were then excised and eluted from parallel unstained separations for structural analysis.

(>94.6%) of the respective neutral desulfated analog and, as representatively presented in Fig. 1B, with no demonstrable change in oligosaccharide size. For each respective purified desulfated product (DS) this high yield, the absence of detectable sulfate, and no change in the carbohydrate molar ratios presented in Table II, when compared to Table I, indicated that desulfation of the monosulfated oligosaccharides with methanolic hydrogen chloride was efficient and produced few side-products.

Permethylation analysis of S-VI–S-XXVII and desulfated analogs. — Very little (<2%) or no desulfation of sulfated oligosaccharides occurred as a result of the methylation procedure. In addition to confirming the carbohydrate and Bio-Gel P-2 findings for both composition and size of each oligosaccharide, an initial examination of the permethylation analyses of monosulfated oligosaccharides S-VI–S-XXVII and their parallel desulfated analogs DS-VI–DS-XXVII, Tables I and II, respectively, provide further structural details. Notably the findings of 2-acetamido-2-deoxy-1,4,5,6-tetra-*O*-methyl-, 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-methylamino-, 2-acetamido-2-deoxy-1,4,5-tri-*O*-methyl-, and 2-deoxy-1,4,5-tri-*O*-methyl-2-methylamino-D-galactitol indicate

respectively, previously purified by Whatman high-voltage paper electrophoresis, were individually applied and eluted from the column. The elution profiles of the desulfated analogs of each representative sulfated oligosaccharide are also shown.

TABLE I

Methylation analysis of sulfated oligosaccharides from human tracheobronchial glycoproteins[a,b]

Sulfated Oligosaccharide	*GlcN(Me)Ac*	*Gal*	*GalN(Me)Ac-ol*[c]	*Sulfate*[d]
S-VI	4 (0.98)	2,3,4,6 (0.93)[c]	1,4,5,6 (1)	0.89
S-VII	3 (1.08)	2,3,4,6 (0.96)[c]	1,4,5,6 (1)	0.92
S-VIII	4,6 (1.03)	2,3,4 (0.89)[c] 2,3,4,6 (0.92)[c]	1,4,5 (1)	0.98
S-IX	4,6 (1.05)	2,3,4 (0.88)[c] 2,3,4,6 (0.92)[c]	1,4,5 (1)	0.96
S-X	3,6 (0.97)	2,3,4 (0.90)[c] 2,3,4,6 (0.96)[c]	1,4,5 (1)	0.93
S-XI	3,6 (1.11)	2,3,4 (0.88)[c] 2,3,4,6 (0.92)[c]	1,4,5 (1)	0.90
S-XII	3 (1.08) 3,4,6 (0.97)[c]	2,3,4,6 (0.86)[c]	1,4,5 (1)	0.94
S-XIII	3 (0.89)	2,3,4,6 (1.84)[c]	1,4,5 (1)	0.87
S-XIV	3,6 (1.13)	2,4 (0.95) 2,3,4,6 (0.93)[c]	1,4,5,6 (1)	0.93
S-XV	3 (1.07)	2,4,6 (0.95) 2,3,4,6 (0.97)[c]	1,4,5,6 (1)	0.90
S-XVI	3,6 (1.85)	2,3,4 (0.84)[c] 2,3,4,6 (0.90)[c]	1,4,5 (1)	0.87
S-XVII	3,6 (0.96) 4,6 (1.05)	2,3,4 (0.86)[c] 2,3,4,6 (0.93)[c]	1,4,5 (1)	0.86
S-XVIII	3,6 (1.03) 4,6 (1.08)	2,3,4 (0.91)[c] 2,3,4,6 (0.91)[c]	1,4,5 (1)	0.90
S-XIX	3,6 (1.07) 4,6 (0.96)	2,4 (0.84) 2,3,4,6 (1.92)[c]	1,4,5 (1)	0.94
S-XX	3,6 (2.05)	2,3,4 (0.90)[c] 2,4,6 (1.04) 2,3,4,6 (0.97)[c]	1,4,5 (1)	0.95
S-XXI	3,6 (2.09)	2,3,4 (0.88)[c] 2,4,6 (1.07) 2,3,4,6 (0.94)[c]	1,4,5 (1)	0.96
S-XXII	3,6 (1.90)	2,3,4 (0.90)[c] 2,4,6 (0.91) 2,3,4,6 (0.91)[c]	1,4,5 (1)	0.91
S-XXIII	3,6 (1.10) 4,6 (0.94)	2,3,4 (0.91)[c] 2,4,6 (0.90) 2,3,4,6 (0.96)[c]	1,4,5 (1)	0.90
S-XXIV	3,6 (1.86) 4,6 (0.90)	2,3,4 (0.86)[c] 2,4,6 (0.93) 2,3,4,6 (0.93)[c]	1,4,5 (1)	0.86
S-XXV	3,6 (2.91)	2,3,4 (0.87)[c] 2,4,6 (0.92) 2,3,4,6 (0.93)[c]	1,4,5 (1)	0.86
S-XXVI	3,6 (2.89)	2,3,4 (0.89)[c] 2,4,6 (0.91) 2,3,4,6 (0.94)[c]	1,4,5 (1)	0.88
S-XXVII	3,6 (2.11) 4,6 (1.05)	2,3,4 (0.86)[c] 2,4,6 (1.03) 2,3,4,6 (0.94)[c]	1,4,5 (1)	0.93

[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 sulfated oligosaccharide. [b] Purified, sulfated-reduced oligosaccharides, isolated by Bio-Gel P-2 chromatography and high-voltage electrophoresis, were per-*O*-methylated, subjected to acid hydrolysis, derivatized to their respective alditol acetates, and analyzed by g.l.c.. [c] Residue was destroyed when the respective oligosaccharide was pretreated with sodium metaperiodate. [d] Sulfate was determined both colorimetrically by the benzidine procedure of Antonopoulos[19] and by g.l.c.[20].

