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Structure of sialyl-oligosaccharides isolated from bronchial mucus glycoproteins of patients (blood group O) suffering from cystic fibrosis

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The carbohydrate chains of the bronchial-mucus glycoproteins of six cystic fibrosis patients with blood group O were released by alkaline borohydride treatment. Low-molecular-mass, monosialyl oligosaccharide-alditols were isolated by anion-exchange chromatography and fractionated by high-performance liquid chromatography. Structural characterization was performed by 500-MHz ^{1}H-NMR spectroscopy in combination with quantitative sugar analysis. The established structures range in size from tetra- up to heptasaccharides. They are all sialyl analogs of neutral oligosaccharides that were characterized previously [Lamblin G., Boersma A., Lhermitte M., Roussel P., Mutsaers J. H. G. M., Van Halbeek H. & Vliegenthart J. F. G. (1984) *Eur. J. Biochem. 143*, 227–236]. The NeuAc residue was found to occur either in α(2→3)-linkage to Gal, or in α(2→6)-linkage to GalNAc-ol or Gal.

Human bronchial mucus is secreted as a gelatinous layer on the surface of the tracheobronchial airway epithelium and is continuously moved towards the pharynx where it is swallowed. It acts as a medium for protection and lubrication of the mucosa and for transport of inhaled particles such as dust, pollen, viruses and bacteria to clear the bronchial epithelium.

Bronchial mucins, as most mucins [1], contain about 70% carbohydrate which is O-glyosidically linked to the peptide backbone as neutral and acidic oligosaccharide chains. The acid functions stem from the presence of sialic and/or sulfate residues [2].

Any change in the structure of these oligosaccharides may modify the rheological properties of the bronchial mucus and lead to a non-efficient mucociliary clearance and to destruction phenomena found in cystic fibrosis or other bronchial diseases where hypersecretion of mucus is a pre-eminent feature of the pathological process. Increased adherence of inhaled particles may be a concomitant phenomenon.

Since it is difficult to obtain the amount of normal human bronchial secretion required for detailed carbohydrate structural analysis [3], we have taken mucins secreted by patients suffering from cystic fibrosis (CF) for our structural studies. In the future, the results of the structural characterization of their carbohydrates will be compared to mucins secreted by patients with other chronic bronchial hypersecretion diseases.

Previously, we have isolated and characterized several low-molecular-weight oligosaccharides that were obtained from the carbohydrate material released from the mucins occurring in the sputum of six CF patients with blood group O [4–6]. The structures of 20 neutral and 5 sialyl-oligosaccharides were determined by 500-MHz ^{1}H-NMR spectroscopy in conjunction with sugar composition analysis. The present paper deals with the determination of the structure of another 13 sialyl-oligosaccharides isolated from the same pool of CF bronchial mucins.

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Abbreviations. Fuc, L-fucose; GalNAc, *N*-acetyl-D-galactosamine; GalNAc-ol, *N*-acetyl-D-galactosaminitol; NeuAc, *N*-acetylneuraminic acid; CF, cystic fibrosis.

MATERIALS AND METHODS

Isolation and purification of human bronchial sialyl-oligosaccharides

Mucin glycoproteins were isolated from the sputum of six patients (blood group O) suffering from cystic fibrosis as described [2]. Briefly, gelatinous bronchial mucus from the sputum was solubilized by the action of 2-mercaptoethanol (1%, by vol.) and fractionated on an Ecteola-cellulose column with stepwise elution by 0.1 M NaCl, by 0.1 M NaCl with 0.01 M HCl and by 0.7 M NaCl with 0.01 M HCl [2]. Some *N*-acetylneuraminic acid residues are lost at room temperature during elution with solutions containing 0.01 M HCl: in our experimental conditions, the loss does not exceed 5% of the *N*-acetylneuraminic acid content. Subsequently, the most acidic fraction (62%) was purified by gel filtration on a Sepharose 4B column.

Alkaline borohydride treatment of these acidic glycoproteins with 0.05 M NaOH in the presence of 2 M $NaBH_4$ [7] afforded a mixture of glycopeptides and oligosaccharide-alditols. These were fractionated by ion-exchange chromatography on Dowex AG1X2 according to their acidity and by gel filtration on Bio-Gel P4 according to their molecular size. Again, some sialic acid residues may be lost during elution with 0.5 M formic acid: if the eluate is kept less than 3 h at room temperature before being neutralized, the loss does not exceed 3% of the *N*-acetylneuraminic acid content. Four pools of oligosaccharide-alditols were obtained, one of which (namely IIc) contains low-molecular-mass sialyl-oligosaccharide-alditols [8, 9].

Fraction IIc (30 mg) was then fractionated on a DAX4 column, eluted with 0.5 M ammonium borate buffer pH 8.0, as described before [6]. Two main (a, b) and three minor (c–e) fractions were obtained (Fig. 1). Fractions a–d were subfractionated by HPLC using a Varian Model 5000 liquid chromatograph. The apparatus was equipped with a Lichrosorb-NH_2 column (4 × 250 mm, particle size 5 μm; Merck); elution was by a linear gradient of 80/20 to 50/50 (v/v) acetonitrile/water containing 2.5 mM ammonium bicarbonate, during 70 min, at room temperature at a flow rate of 1 ml/min as described [10]. Elution profiles obtained for each fraction (a–d) are shown in Figs 2–5).

To improve the purity of some oligosaccharides which were not well separated from adjacent peaks, an additional HPLC step was carried out on the same column of Lichrosorb-NH_2 eluted by a gradient of 65/35 to 50/50 acetonitrile/water containing 2.5 mM ammonium bicarbonate, during 60 min, at room temperature at a flow rate of 1 ml/min. In some cases the gradient was stopped 5 min before the elution of the peak to allow a better separation of the minor contaminating peaks (data not shown).

Analytical methods

For quantitative sugar analysis, the sialyl-oligosaccharide-alditols were methanolyzed by 1.5 M methanolic HCl for 24 h at 85°C, *N*-reacetylated with acetic anhydride/pyridine (1:1, v/v), *O*-deacetylated with 0.5 M methanolic ammonia for 1 h at 65°C and finally trimethylsilylated with Sylon HTP (Supelco, Bellafonte, PA), in the presence of *myo*-inositol as internal standard. The trimethylsilylated methyl glycosides were analyzed by gas-liquid chromatography (GLC), using a Hewlett-Packard 5840 gas chromatograph equipped with dual-flame ionisation detectors and a glass column (180 × 0.3 cm) containing 3% OV17 on Chromosorb, 80–100 mesh (Supelco, Bellafonte, PA). The oven temperature was programmed from 120°C up to 250°C, at a rate of 8°/min [6].

Prior to ^{1}H-NMR spectroscopic analysis, the underivatized sialyl-oligosaccharide-alditols were repeatedly treated with D_2O at pD 7 and room temperature. After each exchange treatment, the material was lyophilized. Finally, each sample was redissolved in 0.4 ml D_2O (99.96 atom % D, Aldrich, Milwaukee, WI). 500-MHz ^{1}H-NMR spectroscopy was performed on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University, The Netherlands), operating under control of an Aspect-2000 computer. The probe temperature was kept at 27°C. Further experimental details have been described [4, 11, 12]. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation, followed by zerofilling from 8 K or 16 K to 32 K addresses and complex Fourier transformation. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225 in D_2O at 27°C), with an accuracy of 0.002 ppm.

RESULTS

Preparation and fractionation of sialyl-oligosaccharides-alditols from CF sputum

As part of an extensive fractionation of oligosaccharides-alditols isolated from reduced fibrillar mucus of six CF patients with blood group O activity, a pool (denoted IIc) comprising low-molecular-mass (mono)sialyl-oligosaccharides was obtained [8, 9]. Fractionation of IIc on a DAX4 anion exchange column gave rise to two major (a and b) and three minor (c, d and e) fractions (Fig. 1). Fraction e has not been studied further since it contained hardly any carbohydrate material. The other fractions, obtained in ratios 25:45:20:10 (w/w), were then subfractionated by HPLC. The yields of the various subfractions are listed in Table 1. The HPLC elution profiles are depicted in Figs 2–5. Identification of fraction a (structure a-1–a-4 and a-6, see Scheme 1) has been reported [6].

