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Isolation and structural characterization of novel sialylated oligosaccharide-alditols from respiratory-mucus glycoproteins of a patient suffering from bronchiectasis

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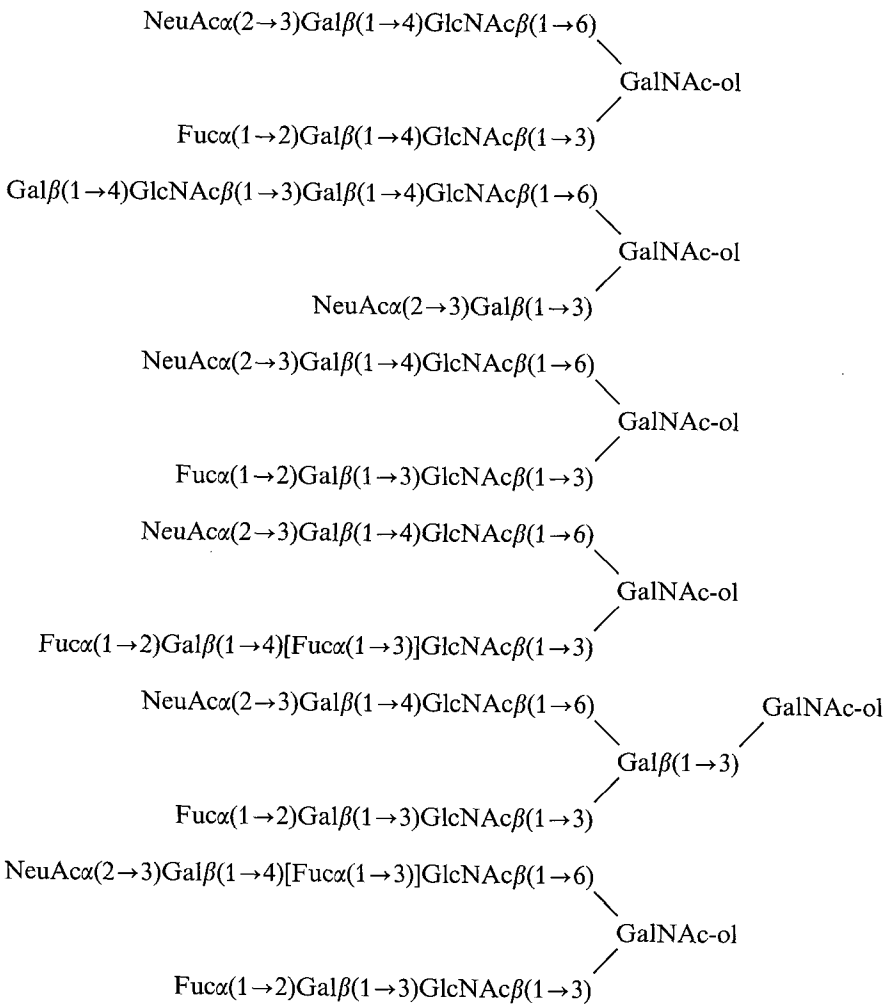
The carbohydrate chains of the respiratory-mucus glycoproteins of a patient (blood group O) suffering from bronchiectasis due to Kartagener's syndrome, were released by alkaline borohydride treatment of a pronase digest. The structures of 82 neutral and low-molecular-mass sialylated oligosaccharides have been described previously [van Kuik A., de Waard P., Vliegenthart J. F. G., Klein A., Carnoy C., Lamblin G. Roussel P. (1991) *Eur. J. Biochem. 198*, 169–182]. In the present work, medium-size sialylated oligosaccharides were obtained after ion-exchange chromatography and were subsequently separated into 36 fractions utilizing gel filtration, HPLC on normal-phase alkylamine-bonded silica and reverse-phase HPLC. From these fractions, the following six sialylated hepta- and octa-saccharide-alditols have been characterized by employing 500-MHz ^{1}H-NMR spectroscopy, in conjunction with fast-atom-bombardment mass spectroscopy and methylation analysis.



Human respiratory mucins consist of a broad family of glycoproteins synthesized by the respiratory mucosa. *O*-Glycosylation of the peptide backbone is responsible for 80% of the mass of the mucin molecule [1]. This glycosylation is characterized by the extreme diversity of the carbohydrate chains, previously called 'microheterogeneity'. This diversity is supposed to represent a 'mosaic' of binding sites recognized by adhesins or hemagglutinins of microorganisms. The attachment of microorganisms to the mucin carbohydrate chains would be the first event for their subsequent clearance by the mucocillary system [1]. Sialic acid, an outermost sugar of mucins, is often implicated in the recognition of bacterial adhesins of *Salmonella sanguis* [2] and *Escherichia coli* [3], and of hemagglutinins of influenza virus [4].

We have previously isolated 82 different neutral and low-molecular-mass sialylated oligosaccharide-alditols, from the respiratory mucins of the sputum of a patient, with blood group O, suffering from a Kartagener's syndrome [5–9]. Here we present the fractionation and the purification by HPLC of six sialylated hepta- or octa-saccharide-alditols from the mucin of the same patient. Their structural characterization was determined by a combination of 500-MHz ^{1}H-NMR spectroscopy, methylation analysis and fast-atom-bombardment (FAB) MS.

MATERIALS AND METHODS

Materials

Pronase was from Calbiochem (Behring Diagnostics, La Jolla, CA, USA); Sepharose CL-2B was from Pharmacia (Uppsala, Sweden); guanidinium chloride was from Fluka (Buchs, Switzerland); AG50WX8 (100–200 mesh) and AG1X2 (100–200 mesh) ion-exchange resins, Bio-Gel P-4 (200–400 mesh) were from Bio-Rad Laboratories (Richmond, CA, USA). HPLC was performed with a Spectroflow 400 solvent-delivery system equipped with a Spectroflow 783 detector (Kratos, Ramsey, NY, USA); the Lichrosorb-NH_2 column was from Merck (Darmstadt, FRG); Ultrasphere ODS was from Beckman (Berkeley, CA, USA); HPLC solvents and ammonium bicarbonate were from Carlo Erba (Milano, Italy).

Collection and preparation of mucin glycopeptides

Human sputum (2100 ml) was collected everyday from a patient with blood-group O suffering from bronchiectasis due to Kartagener's syndrome. It was kept frozen until use. These bronchial secretions were thawed at 4 °C, diluted 1:12 with deionized water and stirred overnight at 4 °C. The diluted mucus was then centrifuged at $3000 \times g$ for 30 min. The lyophilized mucus supernatant was digested with pronase, and fractionated by chromatography on Sepharose CL-2B in order to prepare mucin glycopeptides (fraction P2) as described previously [5].

