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Further characterization, by a combined high-performance liquid chromatography/^{1}H-NMR approach, of the heterogeneity displayed by the neutral carbohydrate chains of human bronchial mucins

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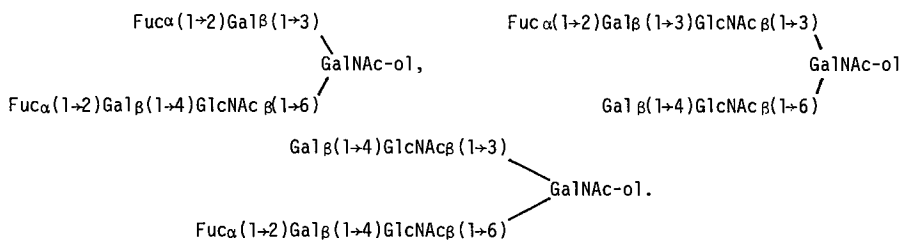
From bronchial mucins of cystic fibrosis patients with blood group O, the carbohydrate chains were released in the form of oligosaccharide-alditols by alkaline borohydride treatment. Application of high-performance liquid chromatography directly on the pool of neutral oligosaccharides afforded 23 fractions. Twenty oligosaccharide structures were characterized by employing 500-MHz ^{1}H-NMR spectroscopy in conjunction with sugar analysis. Thirteen among these had been revealed before to occur in human bronchial mucins [Van Halbeek, H., Dorland, L., Vliegenthart, J. F. G., Hull, W. E., Lamblin, G., Lhermitte, M., Boersma, A., and Roussel, P. (1982) *Eur. J. Biochem. 127*, 7–20], when paper-chromatographic fractionation of this pool of neutral oligosaccharides was employed. High-performance liquid chromatography enabled to obtain another seven pentasaccharide and hexasaccharide alditols; the largest-size representatives are:



Thereby, this approach afforded deeper insight into the structural heterogeneity displayed by the carbohydrate chains of bronchial mucins.

The tracheobronchial secretion is an important component of the mucociliary system which protects the airway mucosa by removing the dust particles that are inhaled. It contains highly viscous, mucous glycoproteins (so-called mucins) the carbohydrate moiety of which determines, to a large extent, the characteristic rheological properties of the respiratory mucus necessary for the efficiency of the system. Under pathological conditions characterized by bronchial hypersecretion like, for example, cystic fibrosis, the functioning of the mucociliary system is disturbed, leading to bronchial obstruction which may induce infection of the airways and increase the gravity of the disease.

In order to determine whether these phenomena are due to an alteration of the structure of the bronchial mucins, we decided to investigate the carbohydrate structure of bronchial mucous glycoproteins obtainable from patients suffering from cystic fibrosis. In a previous study [1], the isolation and characterization of 14 neutral oligosaccharides from this source was described. The applied isolation procedure comprised anion-exchange chromatography of the pool of neutral oligosaccharides, followed by preparative paper chromatography of the various fractions. Since this procedure consumed rather large amounts of time and material [1], it was decided to try an alternative approach, namely, purification of the oligosaccharides by direct high-performance liquid chromatography (hplc) of the neutral pool.

Here, we describe the separation by hplc of at least 20 oligosaccharide fractions from cystic-fibrosis mucins. For characterization of the structures of the constituting oligosaccharides, 500-MHz ^{1}H-NMR spectroscopy was applied in conjunction with carbohydrate analysis, as this approach had turned out to be most successful in elucidating this type of mucin carbohydrate structures [1]. Several of the hplc fractions will be demonstrated to contain oligosaccharides whose structures had not been revealed before for cystic-fibrosis mucins.

A preliminary account of this investigation has been presented [2].

Abbreviations. Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; GalNAc-ol, *N*-acetyl-D-galactosaminitol; hplc, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