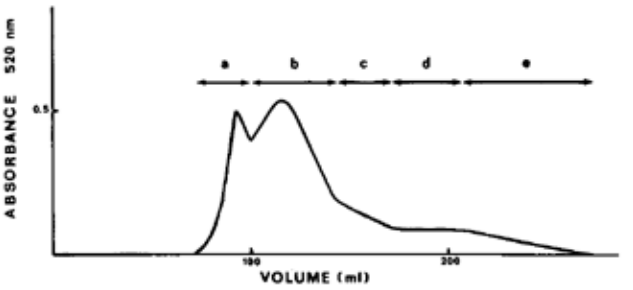


Fig. 1. *Elution profile of the fractionation of CF bronchial mucin sialyl-oligosaccharide-alditols (pool IIc) on a DAX4 anion-exchange column (30 × 0.9 cm) eluted with 0.5 M ammonium borate buffer pH 8.0.* Fractions (2 ml) were collected and analyzed for hexose (absorbance at 520 nm) as described before [22]

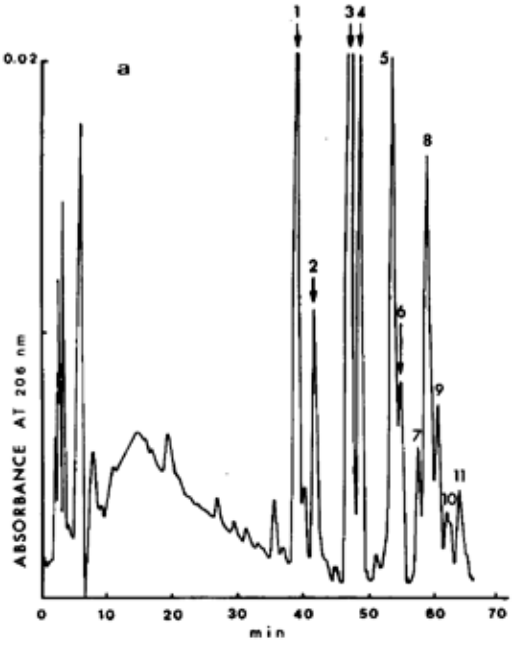


Fig. 2. *Elution profile of the HPLC separation of DAX4 fraction a obtained from CF sialyl-oligosaccharide-alditols (IIc) on a Lichrosorb-NH_2 column [6]*

Structure determination of the sialyl-oligosaccharide-alditols present in DAX4 fractions b, c and d

Sugar analysis of the fractions obtained by HPLC indicated that samples b-1, b-12, b-13, c-1, c-2, c-8, c-10, d-1, d-2, d-5 and d-6 did not contain GalNAc-ol in detectable amount. They were not studied further. The sugar analysis of

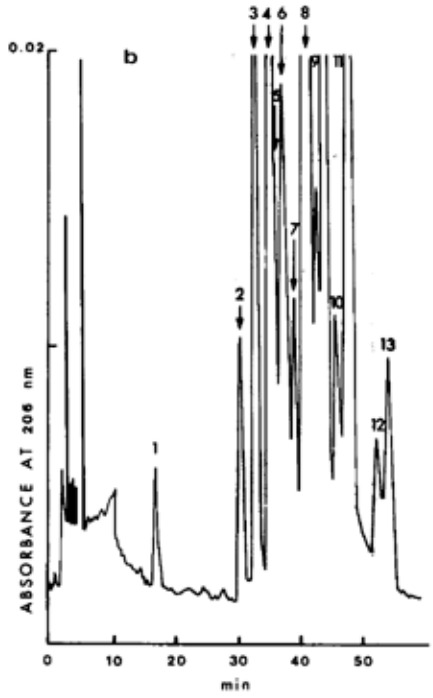


Fig. 3. *Elution profile of the HPLC separation of DAX4 fraction b obtained from CF bronchial sialyl-oligosaccharide-alditols (IIc) on a Lichrosorb-NH_2 column.* Details are given in Experimental Procedures. Arrows indicate those fractions of which structural characterization of constituent oligosaccharides was successfully completed

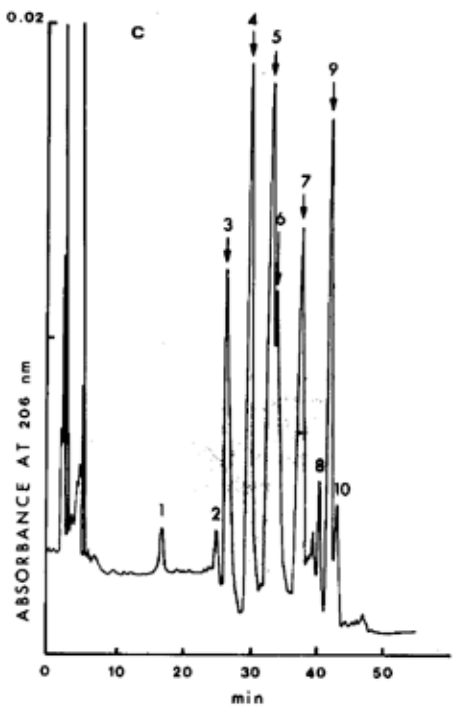


Fig. 4. *Elution profile of the HPLC separation of DAX4 fraction c obtained from CF bronchial sialyl-oligosaccharides.* Details as in Fig. 3

the remaining fractions (Table 1) indicates the presence of GalNAc-ol, GlcNAc, Gal and (except for c-3) NeuAc. Besides these, about 50% of these fractions contain Fuc. Based on the assumption that one residue of GalNAc-ol per oligo-

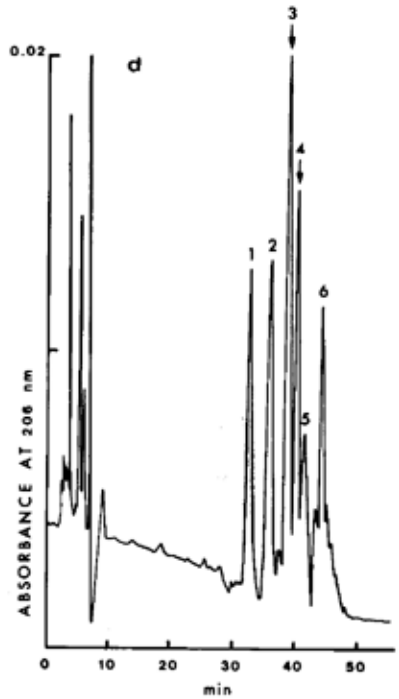


Fig. 5. *Elution profile of the HPLC separation of DAX4 fraction d obtained from CF bronchial sialyl-oligosaccharides.* Details as in Fig. 3

saccharide-alditol molecule occurs, the fractions obtained comprise monosialyl tetra- up to heptasaccharides. They were then subjected to 500-MHz ^{1}H-NMR spectroscopic analysis.

The 500-MHz ^{1}H-NMR spectra of the investigated fractions indicated the presence of carbohydrate material. From the spectra of the fractions b-9, b-10 and b-11 no primary structural assignments of constituting oligosaccharide-alditols could be deduced. This was due to the low amount of carbohydrate material available, to the interference of signals in the spectrum stemming from non-carbohydrate contaminants and/or to the apparent heterogeneity of carbohydrate structures present.

The structures of the oligosaccharide-alditols that could be deduced from the 500-MHz ^{1}H-NMR spectra of the respective fractions, together with those of some reference compounds, are summarized in Schemes 2, 3 and 4. The oligosaccharide-alditol structures in these schemes are arranged according to a common structural element and the discussion of the 500-MHz ^{1}H-NMR spectra will be done likewise. The chemical shifts of the structural-reporter-groups for these compounds are compiled in Tables 2–4. The molar carbohydrate composition of the fractions that were successfully analyzed by ^{1}H-NMR has been summarized in Table 1.

Structures with the element
Galβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol

The structures of this group (see Scheme 2) are recognized from the ^{1}H-NMR spectra by the chemical shift values of H-2 and H-5 of GalNAc-ol, at about δ 4.39 and δ 4.26–4.28, respectively [4, 5]. Further characteristics of the common element are the chemical shift values of the NAc signal of GalNAc-ol at δ 2.064–2.067, of H-1 of GlcNAc^6 at δ 4.54–

Table 1. *Molar composition of bronchial sialyl-oligosaccharide-alditol fractions separated by HPLC*
The molar carbohydrate composition of the oligosaccharide-alditols was calculated on the basis of one residue of GalNAc-ol per molcule

Oligosaccharide-alditol fraction	Molar ratio of monosaccharides					Yield
	Fuc	Gal	GlcNAc	GalNAc-ol	NeuAc	
						µg
b-2		1.1	1.1	1	0.9	148
b-3		0.9	1.4	1	0.7	256
b-4		1.9	1.1	1	1.1	430
b-5		2.5	1.2	1	1.1	124
b-6	0.6	1.5	0.9	1	1.1	940
b-7		2.4	1.8	1	1.0	300
b-8	0.9	2.6	1.5	1	1.1	1320
c-3		1.2	0.8	1	0.2	62
c-4	0.1	0.9	0.8	1	1.1	62
c-5		1.8	0.7	1	0.8	168
c-6	0.1	1.6	0.9	1	0.6	85
c-7.1	0.8	2.5	1.4	1	1.0	70
c-9	0.9	1.9	1.5	1	1	69
d-3	0.8	1.1	0.9	1	0.7	53
d-4	0.8	1.9	0.9	1	1.3	59

4.56, and of the NAc signal of GlcNAc[6] at δ 2.055–2.065 (see Table 2). The chemical shift values of the other structural-reporter groups in the common element experience a major influence of extension of this element and are indicative of the nature of the extensions.

Structures c-3 and b-4/c-5. The ^{1}H-NMR spectrum of fraction c-3 is identical to that observed earlier for fraction 7 in the series of neutral oligosaccharides resulting from the paper-chromatographic working-up procedure of pool Ic from cystic fibrosis sputum [4]. The structure of the oligosaccharide-alditol in fraction c-3 is thereby determined as Galβ(1→3)-[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol. This oligosaccharide may have arisen by the loss of *N*-acetylneuraminic acid from a sialylated oligosaccharide during the elution from the AG1X2 column with 0.5 M formic acid. The spectra and the sugar composition of the fractions b-4 and c-5 are identical. The same ^{1}H-NMR spectral features were reported previously for an acidic oligosaccharide unit derived from cow colostrum κ-casein [13], the structure of which was then determined to be NeuAcα(2 → 3)Galβ(1 → 3)[Galβ(1 → 4)GlcNAcβ(1 → 6)]-GalNAc-ol. A detailed description of the deduction of the primary structures of these oligosaccharide-alditols from the ^{1}H-NMR spectra has been given [4, 13]. It should be noted that compound b-4/c-5 is an analog of c-3, being sialylated on Gal[3].