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Abbreviations. NeuAc, *N*-acetyl-neuraminic acid; Fuc, L-fucose; GalNAc, *N*-acetyl-D-galactosamine; GalNAc-ol, *N*-acetyl-D-galactosaminitol; FAB, fast-atom-bombardment.

Purification of oligosaccharides

Alkaline borohydride reductive degradation of 3 g bronchial glycopeptides (fraction P2) was performed according to [5] and led to an heterogeneous population of glycopeptides and reduced oligosaccharides. This mixture was fractionated by ion-exchange chromatography and by gel filtration on Bio-Gel P4 as reported [10], yielding fraction IIb which corresponds to a pool of medium-size sialylated oligosaccharide-alditols.

Fractionation of sialylated oligosaccharide-alditols of fraction IIb was carried out by HPLC on a Lichrosorb-NH_2 column (25×0.46 cm internal diameter; particle size 5 μm; Fig. 2). Elution was performed with a linear gradient of 62/38 to 44/56 (by vol.) acetonitrile/water containing 2.5 mM ammonium bicarbonate, during 70 min at room temperature and at a flow rate of 1 ml/min. Further separations were realized by reverse-phase chromatography on an Ultrasphere ODS column (25×0.46 cm; particle size 5 μm); elution was performed isocratically with water at a flow rate of 0.5 ml/min at room temperature. Oligosaccharides were detected by absorption at 206 nm.

Analysis of permethylated oligosaccharide-alditols

The oligosaccharide-alditols were permethylated with methyl iodide, solid NaOH and methylsulfoxide, according to [11]. The permethylated oligosaccharide-alditols were analyzed by FAB-MS.

A Kratos concept EBEB high-resolution mass spectrometer (Kratos Analytical Instrument, Urmstom, Manchester, UK) equipped with a DS 90 (DGDG/30) data system was used. The mass spectrometer was operated at an accelerating potential of 8 keV. An Ion Tech model B 11 NF saddle field fast atom source, energized with the B 50 current regulated power supply, was used with xenon as the bombarding atom (operating conditions: 7.3 kV, 1.2 mA). The mass range 2000–200 Da was scanned at 10 s/decade. The permethylated oligosaccharide-alditols were dissolved in methanol and loaded on the copper tip with a solution of thioglycerol containing 0.5 M sodium acetate as matrix.

The partially methylated and acetylated methyl glycosides were identified by GC-MS according to [12]. Combined GC-MS was carried out on a capillary column CP-SIL 5CB (50 mm × 0.32 mm internal diameter) with a temperature program (150 °C to 280 °C, 5 °C/min) and a Riber Mag 10-10 mass spectrometer. The ionizing potential was 70 eV and the mass range 40–950 Da. The monosaccharides were identified by comparison of their relative retention times and their mass spectra with those of authentic standards.

^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis, the HPLC-fractionated oligosaccharide-alditols were repeatedly treated with ^2H_2O at room temperature, with intermediate lyophilization. Before analysis, each sample was redissolved in 0.5 ml ^2H_2O (99.96 atom % ^{2}H, Aldrich). 500-MHz ^{1}H-NMR spectroscopy was performed on a Bruker AM-500 spectrometer (Department of NMR Spectroscopy, University of Utrecht). Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation. The probe temperature was kept at 27 °C. Chemical shifts (δ) are expressed downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 in ^2H_2O).

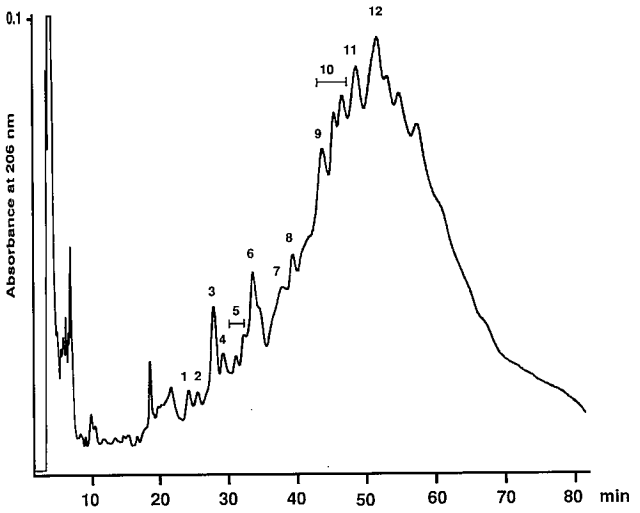

Fig. 1. HPLC elution profile of bronchial oligosaccharide-alditols of fraction IIb on a 5-μm Lichrosorb-NH_2 column, eluted with a gradient of acetonitrile/water (62/38 to 44/56, by vol.) containing 2.5 mM ammonium bicarbonate.

RESULTS

Isolation and purification of sialylated oligosaccharide-alditols

Bronchial mucus glycopeptides (fraction P2, cf. [5]) were prepared from the sputum of a patient with Kartagener's syndrome. Fraction P2 (3 g) was submitted to alkaline-borohydride degradation and then separated into four fractions (I–IV) [5]. Fraction II, which was eluted from Dowex AG1X2 with 0.5 M formic acid, was subfractionated into IIa, IIb and IIc by chromatography on Bio-Gel P4 [8]. Fraction IIb (30 mg) contained medium-size sialylated oligosaccharide-alditols. HPLC on Lichrosorb-NH_2 was employed to fractionate IIb into 12 fractions, denoted 1–12 (Fig. 1). Fractions 1, 3, 4, 5 and 6 were subfractionated on a reverse-phase column and 36 fractions were obtained by this procedure, 1.1–1.9, 3.1–3.7, 4.1–4.6, 5.1–5.6, 6.1–6.8 (Fig. 2A, 2B, 2C, 2D and 2E) respectively. Fractions 7–12 could not be resolved by this second HPLC procedure and were too heterogeneous for further structural characterization. Fraction 2 did not contain sufficient material for structural studies.