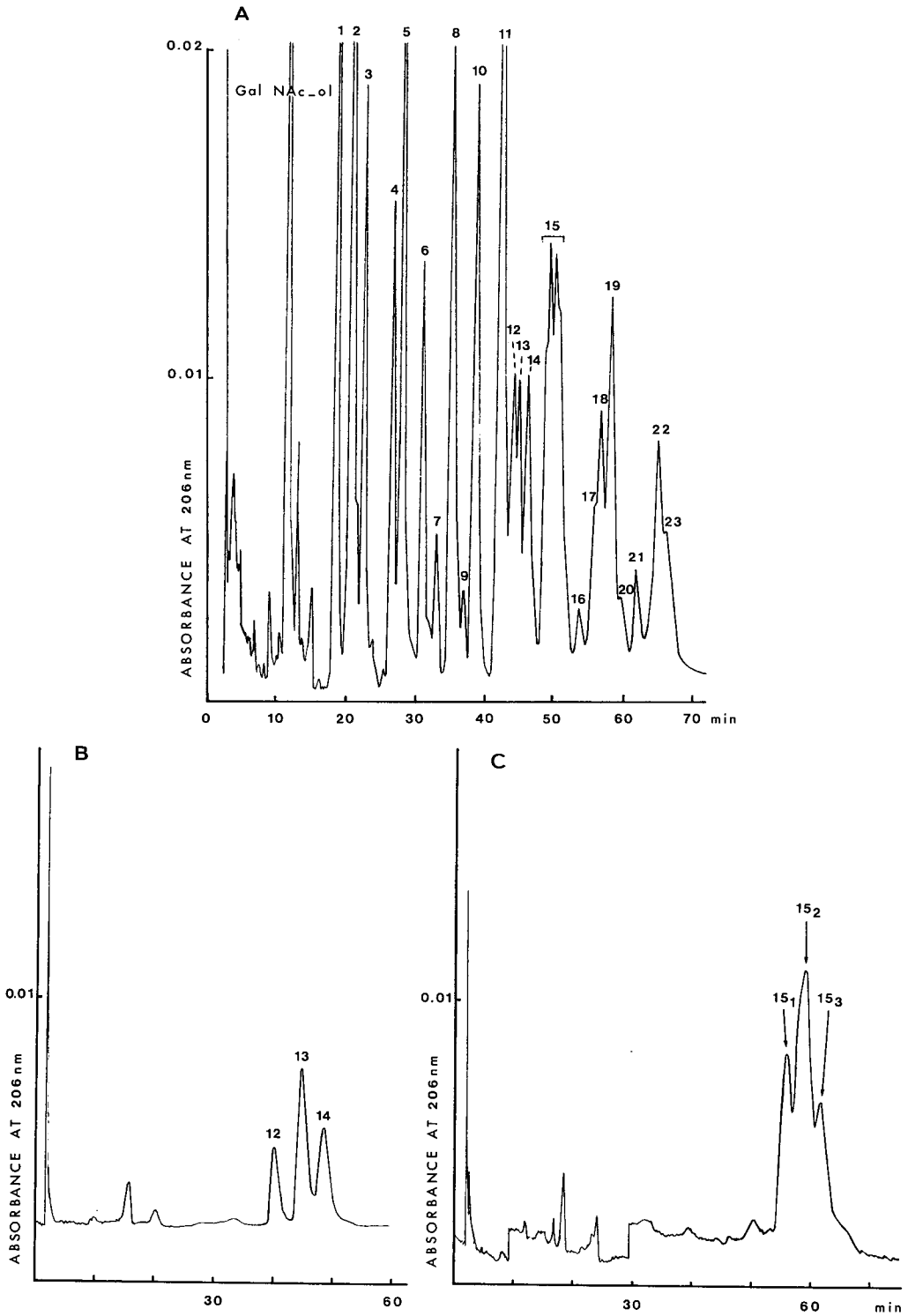


Fig. 1. *Separation of neutral human bronchial oligosaccharide-alditols by high-performance liquid chromatography.* (A) Initial separation on a Lichrosorb-NH_2 [5] column, eluted with a linear acetonitrile/water gradient (17:3 up to 3:2, v/v). (B) Repurification of combined fractions **12** and **13** by a second hplc run; elution was performed isocratically by acetonitrile/water (77:23, v/v). (C) Subfractionation of fraction **15** by a second hplc run; elution was performed isocratically by acetonitrile/water (3:1, v/v)

MATERIALS AND METHODS

Isolation of bronchial oligosaccharides

Alkaline borohydride reductive degradation of acidic bronchial mucins obtained [3] from six patients suffering from cystic fibrosis resulted in a mixture of reduced oligosaccharides and glycopeptides [4]. These were fractionated by ion-exchange chromatography (Dowex AG1X2) and gel-filtration (Bio-Gel P4), according to acidity and molecular size, respectively. By this procedure, four pools of oligosaccharide-alditols were obtained: one consisting of neutral

oligosaccharide-alditols (Ic), another of sialylated oligosaccharide-alditols (IIc) and two pools of sulfated oligosaccharide-alditols [1, 3].

Fractionation of Ic was performed by hplc using a Waters liquid chromatograph model 6000A equipped with a universal injector, model U6K, essentially as described previously [5]. The apparatus was provided with a Lichrosorb-NH_2 column (4 × 250 mm, particle size 5 μm, Merck). The column was run with a linear gradient of acetonitrile/water (17:3 up to 3:2, v/v), for 90 min at room temperature at a flow rate of 1 ml/min. Oligosaccharide peaks were detected by absorption at 206 nm (see Fig. 1A), collected in a vial, evaporated under nitrogen at room temperature and lyophilized. Some of the oligosaccharide fractions obtained were not well separated from the adjacent peaks; they were repurified isocratically by hplc on the same column, again at room temperature. A mixture of acetonitrile/water in the ratio 77:23, at a flow rate of 1 ml/min was used for improved separation of peaks **12** and **13** (see Fig. 1B); a 75:25 mixture at a flow rate of 1.2 ml/min, could resolve fraction **15** into three subfractions (see Fig. 1C). The composition of the eluents was chosen on the basis of the retention time of the oligosaccharide peaks in the first hplc run.

Analytical methods

The molar ratios of neutral sugars and *N*-acetylhexosamines in the oligosaccharide-alditol fractions separated by hplc were determined after methanolysis by gas-liquid chromatography of their trimethylsilyl derivatives as described [1,6]. Prior to ^{1}H-NMR spectroscopic analysis, the hplc-fractionated neutral oligosaccharide-alditols were repeatedly treated with ^2H_2O at room temperature. After each exchange treatment, the materials were lyophilized. Finally, each sample was redissolved in 0.4 ml ^2H_2O (99.996 atom % ^{2}H, Aldrich). 500-MHz ^{1}H-NMR spectroscopy was performed on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands), operating under control of an Aspect-2000 computer. Experimental details have been described [1,6–8]. Resolution-enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation. The probe temperature was kept at 22.0 (±0.1) °C. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone (δ = 2.225 ppm in ^2H_2O at 22 °C), with an accuracy of 0.002 ppm.

Table 1. *Molar carbohydrate composition of the neutral bronchial oligosaccharide-alditol fractions obtained by hplc of pool Ic from cystic fibrosis sputum*

The molar composition of the oligosaccharide-alditols was calculated on the basis of one residue of GalNAc-ol per molecule, taking into account the partial dehydration of the alditol under the applied methanolysis conditions [9,10]. The amounts of fractions **7**, **9**, **16**, **20**, **21** and **23** were too low to be analyzed

Oligosaccharide-alditol fraction	Molar ratios of monosaccharides			
	Fuc	Gal	GlcNAc	GalNAc-ol
1	–	–	0.8	1
2	–	0.8	–	1
3	0.6	0.7	–	1
4	–	1.0	0.7	1
5	–	0.9	0.7	1
6	–	1.0	0.9	1
8	1.0	1.1	0.7	1
10	–	1.4	1.4	1
11	–	1.8	0.8	1
12+13	0.7	1.7	1.1	1
14	0.5	1.7	1.2	1
15	0.7	1.8	1.1	1
17	0.7	1.9	1.5	1
18	1.0	1.6	1.6	1
19	0.9	2.3	1.5	1
22	1.8	2.8	1.7	1

RESULTS

Separation of neutral bronchial oligosaccharides by high-performance liquid chromatography

High-performance liquid chromatography was employed to fractionate the pool of neutral oligosaccharides (Ic) isolated from acidic bronchial mucins from six patients with blood-group O activity, suffering from cystic fibrosis. Upon running the Lichrosorb-NH_2 column with a linear gradient of acetonitrile/water (17:3 to 3:2, v/v), separation of 23 oligosaccharide fractions could be achieved (see Fig. 1A). Since peaks **12** and **13** were not adequately separated from each other, the mixture of them was rechromatographed, but now isocratically with a 77:23 (v/v) mixture of acetonitrile/water (see Fig. 1B). The assembly of peaks denoted **15** in Fig. 1A, was subfractionated into three fractions, **15.1** to **15.3**, by rerunning **15** isocratically using acetonitrile/water in the ratio 75:25 (see Fig. 1C).

Structure determination

Table 1 lists the molar carbohydrate compositions of the major oligosaccharide-alditol fractions obtained on the first hplc run of the fraction Ic, and pooled as indicated in Fig. 1A. The amounts of the minor fractions **7**, **9**, **16**, **20**, **21** and **23** were too low to permit any kind of structural analysis.

