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Structures of the α(1-3)-galactose-containing asparagine-linked glycans of a Lewis lung carcinoma cell subline resistant to *Aleuria aurantia* agglutinin: elucidation by ^{1}H-NMR spectroscopy

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Four bi-antennary glycan fractions of the *N*-acetyllactosamine-type, derived from a Lewis lung carcinoma (LL_2) cell subline resistant to the *Aleuria aurantia* agglutinin were studied by 400 MHz ^{1}H-NMR spectroscopy. By this method, their antennae were found to be terminated either by α(2-3 or 6)-linked *N*-acetylneuraminic acid or α(1-3)-linked galactose residues. The primary structure of glycans of these four glycopeptide or derived oligosaccharide-alditols has been determined in full detail.

Keywords: ^{1}H-NMR, asparagine-linked glycans, lectin resistance

Abbreviations: NAc, *N*-acetyl group; NGc, *N*-glycolyl group; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Man, mannose; Gal, galactose; Fuc, fucose; Con A, concanavalin A; LCA, *Lens culinaris* agglutinin; AAA, *Aleuria aurantia* agglutinin; WGA, Wheat germ agglutinin; RCA II, *Ricinus communis* agglutinin II; PBS, phosphate buffered saline, 0.01 M Na_2HPO_4/0.14 M NaCl, pH 7.2; HPLC, high performance liquid chromatography; EMEM, Eagle's Minimal Essential Medium; Lec^R, lectin resistant; MG, α-methylglycoside.

Numerous studies on cell surface glycoconjugates (glycoproteins and glycolipids) of normal and transformed cells have revealed characteristic changes in their expression associated with neoplastic transformation [1–3]. Increased branching of the *N*-acetyllactosamine type glycans and a higher degree of sialylation are typical examples of altered glycosylation of glycoproteins [1, 4, 5]. Changes in the organization and expression of carbohydrate chains of glycoproteins from metastatic and non-metastatic tumour cells have been reported but, in most cases, were not structurally characterized [6, 7]. To investigate this topic further, we developed a mouse tumour cell model: the Lewis lung carcinoma (LL_2) *in vitro* cell line and five lectin resistant (Lec^R) variant sublines with altered metastatic ability and preserved tumorigenicity [8, 9]. Applying a protocol of fractionation based on the use of immobilized *N*-glycosylproteins of the parent LL_2 line was compared to lectins [4], the distribution of radiolabeled glycans from those from three wheat germ agglutinin resistant (WGA^R) variants, a *Ricinus communis* agglutinin II resistant ($RCA\ II^R$) variant and an *Aleuria aurantia* agglutinin resistant (AAA^R) variant. The results revealed that low-metastatic WGA^R and $RCA\ II^R$ variants possessed less highly branched tri- and tetra-antennary glycans of the *N*-acetyllactosamine type with a simultaneous increase in bi-antennary *N*-acetyllactosamine type, oligomannosidic type or hybrid type glycans as compared to the parent metastasizing LL_2 cell line [10]. These findings imply that cell surface carbohydrate changes may possibly be relevant for metastasis.

However, during this study, we found that the AAA^R variant, with reduced spontaneous metastatic ability after subcutaneous (sc) administration, but with increased experimental metastatic ability after intravenous (iv) inoculation, exhibited apparently the same glycan pattern as the parent LL_2 line.

In order to understand the mechanism of the resistance of

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the LL_2 cells to the *A. aurantia* agglutinin, a fucose-specific lectin [11], and to search for specific carbohydrate determinants which could play a specific role in the increased experimental metastatic ability, analysis by 400 MHz ^{1}H-NMR spectroscopy of the primary structure of glycans of *N*-glycosylpeptides from the AAA^R variant was undertaken.

During this investigation, we detected the Galα1-3Galβ1-4GlcNAc moiety in some glycans. Here we report the detailed characterization of this structural element in four membrane α(1-6)-fucosylated bi-antennary glycopeptides isolated from the AAA^R variant.

Materials and methods

Cells

Lewis lung carcinoma LL_2 AAA^R cells were cultivated in EMEM containing 10% fetal bovine serum (both from Flow, Irvine, Scotland), 2 mM L-glutamine and antibiotics, at 37°C in a humidified atmosphere of 5% CO_2/95% air, as described previously [8]. This cell line was selected for its resistance to 10 μg ml^{-1} of the *A. aurantia* agglutinin through ten cycles of lectin treatment [9].