TABLE II

Methylation analysis of the desulfated (D.S.) oligosaccharides produced from the respective sulfated oligosaccharide from human tracheobronchial glycoproteins[a,b]

Desulfated Oligosaccharide	*GlcN(Me)Ac*	*Gal*	*GalN(Me)Ac-ol*[c]	*Sulfate*[d]
DS-VI	4,6 (1.13)	2,3,4,6 (0.90)[e]	1,4,5,6 (1)	n.d.
DS-VII	3,5 (1.14)	2,3,4,6 (0.92)[e]	1,4,5,6 (1)	n.d.
DS-VIII	4,6 (1.11)	2,3,4,6 (1.91)[e]	1,4,5 (1)	n.d.
DS-IX	4,6 (1.08)	2,3,4,6 (1.93)[e]	1,4,5 (1)	n.d.
DS-X	3,6 (0.97)	2,3,4,6 (1.85)[e]	1,4,5 (1)	n.d.
DS-XI	3,6 (0.96)	2,3,4,6 (1.90)[e]	1,4,5 (1)	n.d.
DS-XII	3,6 (0.96) 3,4,6 (1.07)[e]	2,3,4,6 (0.89)[e]	1,4,5 (1)	n.d.
DS-XIV	3,6 (1.10)	2,4,6 (0.85) 2,3,4,6 (0.93)[e]	1,4,5,6 (1)	n.d.
DS-XV	3,6 (1.12)	2,4,6 (0.85) 2,3,4,6 (0.88)[e]	1,4,5,6 (1)	n.d.
DS-XVI	3,6 (1.93)	2,3,4,6 (1.90)[e]	1,4,5 (1)	n.d.
DS-XVII	3,6 (1.08) 4,6 (1.12)	2,3,4,6 (1.94)[e]	1,4,5 (1)	n.d.
DS-XVIII	3,6 (0.94) 4,6 (1.15)	2,3,4,6 (1.82)[e]	1,4,5 (1)	n.d.
DS-XIX	3,6 (0.95) 4,6 (0.96)	2,4,6 (0.90) 2,3,4,6 (0.87)[e]	1,4,5 (1)	n.d.
DS-XX	3,6 (2.13)	2,4,6 (1.04) 2,3,4,6 (1.91)[e]	1,4,5 (1)	n.d.
DS-XXI	3,6 (2.14)	2,4,6 (0.92) 2,3,4,6 (1.89)[e]	1,4,5 (1)	n.d.
DS-XXII	3,6 (2.02)	2,4,6 (0.92) 2,3,4,6 (1.80)[e]	1,4,5 (1)	n.d.
DS-XXIII	3,6 (1.04) 4,6 (0.98)	2,4,6 (0.88) 2,3,4,6 (1.79)[e]	1,4,5 (1)	n.d.
DS-XXIV	3,6 (1.97) 4,6 (1.10)	2,4,6 (0.94) 2,3,4,6 (1.87)[e]	1,4,5 (1)	n.d.
DS-XXV	3,6 (2.94)	2,4,6 (0.92) 2,3,4,6 (1.83)[e]	1,4,5 (1)	n.d.
DS-XXVI	3,6 (2.80)	2,4,6 (0.93) 2,3,4,6 (1.91)[e]	1,4,5 (1)	n.d.
DS-XXVII	3,6 (1.96) 4,6 (1.12)	2,4,6 (1.03) 2,3,4,6 (1.79)[e]	1,4,5 (1)	n.d.

[a] Data expressed as the molar ratio of the identified partially methylated alditol acetates relative to the respective permethylated GalN(Me)Ac-ol found for each desulfated oligosaccharide analog. [b] Desulfated oligosaccharides were produced by treating the respective sulfated oligosaccharide in Table I with 0.06M 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 analysed by g.l.c. [c] Residue destroyed when the respective oligosaccharide was pretreated with sodium metaperiodate. [d] n.d., not detected.