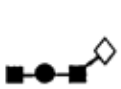
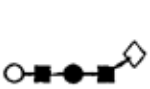
Structure b-5/c-6.1. The ^{1}H-NMR spectrum of fraction b-5 and that of the main component in fraction c-6 were identical (Fig. 6). The spectrum of fraction b-5 together with its carboyhdrate composition are indicative of a pentasaccharide-alditol containing Gal, GlcNAc, NeuAc and GalNAc-ol in a molar ratio of 2:1:1:1. From the chemical shift values of H-2 (δ 4.392) and H-5 (δ 4.282) of GalNAc-ol, the core structure is established as Galβ(1→3)[GlcNAcβ(1→6)]-GalNAc-ol [4]. NeuAc is present in terminal position, α(2→3) linked to an *N*-acetyllactosamine unit, as is evident from the values for its H-3*eq* (δ 2.755) and H-3*ax* (δ 1.799). This identifies the structure as Galβ(1→3)[NeuAcα(2→3)Galβ-(1→4)GlcNAcβ(1→6)]GalNAc-ol. This structure can, analogous to b-4/c-5, be regarded as an α(2→3)sialyl extension of compound c-3, where the extension is now in the 6 branch. The effects of this NeuAc attachment to the *N*-acetyllactosamine unit are easily recognized: for H-1 of GlcNAc[6], $\Delta\delta$ = −0.006, for H-1 of Gal[4], $\Delta\delta$ = +0.079 and for the NAc of GlcNAc[6], $\Delta\delta$ = −0.003 ppm (see Table 2). The primary structure of the minor component in fraction c-6 could not be deduced from the ^{1}H-NMR spectrum, due to the low amount of material.

Structure d-4. Sugar analysis of fraction d-4 (Table 1) indicated the presence of a hexasaccharide-alditol consisting of Gal, GlcNAc, Fuc, NeuAc and GalNAc-ol (2:1:1:1:1). The ^{1}H-NMR spectrum of this fraction indicated the presence of only small amounts of sugar. However, the presence of a hexasaccharide-alditol could be confirmed on the basis of some characteristic sigals in the spectrum. The chemical shift values of H-1 (δ 5.308) and CH_3 (δ 1.227) of Fuc are strong indications for the structural element Fucα(1→2)Galβ(1→4)-GlcNAcβ(1→6) [5]. The values for the anomeric protons of Gal[4] (δ 4.536) and of GlcNAc (δ 4.536) are in accordance with this structural element. The presence of NeuAc in α(2→3) linkage to Gal[3] is evidenced by the values for its H-3*ax* (δ 1.800) and H-3*eq* (δ 2.772) [13]. Combination of these data leads to the conclusion, that the structure of the oligosaccharide-alditol present in fraction d-4 is NeuAcα(2 → 3)Galβ-(1 → 3)[Fucα(1 → 2)Galβ(1 → 4)GlcNAcβ(1 → 6)]GalNAc-ol. The chemical shift values of H-2 and H-5 of GalNAc-ol of δ 4.387 and δ 4.271, respectively, support this structure. In fact d-4 is the α(1→2)fucosylated analog of compound b-4/c-5 and the corresponding shift effects on the 1−6 branch can be observed [5]: for H-1 of GlcNAc, $\Delta\delta$ = −0.022 and for H-1 of Gal[4], $\Delta\delta$ = +0.069 (see Table 2).

Structure b-8.1. The ^{1}H-NMR spectrum of fraction b-8 (Fig. 7), together with its sugar composition (see Table 1), indicate the main component in this fraction to be a hexasaccharide-alditol containing Gal, GlcNAc, Fuc, NeuAc and GalNAc-ol in a ratio of 2:1:1:1:1. The core of the main oligosaccharide-alditol consists of Galβ(1→3)[GlcNAcβ-(1→6)]GalNAc-ol as was deduced from the chemical shift values of H-2 (δ 4.385) and H-5 (δ 4.257) of GalNAc-ol. The core Gal is not terminal, since the concomitant chemical shift, i.e. δ 4.463 ppm, is not observed [4, 5]. A doublet in the anomeric region at δ 4.529 ppm can be assigned to Gal[3] being NeuAcα(2→3)-substituted. The ^{1}H-NMR parameter of this

Table 2. *^{1}H chemical shifts of structural-reporter groups of constituent monosaccharides for oligosaccharide-alditols present in HPLC fractions b-1–b-8, c-1–c-9, d-3 and d-4 possessing the Galβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol core or the Galβ(1→4)GlcNAcβ(1→3)-Galβ(1→3)GalNAc-ol core, all being derived from bronchial mucus glycoproteins of patients with cystic fibrosis, together with compound 4a$_2$ from [4]*

A superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. Chemical shifts are given relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using internal acetone at δ = 2.225) in D_2O at 27°C, acquired at 500 MHz. For complete structures of the compounds, see scheme 2. In the table heading the structures are represented by the short-hand symbolic notation defined previously [4, 14]; ◇ = GalNAc-ol; ■ = Gal; ● = GlcNAc; □ = Fuc; △ = NeuAc^3 and ○ = NeuAc^6. The type of linkage in ■ – ● is (1→4) unless otherwise indicated. n. d. indicates values could not be determined merely by inspection of the spectrum

Residue	Reporter group	Chemical shift in						
		c-3	b-4/c-5	b-5/c-6.1	d-4	b-8.1	4a$_2$	b-6.2
								
		ppm						
GalNAc-ol	H-2	4.393	4.389	4.392	4.387	4.385	4.400	4.398
	H-3	4.060	4.069	4.059	4.068	4.068	4.051	4.049
	H-4	3.460	3.475	3.463	n. d.	3.425	3.497	n. d.
	H-5	4.283	4.271	4.282	4.271	4.257	4.185	4.191
	NAc	2.067	2.065	2.065	2.067	2.066	2.047	2.049
Gal$_3$	H-1	4.463	4.532	4.461	4.532	4.529	4.464	4.464
	H-3	3.6–3.7	4.114	3.6–3.7	4.113	4.113	n. d.	n. d.
	H-4	3.901	3.922	3.899	3.926	3.927	4.126	4.130
GlcNAc^6	H-1	4.557	4.558	4.551	4.536	4.558	–	–
	H-6	3.998	3.993	4.008	3.998	4.004	–	–
	NAc	2.064	2.065	2.061	2.064	2.055	–	–
Gal^4	H-1	4.469	4.467	4.548	4.536	4.445	4.481	4.458
	H-3	3.6–3.7	3.6–3.7	4.115	3.6–3.7	3.6–3.7	3.927	n. d.
GlcNAc$_3$	H-1	–	–	–	–	–	4.688	4.709
	H-6	–	–	–	–	–	3.954	n. d.
	NAc	–	–	–	–	–	2.042	2.061
Fuc^2	H-1	–	–	–	5.308	–	–	–
	H-5	–	–	–	4.23	–	–	–
	CH_3	–	–	–	1.227	–	–	–
Fuc^3	H-1	–	–	–	–	5.106	–	–
	H-5	–	–	–	–	4.828	–	–
	CH_3	–	–	–	–	1.173	–	–
NeuAc^3	H-3*ax*	–	1.801	1.799	1.800	1.800	–	–
	H-3*eq*	–	2.773	2.755	2.772	2.773	–	–
	NAc	–	2.032	2.030	2.033	2.033	–	–
NeuAc^6	H-3*ax*	–	–	–	–	–	–	1.723
	H-3*eq*	–	–	–	–	–	–	2.671
	NAc	–	–	–	–	–	–	2.029

NeuAc are another indication of this element (compare structure d-4). The structural-reporter-group resonances of Fuc, namely H-1 (δ 5.106), H-5 (δ 4.828) and CH_3 (δ 1.173) point to the partial structure Galβ(1→4)[Fucα(1→3)]-GlcNAcβ(1→6) [4, 5]. The terminal position of Gal^4 is supported by its anomeric doublet at δ = 4.445 ppm. Combination of these data indicates the structure of the main oligosaccharide-alditol in fraction b-8 to be: NeuAcα(2→3)Galβ-(1→3)[Galβ(1→4)[Fucα(1→3)]GlcNAcβ(1→6)]GalNAc-ol. This is an α(1→3)-fucosylated analog of compound b-4/c-5. The corresponding shift effects on the ^{1}H-NMR characteristics of b-5/c-6.1 are readily recognized [4]: an upfield shift for Gal^4 H-1 and for the NAc signal of GlcNAc^6 of 0.022 and 0.008 ppm, respectively, and a downfield shift for H-6 of GlcNAc^6 of 0.011 ppm. No effect on GlcNAc^6 H-1 is observed. The ^{1}H-NMR spectrum indicates the presence of a minor compound (15%) of which the structure could be unraveled as well (see below, b-8.2).