Preparation of α(1-6)-fucosylated bi-antennary glycopeptides

Cell pellets (10^9 unlabeled LL_2 AAA^R cells mixed with 10^7 LL_2 AAA^R cells, metabolically labeled with D-[6-^{3}H]-glucosamine hydrochloride (from The Radiochemical Centre, Amersham, UK) were delipidated according to the procedure of Oliver and Hemming [12]. Delipidated material was digested exhaustively for 72 h with Pronase (grade B, Calbiochem, San Diego, CA, USA) according to the method of Muramatsu *et al.* [13]. Insoluble material was removed by centrifugation at 48 000 × **g** for 2 h at 4°C and the supernatant was fractionated by gel filtration on a 100 × 1.6 cm column of Ultrogel ACA 202 (IBF, Villeneuve la Garenne, France) equilibrated with 0.01 M Tris/HCl buffer, pH 7.4, 0.17 M NaCl, 0.02% NaN_3 at a flow rate of 18 ml h^{-1}. Fractions (6 ml) were collected and 10 μl aliquots counted in 4 ml of Aqualyte liquid scintillation cocktail (J. T. Baker Chemicals, Deventer, The Netherlands) in a Beckman LS-1800 scintillation counter. The column was calibrated with a mixture of *N*-glycosylpeptides as described previously [10]. The eluted fractions containing *N*-glycosylpeptides (fraction F2) were pooled and desalted on a Bio-Gel P-2 (Bio-Rad, France) column (2.5 × 60 cm) equilibrated with water. Desalted fractions were concentrated in a rotary evaporator under reduced pressure and applied to a 2.5 × 15 cm column of Concanavalin A-Sepharose (Pharmacia, Les Ulis, France) equilibrated with 0.005 M sodium acetate buffer, pH 5.2, containing 0.1 M NaCl and 1 mM $MnCl_2$, $CaCl_2$ and $MgCl_2$. Elution was carried out first with the above buffer, allowing the recovery of *N*-glycosylpeptides containing highly branched glycans which passed unretained through the column (fraction FNR-C). Then, bi-antennary *N*-glycosylpeptides of the *N*-acetyllactosamine type were eluted with 10 mM α-methylglucoside in the above buffer (fraction FE-C 0.01 M). Finally, glycopeptides of the oligomannosidic type were eluted with the same buffer containing 0.3 M α-methylglucoside (fraction FE-C 0.3 M). After counting 10 μl aliquots, fractions containing glycopeptides with highly branched glycans (fraction FNR-C) and those containing glycopeptides with bi-antennary glycans (fraction FE-C 0.01 M) were pooled separately and desalted as described above. Desalted fractions were concentrated and applied on a 2 × 10 cm column of *Lens culinaris* agglutinin-Sepharose (LCA-Sepharose) prepared as described previously [14] and equilibrated in PBS buffer, pH 7.2. Elution was carried out first with the above buffer to elute unreactive glycopeptides. Then, elution of LCA-reactive glycopeptides containing an α-L-fucose residue in the C-6 position of the *N*-acetylglucosamine residue involved in the *N*-glycosylamine linkage was obtained with the same buffer containing 0.15 M α-methylglucoside. Affinity chromatography on both Con A- and LCA-Sepharose as carried out at room temperature and at a flow rate of 10 ml h^{-1}. Fractions (1.5 ml) were collected and 20 μl aliquots counted as described above.

Alkaline cleavage of N-glycosidic linkages of the α(1-6)-fucosylated bi-antennary glycopeptides

Alkaline cleavage of the *N*-glycosidic linkages of the α(1-6)-fucosylated bi-antennary glycopeptides was performed according to the method of Lee and Scocca [15]. Briefly, 2 ml of a 1 M NaOH/1 M $NaBH_4$ solution were added to the glycopeptide mixture and the solution was heated under reflux for 6 h at 100°C. The mixture was cooled in an ice-bath and then adjusted to pH 6.0 with 50% acetic acid and desalted on a Bio-Gel P-2 column (2 × 60 cm) equilibrated with water. The oligosaccharide-alditols were revealed by orcinol-H_2SO_4 assay [16], pooled and lyophilized. Re-N-acetylation of oligosaccharide-alditols was carried out in 1 ml of saturated $NaHCO_3$ solution, by addition of five aliquots of 10 μl of acetic anhydride at 5 min intervals. The reaction mixture was desalted on a Dowex 50-X8 (25–50 mesh, H^+) column (1 × 5 cm) and then on a Bio-Gel P-2 column (2 × 60 cm) equilibrated with water.

High performance liquid chromatography of α(1-6)-fucosylated bi-antennary oligosaccharide-alditols

Anion-exchange chromatography of oligosaccharide-alditols was carried out with a Spectra-Physics liquid chromatograph model SP 8700 (Spectra-Physics, Les Ulis, France) using a Micropak AX-10 column (0.4 × 25 cm) (Varian Associates, Walnut Creek, CA, USA) by applying a modification of the procedure of Baenziger and Natowicz [17]. Samples were injected in 500 μl of water and elution was carried out for 10 min at a flow rate of 60 ml h^{-1} with water; then an increasing linear gradient of KH_2PO_4, pH 4.0 from 0 to 25 mM for 10 min, followed by a 5 min plateau at this

concentration, was applied. Finally, an increasing linear gradient of KH_2PO_4, pH 4.0 from 25 mM to 100 mM in 30 min was applied, followed by a linear increase of the phosphate concentration up to 400 mM over 20 min. Fractions of 1 ml were collected and radioactivity of 5 µl aliquots counted as described above.

Methylation analysis

This was done as described by Paz Parente *et al.* [18] allowing the methylation of microquantities. The methyl-derivatives were identified after gas-liquid chromatography/mass spectrometry analysis as described by Fournet *et al.* [19].

400 MHz ^{1}H-NMR spectroscopy

Desalted glycopeptides or oligosaccharide-alditols were repeatedly treated with ^2H_2O (99.95%, Commissariat à l'Energie Atomique, Saclay, France) at pH 6–7 and room temperature with intermediate lyophilization. 400 MHz ^{1}H-NMR Spectroscopy was performed using a Bruker AM-400 W spectrometer operating in the pulsed Fourier-Transform mode at a probe temperature of 27°C. Chemical shifts (δ) are expressed downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm in ^2H_2O at 27°C).

Results

Fractionation of glycopeptides from Lewis lung carcinoma LL_2 AAA^R cell variant

The mixture of glycopeptides obtained after Pronase digestion of the delipidated cell pellet was fractionated according to Fig. 1. As reported previously [10], two major fractions, F1 and F2, were separated by gel filtration on Ultrogel ACA 202 (data not shown). Fraction F1, eluted at the void volume, contained proteoglycans and large glycopeptides with *O*-glycosidically linked glycans and represented 36% of the total membrane glycopeptides. The second fraction (F2) contained the *N*-glycosylpeptides representing 64% of the total membrane glycopeptides. *N*-Glycosylpeptides from fraction F2 were further fractionated by affinity chromatography on Con A and subsequently on LCA-Sepharose.