500-MHz ^{1}H-NMR spectra were recorded of the major hplc (sub)fractions (Fig. 1A–C), in order to establish the primary structures of the constituent oligosaccharide-alditols. The spectra of fractions **2**, **3**, **6**, **11**, **12**, **14** and **15.3** showed the H-2 signal of GalNAc-ol at $\delta \approx 4.39$ ppm, reflecting the substitution of GalNAc-ol by Gal in $\beta(1\rightarrow3)$-linkage [1]. The spectra of fractions **1**, **4**, **5**, **8**, **13**, **17** and **19** possessed the GalNAc-ol H-2 signal at $\delta \approx 4.29$ ppm; the latter value is known to be characteristic of substitution of GalNAc-ol by GlcNAc in $\beta(1\rightarrow3)$-linkage [1]. Another series of spectra contained GalNAc-ol H-2 signals simultaneously at the two aforementioned positions in an intensity ratio mostly different from 1:1. In those cases, namely, for fractions **10**, **15.1**, **15.2**, **18** and **22**, mixtures of oligosaccharide-alditols were involved differing in the type of core substituent present at C-3 of GalNAc-ol. According to the chemical shift of GalNAc-ol H-2, the ^{1}H-NMR data of the pertinent structural-reporter groups of the compounds identifiable in fractions **1**–**22**, have been compiled either in Table 2 or in Table 3.

The spectra of the relatively fast-eluting, smaller-size oligosaccharide-alditols **1**–**11** and also those of **14** and **15.2** had been observed for the series of compounds resulting from the paper-chromatographic working-up procedure of pool Ic from cystic fibrosis sputum [1]. A detailed description of the deduction of the primary structures of these oligosaccharide-

Table 2. *^1H chemical shifts of structural-reporter groups of constituent monosaccharides for the hplc-fractionated, neutral bronchial oligosaccharide-alditols possessing the Galβ(1→3)GalNAc-ol core unit*

Chemical shifts are relative to internal DSS (using internal acetone at $\delta = 2.225$ ppm) in ^2H_2O at 22 °C (δ $HO^2H = 4.811$ ppm), acquired at 500 MHz. For the complete structures of the compounds, see Schemes 1 and 2. In the table-heading, the structures are represented by short-hand symbolic notation (cf. [1]); ◇ = GalNAc-ol; ■— = Gal; ●— = GlcNAc and □— = Fuc

Residue	Reporter group	Chemical shift in compound									
		2	3	6	10B	11	12	14	15.1A	15.2B	15.3
		ppm									
GalNAc-ol	H-2	4.396	4.398	4.397	4.396	4.399	4.405	4.399	4.398	4.395	4.400
	H-3	4.063	4.089	4.061	4.049	4.061	4.085	4.049	4.060	4.059	4.083
	H-4	3.501	3.521	3.463	3.496	3.460	3.499	3.488	3.464	3.446	3.496
	H-5	4.197	4.164	4.282	4.182	4.285	4.269	4.187	4.287	4.270	4.265
	NAc	2.050	2.046	2.067	2.045	2.067	2.055	2.046	2.066	2.066	2.053
Gal^3 [a]	H-1	4.476	4.583	4.464	4.463	4.463	4.573	4.464	4.463	4.461	4.576
	H-4	3.898	3.925	3.898	4.125	3.898	3.923	4.129	3.90	3.90	3.92
$GlcNAc^6$	H-1	-	-	4.535	-	4.557	4.570	-	4.535	4.557	4.55 [b]
	H-6	-	-	3.931	-	3.996	3.998	-	3.99	4.003	3.999
	NAc	-	-	2.067	-	2.066	2.055	-	2.066	2.056	2.058
Gal^4	H-1	-	-	-	4.479	4.469	4.469	4.458	4.536	4.442	4.54 [b]
	H-4	-	-	-	3.92	3.923	3.923	3.897	3.98	3.92	3.92
$GlcNAc^3$	H-1	-	-	-	4.688	-	-	4.686	-	-	-
	H-6	-	-	-	3.95	-	-	3.957	-	-	-
	NAc	-	-	-	2.043	-	-	2.032	-	-	-
Fuc^2	H-1	-	5.253	-	-	-	5.221	-	5.309	-	5.216 [c] / 5.306 [d]
	H-5	-	4.278	-	-	-	4.278	-	4.227	-	4.27 [e] / 4.226
	CH_3	-	1.243	-	-	-	1.244	-	1.233	-	1.244 / 1.229
Fuc^3	H-1	-	-	-	-	-	-	5.139	-	5.106	-
	H-5	-	-	-	-	-	-	4.83	-	4.83	-
	CH_3	-	-	-	-	-	-	1.176	-	1.171	-

[a] A superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked (cf. [1]).
[b] Values could not be determined more accurately, because of the presence of a non-carbohydrate contaminant.
[c] Left-hand set of chemical shift values in this column belong to structural-reporter groups of $Fuc^{2,3}$.
[d] Right-hand set of chemical shift values in this column belong to structural-reporter groups of $Fuc^{2,4}$.
[e] Value could not be determined more accurately due to partial overlap of the signal with that of H-5 of GalNAc-ol.

alditols has been published [1]. The structures of the compounds present in the aforementioned fractions are summarized in Scheme 1.

The NMR data listed for these compounds in Tables 2 and 3 differ slightly from those reported before [1] because the spectra of the hplc-purified fractions were recorded at 22 °C instead of 27 °C. This enabled the undisturbed observation of the β-anomeric-proton region, from $\delta = 4.40$ ppm up to $\delta = 4.75$ ppm, since the residual HO^2H signal was found at $\delta \approx 4.81$ ppm, at 22 °C. Compound **14**, which used to be present as the main component ($3a_1$) of a mixture of oligosaccharide-alditols [1], was now found to be pure. Fractions **10** and **15.2** (even after a second hplc run, see Fig. 1C) contained mixtures of oligosaccharide-alditols; those from fraction **10** were denoted **10A** and **10B** (ratio 3:2) corresponding to compounds $4a_1$ and $4a_2$; those from fraction **15.2** were denoted **15.2A** and **15.2B** (ratio 1:1), corresponding to compounds $6a_1$ and $6a_2$, respectively (see Scheme 1); it is remarkable that within these pairs, the compounds were not separated from each other by paper chromatography, either [1].

The spectra of the remaining hplc fractions investigated had not been observed before. The structure elucidation of their constituting oligosaccharide-alditols is discussed below.