Structures with the element GlcNAcβ(1→3)[NeuAcα(2→6)]GalNAc-ol

The structures of Scheme 3 and Table 3 have in common the element GlcNAcβ(1→3)[NeuAcα(2→6)]GalNAc-ol. A trisaccharide comprising the common element has recently been obtained in the oligosaccharide-alditol fractionation of sputum of a patient suffering from bronchiectasis (H. van Halbeek, unpublished results). For comparison the ^{1}H-NMR parameters of this compound (R1) are added to Table 3. Typical chemical shifts for the oligosaccharide-alditols in this

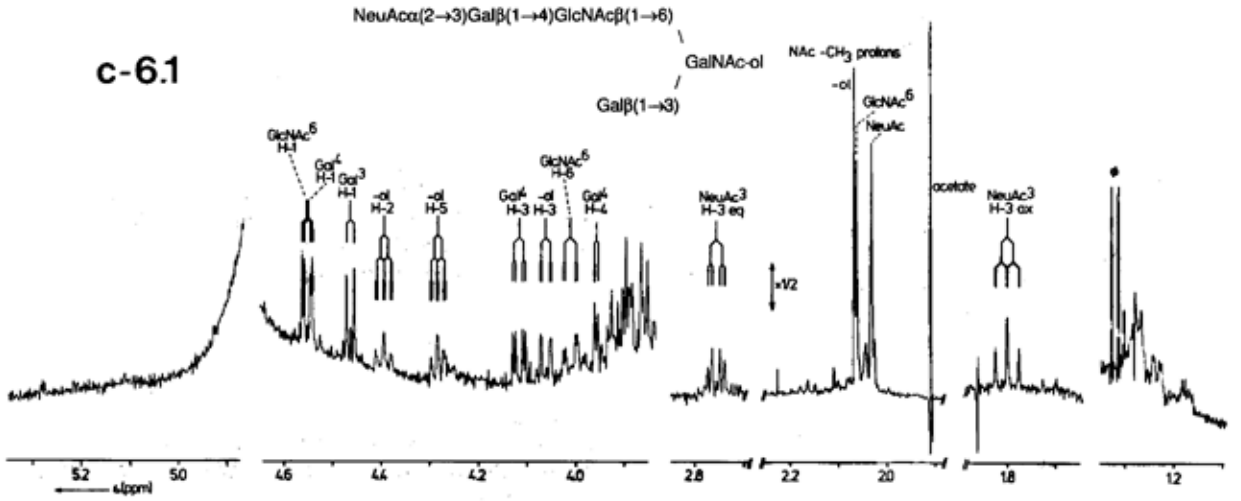


Fig. 6. *500-MHz ^{1}H-NMR spectrum (D_2O, pD 7, 27°C) of CF bronchial sialyl-oligosaccharide-alditol fraction c-6.* The relative intensity scale of the *N*-acetyl methyl proton region ($1.85 < \delta < 2.30$) deviates from that of the other parts of the corresponding spectrum. Resonances marked by ∅ stem from non-protein, non-carbohydrate contaminants

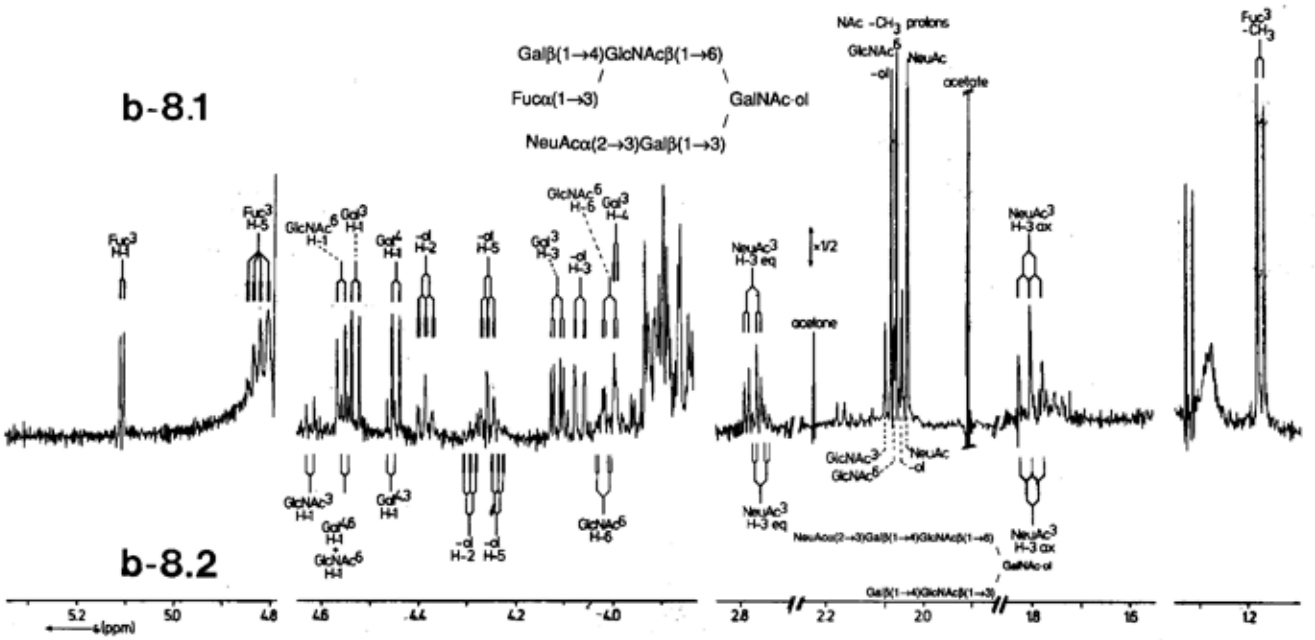


Fig. 7. *500-MHz ^{1}H-NMR spectrum (D_2O, pD 7, 27°C) of CF bronchial sialyl-oligosaccharide-alditol fraction b-8.* The relative intensity scale of the *N*-acetyl methyl proton region ($1.85 < \delta < 2.30$) deviates from that of the other parts of the corresponding spectrum. Resonances marked by ∅ stem from non-protein, non-carbohydrate contaminants

group are those of H-2 and H-5 of GalNAc-ol at δ 4.24–4.26 and δ 4.15–4.19, respectively, and of H-3*ax* and H-3*eq* of NeuAc at δ 1.697 and δ 2.733 respectively (see Table 3). The latter two values are constant elements in the ^{1}H-NMR spectra and can be regarded as markers for this group of carbohydrate structures. The chemical shift values of the structural-reporter group of GlcNAc^3 in the common element are largely influenced by the remainder of the structure.

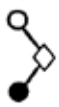
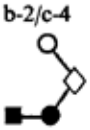
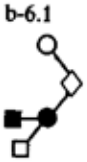
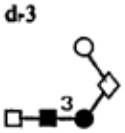
Structure b-2/c-4. The ^{1}H-NMR spectrum of the fractions b-2 and c-4 are identical (see Fig. 8). From the ^{1}H-NMR parameters and the carbohydrate composition (Table 1) it can be concluded that the compound present is a tetrasaccharide-alditol containing Gal, GlcNAc, NeuAc and GalNAc-ol. The shift values of H-3*ax* (δ 1.697) and H-3*eq* (δ 2.733) of NeuAc, together with the chemical-shift values of H-2 (δ 4.261) and H-5 (δ 4.187) of GalNAc-ol are indicative of the core GlcNAcβ(1→3)[NeuAcα(2→6)]GalNAc-ol. Structure b-2/c-4 has GlcNAc extended with a Gal residue, which is evident from two doublets present in the anomeric region representing the β-linked GlcNAc (δ 4.633) and the β-linked Gal (δ 4.465) residues. The latter value is indicative of a Gal residue in a terminal position, linked to GlcNAc [4]. From the chemical shifts of the GlcNAc^3 structural-reporter groups, that is, H-1 (δ 4.633) and NAc (δ 2.067), in combination with that of the Gal H-1 doublet (δ 4.465), it is evident that the Gal residue is β(1→4)-linked to GlcNAc^3 [4–6]. Also the features of

the GlcNAc H-6 signal are in full agreement with a $\beta(1\rightarrow4)$ linkage, while the absence of a doublet of doublets for H-3 of GlcNAc[3] at 3.91 ppm excludes the possibility of a $\beta(1\rightarrow3)$ linkage. These findings determine the structure present in fraction b-2 and c-4 as Galβ(1→4)GlcNAcβ(1→3)[NeuAcα(2→6)]GalNAc-ol.

Structure b-6.1. The ^{1}H-NMR spectrum of the fraction b-6 (see Fig. 9) shows the presence of a major oligosaccharide-alditol and of a minor compound (20%). The spectrum together with the results of the sugar analysis (Table 1) indicate the major component to be a pentasaccharide-alditol containing Gal, GlcNAc, Fuc, NeuAc and GalNAc-ol. The chemical shift values for H-3*eq* (δ 2.733) and H-3*ax* (δ 1.697) of NeuAc, in combination with the chemical shift values for H-2 (δ 4.247) and H-5 (δ 4.175) of GalNAc-ol, are indicative of the core GlcNAcβ(1→3)[NeuAcα(2→6)]GalNAc-ol. The observable structural-reporter-group resonances of Fuc, namely H-1 (δ 5.132) and CH_3 (δ 1.176) are found at positions that are characteristic of the Galβ(1→4)[Fucα(1→3)]GlcNAcβ(1→.) structural element [4, 5, 14]. The combination of these data defines the structure of the main carbohydrate constituent in fraction b-6 to be Galβ(1→4)[Fucα(1→3)]GlcNAcβ-(1→3)[NeuAcα(2→6)]GalNAc-ol. This structure is an extension of b-2/c-4 with a Fuc residue in $\alpha(1\rightarrow3)$ linkage and the corresponding shift effects on the structural-reporter groups of b-2/c-4 are accordingly [6]: for H-2 and H-5 of GalNAc-ol, $\Delta\delta = -0.014$ and -0.012 respectively, for NAc of GalNAc-ol, $\Delta\delta = -0.009$, for H-1 of GlcNAc[3], $\Delta\delta = +0.012$ and for NAc of GlcNAc[3], $\Delta\delta = -0.009$. From the ^{1}H-NMR spectrum the primary structure of the minor component in fraction b-6 could be deduced as well (see below, b-6.2).