Con A-Sepharose affinity chromatography resulted in three major fractions (Fig. 2). According to the well known interactions between immobilized Con A and glycans (14, 20], the non-retained fraction (FNR-C) contained glycopeptides with tri- and tetra-antennary glycans of the *N*-acetyllactosamine type and/or more branched glycans and/or bi-antennary glycans with a bisecting *N*-acetylglucosamine residue. This fraction represented 27.10% of the total membrane glycopeptides. The weakly retained fraction eluted with 0.01 M α-methylglucoside (FE-C 0.01 M) contained glycopeptides with glycans of the bi-antennary *N*-acetyllactosamine type and represented 23.90% of the total membrane glycopeptides. The strongly retained fraction eluted with 0.3 M α-methylglucoside (FE-C 0.3 M) contained mainly glycopeptides with oligomannosidic type glycans (13.60% of the total membrane glycopeptides).

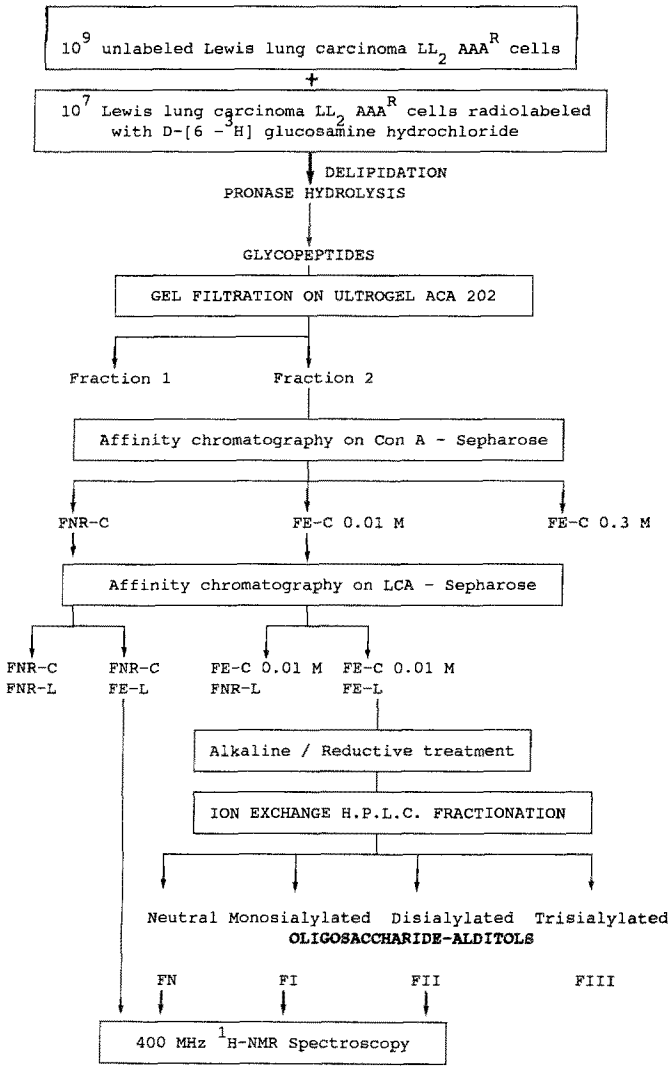


Figure 1. Fractionation scheme of glycopeptides obtained after Pronase digestion of delipidated cell pellet. For details see the Materials and methods section.

The Con A-Sepharose non-retained (FNR-C) and weakly bound (FE-C 0.01 M) glycopeptides were further fractionated on a LCA-Sepharose column in order to separate unbound glycopeptides with glycans devoid of α(1-6)-linked fucose residue in the C-6 position of the *N*-acetylglucosamine in the GlcNAc-Asn linkage (FNR-C/FNR-L and FE-C 0.01 M/FNR-L) and bound glycopeptides possessing this α(1-6)-linked fucose residue (FNR-C/FE-L and FE-C 0.01 M/FE-L), and eluted with 0.15 M α-methylglucoside in PBS [14, 20].

The Con A-non reactive but LCA-bound glycopeptide fraction (FNR-C/FE-L) which represented 1.2% of the total