Structure determination

500-MHz ^{1}H-NMR spectra of the 36 fractions obtained by HPLC have been recorded; 26 of them contained too low amounts of material to permit NMR analysis. Spectra of fractions 3.4, 5.3, 5.4, and 6.6 indicated the presence of carbohydrate material, but the chemical shifts of the structural reporters groups could not be interpreted in terms of primary structures, since these fractions were too heterogeneous. For the remaining fractions, FAB-MS and methylation analysis were used to corroborate the NMR data. The interpretation of the FAB-MS data was guided by the sugar analysis data of glycopeptide fraction P2 [5]. The data of the methylation analysis are compiled in Table 1, and the ^{1}H-NMR chemical shifts of the structural-reporter groups are listed in Tables 2 and 3.

Fraction 1.7. The ^{1}H-NMR spectrum of this fraction (Fig. 3) shows that it consisted of a single oligosaccharide.

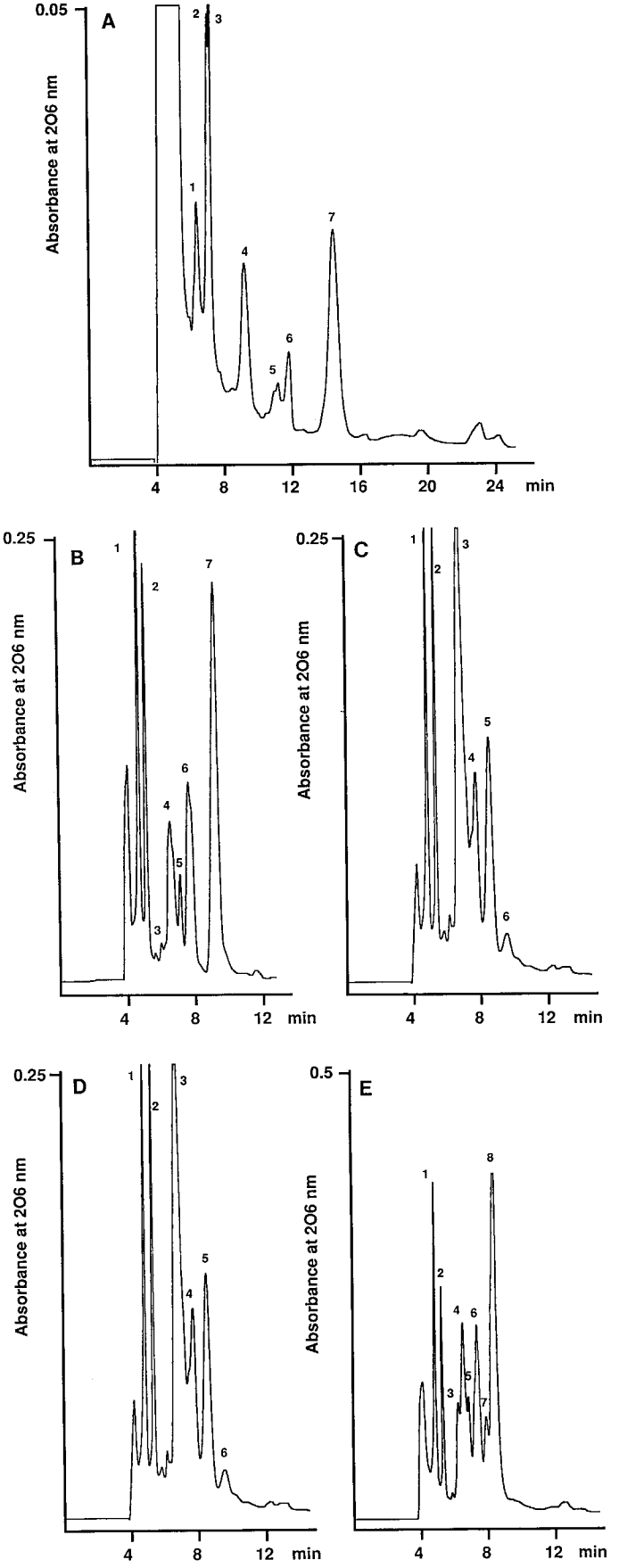

Fig. 2. Subfractionation of fractions 1 (A), 3 (B), 4 (C), 5 (D), 6 (E) by a second HPLC run on an Ultrasphere ODS column, eluted isocratically with water.

Table 1. Methyl glycosides present in the methanolysates of the permethylated oligosaccharide fractions. Due to the low amounts of oligosaccharide-alditols, the results could not been expressed in accurate molar ratios relative to GalNAc-ol derivatives; therefore, the major deivatives were indicated as + +, and the minor ones as +.

Monosaccharide methylethers	Present in oligosaccharide fractions					
	1.7	3.6	3.7	4.5	5.5	6.8
2,3,4-Me_3Fuc	+	+	+	+	+	+
2,3,4,6-Me_4Gal		+				
2,4,6-Me_3Gal	+	+ +	+	+	+	+
3,4,6-Me_3Gal	+	+	+	+	+	+
2,4-Me_2Gal					+	
3,6-Me_2GlcNAc(Me)	+	+ +	+	+	+	
4,6-Me_2GlcNAc(Me)			+		+	+
6-MeGlcNAc(Me)		+		+		+
4,7,8,9-Me_4NeuAc(Me)	+	+ +	+	+	+	+
1,4,5-Me_3GalNAc(Me)-ol + 1,4,5-Me_3-3,6-anhydroGalNAc(Me)-ol	+	+ +	+	+		+
1,4,5,6-Me_4GalNAc(Me)-ol					+	