one and two glycosidic linkage points, respectively, located on the 2-acetamido-2-deoxy-D-galactose residue involved in the glycopeptide linkage of the mucous glycoproteins. Also, the strong binding of these oligosaccharides to RCA-I lectin is explained by the fact that each monosulfated oligosaccharide (Table I) possessed at least one single unsubstituted D-galactose residue at a nonreducing terminus, as indicated by the presence of 2,3,4,6-tetra-*O*-methyl-D-galactose. A comparison of the methylation data of the sulfated oligosaccharides (Table I) with that of the parallel desulfated analogs (Table II) yields important glycosidic and sulfate ester linkage information. First, the methylation results for 2-acetamido-2-deoxy-D-galactitol for each respective pair of sulfated and desulfated oligosaccharides were identical in both Tables, indicating that a sulfate ester did not reside on this carbohydrate residue. Second, excluding the C-1 carbon for each sugar residue, 2-acetamido-2-deoxy-D-galactitol was the only carbohydrate to display two glycosidic linkage sites. This indicates that all existing branch sites were present on 2-acetamido-2-deoxy-D-galactose and that S-VI, S-VII, S-XIV, and S-XV which possessed 2-acetamido-2-deoxy-1,4,5,6-tetra-*O*-methyl-D-galactitol and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-methylamino-D-galactitol were unbranched linear oligosaccharides and that the remaining oligosaccharides which possess 2-acetamido-2-deoxy-1,4,5-tri-*O*-methyl- and 2-deoxy-1,4,5-tri-*O*-methyl-2-methylamino-D-galactitol had linkages on C-3 and C-6. Third, for fifteen of the monosulfated oligosaccharides, the elimination of 2,3,4-tri-*O*-methyl-D-galactose (Table I) and the concomitant molar increase in 2,3,4,6-tetra-*O*-methyl-D-galactose (Table II), as a result of desulfation, indicates the site of sulfation to be C-6 of D-galactose. Fourth, the absence of other linkages on these sulfated D-galactose residues show that they occupied nonreducing termini, and from their relative molar concentrations it is determined that each of these fifteen oligosaccharides possessed a single D-galactose-6-sulfate residue. Fifth, analogous comparisons between sulfated oligosaccharides and their desulfated analogs, Tables I and II, respectively, reveal the losses of 2,4-di-*O*-methyl-D-galactose to 2,4,6-tri-*O*-methyl-D-galactose (S-XIV and S-XIX), of 2-acetamido-2-deoxy-3-*O*-methyl- and 2-deoxy-3,6-di-*O*-methyl-2-methylamino-D-glucose (S-VII, S-XII, S-XIII, and S-XV) and of 2-acetamido-2-deoxy-4-*O*-methyl- and 2-deoxy-4-*O*-methyl-2-methylamino-D-glucose to 2-acetamido-2-deoxy-4,6-di-*O*-methyl- and 2-deoxy-4,6-di-*O*-methyl-2-methylamino-D-glucose (S-VI) as a result of desulfation, indicating a C-6 sulfation site on each of these sugar residues. Sixth, since these latter sulfated sugar units possessed glycosidic linkages at carbon positions 3, 4, and 3, respectively, it is concluded that these residues were found within the oligosaccharide structure and did not occupy nonreducing termini. Seventh, with the exception of S-XII which possessed a 2-acetamido-2-deoxy-D-glucose residue at one of its nonreducing termini, as indicated by the finding of 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl- and 2-deoxy-3,4,6-tri-*O*-methyl-2-methylamino-D-glucose (Tables I and II), all other nonreducing termini were found to be occupied with a D-galactose residue. These latter D-galactose units were either unsubstituted or monosulfated as indicated by the findings of 2,3,4,6-tetra-*O*-methyl-D-galactose for sulfated oligomers (Table I) or of the concomitant losses of 2,3,4-tri-*O*-methyl-D-galactose and gains of 2,3,4,6-tetra-*O*-methyl-D-galactose (Tables I and II) due to desulfation, respectively.

Periodate oxidation analysis of S-VI–S-XXVII and desulfated analogs. — The specific carbohydrate residues destroyed by pretreatment with periodate oxidation for each respective sulfated oligosaccharide and its desulfated analog, as subsequently determined by carbohydrate and permethylation analysis, are indicated in Tables I and II. In all cases, residues possessing unsubstituted vicinal hydroxyl groups within the parent molecule were eliminated. The 2-acetamido-2-deoxy-D-galactitol residue of each monosulfated oligosaccharide, following periodate oxidation, was converted to 2-acetamido-2-deoxy-L-threitol, as analyzed by carbohydrate analysis, and to 2-acetamido-2-deoxy-1,4-di-*O*-methyl- and 2-deoxy-1,4-di-*O*-methyl-2-methylamino-L-threitol, as analyzed by permethylation analysis, confirming that each possessed a C-3 branch. These results also confirm the permethylation results by verifying identified, unsubstituted vicinal hydroxyl sites.

Lectin analysis of S-VI–S-XXVII and desulfated analogs. — Following the determination of size, carbohydrate composition, the carbohydrate involved in the glycopeptide linkage, branch locations, sites of sulfation, and sugars occupying nonreducing termini, the carbohydrate sequence for each oligosaccharide was determined by comparative sequential exoglycosidase analysis and lectin affinities. The initial lectin affinities of sulfated oligosaccharides S-VI–S-XXVII and their desulfated analogs are comparatively presented in Table III. As shown, all sulfated oligosaccharides and desulfated analogs initially possessed strong RCA-I affinity, indicating that each contained a nonreducing terminal β-D-galactose residue. This initial RCA-I affinity, though, was completely abolished when either the sulfated oligosaccharide or its respective desulfated analog was treated with β-D-galactosidase. Sulfated oligosaccharides S-XI, S-XIV, S-XVI, S-XVIII, S-XXI, S-XXII, S-XXIV, S-XXV, and S-XXVI also co-demonstrated an affinity for ECA lectin, indicating that these sulfated oligosaccharides also possessed an available β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc binding site. Following desulfation, these oligosaccharides retained their ECA affinity, and, in addition, the desulfated analogs of S-VII, S-X, S-XII, S-XIII, S-XV, S-XVII, S-XX, S-XXIII, and S-XXVII displayed ECA affinity, indicating that they now possess an available β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc binding site, which suggests that this lectin-binding site was originally sulfated and that the presence of the sulfate ester inhibited ECA lectin binding. Only S-XII displayed BSA-II specificity, indicating that it possessed a 2-acetamido-2-deoxy-β-D-glucose-binding site and that this residue remained after desulfation.