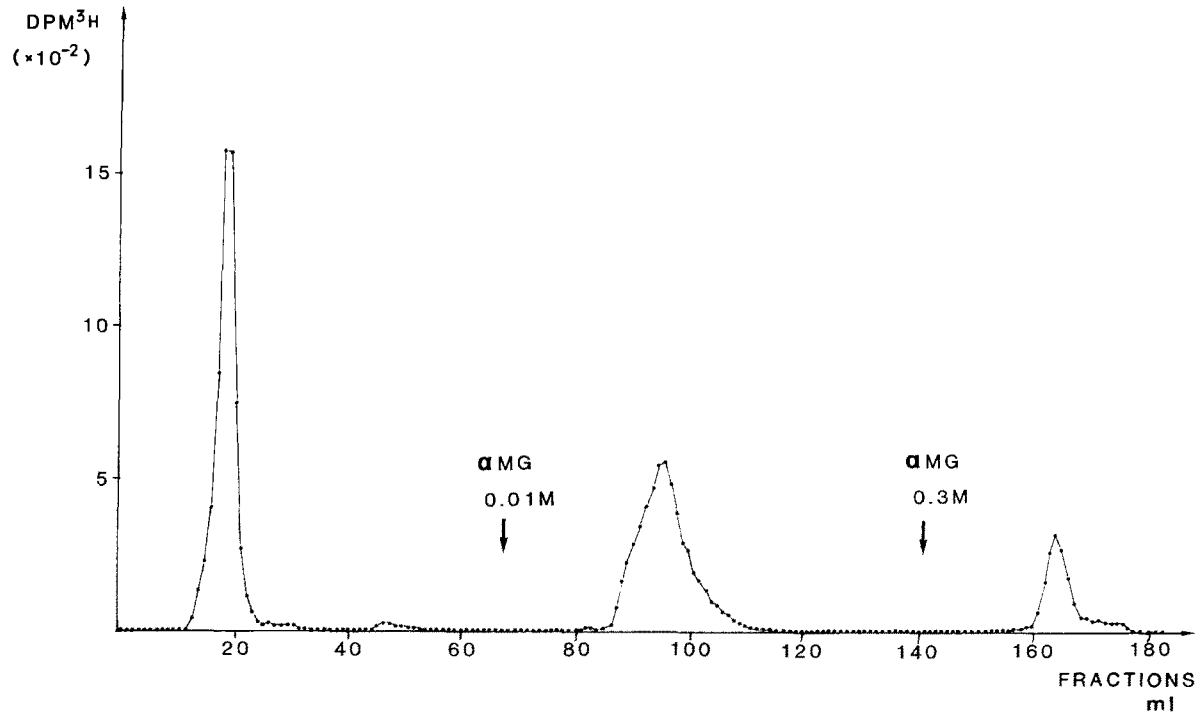


Figure 2. Fractionation pattern of membrane *N*-glycosylpeptides from LL_2 AAA^R cells by affinity chromatography on Con A-Sepharose. Flow rate: 18 ml h^{-1}; fraction volume: 6 ml.

membrane glycopeptides was, after desalting, directly analysed by 400 MHz ^{1}H-NMR spectroscopy.

The Con A-weakly reactive and LCA-bound glycopeptide fraction (FE-C 0.01 M/FE-L) representing 17.8% of total glycopeptides with bi-antennary glycans of the *N*-acetyllactosamine type was submitted to an alkaline cleavage of the *N*-glycosidic linkages according to the method of Lee and Scocca [15]. The released oligosaccharide-alditols were further fractionated according to their charge by anion-exchange HPLC. Four fractions were obtained (Fig. 3): i) the neutral glycans (FN) representing 4.2% of the total material; ii) glycans with one sialic acid residue (F-I) 7.7%; iii) glycans with two sialic acid residues (F-II) 4.8%; and iv) more highly charged oligosaccharide-alditols (F-III) representing 1.1% of the total membrane glycopeptides. This fourth fraction was too heterogeneous to obtain interpretable 400 MHz ^{1}H-NMR spectra.

Structure of the FNR-C/FE-L glycopeptide fraction

The interpretable part of the 400 MHz ^{1}H-NMR spectrum of the FNR-C/FE-L glycopeptide fraction is shown in Fig. 4. The main representative resonances are respectively: at $\delta = 5.144$ ppm, the anomeric proton of the α(1-3)-linked galactose residue and at 5.055 ppm and 5.005 ppm, the significant H-1 signals of Man-**4** and **4′** of a bisecting *N*-acetylglucosamine containing oligosaccharide structure. This is confirmed by the signals observed at 4.465 and 2.064 ppm, corresponding to the H-1 and NAc resonance of GlcNAc-**9**, at $\delta = 1.720$ and 1.800 ppm, the H-3a signal

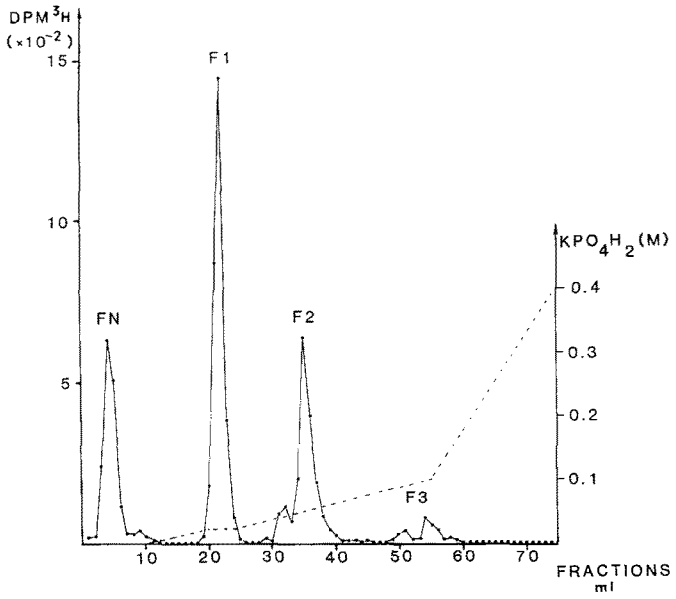


Figure 3. Fractionation pattern of the oligosaccharide-alditols derived from α(1-6)-fucosylated bi-antennary glycopeptides by anion-exchange HPLC on a Micropak AX-10 column. Neutral oligosaccharide-alditols were eluted with water and sialylated oligosaccharide-alditols with a monopotassium dihydrogen phosphate pH 4.0 gradient. FN: neutral glycans, F-I, F-II and F-III, mono-, di- and trisialylated glycans, respectively. Injection volume: 0.5 ml; flow rate: 60 ml h^{-1}; fraction volume: 1 ml.