Structure d-3. Sugar analysis of fraction d-3 (Table 1) suggests it to contain a pentasaccharide-alditol consisting of Gal, GlcNAc, Fuc, NeuAc and GalNAc-ol. This is also evident from the ^{1}H-NMR spectrum, despite the low amount of material and signals stemming from non-carbohydrate material. The *N*-acetyl signal at 2.107 ppm is characteristic of the structural element Fucα(1→2)Galβ(1→3)GlcNAcβ-(1→3)GalNAc-ol with GalNAc-ol bearing a substituent at C-6 [5]. The values of the structural-reporter groups H-1 (δ 5.208) and CH_3 (δ 1.235) of Fuc support this structure. The values for H-3*ax* (δ 1.697) and H-3*eq* (δ 2.734) of NeuAc are indicative of the core GlcNAcβ(1→3)[NeuAcα(2→6)]-GalNAc-ol. Combination of these two clearly recognizable

Table 3. ^{1}*H chemical shifts of structural-reporter groups of constituent monosaccharides for oligosaccharide-alditols present in HPLC fractions b-1–b-8, c-1–c-9, d-3 and d-4 possessing the GlcNAcβ(1→3)[NeuAcα(2→6)]GalNAc-ol core, all being derived from bronchial mucus glycoproteins of patients with cystic fibrosis, together with compound R1 (see text)*
Details are as in Table 2

Residue	Reporter group	Chemical shift in			
		R1 	b-2/c-4 	b-6.1 	d-3 
		ppm			
GalNAc-ol	H-2	4.260	4.261	4.247	4.237
	H-5	4.185	4.187	4.175	4.147
	NAc	2.035	2.034	2.025	2.033
GlcNAc[3]	H-1	4.608	4.633	4.645	4.652
	H-6	3.985	4.009	4.025	3.942
	NAc	2.079	2.076	2.067	2.107
Gal	H-1	–	4.465	4.447	4.577
	H-3	–	3.6–3.7	n. d.	n. d.
Fuc[2]	H-1	–	–	–	5.208
	H-5	–	–	–	4.27
	CH_3	–	–	–	1.235
Fuc[3]	H-1	–	–	5.132	–
	H-5	–	–	n. d.	–
	CH_3	–	–	1.176	–
NeuAc[6]	H-3*ax*	1.697	1.697	1.697	1.697
	H-3*eq*	2.733	2.733	2.733	2.734
	NAc	2.031	2.031	2.034	2.030

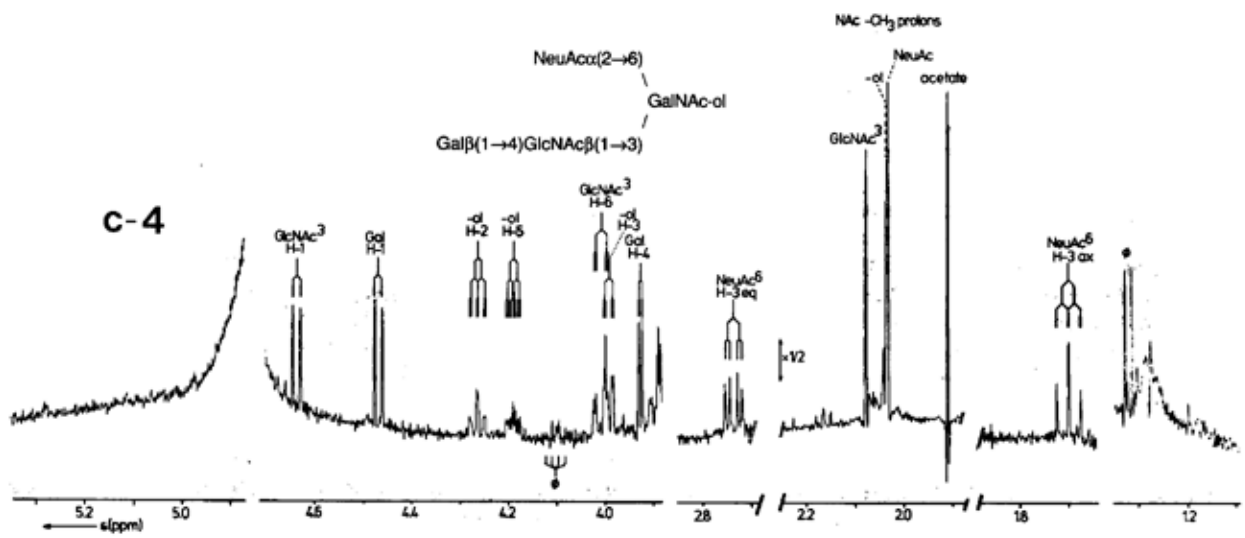


Fig. 8. *500-MHz ^{1}H-NMR spectrum (D_2O, pD 7, 27°C) of CF bronchial sialyl-oligosaccharide-alditol fraction c-4.* The relative intensity scale of the *N*-acetyl methyl proton region ($1.85 < \delta < 2.30$) deviates from that of the other parts of the corresponding spectrum. Resonances marked by ∅ stem from non-protein, non-carbohydrate contaminants

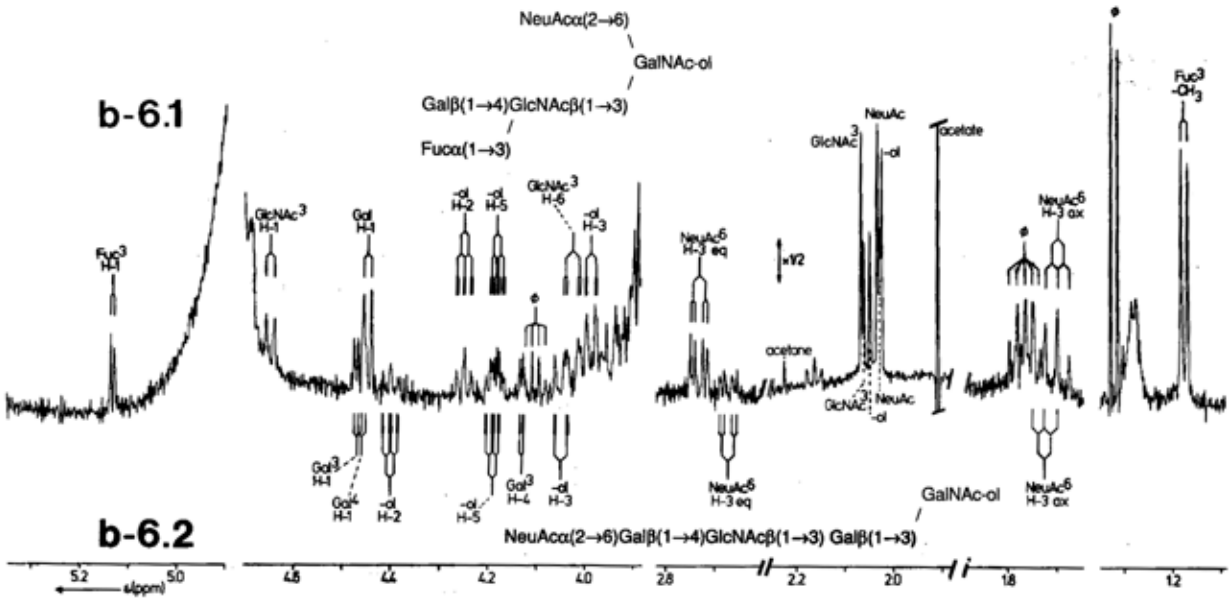


Fig. 9. *500-MHz ^{1}H-NMR spectrum (D_2O, pD 7, 27°C) of CF bronchial sialyl-oligosaccharide-alditol fraction b-6.* The relative intensity scale of the *N*-acetyl methyl proton region ($1.85 < \delta < 2.30$) deviates from that of the other parts of the corresponding spectrum. Resonances marked by ∅ stem from non-protein, non-carbohydrate contaminants

elements determines the structure of the oligosaccharide-alditol in fraction d-3 as Fucα(1→2)Galβ(1→3)GlcNAcβ(1→3)[NeuAcα(2→6)]GalNAc-ol.

Structures with the element GlcNAcβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol

The structures in Scheme 4 have in common the element GlcNAcβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol. The latter structure could be identified as such by ^{1}H-NMR spectroscopy of a fraction of neutral bronchial oligosaccharide-alditols of patients suffering from cystic fibrosis [4]. The characteristic ^{1}H-NMR chemical shifts of this compound (4a$_1$) have been included in Table 4. Chemical shift values for H-2 and H-5 of GalNAc-ol for structures containing this element are δ 4.26–4.29 and δ 4.21–4.24 respectively [4, 5], the exact value determined by substitutions in this core. Other typical features in the ^{1}H-NMR spectra are the NAc signal of GalNAc-ol at δ 2.041–2.046, the H-1 of GlcNAc^6 at δ 4.55–4.57 and the NAc signal of GlcNAc^6 at δ 2.057–2.066 ppm. ^{1}H chemical shift values of the structural-reporter groups of GlcNAc^3 and Gal^{4,6} are specific for substitutions in these sugars.

Structure b-3. The ^{1}H-NMR spectrum of fraction b-3 was identical to that of subfraction A-2 of fraction a of the pool of smaller oligosaccharides from cystic fibrosis sputum [6] (see also Scheme 1). Therefore the structure b-3 is GlcNAcβ(1→3)[NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol, which is in accordance with the sugar composition (Table 1).