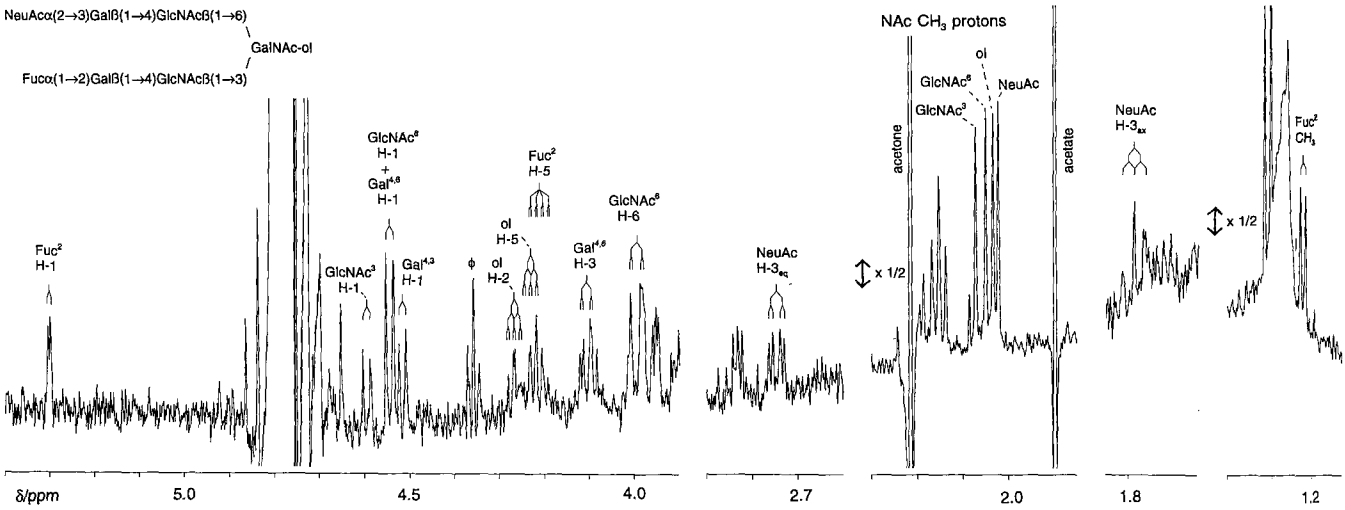

Fig. 3. Resolution-enhanced 500-MHz ^{1}H-NMR spectrum (^2H_2O, 27°C) of fraction 1.7, obtained from the pool of oligosaccharide-alditols IIb from the sputum of a patient with Kartagener's syndrome. The relative intensity scale of the *N*-acetyl and Fuc methyl proton region of the spectrum differs from that of the other parts, as indicated. Signals marked by ϕ stem from a frequently occurring, non-protein, non-carbohydrate, contaminant.

Comparison of the ^{1}H-NMR features (Table 2) of fraction 1.7 with those of the previously characterized compound **9a3b** from Bio-Gel P4 fraction IIc, NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)[Galβ(1→4)GlcNAcβ(1→3)]GalNAc-ol [7], revealed that they have the NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)GalNAc-ol branch in common. In addition fraction 1.7 showed essentially the same structural-reporter group signals for the Fucα(1→2)Galβ(1→4)GlcNAcβ(1→3)-GalNAc-ol branch as reported previously for compound **18.2** from Bio-Gel P4 fraction Ic, Galβ(1→4)GlcNAcβ(1→6)-[Fucα(1→2)Galβ(1→4)GlcNAcβ(1→3)]GalNAc-ol [6]. The structure of compound 1.7 can be conceived either as an extension of compound **9a3b** by a α1→2 linked fucose residue or as compound **18.2** with an α2→3-linked *N*-acetylneuraminic acid residue. FAB-MS analysis of the permethylated fraction 1.7 demonstrated the presence of a high-intensity ion $(M+Na)^+$ which is observed at *m/z* 1764, supporting an heptasaccharide-alditol constituted of GalNAc-ol, Gal, GlcNAc, Fuc, NeuAc in a ratio of 1:2:2:1:1. The data of the methylation analysis indicated the substitution of *N*-acetylgalactosaminitol at C-3 and C-6 that was confirmed by the presence of trimethyl anhydrogalactosaminitol [13]. The results of the methylation analysis (Table 1) are in agreement with this novel structure, which is presented in Scheme 1.

Fraction 4.5. From the comparison of the ^{1}H-NMR spectrum of fraction 4.5 (Fig. 4) with that of fraction 1.7, it appears that the single component of fraction 4.5 can be conceived as an extension of 1.7 by an α(1→3)-linked fucose residue to GlcNAc^3. The presence of Fuc^3 was concluded from comparing the ^{1}H-NMR features of fraction 4.5 (Table 2) with those of the branch Fucα(1→2)Galβ(1→4) [Fucα(1→3)] GlcNAcβ(1→3)GalNAc-ol from compound **23b** isolated from Bio-Gel fraction Ic, Fucα(1→2)Galβ(1→4)GlcNAcβ (1→6)[Fucα(1→2)Galβ(1→4)[Fucα(1→3)]GlcNAcβ(1→3)] GalNAc-ol [6]. FAB-MS analysis of the permethylated fraction 4.5 shows the presence of a high-intensity ion $(M+Na)^+$ which is observed at *m/z* 1939. This supported an octasaccharide-alditol composed of GalNAc-ol, Gal, GlcNAc, Fuc,

Table 2. ^{1}H-chemical shifts of structural-reporter groups of constituent monosaccharides for the HPLC-fractionated oligosaccharide-alditols 1.7–3.6. Chemical shifts are relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using internal acetone at 2.225 ppm) in ^2H_2O at 27°C, acquired at 500 MHz. For the complete structures of the compounds, see Scheme 1. In the table, the structures are represented by shorthand symbolic notation; ◇ = GalNAc-ol; ● = GlcNAc; ■ = Gal; □ = Fuc and △ = NeuAc. The position of the linkage in this notation is specified by the angle of the connecting bars as follows: 

; n.d., value could not be determined merely by inspection of the spectrum. A superscript at the name of a sugar indicates to which position of the adjacent monosaccharide it is glycosidically linked. Frequently, more than one superscript is used to discriminate between identically linked residues, by indicating the types of the next linkages in the sequence in the direction of GalNAc-ol. Data for compounds **9a3b** [7], **18.2** [6], **23b** [6], and **A-4** [14] have been added as references.

Residue	Reporter group	Chemical shift in compound							
		9a3b	18.2	1.7	23b	4.5	A-4	3.6a	3.6b
									