Lectin analysis was also performed on the sulfated oligosaccharide product produced following each exoglycosidase treatment. In all cases, lectin affinities displayed for each exoglycosidase product were representative of the newly uncovered carbohydrate or disaccharide occupying the nonreducing termini (Table III). It was also determined that each sulfated oligosaccharide demonstrated negligible affinity for the lectins tested when the sulfated residue occupied a nonreducing terminus (Table III), and coexisting neutral oligosaccharide branches were enzymatically removed. In contrast, lectin affinities were found at each enzymatic step for the desulfated analogs. These data indicate that the affinity that a lectin has for a specific carbohydrate is inhibited when the carbohydrate is sulfated.

TABLE III

Sequential exoglycosidase treatment and lectin affinity data of sulfated and their respective desulfated oligosaccharides.

Oligosaccharide	*Initial lectin affinities*[a,b]	*Sequence of glycosidically released carbohydrates (more ratio, lectin affinity of resulting oligosaccharide)*[b,c]
S-VI	R	Galβ1→3 (0.95)
DS-VI	R	Galβ1→3 (0.86,B) GlcNacβ (1.11) + GalNAc-ol (1)
S-VII	R	Galβ1→4 (0.85)
DS-VII	R,E	Galβ1→4 (0.83,B) GlcNAcβ (1.09) + GalNAc-ol (1)
S-VIII	R	Galβ1→3 (0.94,B) GlcNAcβ (0.97)
DS-VIII	R	Galβ1→3 (1.93,B) GlcNAcβ (1.06) + GalNAc-ol (1)
S-IX	R	Galβ1→3 (0.92)
DS-IX	R	Galβ1→3 (1.85,B) GlcNAcβ (1.11) + GalNAc-ol (1)
S-X	R	Galβ1→3 (1.03)
DS-X	R,E	Galβ1→3 (0.95) Galβ1→4 (1.01,B) GlcNAcβ (1.18) + GalNAc-ol (1)
S-XI	R,E	Galβ1→4 (0.96,B) GlcNAcβ (1.11)
DS-XI	R,E	Galβ1→3 (0.92) Galβ1→4 (0.96,B) GlcNAcβ (1.04) + GalNAc-ol (1)
S-XII	R,B	Galβ1→4 (0.94,B) GlcNAcβ (1.18)
DS-XII	R,E,B	Galβ1→4 (0.85,B) GlcNAcβ (2.14) + GalNAc-ol (1)
S-XIII	R	Galβ1→3 (0.99) Galβ1→4 (0.94)
DS-XIII	R,E	Galβ1→3 (0.95) Galβ1→4 (0.96,B) GlcNAcβ (1.09) + GalNAc-ol (1)
S-XIV	R,E	Galβ1→4 (1.05,B) GlcNAcβ (1.00)
DS-XIV	R,E	Galβ1→4 (0.90,B) GlcNAcβ (1.07,R) Galβ1→3 (0.93) + GalNAc-ol (1)
S-XV	R	Galβ1→4 (0.93)
DS-XV	R,E	Galβ1→4 (0.86,B) GlcNAcβ (1.15,R) Galβ1→3 (0.92) + GalNAc-ol (1)
S-XVI	R,E	Galβ1→4 (0.93,B) GlcNAcβ (1.14)
DS-XVI	R,E	Galβ1→4 (1.94,B) GlcNAcβ (2.01) + GalNAc-ol (1)
S-XVII	R	Galβ1→3 (0.91,B) GlcNAcβ (1.07)
DS-XVII	R,E	Galβ1→3 (1.01,B) Galβ1→4 (0.96,B) GlcNAcβ (2.00) + GalNAc-ol (1)
S-XVIII	R,E	Galβ1→4 (1.06,B) GlcNAcβ (1.16)
DS-XVIII	R,E	Galβ1→3 (0.93,B) Galβ1→4 (0.97,B) GlcNAcβ (2.09) + GalNAc-ol (1)
S-XIX	R	Galβ1→3 (1.79,B) GlcNAcβ (0.93)
DS-XIX	R	Galβ1→3 (1.83,B) GlcNAcβ (0.97,E) Galβ1→4 (0.91,B) GlcNAcβ (1.12) + GalNAc-ol (1)
S-XX	R	Galβ1→3 (1.03)
DS-XX	R,E	Galβ1→3 (0.92) Galβ1→4 (0.95,B) GlcNAcβ (1.01,E) Galβ1→4 (0.85,B) GlcN (0.96) + GalNAc-ol (1)
S-XXI	R,E	Galβ1→4 (0.93,B) GlcNAcβ (1.07,R) Galβ1→3 (1.02)

TABLE III (continued)