Novel bronchial oligosaccharides, of the Galβ(1→3)GalNAc-ol core type

The 500-MHz ^1H-NMR spectrum of hplc fraction **12** is depicted in Fig. 2. It shows the characteristic features of a mucin-type oligosaccharide-alditol containing the branched Galβ(1→3)[GlcNAcβ(1→6)]GalNAc-ol core element. This could be derived from the chemical shifts of GalNAc-ol H-2 ($\delta = 4.405$ ppm) and H-5 ($\delta = 4.269$ ppm) (compare [1]). The $GlcNAc^6$ residue is substituted by Gal in β(1→4)-linkage, as became evident from the chemical shift of its H-1 ($\delta = 4.570$ ppm; $J_{1,2} = 8.3$ Hz) [1, 7]. In addition, a Fuc residue is present in compound **12** (see also Table 1); it is α(1→2)-linked to Gal^3. This could be deduced from the typical set of chemical shift values for the Fuc structural-reporter groups (see Table 2). The position of the Fuc CH_3 doublet ($\delta = 1.24$ ppm) is indicative, already on its own, of the (1→2)-type of attachment to Gal, whereas the chemical shifts of H-1 and H-5 exclude the possibility of Fuc^2 being attached to Galβ(1→4)GlcNAcβ(1→·) [9, 11, 12] (compare also compound **15.1A**; see below). The deviation of the Fuc H-1 chemical shift for compound **12** as compared to compound **3** (see Table 2) is ascribed to the attachment of the *N*-acetyl-

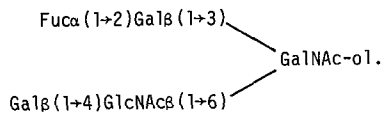
Table 3. *^1H chemical shifts of structural-reporter groups of constituent monosaccharides for the hplc-fractionated, neutral bronchial oligosaccharide-alditols possessing the GlcNAcβ(1→3)GalNAc-ol core unit*

Chemical shifts are relative to internal DSS (using internal acetone at δ = 2.225 ppm) in ^2H_2O at 22 °C (δHO^2H = 4.811 ppm), acquired at 500 MHz. For the complete structures of the compounds, see Schemes 1 and 2. In the table-heading, the structures are represented by short-hand symbolic notation (cf. [1]); ◇ = GalNAc-ol; ●— = GlcNAc; ■— = Gal and □— = Fuc. The type of linkage in ■——● is specified to be either β(1→4) or β(1→3)

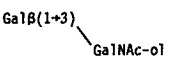
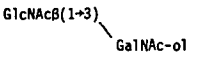
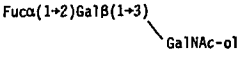
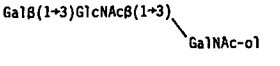
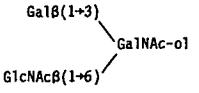
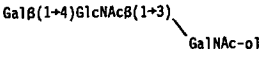
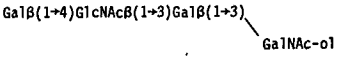
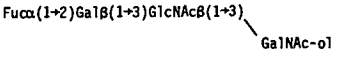
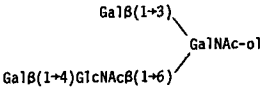
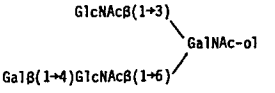
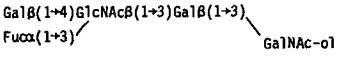
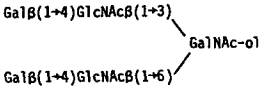
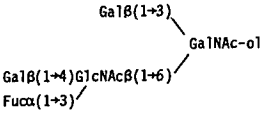
Residue	Reporter group	Chemical shift in compound									
		1	4	5	8	10A	13	15.1B	15.2A	17	19
		ppm									
GalNAc-ol	H-2	4.289	4.290	4.291	4.264	4.279	4.285	4.287	4.284	4.286	4.264
	H-5	4.145	4.141	4.146	4.114	4.237	4.233	4.233	4.242	4.234	4.217
	NAc	2.037	2.034	2.038	2.036	2.045	2.043	2.043	2.045	2.045	2.041
$GlcNAc^{3(a)}$	H-1	4.601	4.654	4.631	4.654	4.597	4.595	4.648	4.623	4.622	4.654
	NAc	2.085	2.073	2.083	2.111	2.081	2.080	2.069	2.079	2.079	2.109
$Gal^{3,3}$	H-1	-	4.461	-	4.577	-	-	4.453	-	-	4.566
	H-4	-	3.926	-	3.889	-	-	3.92	-	-	3.98
$Gal^{4,3}$	H-1	-	-	4.454	-	-	-	-	4.451	4.454	-
	H-4	-	-	3.923	-	-	-	-	3.923	3.92	-
$GlcNAc^{6}$	H-1	-	-	-	-	4.563	4.541	4.559	4.558	4.542	4.566
	NAc	-	-	-	-	2.061	2.064	2.062	2.062	2.067	2.059
$Gal^{4,6}$	H-1	-	-	-	-	4.472	4.539	4.472	4.473	4.541	4.468
	H-4	-	-	-	-	3.92	3.98	3.92	3.923	3.98	3.92
$Fuc^{2,3}$	H-1	-	-	-	5.208	-	-	-	-	-	5.210
	H-5	-	-	-	4.273	-	-	-	-	-	4.271
	CH_3	-	-	-	1.230	-	-	-	-	-	1.232
$Fuc^{2,4}$	H-1	-	-	-	-	-	5.305	-	-	5.305	-
	H-5	-	-	-	-	-	4.23	-	-	4.23	-
	CH_3	-	-	-	-	-	1.232	-	-	1.235	-

(a) A superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked (cf. [1]). A second superscript is used to discriminate between identically-bound Gal or Fuc residues; it indicates the type of linkage of the adjacent monosaccharide.

lactosamine unit in β(1→6)-linkage to GalNAc-ol; this demonstrates again [12] that the chemical shift of H-1 of Fuc is extremely sensitive to structural changes even if somewhat remote from its direct environment. Comparison of the NMR data for compound **12** with those for its afuco analogue **11** (see Table 2) reveals that H-1 of Gal^3 ($J_{1,2}$ = 8.0 Hz) shifted downfield ($\Delta\delta$ = 0.11 ppm) towards δ = 4.573 ppm, while the position of H-1 of Gal^4 (δ = 4.469 ppm) remained unaltered. The former shift increment, together with the effects observed upon fucosylation of compound **11** for GalNAc-ol H-3($\Delta\delta$ = 0.024 ppm), H-4 ($\Delta\delta$ = 0.039 ppm) and H-5 ($\Delta\delta$ = −0.016 ppm), are in excellent agreement with the corresponding shift alterations in the step from compound **2** to **3** (see Table 2). This coroborates, once more, the location of Fuc at Gal^3, rather than at Gal^4. So, the structure of compound **12** was established to be the following pentasaccharide-alditol:



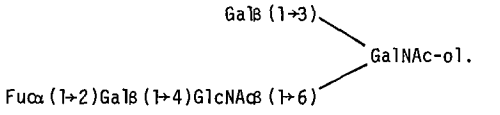
The 500-MHz ^1H-NMR spectrum of fraction **15.1** is presented in Fig. 3. The most remarkable feature of this spectrum is the simultaneous occurrence of two GalNAc-ol H-2 signals, one at δ = 4.398 ppm, and the other at δ = 4.287 ppm. The latter signal coincides with a GalNAc-ol H-5 signal (see Fig. 3). This situation is similar to that described [1] for the mixture of compounds **15.2A** and **15.2B** ($6a_1$ and $6a_2$ in [1]). The intensity ratio of the signals at δ = 4.398 ppm and 4.287 ppm in Fig. 3, being 2:3, may indicate that the sample consists of a mixture of two oligosaccharide-alditols with a different type of core. The compound that gives rise to GalNAc-ol H-2 at δ = 4.398 ppm and to H-5 at δ = 4.287 ppm, is the major (67%) component: it is denoted **15.1A**. As with compound **12**, its core consists of the Galβ(1→3) [GlcNAcβ(1→6)]GalNAc-ol trisaccharide element. The minor (33%) constituent of the mixture, **15.1B**, for which H-2 of GalNAc-ol resonates at δ = 4.287 ppm, will be dealt with below. Based on the relative intensities of the signals in the anomeric proton regions of the spectrum, the two almost coincident doublets at $\delta \approx$ 4.53 ppm, the doublet at δ = 4.463 ppm, and the doublet at δ = 5.309 ppm are ascribed to compound **15.1A**, suggesting that another Fuc-containing

hplc fraction	Former code [1]	Structures of the Galβ(1→3)GalNAc-ol core type	hplc fraction	Former code [1]	Structures of the GlcNAcβ(1→3)GalNAc-ol core type
2	(4b)		1	(2)	
3	(9)		4	(5b)	
6	(5a)		5	(3b)	
10B	($4a_2$)		8	(6b)	
11	(7)		10A	($4a_1$)	
14	($3a_1$)		15.2A	($6a_1$)	
15.2B	($6a_2$)				

Scheme 1. *Structures of 13 neutral bronchial oligosaccharide-alditols, obtained by direct hplc of fraction Ic from cystic fibrosis sputum.* For comparison, the codes for the compounds which were used in the paper chromatography working-up procedure of pool Ic [1], have been included between brackets

pentasaccharide-alditol is concerned. The chemical shifts of these and other structural-reporter groups of **15.1A** have been included in Table 2.

Comparison of the data for **15.1A** with those for **6** and **11** (Table 2) reveals that the aforementioned signal at $\delta =$ 4.463 ppm can be attributed to the anomeric proton of terminal Gal[3] linked to GalNAc-ol. The Fuc residue is $\alpha(1 \rightarrow 2)$-linked to Gal[4] forming part of the Gal $\beta(1 \rightarrow 4)$ GlcNAc unit that is $\beta(1 \rightarrow 6)$-linked to GalNAc-ol. This conclusion is based upon the typical chemical shifts of Fuc H-1 ($\delta = 5.309$ ppm), H-5 ($\delta = 4.227$ ppm) and CH_3 ($\delta =$ 1.233 ppm) [7,9,11,12]. The shift increments shown by Gal[4] H-1 ($\Delta\delta = 0.067$ ppm) and GlcNAc[6] H-1 ($\Delta\delta = -0.022$ ppm) in the step from compound **11** to **15.1A** support this location of Fuc[2] [7,9,11]. It should be mentioned that the doublets at $\delta = 4.535$ ppm ($J_{1,2} = 8.6$ Hz) and at $\delta = 4.536$ ppm ($J_{1,2} = 7.3$ Hz) were assigned to H-1 of GlcNAc[6] and Gal[4] respectively, on the basis of the pronounced difference in coupling constant between them [1]. Thus, compound **15.1A** was established to be a positional isomer of **12**, differing in the location of the $\alpha(1 \rightarrow 2)$-linked Fuc residue:



The 500-MHz ^{1}H-NMR spectrum of fraction **15.3** revealed the oligosaccharide-alditol to belong also to the Galβ $(1 \rightarrow 3)$[GlcNAc$\beta(1 \rightarrow 6)$]GalNAc-ol core type (see Table 2, in particular δH-2 and δH-5 of GalNAc-ol). Moreover, it showed two sets of Fuc structural-reporter-group signals of equal intensity; their chemical shifts are listed in Table 2. One of the sets has been mentioned before to be characteristic of Fuc$\alpha(1 \rightarrow 2)$-linked to Gal[3]; the other one is the set typical for Fuc$\alpha(1 \rightarrow 2)$-linked to Gal[4] of an *N*-acetyllactosamine unit. The shift increments and decrements of Gal and GlcNAc structural reporters discussed above for the steps from **11** to **12**, and from **11** to **15.1A** respectively, are found together, when comparing