		ppm							
GalNAc-ol	H-2	4.28	4.27	4.278	4.26	4.279	4.386	4.388	4.28
	H-4	n.d.	n.d.	n.d.	n.d.	n.d.	3.438	3.438	n.d.
	H-5	4.23	4.23	4.23	4.23	4.23	4.268	4.269	4.23
	NAc	2.043	2.043	2.043	2.038	2.038	2.065	2.064	2.035
GlcNAc^6	H-1	4.558	4.560	4.554	4.538	4.555	4.556	4.556	4.556
	H-6	4.009	n.d.	4.007	n.d.	n.d.	3.992	n.d.	n.d.
	NAc	2.059	2.062	2.059	2.070	2.062	2.061	2.064	2.064
Gal^{4,6}	H-1	4.551	4.475	4.554	4.538	4.550	4.459	4.457	4.556
	H-3	4.115	n.d.	4.114	n.d.	n.d.	n.d.	n.d.	n.d.
	H-4	3.960	n.d.	n.d.	3.89	n.d.	4.149	4.151	n.d.
GlcNAc^3	H-1	4.632	4.603	4.605	4.617	4.620[a]	4.707	4.70	n.d.
	NAc	2.078	2.083	2.082	2.070	2.070	2.036	2.035	2.070
Gal^{4,3}	H-1	4.456	4.523	4.526	4.481	4.483	4.478	4.480	4.480
	H-4	3.68	3.89	3.893	n.d.	n.d.	n.d.	n.d.	n.d.
Fuc^{2,4,6}	H-1				5.306				
	H-5				4.23				
	CH_3				1.237				
Fuc^2	H-1		5.305	5.310	5.279	5.280			5.281
	H-5		4.23	4.23	4.24	4.257			n.d.
	CH_3		1.229	1.234	1.276	1.275			1.277
Fuc^3	H-1				5.118	5.117			5.117
	H-5				4.867	4.877			4.88
	CH_3				1.237	1.238			1.239
Gal^3	H-1						4.530	4.532	
	H-3						4.114	4.115	
NeuAc	H-3_{ax}	1.800		1.798		1.799	1.800	1.801	1.801
	H-3_{eq}	2.758		2.758		2.757	2.774	2.770	2.766
	NAc	2.033		2.031		2.032	2.034	2.035	2.035

[a] Virtual coupling.

NeuAc in a ratio of 1:2:2:2:1. The data of the methylation analysis (Table 1) were in agreement with this novel structure (Scheme 1).

Fraction 3.6. The ^{1}H-NMR spectrum of fraction 3.6 (Fig. 5) showed a mixture of two components. This is concluded from the Fuc H-1 signals at δ 5.281 and 5.117, which have a lower intensity than the anomeric signals in the region 4.3–4.7 ppm, and from the presence of two high-intensity ions in the FAB-MS spectrum. The structural-reporter groups of the major component (denoted 3.6a) resonated essentially at the same position as those described for compound **A-4** [14] which has been isolated from the secretory IgA hinge region. A high-intensity $(M+Na)^+$ ion at m/z 1793, observed by FAB-MS analysis, confirmed the identification of this compound in agreement with the methylation analysis data (Table 1). The structure 3.6a is presented in Scheme 1. The ^{1}H-NMR features of the minor component (denoted 3.6b) have been observed previously, for fraction 4.5. The equivalence of compound 3.6b and 4.5 is supported by an $(M+Na)^+$ ion at m/z 1939, and the outcomes of the methylation analysis.

Fraction 3.7. This fraction contained a single component (see Table 3; ^{1}H-NMR spectrum not shown) with the structure NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)[Fucα(1→2)Galβ(1→3) GlcNAcβ (1→3)] GalNAc-ol (Scheme 1), which has already been observed for structure **10c** from Bio-Gel-P4 fraction IIc [7]. FAB-MS analysis of the permethylated fraction, showed the presence of a high-intensity ion $(M+Na)^+$ at m/z 1764, confirming the presence of the heptasaccharide-alditol

Table 3. ^{1}H-chemical shifts of structural-reporter groups of constituent monosaccharides for the HPLC-fractionated oligosaccharide-alditols 3.7–6.8. Chemical shifts are relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using internal acetone at 2.225 ppm) in ^2H_2O at 27°C, acquired at 500 MHz. For explanation of the notation, see Table 2. n.d., value could not be determined merely by inspection of the spectrum. A superscript at the name of a sugar indicates to which position of the adjacent monosaccharide it is glycosidically linked. Frequently, more than one superscript is used to discriminate between identically linked residues, by indicating the types of the next linkages in the sequence in the direction of GalNAc-ol. Data for compounds **10c** [7], **4.8** [9], and **A4** [15] have been added as references.

Residue	Reporter group	Chemical shift in compound					
		10c	3.7	4.8	5.5	**A4**	6.8
							
		ppm					
GalNAc-ol	H-2	4.257	4.256	4.387	4.384	4.278	4.256
	H-3	3.983	3.985	4.003	n.d.	3.982	n.d.
	H-5	4.204	4.204	4.143	4.143	4.227	4.190
	NAc	2.042	2.041	2.046	2.047	2.044	2.041
GlcNAc^6	H-1	4.564	4.564	4.582	4.600	4.561	4.561
	H-6	4.003	4.003	n.d.	n.d.	4.019	n.d.
	NAc	2.057	2.057	2.055	2.047	2.049	2.050
Gal^{4,6}	H-1	4.547	4.548	4.530	4.545	4.519	4.516
	H-3	4.115	4.115	3.88	4.109	4.085	4.086
GlcNAc^3	H-1	4.650	4.653	4.599	4.600	4.595	4.652
	H-6	3.948	3.948	n.d.	n.d.	3.947	n.d.
	NAc	2.108	2.107	2.060	2.058	2.080	2.105
Fuc^{2,3,3}	H-1	5.211	5.211	5.192	5.190		5.209
	H-5	4.273	4.272	4.291	4.292		4.272
	CH_3	1.231	1.231	1.235	1.235		1.229
Fuc^{2,4,6}	H-1			5.313			
	H-5			4.221			
	CH_3			1.229			
Fuc^3	H-1					5.108	5.102
	H-5					4.820	4.821
	CH_3					1.169	1.169
Gal^3	H-1			4.452			4.450
	H-4			4.104			4.102
Gal^{3,3}	H-1	4.564	4.564	4.560	4.568		4.651
	H-4	3.961	3.961	3.89	3.960		n.d.
NeuAc	H-3_{ax}	1.799	1.799		1.794	1.795	1.796
	H-3_{eq}	2.758	2.758		2.764	2.764	2.756
	NAc	2.032	2.031		2.032	2.031	2.031

and, together with the data of the methylation analysis (Table 1), gave further proof of the structure.