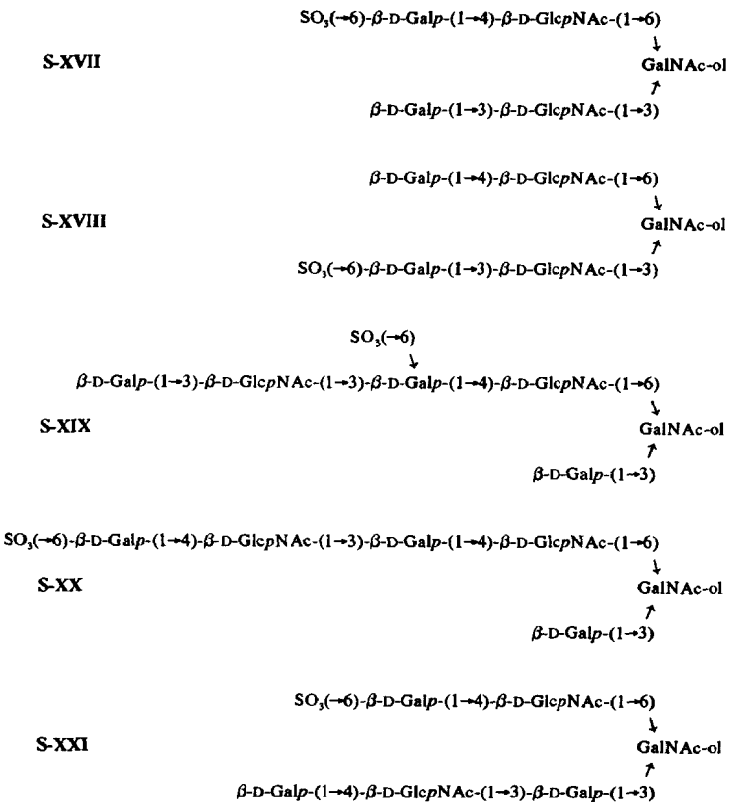
Oligosaccharide	*Initial lectin affinities*[a,b]	*Sequence of glycosidically released carbohydrates (more ratio, lectin affinity of resulting oligosaccharide)*[b,c]
DS-XXI	R,E	Galβ1→4 (1.95,B) GlcNAcβ (2.10,R) Galβ1→3 (1.07) + GalNAc-ol (1)
S-XXII	R,E	Galβ1→4 (0.89,B) GlcNAcβ (1.06,E) Galβ1→4 (0.92,B) GlcNAcβ (1.04)
DS-XXII	R,E	Galβ1→3 (1.06) Galβ1→4 (0.97,B) GlcNAcβ (0.97,E) Galβ1→4 (0.90,B) GlcNAcβ (1.07) + GalNAc-ol (1)
S-XXIII	R	Galβ1→3 (1.05,B) GlcNAcβ (1.09)
DS-XXIII	R,E	Galβ1→3 (0.92,B) Galβ1→4 (1.02,B) GlcNAcβ (2.07,R) Galβ1→3 (0.84) + GalNAc-ol (1)
S-XXIV	R,E	Galβ1→4 (0.94,B) GlcNAcβ (0.96,E) Galβ1→4 (1.00,B) GlcNAcβ (1.01)
DS-XXIV	R,E	Galβ1→3 (0.98,B) Galβ1→4 (0.96,B) GlcNAcβ (2.11,E) Galβ1→4 (0.91,B) GlcNAcβ (1.13) + GalNAc-ol (1)
S-XXV	R,E	Galβ1→4 (0.94,B) GlcNAcβ (1.05)
DS-XXV	R,E	Galβ1→4 (1.86,B) GlcNAcβ (2.06,E) Galβ1→4 (0.89,B) GlcNAcβ (0.96) + GalNAc-ol (1)
S-XXVI	R,E	Galβ1→4 (1.01,B) GlcNacβ (1.14,E) Galβ1→4 (0.97,B) GlcNAcβ (1.08)
DS-XXVI	R,E	Galβ1→4 (1.93,B) GlcNAcβ (1.95,E) Galβ1→4 (0.84,B) GlcNAcβ (1.06) + GalNAc-ol (1)
S-XXVII	R	Galβ1→3 (1.10,B) GlcNAcβ (1.07)
DS-XXVII	R,E	Galβ1→3 (0.97,B) Galβ1→4 (1.04,B) GlcNAcβ (2.14,E) Galβ1→4 (0.93,B) GlcNAcβ (1.12) + GalNAc-ol (1)

[a] Initial lectin affinities for each untreated sulfated and desulfated oligosaccharide determined prior to subjection to sequential exoglycosidase digestion. Named lectins for each oligosaccharide indicates that >90% of the applied oligosaccharide bound to the indicated column. [b] Lectin specificities: R = RCA-I, β-D-galactose; E = ECA, β-D-Gal*p*-(1→4)-β-D-Glc*pN*Ac; B = BSA-II, 2-acetamido-2-deoxy-β-D-glucose. [c] Each reduced sulfated oligosaccharide was subjected to sequential alternating exoglycosidase treatment with *exo*-(1→3)-β-D-galactosidase, *exo*-1,4-β-D-galactosidase, and *exo*-2-acetamido-2-deoxy-β-D-glucosidase. Data expressed, in order from left to right, as the sequentially identified enzymatically liberated carbohydrate (mole ratio of the liberated sugar relative to 2-acetamido-2-deoxy-D-galactitol of the respective oligosaccharide's concentration, lectin affinity identified for the oligosaccharide product). Termination of each reported sequence indicates that no further glycosidase digestion or lectin binding was noted. At each enzymatic step the liberated carbohydrate was quantitated by g.l.c. as the alditol acetate and the resulting oligosaccharide product subjected to lectin affinity analysis. The absence of a lectin entry indicates that the oligosaccharide product did not bind to RCA-I, ECA, or BSA-II. In all cases, ECA-binding oligosaccharides also displayed RCA-I lectin affinity (not shown for sequence data).