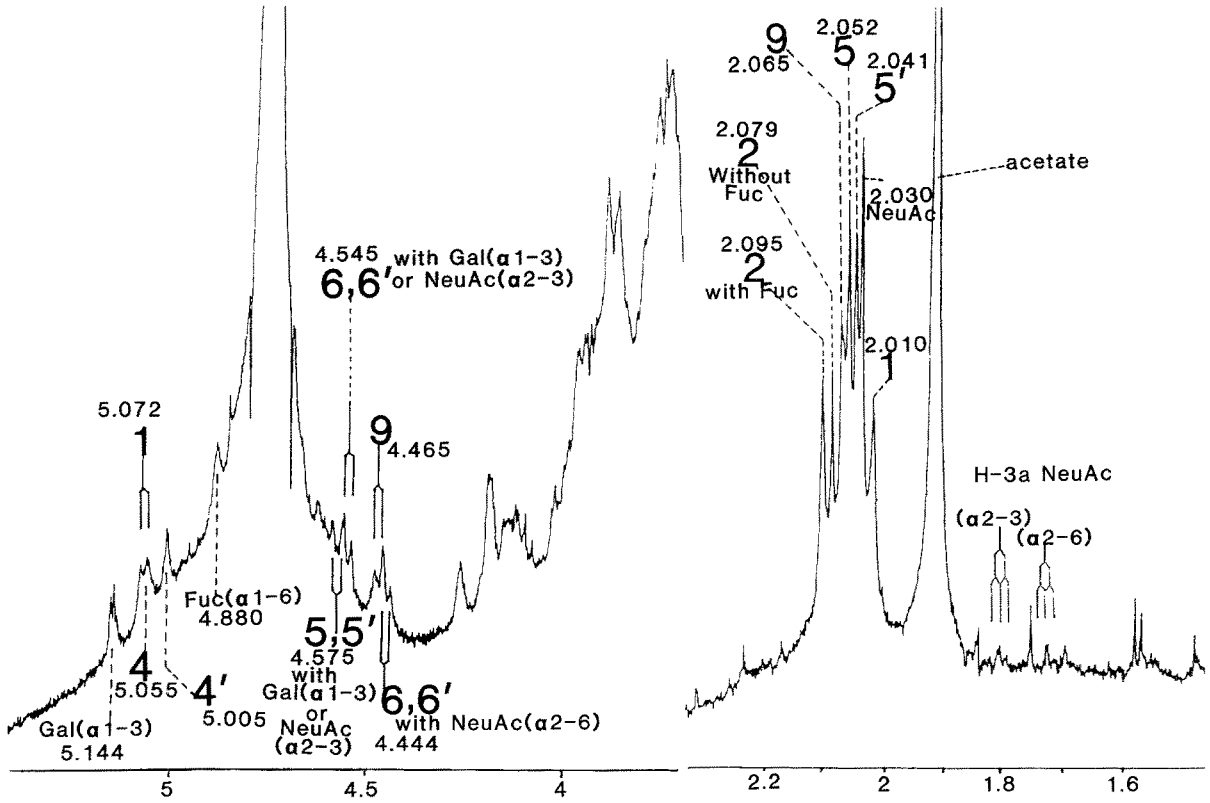


Figure 4. 400 MHz ^{1}H-NMR spectrum of the Con A-non reactive, LCA-bound glycopeptide fraction (FNR-C/FE-L).

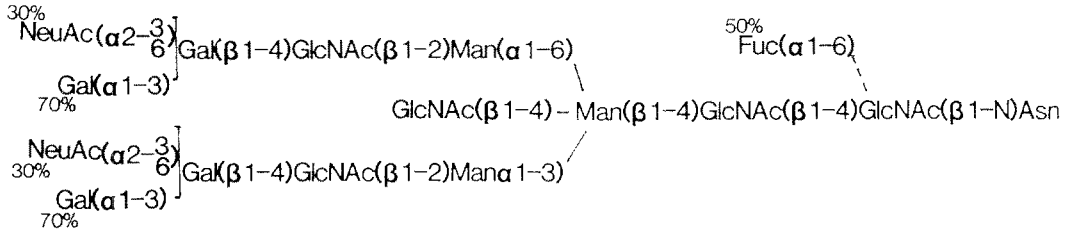


Figure 5. Structure of the glycans from the Con A-non reactive, LCA-bound glycopeptide fraction (FNR-C/FE-L).

of α(2-6)- and α(2-3)-linked *N*-acetylneuraminic acid. The absence of α(2-6)- or α(2-3)-linked *N*-glycolylneuraminic acid residues in a terminal position is reflected mainly by the absence of an NGc signal at $\delta = 4.118$ ppm [21]. Figure 4 gives some other parameters reliably assigned to H-1 of Gal-**6**′ and NAc of GlcNAc-**5**/-**5**′. The overlap of resonances relevant from sialo- and asialo-Gal residues does not allow more precise assignment of these NMR parameters. Indeed, especially for the NAc signals, the simultaneous presence of α-Gal and GlcNAc-**9** results in increments of low intensity which cannot be accurately determined on such a complex spectrum. Nevertheless, these results are sufficient for proposing a general structure as shown in Fig. 5.

Structure of the FE-C 0.01 M/FE-L glycopeptide fraction

In a preliminary attempt to obtain structural information, the 400 MHz ^{1}H-NMR spectrum of the FE-C 0.01 M/FE-L glycopeptide fraction was obtained (data not shown). This spectrum was too complex to propose the glycan structures. However, the absence of α(2-6)- or α(2-3)-linked *N*-glycolylneuraminic acid residues at a terminal position is also reflected here by the absence of an NGc signal at $\delta = 4.118$ ppm. This point is important since in our fractionation procedure, the alkaline cleavage of *N*-glycosidic linkages removes all *N*-acyl groups (acetyl and glycolyl) and since re-*N*-acetylation is performed with acetic anhydride, the liberated sialyloligosaccharide-alditols contain only *N*-acetylneuraminic acid.