Fraction 5.5. The ^{1}H-NMR spectrum of this fraction (Fig. 6) indicated that its single component contained the Fucα(1→2) Galβ(1→3) GlcNAcβ(1→3) Galβ(1→3) GalNAc-ol structural element as indicated by the comparison with compound **4.8** isolated from fraction Ib, Fucα(1→2)Galβ-(1→4)GlcNAcβ(1→6)[Fucα(1→2)Galβ(1→3)GlcNAcβ(1→3)-Galβ(1→3)]GalNAc-ol [9]. The presence of the NeuAcα-(2→3)Galβ(1→4) structural element in 5.5 can be inferred from the comparison of its ^{1}H-NMR features (Table 3) with those of fractions 1.7, 3.7, and 4.5. The presence of a second GlcNAc residue in 5.5 can be deduced from the signals observed at 4.600 and 2.047 ppm, which have twice the intensity of the other GlcNAc signals. From the methylation-analysis data (Table 1) it was apparent this GlcNAc residue was substituted at C-4, and was linked to C-6 of Gal^3. FAB-MS analysis of the permethylated fraction revealed the presence of a high-intensity ion $(M+Na)^+$ which is observed at m/z 1969. This indicates an octasaccharide-alditol constituted of GalNAc-ol, Gal, GlcNAc, Fuc, NeuAc in a ratio of 1:3:2:1:1. The data of the methylation analysis are compiled in Table 1. These data supported a novel structure which is presented in Scheme 1.

Fraction 6.8. Comparison of ^{1}H-NMR spectrum of the single component of fraction 6.8 (Fig. 7) with reference compound **A-4**, NeuAcα(2→3)Galβ(1→4)[Fucα(1→3)]GlcNAc β(1→6)[GlcNAcβ(1→3)]GalNAc-ol [15], which has been isolated from the bronchial mucus of patients suffering from cystic fibrosis, demonstrated the presence of the NeuAcα-(2→3)Galβ(1→4)[Fucα(1→3)]GlcNAcβ(1→6)GalNAc-ol structural element. This element is extended with Fucα(1→2)Galβ-(1→3)GlcNAcβ(1→3) linked to GalNAc-ol, which can be concluded from the comparison of the ^{1}H-NMR features

1.7

NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)
GalNAc-ol
Fucα(1→2)Galβ(1→4)GlcNAcβ(1→3)

4.5/3.6b

NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)
GalNAc-ol
Fucα(1→2)Galβ(1→4)[Fucα(1→3)]GlcNAcβ(1→3)

3.6a

Galβ(1→4)GlcNAcβ(1→3)Galβ(1→4)GlcNAcβ(1→6)
GalNAc-ol
NeuAcα(2→3)Galβ(1→3)

3.7

NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)
GalNAc-ol
Fucα(1→2)Galβ(1→3)GlcNAcβ(1→3)

5.5

NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)
GalNAc-ol
Gal(β(1→3)
Fucα(1→2)Galβ(1→3)GlcNAcβ(1→3)

6.8

NeuAcα(2→3)Galβ(1→4)[Fucα(1→3)]GlcNAcβ(1→6)
GalNAc-ol
Fucα(1→2)Galβ(1→3)GlcNAcβ(1→3)

Scheme 1. Structures of sialylated oligosaccharide-alditols obtained by HPLC fractionation of a pool of medium-size sialylated oligosaccharide-alditols from Kartagener's syndrome sputum.

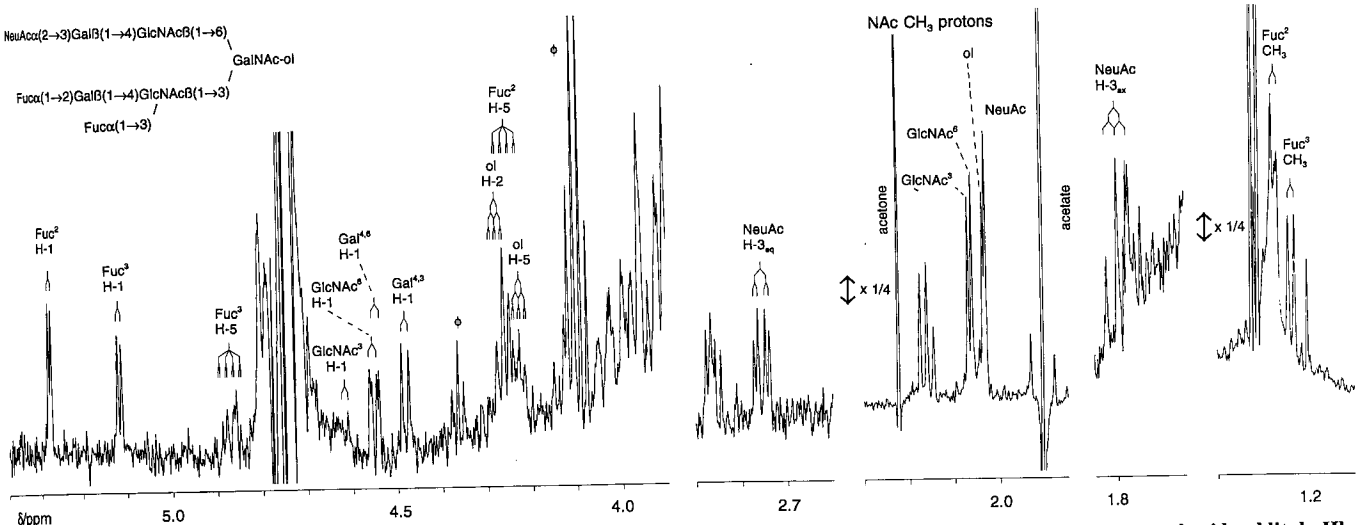

Fig. 4. Resolution-enhanced 500-MHz ^{1}H-NMRspectrum (^2H_2O, 27°C) of fraction 4.5, obtained from the pool of oligosaccharide-alditols IIb from the sputum of a patient with Kartagener's syndrome. The relative intensity scale of the *N*-acetyl and Fuc methyl proton region of the spectrum differs from that of the other parts, as indicated. Signals marked by ϕ stem from a frequently occurring, non-protein, non-carbohydrate, contaminant.

of fraction 6.8 and 3.7 (Table 3). FAB-MS analysis of the permethylated fraction 6.8 showed the presence of a high-intensity ion $(M+Na)^+$ which was observed at m/z 1939. This indicated an octasaccharide-alditol constituted of GalNAc-ol, Gal, GlcNAc, Fuc, NeuAc in a ratio of 1:2:2:2:1, which is in agreement with the ^{1}H-NMR data and the methylation analysis (Table 1). These data pinpoints a novel structure which is presented in Scheme 1.