TABLE IV

Proposed structures for monosulfated oligosaccharides from human tracheobronchial glycoproteins

S-VI	$SO_3(\rightarrow 6)$ ↘ β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-GalNAc-ol
S-VII	$SO_3(\rightarrow 6)$ ↘ β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-GalNAc-ol
S-VIII	β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→6) ↘ $SO_3(\rightarrow 6)$ GalNAc-ol ↘ ↗ β-D-Gal*p*-(1→3)
S-IX	$SO_3(\rightarrow 6)$-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→6) ↘ GalNAc-ol ↗ β-D-Gal*p*-(1→3)
S-X	$SO_3(\rightarrow 6)$-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6) ↘ GalNAc-ol ↗ β-D-Gal*p*-(1→3)
S-XI	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6) ↘ GalNAc-ol ↗ $SO_3(\rightarrow 6)$-β-D-Gal*p*-(1→3)
S-XII	$SO_3(\rightarrow 6)$ ↘ β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6) ↘ GalNAc-ol ↗ β-D-Glc*p*NAc-(1→3)
S-XIII	$SO_3(\rightarrow 6)$ ↘ β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6) ↘ GalNAc-ol ↗ β-D-Gal*p*-(1→3)
S-XIV	$SO_3(\rightarrow 6)$ ↘ β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-GalNAc-ol
S-XV	$SO_3(\rightarrow 6)$ ↘ β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-GalNAc-ol
S-XVI	$SO_3(\rightarrow 6)$-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6) ↘ GalNAc-ol ↗ β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)


S-XVII
SO3(→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
GalNAc-ol
β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)
S-XVIII
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
GalNAc-ol
SO3(→6)-β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)
S-XIX
SO3(→6)
β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
GalNAc-ol
β-D-Galp-(1→3)
S-XX
SO3(→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
GalNAc-ol
β-D-Galp-(1→3)
S-XXI
SO3(→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
GalNAc-ol
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→3)
S-XXII
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
SO3(→6)
GalNAc-ol
β-D-Galp-(1→3)
S-XXIII
β-D-Galp-(1→3)-β-D-GlcpNAc-(1→6)
GalNAc-ol
SO3(→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→3)
S-XXIV
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
GalNAc-ol
SO3(→6)-β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)
S-XXV
SO3(→6)β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
GalNAc-ol
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)
S-XXVI
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
GalNAc-ol
SO3(→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)
S-XXVII
SO3(→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)
GalNAc-ol
β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)

Sequential exoglycosidase analysis. — The results for the exoglycosidase analyses for S-VI–S-XXVII and their desulfated analogs are also comparatively presented in Table III. Based upon these data, the following discussion, and previous analyses presented in Tables I and II, proposed structures for S-VI–S-XXVII are presented in Table IV.

Sequential exoglycosidase analysis of monosulfated branched oligosaccharides where the sulfate ester is on a nonreducing terminus: From previous studies[2] it was determined that the glycosides of D-galactose sulfate (*i.e.*, 2-, 3-, 4-, or 6-sulfate) and 2-acetamido-2-deoxy-D-glucose sulfate (*i.e.*, the 3-, 4-, or 6-sulfate) were very poor substrates for the enzymes β-D-galactosidase and β-2-acetamido-2-deoxy-D-glucosidase, respectively. This inhibition due to substrate sulfation provides for a facile sequencing opportunity in that oligosaccharide branches terminating with a D-galactose sulfate or a 2-acetamido-2-deoxy-D-glucose sulfate residue are resistant to exoglycosidase digestion. In this report, advantage was taken of this resistance by applying it to branched oligosaccharides in which only one branch was sulfated. In this way the nonsulfated branch was enzymatically sequenced in a direct-read fashion (Table III) while the sulfated branch remained intact. The sulfated branch remaining after complete enzymatic digestion of the neutral branch was then subsequently desulfated and analyzed enzymatically. Sulfated oligosaccharides VIII–XI, XVI–XVIII, and XX–XXVII fall into this category. Using S-XXVI as an example for this group, it is known from both the carbohydrate and methylation analyses (Tables I and II) above that it is a heptasaccharide, has two glycosidic linkages on 2-acetamido-2-deoxy-D-galactitol, possesses a sulfate ester on C-6 of one of the two nonreducing terminal galactose residues, and from lectin analysis it binds RCA-I and ECA lectins. Sequential exoglycosidase analysis of S-XXVI, shown in Table III, yielded the sequence β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(→)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(→)- in the near-integral sequential molar ratios of the individual carbohydrates of 1.01:1.14:0.097:1.08, respectively, and collectively represented the neutral branch of the molecule. Subjection of the desulfated analog of S:XXVI (*i.e.*, DS-XXVI) to sequential exoglycosidase analysis, shown in Table III, again confirmed this sequence as well as provided the sequence of the desulfated branch as indicated by the twofold increase in the initial liberation of D-galactose and 2-acetamido-2-deoxy-D-glucose over that observed for the sulfated parent oligosaccharide. This twofold increase reflects both oligosaccharide branches (neutral and previously sulfated) being simultaneously digested with each respective glycosidase treatment. Subtraction of the above neutral branch sequence from the exoglycosidase results obtained in Table III indicates that the sequence of the originally sulfated branch to be β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(→).