Structure of the neutral oligosaccharide-alditols (fraction FN)

The NMR spectrum of the neutral oligosaccharide-alditols fraction is shown in Fig. 6a and the chemical shifts are shown in Table 1. This fraction contains bi-antennary asialo-oligosaccharide-alditols with α(1-6)-linked fucose on

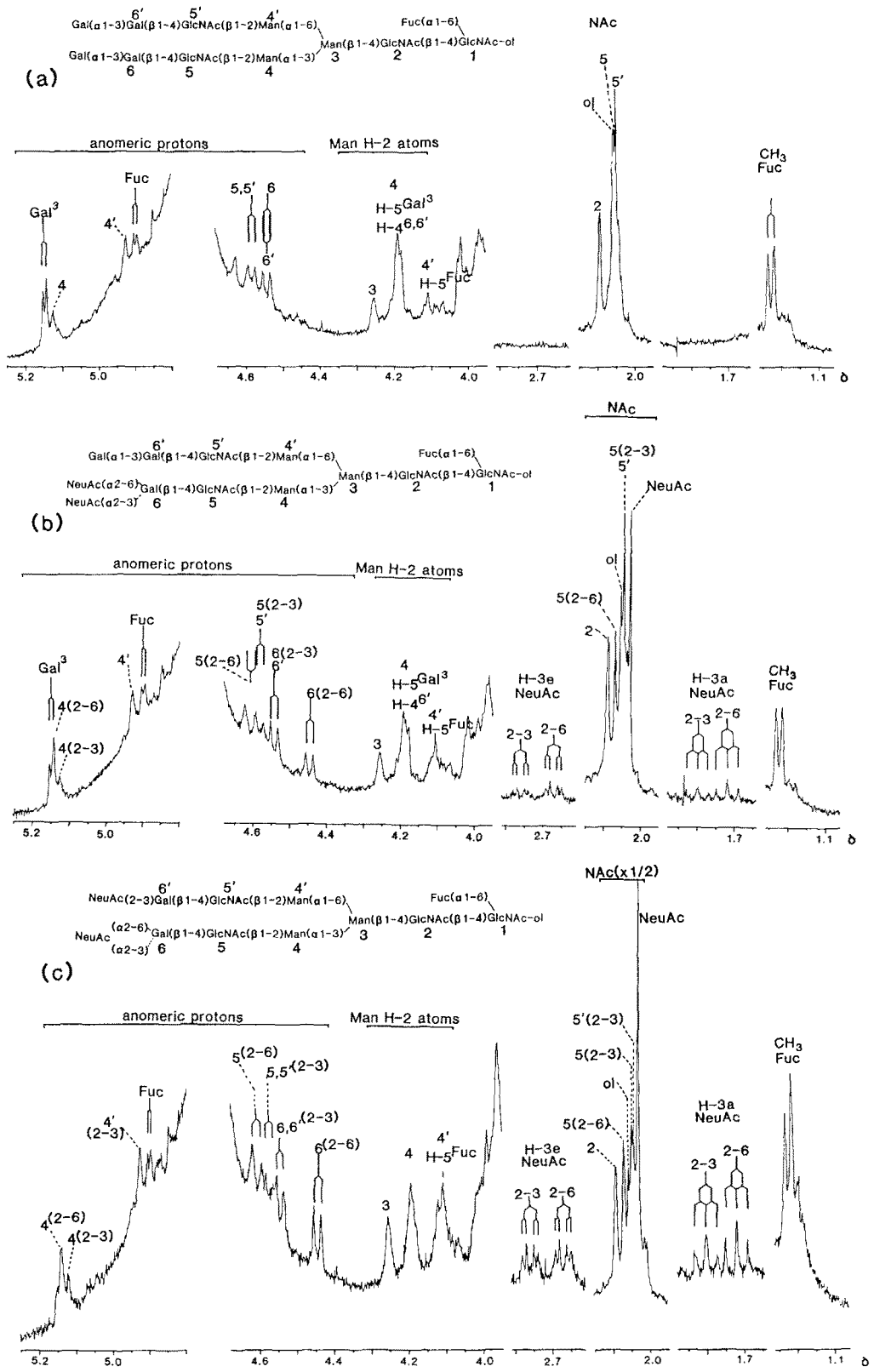
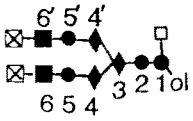
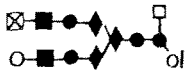
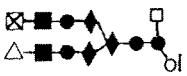
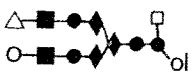
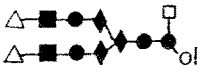


Figure 6. 400 MHz ^{1}H-NMR spectra and structures of the oligosaccharide-alditols derived from the Con A-weakly reactive, LCA-bound glycopeptide fraction (FE-C 0.01 M/FE-L). (a) Neutral fraction (FN); (b) monosialyl oligosaccharide-alditols (F-I); (c) disialyl oligosaccharide-alditols (F-II).

Table 1. ^{1}H Chemical shifts of structural-reporter groups of constituent monosaccharides for oligosaccharide-alditols released from Con A weakly reactive and LCA-bound glycopeptide Fraction (FE-C-0.01 M/FE-L).