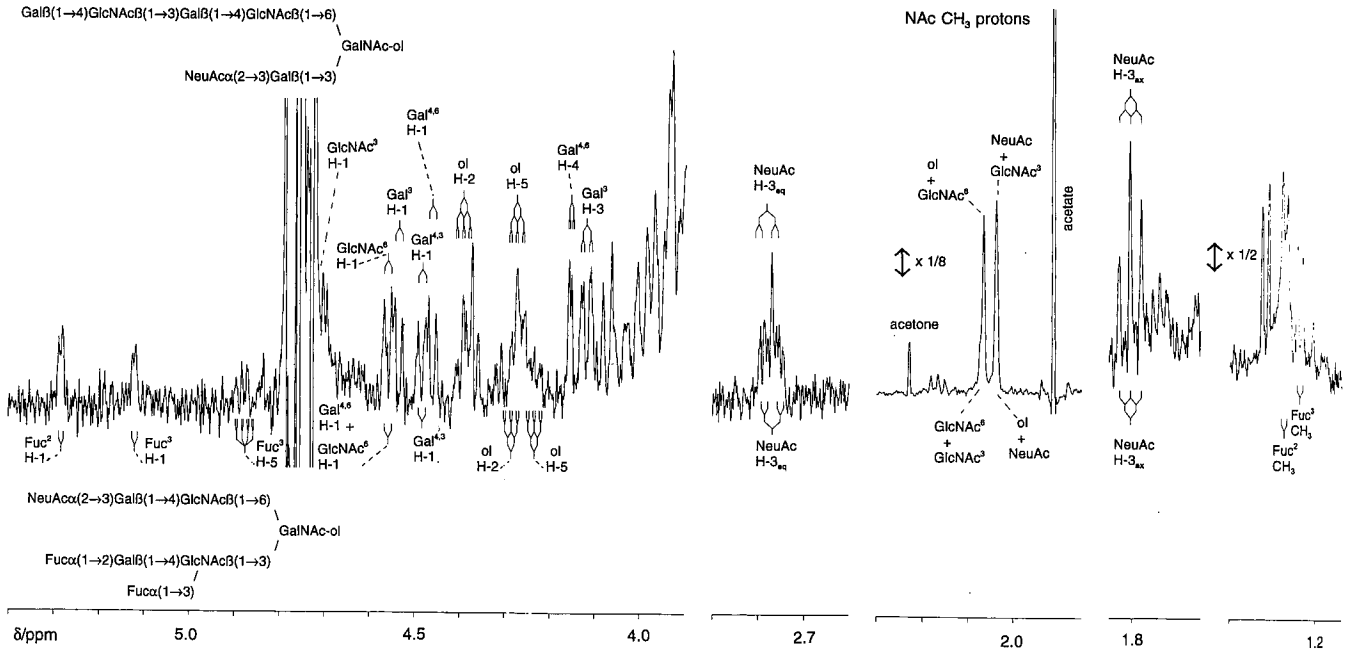

Fig. 5.. Resolution-enhanced 500-MHz ^{1}H-NMR spectrum (^2H_2O, 27°C) of fraction 3.6, obtained from the pool of oligosaccharide-alditols IIb from the sputum of a patient with Kartagener's syndrome. The relative intensity scale of the *N*-acetyl and Fuc methyl proton region of the spectrum differs from that of the other parts, as indicated. Signals attributed to the major component (3.6a) and to the minor component (3.6b) are marked above and below the spectrum respectively.

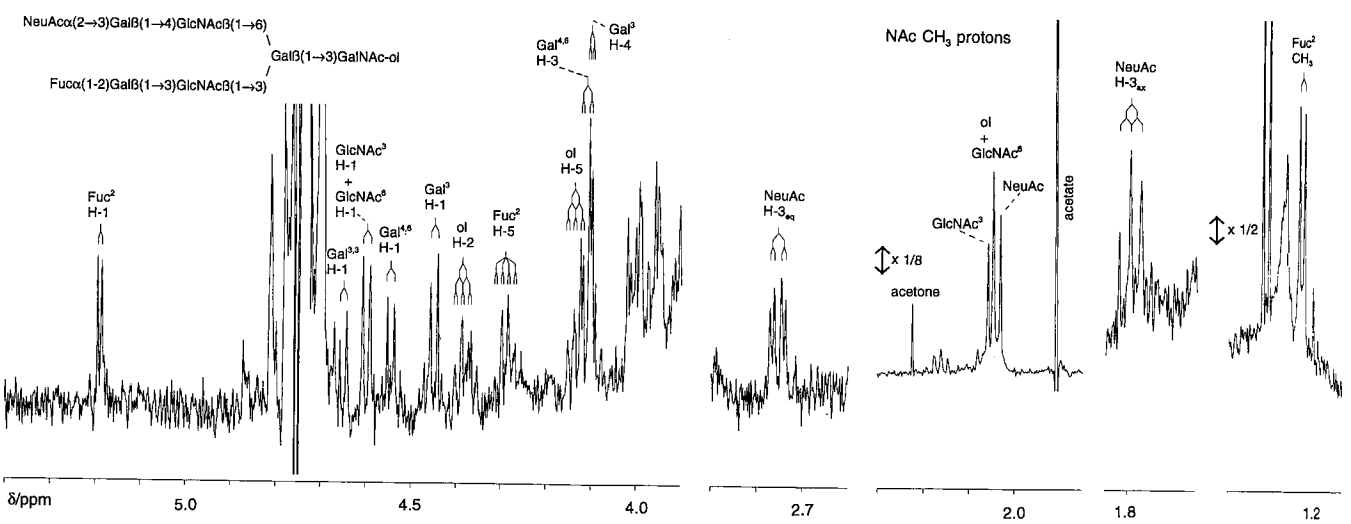

Fig. 6. Resolution-enhanced 500-MHz ^{1}H-NMR spectrum (^2H_2O, 27°C) of fraction 5.5, obtained from the pool of oligosaccharide-alditols IIb from the sputum of a patient with Kartagener's syndrome. The relative intensity scale of the *N*-acetyl and Fuc methyl proton region of the spectrum differs from that of the other parts, as indicated.