Linkage information of the neutral branch of S-XXVI (*i.e.*, β-D-Gal*p*-(→)-β-D-Glc*p*NAc-(→)-β-D-Gal*p*-(→)-β-D-Glc*p*NAc-(→)- to C-6 of 2-acetamido-2-deoxy-D-galactitol was confirmed by the permethylation analysis of the glycosidase-resistant sulfated product from S-XXVI which showed the appearance of 2-acetamido-2-deoxy-1,4,5,6-tetra-*O*-methyl- and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-methylamino-D-galactitol, reflecting the loss of 2-acetamido-2-deoxy-1,4,5-tri-*O*-methyl- and 2-de-

oxy-1,4,5-tri-*O*-methyl-2-methylamino-D-galactitol from the untreated parent sulfated oligosaccharide. Similarly, the C-6 location of the neutral branch to 2-acetamido-2-deoxy-D-galactitol was also shown by permethylation analysis of the respective glycosidase-resistant sulfated product for S-VIII, S-XI, S-XVIII, S-XXII, S-XXIII, S-XXIV, and S-XXVI. In contrast, permethylation analysis of the respective glycosidase-resistant sulfated product from S-IX, S-X, S-XVI, S-XVII, S-XX, S-XXI, S-XXV, and S-XXVII displayed the appearance of 2-acetamido-2-deoxy-1,3,4,5-tetra-*O*-methyl and 2-deoxy-1,4,5-tri-*O*-methyl-2-methylamino-D-galactitol, reflecting the loss of 2-acetamido-2-deoxy-1,4,5-tri-*O*-methyl- and 2-deoxy-1,4,5-tri-*O*-methyl-2-methylamino-D-galactose from the untreated parent sulfated oligosaccharide, indicating a linkage of the neutral branch to C-3 of 2-acetamido-2-deoxy-D-galactitol. Results for each glycosidase-resistant sulfated product and its desulfated analog, acquired by the complete exoglycosidase digestion and permethylation analysis of each enzymatic step, were consistent with the above findings and the structures proposed in Table IV.

Sequential exoglycosidase analysis of monosulfated unbranched and branched oligosaccharides where the sulfate ester does not occupy a nonreducing terminus: In contrast to the sulfated oligosaccharides above, S-VI, S-VII, S-XII–S-XV, and S-XIX, which represent the remaining identified oligosaccharides, each possesses a sulfate ester within the oligosaccharide structure and not on a nonreducing terminus. This was determined from the comparative permethylation results discussed above (Tables I and II) and is supported by a comparison of the sequential exoglycosidase analysis of each sulfated oligosaccharide and its respective desulfated analog (Table III). As shown in Table III, for unbranched, sulfated trisaccharides S-VI and S-VII, the liberation of D-galactose from each of their single nonreducing termini and the absence of subsequent lectin binding and exoglycosidase activity suggests that the penultimate sugar is sulfated. The complete sequential exoglycosidase analysis of their desulfated analogs, *i.e.*, DS-VI and DS-VII, was accomplished and their structures determined to be β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(→)-D-Gal*p*NAc-ol and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(→)-D-Gal*p*NAc-ol, respectively. Comparative analysis of the permethylation results presented in Tables I and II for S-VI/DS-VI and S-VII/DS-VII shows that their single 2-acetamido-2-deoxy-D-glucose residue possessed a sulfate ester on C-6, in addition to providing the glycosidic linkage sites for each oligosaccharide sugar residue. Results for each glycosidase-resistant sulfated product and its desulfated analog, acquired by the complete exoglycosidase digestion and permethylation analysis of each enzymatic step, were consistent with the above findings and for the structures proposed for S-VI and S-VII in Table IV.

The proposed structures of the unbranched internally sulfated tetrasaccharides S-XIV and S-XV, shown in Table IV, were determined similarly and clearly demonstrate the utility of sequential exoglycosidase analysis for sulfated oligosaccharides since they are identical structures differing only in their site of sulfation. In Table III, S-XIV showed no further glycosidase digestion following the initial liberation of a single residue of D-galactose and of 2-acetamido-2-deoxy-D-glucose when treated sequentially with *exo*-(1→4)-β-D-galactosidase and *exo*-β-2-acetamido-2-deoxy-D-glucosidase, re-

spectively. Under the same conditions only a single residue of D-galactose was enzymatically released from S-XV. The sites of sulfation of C-6 on a D-galactose residue possessing a glycosidic linkage on C-3 within S-XIV and of C-6 on a 2-acetamido-2-deoxy-D-glucose residue possessing a glycosidic linkage on C-4 within S-XV is confirmed by the permethylation results presented in Tables I and II. These same data also show that S-XIV and S-XV possess the same carbohydrates and glucosidic linkages and are bound β-(1→3) to 2-acetamido-2-deoxy-D-galactitol. Complete sequential exoglycosidase digestion of the DS-XIV and DS-XV analogs in Table III confirmed the carbohydrate sequence, anomeric configuration, and linkages. Detailed analysis of the glycosidase-resistant sulfated products from S-XIV and S-XV and their respective desulfated analogs, determined by the complete exoglycosidase digestion and permethylation analysis of each enzymatic step, were consistent with the above findings and the structures presented in Table IV.

As indicated above, the remaining oligosaccharides possessing an internal sulfate ester, *i.e.*, S-XII, S-XIII, and S-XIX, are branched. Also noted was the finding by permethylation analysis (Tables I and II) that the tetrasaccharide S-XII is unique amongst the oligosaccharides in this study in that it possesses a 2-acetamido-2-deoxy-D-glucose residue at one of its nonreducing termini and a neutral D-galactose residue on the other. This is also confirmed separately by S-XII's initial lectin affinities for BSA-II and RCA-I (Table III), respectively. Sequential exoglycosidase digestion (Table III) and permethylation of the product supports the proposed structure of S-XII in Table IV. The lack of ECA lectin binding for S-XII and the presence of ECA binding with its desulfated analog (Table III) indicated that S-XII possessed a β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc unit within its structure and demonstrated that the single D-galactose residue of S-XII is associated with the 2-acetamido-2-deoxy-D-glucose residue that was sulfated on C-6. That C-6 of one of the 2-acetamido-2-deoxy-D-glucose residues possesses a sulfate ester was also confirmed by methylation analysis of the parent molecule (Tables I and II) and by permethylation analysis of the glycosidase-resistant sulfated product and its respective desulfated analog (not shown). The finding of 2-acetamido-2-deoxy-1,3,4,5-tetra-*O*-methyl-D-galactitol by permethylation analysis of the glycosidase-resistant sulfated product also indicated that the sulfated chain glycosidically bound to C-6 of 2-acetamido-D-galactitol. Complete sequential exoglycosidase digestion and permethylation analysis of the respective desulfated analog DS-XII produced from the glycosidase-resistant sulfated product of S-XII were consistent with the findings in Tables I–III and the proposed structure in Table IV.