Structure b-8.2. From the lower intensity signals in the ^{1}H-NMR spectrum of fraction b-8 (see Fig. 7) it could be concluded that the minor component had the core structure GlcNAcβ(1→3)[GlcNAcβ(1→6)]GalNAc-ol as was indicated by the chemical shift values of H-2 (δ 4.293) and H-5 (δ 4.235) of GalNAc-ol. The resonances of H-3*eq* (δ 2.757) and H-3*ax* (δ 1.80) of NeuAc pointed to NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→.) as partial structure. The H-3*ax* signal was partly obscured by signals stemming from non-carbohydrate material. In the anomeric region of the spectrum two Gal H-1 signals could be observed, one being specific for a terminal Galβ(1→4) residue (δ 4.457), the other demonstrating a Gal residue substituted with NeuAc in α(2→3) linkage (δ 4.551). These observations indicate the structure to be a sialylated analog of Galβ(1→4)GlcNAcβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol (oligosaccharide 6a1 in [4]). When comparing b-8.2 to its asialo analog, the chemical shift value for the H-1 of the terminal Gal shows this sugar to occur in the Galβ(1→4)GlcNAcβ(1→3)GalNAc-ol branch. The downfield shift of 0.077 ppm for H-1 of Gal^{4,6}, together with the upfield shift of the NAc signal of GlcNAc^6 of 0.003 ppm, are characteristic for NeuAcα(2→3) attachment to Gal^{4,6}. From the above findings it can tentatively be concluded that the structure of the minor compound in fraction b-8 is Galβ(1→4)GlcNAcβ(1→3)[NeuAcα(2→3)-Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol. Structure b-8.2 can also be thought of as an extension of b-3 with a Galβ(1→4) residue attached to GlcNAc^3. The major shift effects corresponding to this type of extension are observed on H-1 and NAc of GlcNAc^3: $\Delta\delta$ = +0.023 and −0.002 ppm, respectively [4].

Structure b-7. The ^{1}H-NMR spectrum of fraction b-7 in combination with the sugar composition (compare Table 1) shows the presence of a hexasaccharide-alditol containing Gal, GlcNAc, NeuAc and GalNAc-ol in a ratio of 2:2:1:1. The GalNAc-ol residue is substituted by GlcNAc residues in β(1→3) and β(1→6) linkage as can be inferred from the values of H-2 (δ 4.286) and H-5 (δ 4.238) of GalNAc-ol. The values of H-3*ax* (δ 1.789) and of H-3*eq* (δ 2.757) of NeuAc are typical for NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→.). Similar to b-8.2, in the ^{1}H-NMR spectrum two Gal H-1 signals are observed, one pointing to a terminal Galβ(1→4) (δ 4.475), the other to Gal extended with NeuAc in α(2→3) linkage (δ 4.533). These data suggest that the structure is yet another sialylated analog of Galβ(1→4)GlcNAcβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol [4]. Comparing b-7 to the asialo analog, the presence of the terminal Galβ-

Table 4. *^{1}H chemical shifts of structural-reporter groups of constituent monosaccharides for oligosaccharide-alditols present in HPLC fractions b-1–b-8, c-1–c-9, d-3 and d-4 possessing the GlcNAcβ(1→3)[Galβ(1→4)GlcNacβ(1→6)]GalNAc-ol core, all being derived from bronchial mucus glycoproteins of patients with cystic fibrosis, together with compound 4a$_1$ from [4]*
Details are as in Table 2

Residue	Reporter group	Chemical shift in					
		4a$_1$	b-3	b-8.2	b-7	c-7.1	c-9.1
		ppm					
GalNAc-ol	H-2	4.282	4.278	4.293	4.286	4.278	4.256
	H-5	4.239	4.235	4.235	4.238	4.238	4.205
	NAc	2.045	2.044	2.044	2.046	2.042	2.041
GlcNAc^3	H-1	4.599	4.599	4.622	4.615	4.648	4.651
	NAc	2.081	2.080	2.078	2.076	2.068	2.108
GlcNAc^6	H-1	4.564	4.557	4.551	4.568	4.552	4.564
	NAc	2.061	2.058	2.059	2.066	2.058	2.057
Gal^{x,3}	H-1	–	–	4.457	4.533	4.451	4.564
	H-3	–	–	3.6–3.7	4.112	n. d.	n. d.
Gal^{4,6}	H-1	4.473	4.551	4.551	4.475	4.554	4.546
	H-3	3.6–3.7	4.113	4.113	n. d.	4.114	4.114
Fuc^2	H-1	–	–	–	–	–	5.209
	H-5	–	–	–	–	–	4.272
	CH_3	–	–	–	–	–	1.230
NeuAc^3	H-3*ax*	–	1.798	1.80	1.798	1.799	1.800
	H-3*eq*	–	2.757	2.757	2.757	2.76	2.760
	NAc	–	2.032	2.033	2.032	2.032	2.030

(1→4)GlcNAcβ(1→6)GalNAc-ol branch in b-7 is indicated by the H-1 chemical shift values, i. e. Gal H-1, δ 4.475 and GlcNAc H-1, δ 4.568 and the *N*-acetyl signal of GlcNAc^6 at 2.066 ppm [4]. The difference between the chemical shift of this *N*-acetyl signal and that of the corresponding signal of the asialo analog of 0.004 ppm, is due to extension of the 3-branch. Owing to the attachement of NeuAc in α(2→3) linkage, the signal for Gal^{4,3} H-1 (δ 4.533) shows a typical downfield shift of 0.077, the GlcNAc^3 H-1 shows a downfield shift of 0.009 ppm, and GlcNAc^3 *N*-acetyl signal the characteristic upfield shift of 0.003 ppm. These data demonstrate the structure of the oligosaccharide-alditol present in fraction b-7 to be NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→3)[Galβ-(1→4)-GlcNAcβ(1→6)]GalNAc-ol.

Structure c-7.1. From the intensities of the signals in the ^{1}H-NMR spectrum of fraction c-7 it is apparent that c-7 contains a mixture of oligosaccharide-alditols with two main and at least two minor components. The primary structure of these compounds could not be deduced conclusively from the ^{1}H-NMR spectrum. Therefore this fraction was isocratically rechromatographed on HPLC to give five subfractions. The amount of material obtained after this step was strongly reduced and only the ^{1}H-NMR spectrum of the first subfraction (c-7.1) allowed interpretation of the ^{1}H-NMR spectrum, affording the structure of the major oligosaccharide-alditol in this subfraction. The ^{1}H-NMR spectrum of fraction c-7.1, together with the sugar composition (Table 1) indicate the presence of a hexasaccharide-alditol containing Gal, GlcNAc, NeuAc and GalNAc-ol in a molar ratio of 2:2:1:1 (The presence of 0.8 fucose indicated in Table 1 is probably due to a contamination by an oligosaccharide-alditol with a Y-determinant; data not shown.) The core structure of this oligosaccharide-alditol is GlcNAcβ(1→3)[GlcNAcβ(1→6)]-GalNAc-ol, as is clear from the chemical shift values of H-2 (δ 4.278) and H-5 (δ 4.238) of GalNAc-ol. The chemical shift values for H-1 and NAc of GlcNAc^3 at δ 4.648 and δ 2.068 respectively, are characteristic for a Galβ(1→3) substitution on GlcNAc^3 [5]. The latter Gal residue is in terminal position as can be inferred from its H-1 signal (δ 4.451). The chemical shift of the anomeric signal of a β(1→4)-linked Gal residue (δ 4.554) substituted with an α(2→3)-linked NeuAc residue, in combination with the chemical shift values of H-3*eq* (δ 2.76) and H-3*ax* (δ 1.799) of NeuAc, point to the structural element NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→.). These data show the structure of compound c-7.1 to be Galβ(1→3)GlcNAcβ-(1→3)[NeuAcα(2→3)Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol. This structure can be considered as a extension of b-3 with Gal in β(1→3) linkage to GlcNAc^3. The concomitant shift-effects are comparable to the extension of 10A to 15B in [5]: Δδ for NAc of GalNAc-ol = −0.002, Δδ for H-2 and H-5 of GalNAc-ol are negligible and Δδ for H-1 and NAc of GlcNAc^3 are +0.049 and −0.012 ppm, respectively.