Reporter group	*Residue*	*Compound and schematic structure*				
						
		FN	*FI-1*	*FI-2*	*FII-1*	*FII-2*
H-1 of	2	n.d.	n.d.	n.d.	n.d.	n.d.
	3	n.d.	n.d.	n.d.	n.d.	n.d.
	4	5.123	5.139	5.124	5.138	5.118
	4′	4.924	4.928	4.924	4.924	4.924
	5	4.581	4.60	4.582	4.605	4.574
	5′	4.581	4.582	4.582	4.574	4.574
	6	4.539	4.446	4.543	4.445	4.545
	6′	4.543	4.543	4.543	4.545	4.545
	⊠ Gal^3	5.144	5.144	5.144	–	–
	□ Fuc^6	4.898	4.898	4.898	4.898	4.898
H-2	3	4.253	4.256	4.256	4.254	4.254
	4	4.194	4.19	4.19	4.19	4.19
	4′	4.11	4.11	4.11	4.11	4.11
H-3a	○ NeuAc^6	–	1.718	–	1.718	–
	△ NeuAc^3	–	–	1.798	1.800	1.800
H-3e	NeuAc^6	–	2.676	–	2.676	–
	NeuAc^3	–	–	2.758	2.758	2.758
H-4	6	4.181	–	–	–	–
	6′	4.181	4.19	4.19	–	–
H-5	Gal^3	4.194	4.19	4.19	–	–
	CH_3	(4.09–4.10)	(4.09–4.10)	(4.09–4.10)	(4.09–4.10)	(4.09–4.10)
NAc	1-ol	2.057	2.057	2.057	2.057	2.057
	2	2.089	2.089	2.089	2.09	2.09
	5	2.054	2.072	2.049	2.071	2.049
	5′	2.048	2.049	2.049	2.044	2.044
	NeuAc	2.031	2.031	2.031	2.031	2.031
CH_3	Fuc^6	1.225	1.225	1.225	1.225	1.225

GlcNAc-ol-**1′**, as verified by the downfield shift effect on the NAc proton signal of GlcNAc-**2** observed at $\delta = 2.089$ ppm, and by the presence of H-1 and CH_3 signals of Fuc-**6** at $\delta = 4.898$ and 1.225 ppm, respectively. The presence of the α(1-6)-linked fucose residue on these oligosaccharide-alditols is consistent with the behaviour of bi-antennary glycopeptides on LCA-Sepharose [14].

The H-1 resonances of Gal-**6** and -**6′** present unusual values ($\delta = 4.539$ and 4.543 ppm), which result from a substitution at C-3 with an α-Gal residue (δH-1 = 5.144 ppm) [22]. The C-3 substitution was also confirmed by the methylation analysis, which indicated 2,4,6-tri-*O*-methyl-galactose among the methyl ethers (Table 2). For Gal-**6**, -**6′** H-1 and GlcNAc-**5**/**5′** NAc resonances, the NMR parameters relevant to the (1-6)- and (1-3)-antennae were determined by comparison with the results obtained for the monosialyl bi-antennary oligosaccharide-alditol fraction (Fraction F-I) which is exclusively sialylated on the (1-3)-antenna (see below). Consequently, the Gal-**6′** H-1 resonance was found at a lower field than for Gal-**6**, and the GlcNAc-**5′** NAc resonance observed at a higher field than for GlcNAc-**5**. This is in accordance with the general observation made for asialo- or sialo-antennae [23]. The H-1 resonances of Man-**4** and -**4′** are not affected by the presence of external α(1-3)-linked galactose residues and are identical to those observed for a classical asialo-bi-antennary oligosaccharide [23]. The H-2 resonances of Man-**4** and -**4′** were not clearly established. Particularly, the intense signal observed at 4.19 includes the H-5 resonance of the two α(1-3)-linked galactose residues and the H-4 resonance of galactose-**6** and -**6′** together with Man-**4** H-2, as shown by Dorland *et al.* [22].

According to the NMR and methylation analysis, the structure of oligosaccharide-alditols present in Fraction FN is as proposed in Fig. 6a.

Table 2. Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated oligosaccharide-alditols released from Con A weakly reactive and LCA-bound glycopeptide fraction (FE-C 0.01 M/FE-L). The molar ratios were calculated on the basis of one residue of 2,4-Me_2Man.

Monosaccharide methyl ethers	*Molar ratio*		
	FN	*F-I*	*F-II*
2,4-Me_2Man	1	1	1
3,4,6-Me_3Man	2.1	2.2	2.1
2,4,6-Me_3Gal	1.8	1.3	1.4
2,3,4-Me_3Gal	–	0.5	0.5
2,3,4,6-Me_4Gal	1.7	0.8	–
3,6-Me_2GlcN(Me)Ac	2.6	2.5	2.4
1,3,5,6-Me_4GlcN(Me)Ac-ol	0.4	0.4	0.5
2,3,4-Me_3Fuc	0.7	0.8	0.7
4,7,8,9-Me_4NeuAc	–	0.7	1.6

Structure of the monosialyl bi-antennary oligosaccharide-alditols (Fraction F-I)