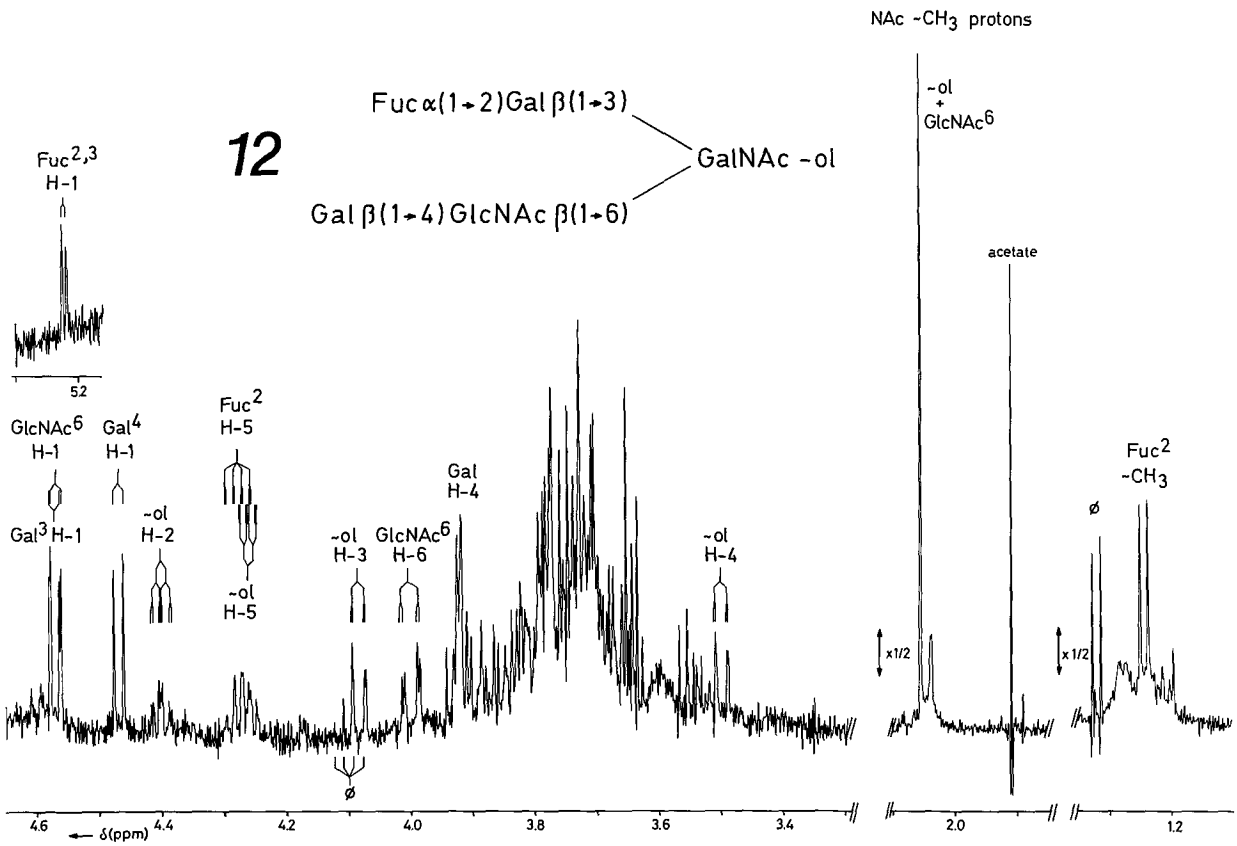
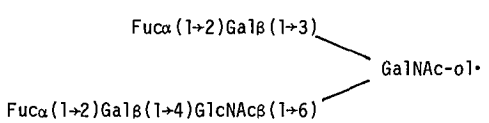


Fig. 2. *Resolution-enhanced 500-MHz ^{1}H-NMR spectrum (^2H_2O, 22 °C) of hplc-fraction 12, obtained from the pool of neutral oligosaccharide-alditols Ic from cystic fibrosis sputum.* The relative-intensity scale of the *N*-acetyl and Fuc methyl proton regions of the spectrum differs from that of the other parts, as indicated. Signals marked by ϕ stem from a frequently occurring, non-protein non-carbohydrate contaminant

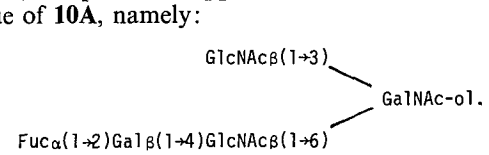
11 to **15.3**. Therefore, compound **15.3** appeared to be the following difucosyl hexasaccharide-alditol:



Novel bronchial oligosaccharides of the GlcNAcβ(1 → 3)GalNAc-ol core type

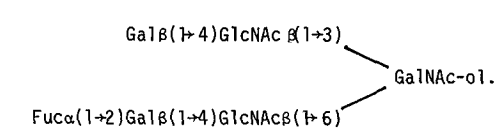
The 500-MHz ^{1}H-NMR spectra of fractions **13**, **17** and **19**, and also the subspectrum of compound **15.1B** in Fig. 3, have in common the GalNAc-ol H-2 and H-5 resonances at $\delta \approx 4.28$ ppm and $\delta \approx 4.23$ ppm, respectively (see Table 3). Therefore, each of the four compounds contains the GlcNAcβ(1→3)[GlcNAcβ(1→6)]GalNAc-ol branching core element [1].

In compound **13**, the core GlcNAc^3 residue is present in terminal position because of the chemical shift of its H-1 signal ($\delta = 4.595$ ppm, $J_{1,2} = 8.3$ Hz; compare with compounds **1** and **10A**, Table 3). The GlcNAc^6 residue bears a Fucα(1→2)Galβ(1→4) moiety, as is evident from comparison of the data for this branch with those for **15.1A** (Table 2). Therefore, compound **13** appeared to be a mono-fucosylated analogue of **10A**, namely:



The effects of extension of **10A** to **13** (Table 3) are completely analogous to those observed upon fucosylation of **11** to **15.1A** (Table 2).

In the spectrum of fraction **17** most of the characteristic features of **13**, in particular those for GalNAc-ol and for the fucosylated *N*-acetyllactosamine unit β(1→6)-linked to GalNAc-ol, were found essentially unaltered (Table 3); however the chemical shift of GlcNAc^3 H-1 has changed considerably towards $\delta = 4.622$ ppm. Furthermore, an additional Gal H-1 signal ($J_{1,2} = 7.6$ Hz) was found at $\delta = 4.454$ ppm. This suggests compound **17** to be an extension of compound **13** with a Gal residue β(1→4)-linked to GlcNAc^3 [1,7]. The chemical shifts of Gal^{4,3} H-1 and GlcNAc^3 H-1 and NAc are exactly the same as those observed for compound **15.2A** ($6a_1$ in [1]), thereby corroborating the β(1→4)-type of linkage between Gal and GlcNAc in this branch. The chemical shift of the terminal Gal^4 residue in compound **17** being $\delta = 4.454$ ppm, substantiates this residue to be linked to GlcNAc^3 (see **15.2A** in Table 3; the values for H-1 of terminal Gal^{4,3} and Gal^{4,6} are clearly different; their assignment has been discussed [1]). By consequence, the Fuc^2 residue is indeed linked to Gal^{4,6} rather than to Gal^{3,4}. In conclusion, the structure of compound **17** was determined to be as follows:



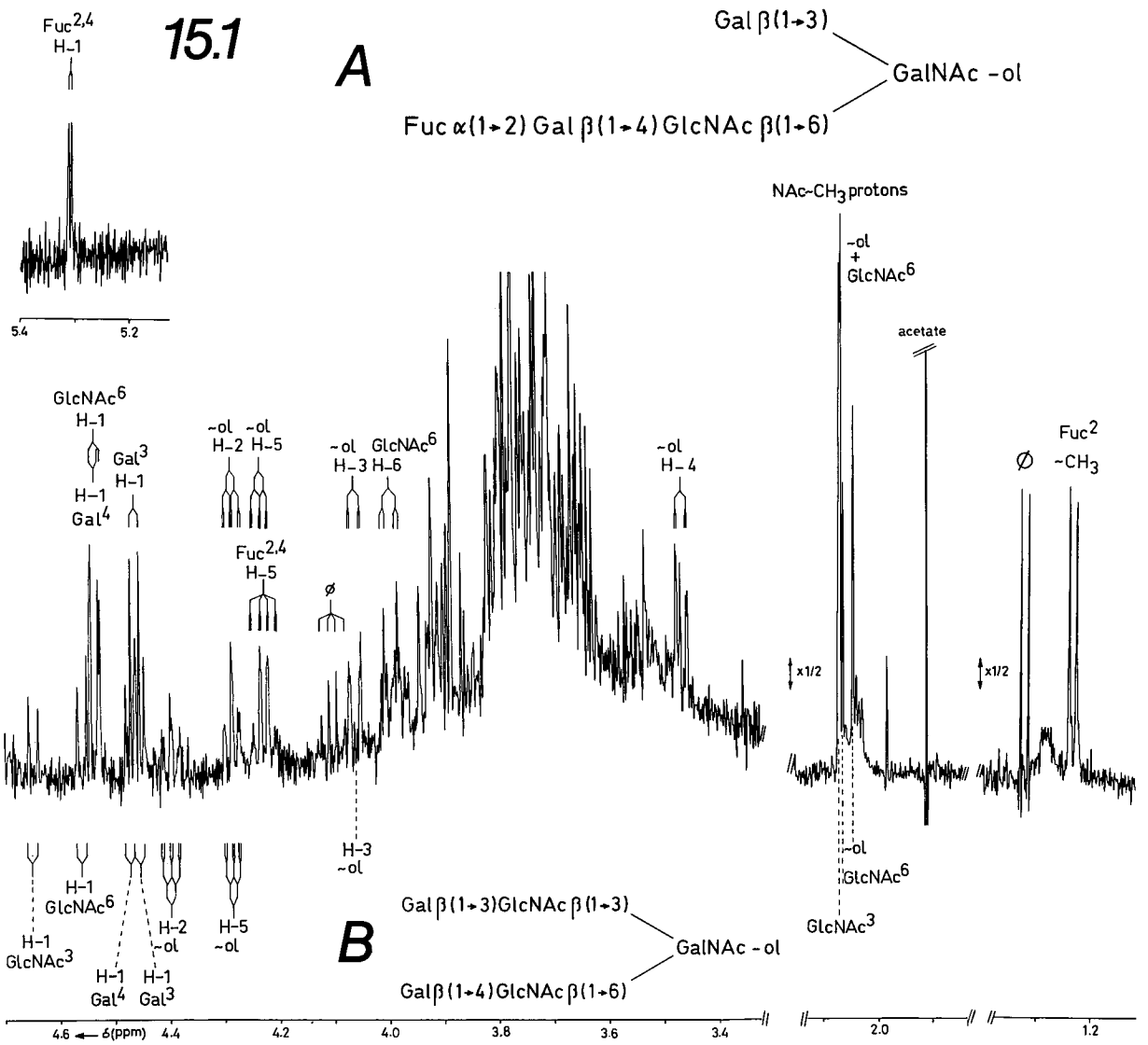
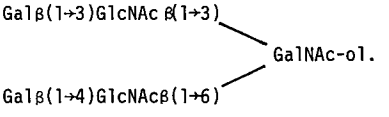


Fig. 3. *Resolution-enhanced 500-MHz ^1H-NMR spectrum (^2H_2O, 22 °C) of hplc-fraction 15.1, being a mixture of the oligosaccharide-alditols 15.1A and 15.1B (in the ratio 2:1), obtained from the pool of neutral oligosaccharide-alditols from cystic fibrosis sputum.* Signals belonging to the main component, **15.1A**, are assigned on top of the spectrum, those attributed to the minor component, **15.1B**, at the bottom. The relative-intensity scale of the *N*-acetyl and Fuc methyl proton regions of the spectrum deviates from that of the other parts, as indicated. Signals marked by ϕ stem from a frequently occurring, non-protein non-carbohydrate contaminant

The 500-MHz ^{1}H-NMR spectrum of fraction **15.1** (see Fig. 3) showed a series of lower-intensity signals belonging to a compound of the GlcNAc$\beta(1\rightarrow3)$[GlcNAc$\beta(1\rightarrow6)$]GalNAc-ol core type (see above). This minor component, **15.1B**, contains two Gal residues in addition to the core GlcNAc residues, because of the presence of four low-intensity anomeric doublets, namely at $\delta = 4.648$ ppm ($J_{1,2} = 8.9$ Hz) for GlcNAc^3, at $\delta = 4.559$ ppm ($J_{1,2} = 8.3$ Hz) for GlcNAc^6, and at $\delta = 4.472$ ppm and 4.453 ppm (both having $J_{1,2} = 7.9$ Hz) for the Gal residues. The former two values indicate that both GlcNAc^3 and GlcNAc^6 are substituted by a Gal residue. The Gal residue linked to GlcNAc^6 is $\beta(1\rightarrow4)$-linked, since the H-1 values of 4.472 ppm and 4.559 ppm are identical to those observed for these residues in compounds **10A** and **15.2A** (see Table 3). In accord with this, the NAc signal at $\delta = 2.062$ ppm is attributed to GlcNAc^6, implying that the NAc signal of GlcNAc^3 is found at $\delta = 2.069$ ppm (see Fig. 3). The latter value indicates GlcNAc^3 to be substituted by Gal in $\beta(1\rightarrow3)$-linkage, because the shift effect observed for this NAc signal when comparing compounds **15.1B** and **10A** ($\Delta\delta = -0.012$ ppm) is the same as that in the step from compound **1** to **4**. Thus compound **15.1B** can be considered as an extension of compound **10A** with Gal^3:



It should be noted that, in fact, the chemical shift for the NAc protons of GlcNAc^3 is the only observable parameter of compound **15.1B** that is significantly different from the corresponding one for compound **15.2A** having Gal^{4,3} instead of Gal^{3,3} (see Table 3).