Structure c-9. The ^{1}H-NMR spectrum of fraction c-9 (Fig. 10) together with the sugar composition (Table 1) indicate the presence of a heptasaccharide-alditol containing Gal, GlcNAc, Fuc, NeuAc and GalNAc-ol in a molar ratio of 2:2:1:1:1. The ^{1}H-NMR spectrum of this fraction indicates also the presence of a minor compound, the structure of which could not be determined due to the low amount of material. The core of the main oligosaccharide-alditol is GlcNAcβ-(1→3)[GlcNAcβ(1→6)]GalNAc-ol, as can be derived from the chemical shift values of H-2 (δ 4.256) and H-5

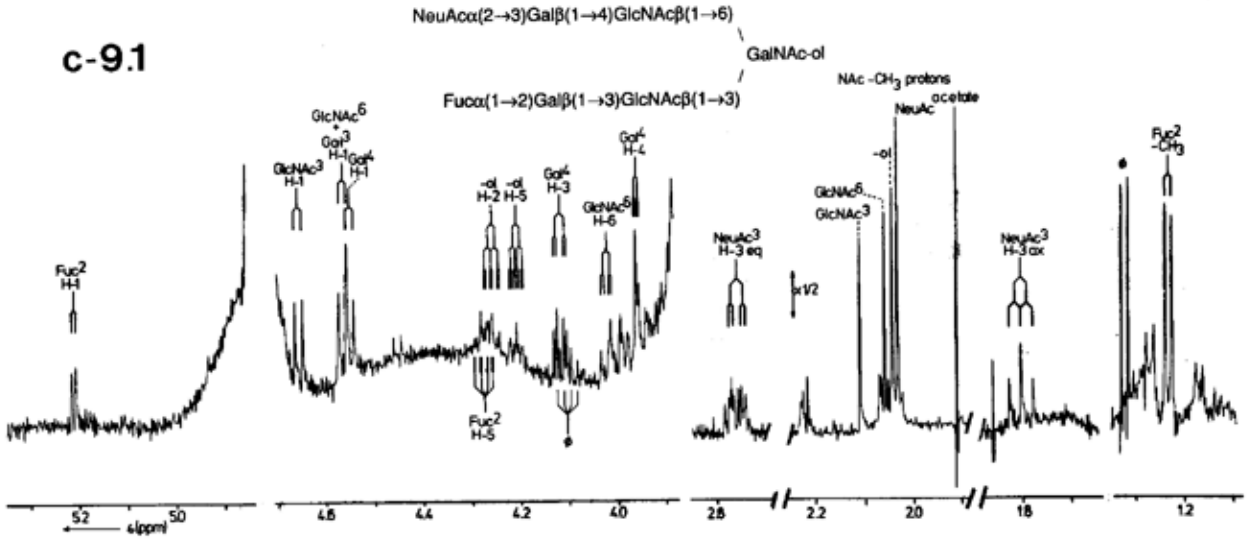


Fig. 10. *500-MHz ^{1}H-NMR spectrum (D_2O, pD 7, 27°C) of CF bronchial sialyl-oligosaccharide-alditol fraction c-9.* The relative intensity scale of the *N*-acetyl methyl proton region ($1.85 < \delta < 2.30$) deviates from that of the other parts of the corresponding spectrum. Resonances marked by ∅ stem from non-protein, non-carbohydrate contaminants

(δ 4.205) of GalNAc-ol. The *N*-acetyl resonance of GlcNAc^3 at 2.108 ppm is typical for the structural element Fucα(1→2)Galβ(1→3)GlcNAcβ(1→3)GalNAc-ol, wherein GalNAc-ol bears a substituent at C-6 [4, 5]. The occurrence of this element is confirmed by the position of H-1 (δ 5.209) and CH_3 (δ 1.230) of the Fuc residue. The presence of NeuAc α(2→3)-linked to the *N*-acetyllactosamine unit is demonstrated by its structural-reporter groups H-3*ax* (δ 1.800) and H-3*eq* (δ 2.760) and supported by the doublet of H-1 of Gal^{4,6} (δ 4.546). Combination of these data determines the structure to be Fucα(1 → 2)Galβ(1 → 3)GlcNAcβ(1 → 3)[NeuAcα(2 → 3)-Galβ(1 → 4)GlcNAcβ(1 → 6)]GalNAc-ol. This compound is the sialylated analog of structure 19 in [5] and the concomitant shift effects are observed: a downfield shift for H-1 of Gal^{4,6} of 0.078 ppm and an upfield shift of 0.002 ppm on NAc of GlcNAc^6.

Structure with the Galβ(1→3)GalNAc-ol core type

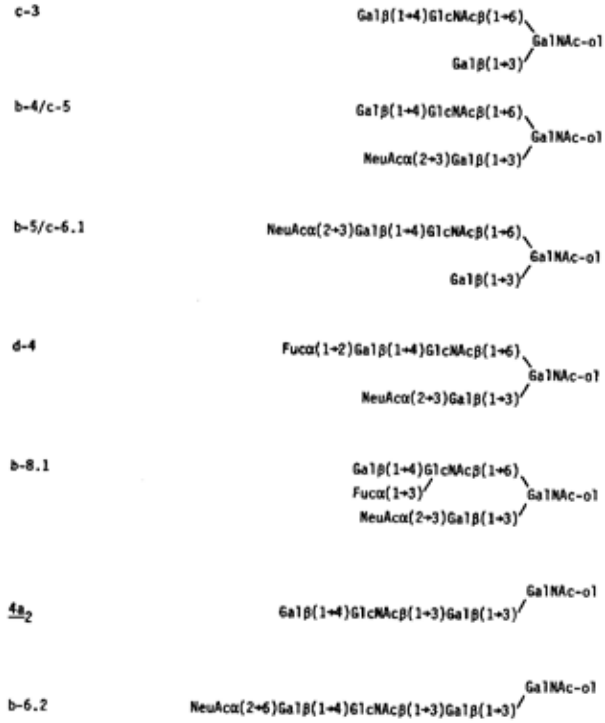
Structure b-6.2. The ^{1}H-NMR spectrum of fraction b-6.2 (see Fig. 9) indicated also the presence of a minor carbohydrate compound (25%), containing Gal, GlcNAc, NeuAc and GalNAc-ol. The core of this structure is Galβ(1→3)GalNAc-ol, as is evident from the values of H-2 (δ 4.398) and H-5 (δ 4.191) of GalNAc-ol [4]. For this structure as well as for a reference compound (structure 4a$_2$ in [4]), the ^{1}H-NMR characteristics and the structure are compiled in Table 2 and Scheme 2. The core Gal^3 residue is substituted at C-3 in β-linkage by a GlcNAc residue, as can be derived from the chemical shift of the Gal^3 H-4 atom (δ 4.130), which is known to be characteristic of the →.)GlcNAcβ(1→3)-Galβ(1→.) sequence [4]. The values of H-3*ax* (δ 1.723) and H-3*eq* (δ 2.671) are indicative of the presence of NeuAc in α(2→6) linkage to a lactosamine unit [14]. These findings suggest the structure of the minor oligosaccharide-alditol compound in fraction b-6 to be NeuAcα(2→6)Galβ-(1→4)GlcNAcβ(1→3)Galβ(1→3)GalNAc-ol. This structure is the α(2→6)-sialylated analog of compound 4a$_2$ in [4] and in fact all structural-reporter-group chemical shift effects are in accordance with this extension: an upfield shift for H-1 of Gal^4 of 0.023 ppm and a downfield shift for H-1 and NAc of GlcNAc^3 of 0.021 ppm and 0.019 ppm respectively.

Scheme 1. *Structures of sialylated oligosaccharide-alditols obtained fromDAX4 fraction a [6] of bronchial mucins of CF patients*

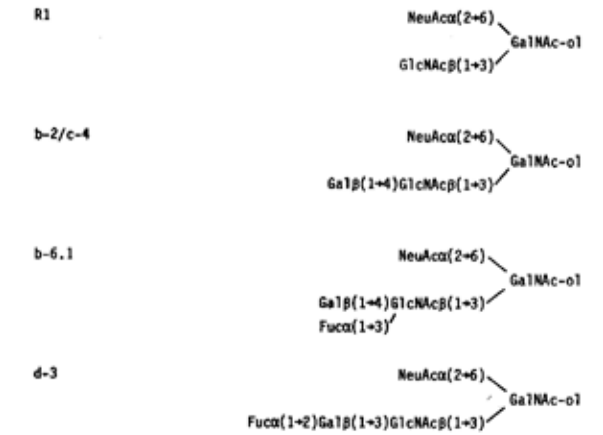
DISCUSSION

Acidic bronchial mucus glycoproteins were obtained from six cystic fibrosis patients with blood-group O [6]. Alkaline borohydride treatment of the purified glycoproteins led to a heterogeneous mixture of glycopeptides and reduced oligosaccharides. Four pools of low-molecular-mass oligosaccharide-alditols were obtained: one consisting of neutral oligosaccharide-alditols (Ic), one of sialylated oligosaccharide-alditols (IIc) and two of sulfated oligosaccharide-alditols [8, 9]. The structure determination of 21 oligosaccharide-alditols from the neutral fraction Ic has already been reported [4, 5]. The low-molecular-mass sialyl-oligosaccharide-alditols (fraction IIc, representing 7% of the total carbohydrate released) were isolated from the pool [6], separated by DAX4 anion-exchange chromatography (subfractions a–e) and subsequently by HPLC on Lichrosorb-NH_2.

Previously, we reported the structure of five compounds fast-eluting from the DAX4 (fraction a): four of them



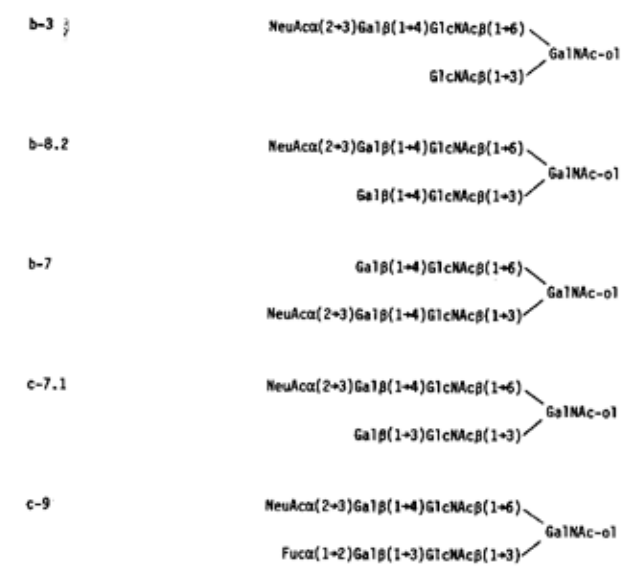
Scheme 2. *Structures of the series of oligosaccharide-alditols with the Galβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol core or Galβ-(1→4)GlcNAcβ(1→3)Galβ(1→3)GalNAc-ol core, obtained from bronchial mucins of patients suffering from cystic fibrosis*



Scheme 3. *Structures of the series of oligosaccharide-alditols with the GlcNacβ(1→3)[NeuAcα(2→6)]GalNAc-ol core, obtained from bronchial mucins of patients suffering from cystic fibrosis*
The structures are compared with the compound R1 recently obtained in the oligosaccharide fractionation of respiratory mucins from a patient suffering from bronchiectasis (H. Van Halbeek, unpublished results)

contained the NeuAcα(2→3)Galβ(1→4)[Fucα(1→3)]-GlcNAcβ(1→.) structure, the so-called sialyl Le^x (antigenic) determinant [6] (see Scheme 1).