Oligosaccharide-alditols of Fraction F-I are bi-antennary, monosialylated structures, fucosylated on GlcNAc-**1**, as previously reported for the above oligosaccharide-alditols. The NMR spectrum of these oligosaccharide-alditols is shown in Fig. 6b. Gal-**6** and Gal-**6′** are substituted by α(1-3)-linked galactose (δH-1 = 5.144 ppm) and α(2-3)- or α(2-6)-linked *N*-acetylneuraminic acid residues (δH-3a at 1.718/1.798 ppm; δH-3e at 2.676/2.758 ppm). The pattern of the NAc proton signals of the GlcNAc-**5** and -**5′** allows us to define the respective position of the substitution. The *N*-acetyl resonance observed at $\delta = 2.049$ ppm is significant for the presence of α-Gal at the extremity of the (1-6)-antenna (compare with oligosaccharide-alditols of Fraction FN, Fig. 6a). The absence of a signal at $\delta = 2.054$ indicates that the (1-3)-antenna is devoid of an α(1-3)-linked galactose residue. Consequently, Gal-**6** is exclusively substituted by α(2-3)- and/or α(2-6)-linked *N*-acetylneuraminic acid residues. These two types of linkage are also confirmed by the doubling of the H-1 signal of Man-**4**, at $\delta = 5.139$ ppm [α(2-6)-linked *N*-acetylneuraminic acid] and $\delta = 5.124$ ppm [α(2-3)-linked *N*-acetylneuraminic acid].

As for the neutral Fraction FN, the Man-**4** H-2 signal is larger than expected, due to the overlap of the H-4 and H-5 resonances of Gal-**6′** and α(1-3)-linked galactose.

Consequently, Fraction F-I was found to contain the two oligosaccharide-alditols shown in Fig. 6b. The same monosialylated glycan structure has been characterized in mouse myeloma immunoglobulin IgM heavy chains but with an α(2-6)-linked *N*-glycolylneuraminic acid residue instead of an α(2-6)- or α(2-3)-linked *N*-acetylneuraminic acid residue [21].

Structure of the disialyl bi-antennary oligosaccharide-alditols (Fraction F-II)

Oligosaccharide-alditols of Fraction F-II are bi-antennary and disialylated structures, fucosylated on the GlcNAc-ol residue. The NMR spectrum of these oligosaccharide-alditols is shown in Fig. 6c. The α(2-3)- or α(2-6)-linked *N*-acetylneuraminic acid groups are attached to Gal-**6**, as may be inferred from the doubling of the chemical shift of the H-1 of Man-**4** ($\delta = 5.138$ and 5.118 ppm). On the other hand, *N*-acetylneuraminic acid is essentially α(2-3)-linked at the Gal-**6′** residue. Indeed, a major signal is observable at $\delta = 4.924$ for H-1 of Man-**4′**, while a signal of very low intensity is hardly discernible at $\delta = 4.946$ ppm. The presence of traces of α(2-6)-linked *N*-acetylneuraminic acid on Gal-**6′** can be also verified by the discrete resonance observed at $\delta = 2.067$ ppm.

Consequently, Fraction F-II contains the two main oligosaccharide-alditols shown in Fig. 6c.

Considering the minor glycans with an α(2-6)-linked *N*-acetylneuraminic acid residue on the Gal-**6′**, we were unable to determine the type of linkage of *N*-acetylneuraminic acid to the second antenna.

Discussion

The primary structure of α(1-6)-fucosylated bi-antennary asparagine-linked oligosaccharides derived from cell membranes of a Lewis lung carcinoma (LL_2) variant resistant to the toxic action of the *A. aurantia* agglutinin have been elucidated by 400 MHz ^{1}H-NMR spectroscopy. During this investigation, the Galα1-3Galβ1-4GlcNAcβ1- structural element was characterized for the first time in four fucosylated bi-antennary glycans isolated from the AAA^R variant, and new sets of ^{1}H-NMR data are provided here. The study of the two other *N*-glycosylpeptide fractions: FNR-C/FNR-L and FE-C 0.01 M/FNR-L which may also contain this structural element is in progress. The Galα1-3Gal epitope has been found previously in various tissues of non-primate mammals [24, 25].

This structural element has been also reported to occur in the carbohydrate chains of membrane glycoproteins of Ehrlich ascites tumor cells [26, 27] as well as in Friend murine leukemia virus glycoproteins [28].

Glycans which terminate in α-galactosyl groups have also been described in glycoproteins such as murine laminin [29], mouse myeloma immunoglobulin IgM heavy chains [21], bovine thyroglobulin [22] and also in some glycolipids [30].

In this study, the presence of bi-antennary monosialylated oligosaccharide-alditols possessing α-Gal exclusively at the terminal position of the α(1-6)-linked mannose antenna can be correlated with the specificity of an Ehrlich tumor cell α(1-3)-galactosyltransferase for the α(1-6)-linked mannose antenna, as was recently demonstrated by Elices and Goldstein [31]. It is also noteworthy that in two of the analysed

glycan fractions, there was a concomitant expression of both α-D-galactosyl and sialic acid residues. This phenomenon has been previously described for several other cell lines [32–35]. Differential expression of these two sugars at the non-reducing terminal position on membrane glycoproteins, by competitive action of glycosyltransferases responsible for the termination of antennae, may induce different cellular phenotypes. Recently Santer *et al.* [36] analysed membrane glycopeptides of NIH 3T3 fibroblasts and their H-*ras* oncogene transformants by 500 MHz ^{1}H-NMR spectroscopy. They found that multi-antennary glycans of the *N*-acetyllactosamine type may be necessary, but not sufficient for complete expression of the transformed phenotype. A large percentage of glycans were multi-antennary prior to transformation and were terminated with α-Gal residues but, after transformation, most of the β-Gal residues were substituted by α(2-3)-linked *N*-acetylneuraminic acid residues. However, the expression of sialylated and β(1-6)-branched glycans was also reported to be closely associated with metastatic potential [5]. The detailed characteristics of these glycan changes occurring at the particular stages of metastatic process seem to be helpful to elucidate the problem.

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