DISCUSSION

Alkaline borohydride reductive treatment of bronchial mucus glycopeptides, isolated from the sputum of a patient suffering from bronchiectasis, resulted in a mixture of glycopeptides and oligosaccharide-alditols. Fractionation by ion-exchange chromatography and gel filtration afforded neutral, monosialylated and more acidic oligosaccharide-alditols fractions. Previously, we studied the neutral low-molecular-mass and medium-size oligosaccharides-alditols (fraction Ic and Ib) and low-molecular-mass monosialylated oligosaccharide-alditols (fraction IIc); these three fractions corresponded to 16.7% of the mucin glycopeptide (fraction P2) and 82 oligosaccharide-alditols were identified. In the present study, a minor fraction (IIb), that contain medium-size monosialylated oligosaccharide-alditols corresponding to 1% of the mucin glycopeptide, was studied. The use of the two-step HPLC fractionation allowed the purification of 36 fractions, only six out of them containing sufficient material for structural analysis. Many fractions contained carbohydrate but structural studies could not be performed, due to the heterogeneity of fraction IIb and to the low amount of the subfractions. However, the structures of six sialylated hepta- and octasaccharide-alditols have been identified. Unfortunately, the

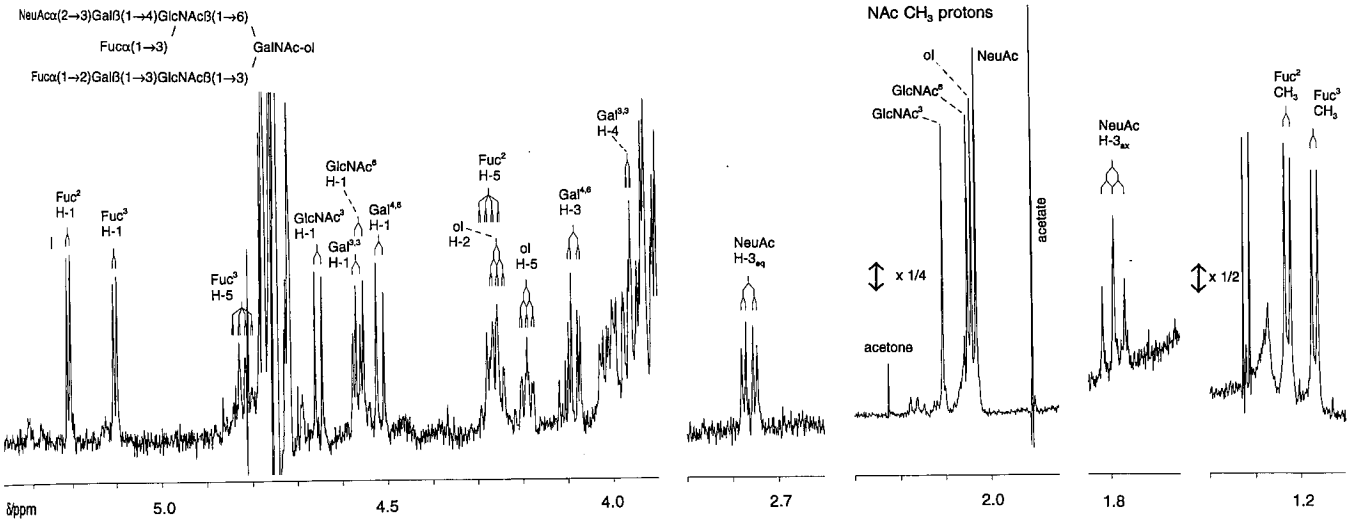

Fig. 7. Resolution-enhanced 500-MHz ^{1}H-NMR spectrum (^2H_2O, 27°C) of fraction 6.8, obtained from the pool of oligosaccharide-alditols IIb from the sputum of a patient with Kartagener's syndrome. The relative intensity scale of the *N*-acetyl and Fuc methyl proton region of the spectrum differs from that of the other parts, as indicated.

fractions containing the higher-molecular-mass oligosaccharides were still very heterogeneous and could not be well separated.

Among these six oligosaccharides, the structures 1.7, 4.5, 5.5 and 6.8 have never been described. The corresponding asialo-oligosaccharides, except oligosaccharides 3.6a and 3.6b, have been described previously in human bronchial mucins [5, 6, 8, 9]. The backbone and core of these carbohydrate chains are typical of mucin structures and the fucose residues diplay a great variety of substitutions as previously seen [5–9]. Sialic acid is α2→3-linked to the galactose residue of the core, of type 1 disaccharide (Galβ1→3GlcNAc), of type-2 disaccharide (Galβ1→4GlcNAc) or of X determinant (or SSEA [16]) resulting in a sialylated X determinant, NeuAc α2→3Galβ1→4[Fucα1→3]GlcNAc.

These six sialylated oligosaccharides increase the number of carbohydrate chains isolated from the bronchial mucins of a single patient to 88. These glycans were purified from four fractions corresponding to small and medium-size neutral and monosialylated oligosaccharide-alditols. These fractions represent 17.7% of the total glycopeptide fraction (P2), but the other fractions have not yet been studied. Therefore, one may estimate the number of different oligosaccharides as around several hundred in the secretion of a single individual.

This diversity might be the result of different phenomena. There are at least two groups of epithelial cells synthesizing mucins in the respiratory mucosa, the goblet cells of the surface and the mucous cells of the submucosal glands. Histochemical studies have suggested differences in the carbohydrate content of the different cell types [17], suggesting differential expression of glycosyltransferases. There is also a large variety of apomucins [18], and peptide structure might influence the action of the different glycosyltransferases. The glycosidases from microorganisms, present in the respiratory tract might, to some extent, degrade the glycans and be responsible for some of the heterogeneity found, but this last hypothesis is unlikely in the case of oligosaccharides terminated with sialic acid.

The extreme diversity of the carbohydrate chains present in human bronchial mucins might represent a patchwork of binding sites for inhaled microorganisms, to clear the airways and play an important role in the defence of the underlying mucosa. In cystic fibrosis, the pathophysiology of bacterial infection by *Pseudomonas aeruginosa*, responsible for much of the morbidity of this disease, might be due to overexpression of binding sites for bacterial adhesins combined with stagnant mucus. Mucin sialic acid is not only implicated in the interaction with extrinsic proteins such as bacterial adhesins [2, 3] but may also interact with basic molecules of the respiratory mucus such as lysozyme and secretory leucocyte proteinase inhibitor (SLPI) [19, 20]. These interactions could play an important role in the protection of mucus and mucosa against proteolysis and in the maintenance of the rheological properties of respiratory mucus. As a matter of fact, respiratory mucins, which can bind more than 100 molecules SLPI/molecule [20], may act as carriers of proteinase inhibitor and, by maintaining a high concentration of protease inhibitors in the vicinity of the surface epithelium, protect the underlying mucosa from proteolytic aggressions.

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