The proposed structures of tetra- and hexa-saccharides S-XIII and S-XIX, respectively, in Table IV, were determined similarly to S-XII. For both monosulfated oligosaccharides a single D-galactose residue bound β-(1→3) was initially enzymatically liberated (Table III). Permethylation analysis of the sulfated product showed that the 2-acetamido-2-deoxy-1,4,5-tri-*O*-methyl-D-galactitol residue of the parent molecules, Tables I and II, was lost and replaced with the concomitant appearance of 2-deoxy-1,3,4,5-tetra-*O*-methyl-2-methylamino- and 2-acetamido-2-deoxy-1,3,4,5-tetra-*O*-methyl-D-galactitol suggesting that this single D-galactose residue was bound β-(1→3)

to 2-acetamido-2-deoxy-D-galactitol. The structures of the sulfated branches of S-XIII and S-XIX, respectively, to C-6 of 2-acetamido-2-deoxy-D-galactitol were then determined by sequential exoglycosidase digestion and permethylation analysis.

Endo-*(1→4)-β-D-galactosidase analysis of monosulfated oligosaccharides.* — All monosulfated oligosaccharides and their desulfated analogs were treated with *endo*-(1→4)-β-D-galactosidase which will hydrolyze β-(1→4) linkages between D-galactose and 2-acetamido-2-deoxy-D-glucose when it occurs within the unbranched sequence β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc. Sulfated oligosaccharides S-XX, S-XXII, S-XXIV, S-XXV, S-XXVI, and S-XXVII and their desulfated analogs demonstrated cleavage within the oligosaccharide branch bound to C-6 of 2-acetamido-2-deoxy-D-galactitol as determined by continued sequential exoglycosidase and permethylation analysis of the products. The neutral trisaccharide sequence β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal was released by the oligosaccharides DS-XX, S-XXII, DS-XXII, S-XXIV, DS-XXIV, DS-XXV, S-XXVI, DS-XXVI, and DS-XXVII. The sulfated trisaccharide sequence SO_3(→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal was liberated from S-XX, S-XXV, and S-XXVII. In contrast to these oligosaccharides, S-XIX demonstrated complete resistance to digestion with *endo*-(1→4)-β-D-galactosidase, but its desulfated analog DS-XIX produced the trisaccharide β-D-Gal*p*-(1→3)-β-DGlc*p*NAc-(1→3)-β-D-Gal suggesting an internal sulfated site that interfered with enzymatic digestion. These findings are consistent with the proposed structures in Table IV.

DISCUSSION

In order to acquire more information on where sulfate esters reside on side-chain oligosaccharides of human tracheobronchial glycoproteins, a sequencing strategy was developed and successfully applied to the structural characterization of twenty-two novel, monosulfated oligosaccharides isolated from highly anionic human respiratory mucins from an individual with cystic fibrosis. To our knowledge, the present findings, in addition to our earlier report[2], represent the only articles presenting complete structural analysis of sulfated oligosaccharides from tracheobronchial mucins. Though none of these unique sulfated oligosaccharides structures from any source have been reported to date, nine of the internal unsulfated structures have been previously identified, consisting primarily of pentasaccharides and smaller, and being isolated from a variety of human and non-human sources[10,11,13,25-32]. The basis for the unambiguous sequencing strategy presented here to determine the complete structural characterization of these monosulfated oligosaccharides takes advantage of the limited sequential digestion of monosulfated oligosaccharides which occurs with specific exoglycosidases and of the complete sequential enzymatic digestion observed for the respective desulfated analog of each parent molecule. Sequential exoglycosidase digestion (with released carbohydrate quantitation and lectin affinity analysis) of (i) the parent molecule, (ii) the desulfated analog and (iii) the desulfated fragment derived from the sulfated glycosidase resistant portion of the parent molecule, yielded the quantita-

tive read sequence of each monosulfated oligosaccharide. The combination of this quantitative sequence information with the compositional and linkage data acquired from the initial and subsequent sequential Bio-Gel P-2 chromatography, carbohydrate and permethylation analysis, and lectin affinities analysis of both the sulfated parent molecule and its respective desulfated analog provided the complete unequivocal structure with sulfate ester location for all of the mucin-derived monosulfated oligosaccharides analyzed. These data confirm that in respiratory mucin side-chain oligosaccharides sulfate esters can be found on C-6 of D-galactose of a nonreducing terminus of either branched or linear monosulfated oligosaccharides and report that sulfate ester location may also be on C-6 of internal residues of D-galactose or 2-acetamido-2-deoxy-D-glucose of either branched or linear monosulfated oligosaccharides. The roles of sulfate esters on tracheobronchial mucous glycoproteins are poorly understood at best. A better understanding their structural locations can only provide important clues to their function and help elucidate the basis of their significant increase in the diseased state.

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