In this study, we have completed the characterization of HPLC-separated subfractions of DAX4 fractions b, c and d. The structures of the 17 oligosaccharide-alditols have been summarized in Schemes 2–4. The recovery of HPLC-purified oligosaccharide-alditols that were structurally characterized by combination of ^{1}H-NMR spectroscopy and sugar analysis, was 40% of pool IIc (fraction b was the most abundant fraction and made up about 70% of the recovered



Scheme 4. *Structures of the series of oligosaccharide-alditols with the GlcNAcβ(1→3)[Galβ(1→4)GlcNAcβ(1→6)]GalNAc-ol core, obtained from bronchial mucins of patients suffering from cystic fibrosis*

oligosaccharides). The efficiency of the DAX4 chromatography step (Fig. 1) is revealed in the relatively small overlap of compounds present in the fractions a–d, i.e. only compounds a-2, b-2, b-4 and b-5 are present in detectable amounts in two different DAX4 fractions. More importantly, all structures containing the sialyl Le^x determinant are only present in fraction a.

HPLC is very convenient for the purification of this size of oligosaccharides. The elution sequence of the sialyl-oligosaccharide-alditols seems to be essentially related to size.

In this study the structural determination of the oligosaccharide-alditols has been carried out by a combination of quantitative sugar analysis and 500-MHz ^{1}H-NMR spectroscopy. Analysis by ^{1}H-NMR is performed by application of empirical rules as developed previously in the structural characterization of neutral and sialic-containing oligosaccharide-alditols [4–6, 13, 14].

Previously reported structures were determined by a combination of ^{1}H-NMR spectroscopy, sugar analysis and/or methylation and/or exoglycosidase treatment. Since then, the sensitivity of structural-reporter-group chemical shifts to differences in primary structures has been well recognized. In this paper all structures are closely related to those reported before; thus, they can be established straightforwardly using the concept of NMR structural-reporter groups only.

The amount of material available precludes application of more advanced ^{1}H-NMR techniques. Therefore, for some NMR signals, the assignment is tentative, e.g. the *N*-acetyl signals of the structures as reported in Table 2. However the primary structures are firmly established since they rely on clearly recognized structural-reporter groups.

The present study emphasizes the wide heterogeneity of the oligosaccharides from human bronchial mucins. From a structural point of view, four common core types were found to occur in the sialyl-oligosaccharides of fraction IIc. Among the 17 sialyl-oligosaccharides described in a previous paper [6] and in this present work, three oligosaccharides had a blood-group O determinant (c-9, d-3 and d-4); 14 oligosaccharides correspond to extensions of neutral oligosaccharides obtained in fraction Ic [4, 5].

N-Acetylneuraminic acid is linked either α(2→6) to *N*-acetylgalactosaminitol (in three oligosaccharides) or α(2→3) to galactose (core Gal^3, or Gal^4 in an *N*-acetyllactosaminyl unit). In oligosaccharide b-6.2, *N*-acetylneuraminic acid is linked α(2→6) to Gal^4 in an *N*-acetyllactosaminyl unit. Such a linkage has already been described in rat sublingual [15] and colonic mucins [16], and also in human colonic mucins [17].

The yield of the different HPLC oligosaccharide-alditol fractions (see Table 1) indicates that bronchial mucins from CF patients seem to have a predominance of oligosaccharides containing sialic acid in α(2→3) linkage. The occurrence of NeuAcα(2→6) attached to an *N*-acetyllactosamine unit in a minor component in a single fraction in the total of HPLC fractions of IIc, may be conceived as an indication of a further heterogeneity in the oligosaccharides present in smaller quantities in fraction IIc.

Oligosaccharide b-2 has been observed in rat and human colonic mucins [16, 17] and oligosaccharide b-4 occurred in a cell-surface sialoglycoprotein of ascites sublines from a rat mammary adenocarcinoma [18]. All other oligosaccharide structures reported in the present work are novel.

The structures of sialylated oligosaccharides described so far are compatible with the presence, within the bronchial mucosa, of the four sialyl-transferases that seem to be responsible for the synthesis of most of the O-linked sialylated oligosaccharides: *N*-acetylgalactosaminide (α2→6)sialyltransferase, galactosyl (β1→3)*N*-acetylgalactosaminide (α2→3)sialyltransferase, galactosyl (β1→3/4)*N*-acetylglucosaminide (α2→3)sialyltransferase and galactoside (α2→6)sialyltransferase [19].

It has been pointed out in previous studies using labeled lectins [20], that the sialylated mucins synthesized by goblet cells and mucous cells of the bronchial mucosa may be different at least in the linkage of the sialic acid. These data suggest that the oligosaccharides described herein could stem from mucins synthesized by both kind of cells.

In cystic fibrosis, pulmonary infection with *Pseudomonas aeruginosa* is a common clinical complication that determines most morbidity and almost all excess mortality. Sialic acid residues have been shown to be involved in the binding of microorganisms such as *Pseudomonas aeruginosa* [21]. Therefore it would be very interesting to determine whether such a pattern in sialylated oligosaccharides and particularly the large predominance of α(2→3) linkages as compared to α(2→6) linkages, is specific for cystic fibrosis or may be also found in the mucins from other bronchial diseases.

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REFERENCES

1. Carlstedt, I., Sheehan, J. K., Corfield, A. P. & Gallagher, J. T. (1985) *Essays Biochem. 20*, 40–76.
2. Lamblin, G., Lhermitte, M., Degand, P., Roussel, P. & Slayter, H. S. (1979) *Biochimie (Paris) 61*, 23–43.
3. Lafitte, J. J., Lamblin, G., Lhermitte, M., Humbert, P., Degand, P. & Roussel, P. (1977) *Carbohydr. Res. 56*, 383–389.
4. Van Halbeek, H., Dorland, L., Vliegenthart, J. F. G., Hull, W. E., Lamblin, G., Lhermitte, M., Boersma, A. & Roussel, P. (1982) *Eur. J. Biochem. 127*, 7–20.
5. Lamblin, G., Boersma, A., Lhermitte, M., Roussel, P., Mutsaers, J. H. G. M., Van Halbeek, H. & Vliegenthart, J. F. G. (1984) *Eur. J. Biochem. 143*, 227–236.
6. Lamblin, G., Boersma, A., Klein, A., Roussel, P., Van Halbeek, H. & Vliegenthart, J. F. G. (1984) *J. Biol. Chem. 259*, 9051–9058.
7. Carlson, D. M. (1968) *J. Biol. Chem. 243*, 616–626.
8. Roussel, P., Lamblin, G., Degand, P., Walker-Nasir, E. & Jeanloz, R. W. (1975) *J. Biol. Chem. 250*, 2114–2122.
9. Lamblin, G., Humbert, P., Degand, P. & Roussel, P. (1977) *Clin. Chim. Acta 79*, 425–436.
10. Lamblin, G., Klein, A., Boersma, A., Nasir-Ud-Din & Roussel, P. (1983) *Carbohydr. Res. 118*, C1–C4.
11. Vliegenthart, J. F. G., Van Halbeek, H. & Dorland, L. (1981) *Pure Appl. Chem. 53*, 45–77.
12. Vliegenthart, J. F. G., Dorland, L., Van Halbeek, H. & Haverkamp, J. (1982) *Cell. Biol. Monogr. 10*, 127–172.
13. Van Halbeek, H., Dorland, L., Vliegenthart, J. F. G., Fiat, A. M. & Jollès, P. (1981) *FEBS Lett. 133*, 45–50.
14. Vliegenthart, J. F. G., Dorland, L. & Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–374.
15. Slomiany, A. & Slomiany, B. L. (1978) *J. Biol. Chem. 253*, 7301–7306.
16. Slomiany, B. L., Murty, V. L. N. & Slomiany, A. (1980) *J. Biol. Chem. 255*, 9719–9723.
17. Podolsky, D. K. (1985) *J. Biol. Chem. 260*, 15510–15515.
18. Hull, S. R., Laine, R. A., Kaizu, T., Rodriguez, I. & Carraway, K. L. (1984) *J. Biol. Chem. 259*, 4866–4877.
19. Sadler, J. E. (1984) in *Biology of carbohydrates* (Ginsburg V. & Robbins, P. W., eds), Vol. 2, pp. 199–288, John Wiley and Sons, New York.
20. Mazzuca, M., Lhermitte, M., Lafitte, J. J. & Roussel, P. (1982) *J. Histochem. Cytochem. 30*, 956–966.
21. Ramphal, R. J. & Pyle, M. (1983) *Infect. Immunol. 41*, 339–344.
22. Lamblin, G., Lhermitte, M., Boersma, A., Roussel, P. & Reinhold, V. (1980) *J. Biol. Chem. 255*, 4595–4598.