The 500-MHz ^{1}H-NMR spectrum of fraction **19** pointed to the presence of a structure similar to **15.1B**, extended by a Fuc residue (compare the data for both compounds listed in

hplc fraction	Structures of the Galβ(1→3)GalNAc-ol core type	hplc fraction	Structures of the GlcNAcβ(1→3)GalNAc-ol core type
12	Fucα(1→2)Galβ(1→3) GalNAc-ol Galβ(1→4)GlcNAcβ(1→6)	13	GlcNAcβ(1→3) GalNAc-ol Fucα(1→2)Galβ(1→4)GlcNAcβ(1→6)
15.1A	Galβ(1→3) GalNAc-ol Fucα(1→2)Galβ(1→4)GlcNAcβ(1→6)	15.1B	Galβ(1→3)GlcNAcβ(1→3) GalNAc-ol Galβ(1→4)GlcNAcβ(1→6)
15.3	Fucα(1→2)Galβ(1→3) GalNAc-ol Fucα(1→2)Galβ(1→4)GlcNAcβ(1→6)	17	Galβ(1→4)GlcNAcβ(1→3) GalNAc-ol Fucα(1→2)Galβ(1→4)GlcNAcβ(1→6)
		19	Fucα(1→2)Galβ(1→3)GlcNAcβ(1→3) GalNAc-ol Galβ(1→4)GlcNAcβ(1→6)

Scheme 2. *Structures of seven neutral bronchial oligosaccharide-alditols, obtained by direct hplc of fraction Ic from cystic fibrosis sputum.* This series of compounds had not been discovered in the paper-chromatography working-up procedure [1]

Table 3). The Fuc structural-reporter-group signals are observed at positions that are indicative of its $\alpha(1\rightarrow2)$-linkage to a Gal residue. Moreover, the H-1 ($\delta=5.210$ ppm) and H-5 ($\delta=4.271$ ppm) chemical shifts indicate that Fuc^2 is attached to Gal^3 rather than to Gal^4 (compare with compounds **8**, **12** and **15.3**, see Tables 2 and 3). In accordance with this extension, H-1 of Gal^{3,3} is found to resonate at $\delta=4.566$ ppm (compare compound **8**), nearly coinciding with H-1 of GlcNAc^6. The location of Fuc^2 at Gal^{3,3} is further evidenced by the highly typical shift increment of NAc of GlcNAc^3 in the step from compound **15.1B** to **19**,namely, $\Delta\delta=0.04$ ppm; the latter was found also in the step from compound **4** to **8** (see Table 3). Also the shift effects observed for H-2 and H-5 of GalNAc-ol are the same for both steps, thereby turning out to be specific for the attachment of Fuc^2 in the Galβ(1→3)GlcNAcβ(1→3) branch. Therefore, the structure of **19** was established to be the following hexasaccharide-alditol:

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

The 500-MHz ^{1}H-NMR spectra of fractions **18** and **22** were too complex to be interpreted in terms of detailed structures, as yet. Anyway, on the basis of the occurrence of GalNAc-ol H-2 signals at $\delta\approx4.39$ as well as at $\delta\approx4.29$ ppm in both spectra, it can be stated that **18** and **22** each consist of at least two compounds with different core substituents at C-3 of GalNAc-ol.

DISCUSSION

Alkaline borohydride treatment of acidic bronchial glycoproteins from six patients suffering from cystic fibrosis afforded a mixture of reduced oligosaccharides and glycopeptides that could be fractionated by ion-exchange chromatography and gel-filtration. This procedure led to a pool of neutral oligosaccharide-alditols (Ic) and three pools of acidic oligosaccharide-alditols. Previously, fractionation of Ic had been carried out by anion-exchange chromatography on DAX4 followed by preparative paper chromatography. The combination of periodic oxidation, methylation analysis including gas chromatography and mass-spectrometric identification of partially methylated alditol acetates, and 500-MHz ^{1}H-NMR spectroscopy have led to the determination of the structure of 14 neutral oligosaccharides [1].

Since both the DAX4 step and paper chromatography consumed much time and material, these two steps were eliminated. As an alternative, an hplc procedure was set up to fractionate directly the neutral oligosaccharides of fraction Ic. Some 20 oligosaccharide fractions have been obtained in amounts amenable for structural analysis by 500-MHz ^{1}H-NMR spectroscopy.

Taking advantage of the empirical rules developed for correlating ^{1}H-NMR features of mucin-type oligosaccharide-alditols with their structures [1], 20 structures could be arrived at just by combination of 500-MHz ^{1}H-NMR spectroscopy and carbohydrate analysis performed on the hplc fractions. Thirteen of the oligosaccharides had been characterized also after the previous purification procedure (see Scheme 1) [1]. In addition, seven neutral oligosaccharides were identified possessing structures that had not been discovered before for cystic-fibrosis mucins. These have been listed in Scheme 2. Most of them appeared to be extensions of smaller structures (Scheme 1) by one or two fucose residues, $\alpha(1\rightarrow2)$-attached to galactose residues, and were in accordance with the blood-group O activity of the patients. For a few hplc fractions, further separation of the components will be necessary to enable determination of their structure.

In general, the hplc retention times of the identified compounds appeared to increase with the number of constituting monosaccharides. Under the applied conditions, Fuc behaves smaller than GlcNAc, which in turn, is apparently smaller than Gal. The apparent sizes of the monosaccharides are modulated by their type of linkage. The latter is evidenced by the behaviour of oligosaccharides **1**, **10A** and **13** having in common a terminal *N*-acetylglucosamine in $\beta(1\rightarrow3)$-linkage to *N*-acetylgalactosaminitol: their respective retention times are shorter than those of the corresponding oligosaccharides possessing galactose instead of the terminal *N*-acetylglucosamine (oligosaccharides **2**, **11** and **15.1A**). Such an accelerating effect of *N*-acetylglucosamine has already been observed [5,13,14].

Within a certain class of size, hplc allowed the separation of structural isomers, for example, of trisaccharides **4**, **5** and **6**; of tetrasaccharides **10B** and **11**; of the monofucosyl pentasaccharides **12**, **14**, **15.1A** and **15.2B**; of the pentasaccharides **15.1B** and **15.2A**; and of monofucosyl hexasaccharides **17** and **19**. Oligosaccharides containing the type-1 sequence Gal$\beta(1\rightarrow3)$GlcNAc$\beta(1\rightarrow3)$GalNAc-ol (see, for example, compounds **4** and **15.1B**) showed a shorter retention time than their type-2 isomers with galactose in $\beta(1\rightarrow4)$-linkage to the *N*-acetylglucosamine (compounds **5** and **15.2A**, respectively).

Once the elution profile of the hplc procedure has been calibrated, this approach on its own is proposed as a suitable and convenient one to compare oligosaccharide profiles from mucins isolated from different bronchial hypersecretions. Finally, these data emphasize once more that the heterogeneity of bronchial-mucin carbohydrates is amazingly large and that one type of hplc procedure alone is not sufficient to separate and characterize all the structural variants, which occur.

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