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Structure of a New Nonasaccharide Isolated from Human Milk: VI^{2}Fuc,V^{4}Fuc,III^{3}Fuc-*p*-Lacto-*N*-hexaose

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The structure of a new nonasaccharide isolated from human milk has been investigated. By using methylation analysis, FAB-MS and ^{1}H- and ^{13}C-NMR spectroscopy as basic methods of structural investigation, this oligosaccharide was identified as VI^2-α-Fuc,V^4-α-Fuc,III^3-α-Fuc-*p*-lacto-*N*-hexaose:

Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc.

Up to now, more than 50 different human milk oligosaccharides have been isolated [1-7] and an increased interest has been shown in them since many have structures similar to cell surface glycolipids and glycoproteins. In addition, some minor oligosaccharide components of human milk were recently characterized as gastrointestinal tumor-associated ganglioside antigens, isolated from human colorectal carcinoma cell lines [8-10]. By analysis of human milk oligosaccharides, we have detected more than 70 sialyl and neutral higher oligosaccharides, by using new, improved HPLC columns. This paper describes the isolation and structural studies of a new nonasaccharide.

Abbreviations: COSY, correlation spectroscopy; DP, degree of polymerisation; FAB-MS, fast atom bombardment-mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; GLC, gas-liquid chromatography.

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Material and Methods

Fractionation of Milk Oligosaccharides

Pooled milk was defatted by centrifugation at 3000 × *g* for 1 h at 4°C and oligosac- were isolated by ultrafiltration on membrane AN 69 (Hemodialyser Hospal, Sodip, Meyzieu, France). After removal of the sialyloligosaccharides on an anion exchanger (Dowex 1-X2; 200-400 mesh; bicarbonate form), the neutral solution obtained was evaporated under reduced pressure and oligosaccharides were separated from lactose by gel filtration on Bio-Gel P-2 (140 × 8 cm, Bio-Rad, Richmond, CA, USA). The whole oligosaccharide fraction (DP-4 to DP-14) was pooled and submitted to preparative paper chromatography for three days. The major oligosaccharides (DP-4 to DP-6) were isolated by this procedure, while the higher oligosaccharides remained at the origin. After elution from the paper, this latter material was submitted to a new preparative paper chromatography developed for 12 days. Eleven fractions were obtained, each of them being heterogeneous when analyzed by HPLC as described below. In particular, fraction VII was subfractionated into 18 components when analyzed by HPLC, and the major one (VII-2) was found to be a nonasaccharide (yield: 60 mg, starting from 20 l of human milk).

Analytical Methods

Descending paper chromatography was performed on Whatman no. 3 paper using pyridine/ethyl acetate/acetic acid/water, 5/5/1/3 by vol, with detection by aniline oxalate reagent [11]. Preparative reversed phase HPLC of native oligosaccharides was performed on a 5 μm ODS Zorbax column (25 cm × 0.94 cm I.D.; Du Pont Instruments, Paris, France). The solvent was distilled water and the flow rate was 0.5 ml/min for 1 h, then 2 ml/min to the end of the analysis. The oligosaccharides were detected at 206 nm.

Methylation Analysis

Oligosaccharides, previously reduced with $NaBH_4$, were methylated [12] and then methanolysed (0.5 N methanolic HCl, 24 h, 80°C). The partially methylated methyl glycosides were *O*- and *N*-acetylated [13] using pyridine/acetic anhydride, 1/5 by vol, and the methylated monosaccharides were analysed by GLC-MS using a capillary column (0.33 mm × 25 m) coated with fused silica CP-SIL 5 CB (temperature programme, 100-240°C, 4°C/min).

Fast-atom Bombardment (FAB) Mass Spectrometry

FAB-MS was performed on a Kratos MS-50 mass spectrometer using xenon atoms (2×10^5 torr) with a kinetic energy equivalent to 9 keV.

The spectrometer was calibrated with CsI. The spectra were recorded in positive-ion mode at 5 kV acceleration voltage. The permethylated oligosaccharide (5-10 μg), dissolved in 1 μl of methanol, was added to the thioglycerol matrix without previous coating with sodium salt.

The 400 MHz ^{1}H-NMR experiments were performed on a BRUKER AM-400 WB spectrometer equipped with a 5 mm ^{1}H/^{13}C mixed probe-head, operating in the pulse Fourier transform mode and controlled by an Aspect 3000 computer. The concentration of the oligosaccharide was 55 mg/0.5 ml $^{2}H_2O$. After three exchanges with $^{2}H_2O$ (99.96% atoms ^{2}H, Aldrich, Milwaukee, IL, USA) and intermediate lyophilisation, the products were analysed with a spectral width of 3000 Hz for 16 K frequency domain points and time domain data points giving a final digital resolution of 0.365 Hz/point.

The 100 MHz ^{13}C-NMR experiments were obtained using the standard BRUKER pulse programme POWGATE with ^{1}H broad band composite-pulse decoupling. The spectral width is 20 000 Hz for 32 K frequency domain points and time domain data giving a final digital resolution of 1.221 Hz/point. A 90° pulse (6 μsec) and a 0.5 sec recycle delay were used. The chemical shifts are given relative to sodium-4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured to the methyl of acetone (δ = 2.225 ppm for ^{1}H and δ = 31.55 ppm for ^{13}C).

The 2D-homonuclear COSY 45 experiments were performed using the standard BRUKER pulse programme COSY. In these experiments the spectral width was 1800 Hz. The ^{1}H ninety degrees pulse was 10.6 sec. 256 W × 2 K data matrix was acquired which was zerofilled prior to Fourier transformation to obtain a 1 K × 2 K spectral data matrix; a sine-bell squared funDction was used in both dimensions. The 2D-homonuclear COSY with simple and double relay transfers was performed using the standard BRUKER pulse programme COSYRCT and the pulse programme COSYDR (Bruno Perly, C.E.A. Saclay, personal communication). For example, two COSYDR experiments were performed using the sequence: D_1-90-D_ϕ-90-D_2-180-D_2-90-D_3-180-D_3-90-FID, where D_1 = 2 sec; 90, 180 = 90°, 180° ^{1}H pulse (90° = 10.6 μsec); D_ϕ = incremental delay (initial = 3 μsec); D_2 = D_3 = 35 ms (80 ms in another experiment). In all experiments a spectral width of 1800 Hz, 256 W × 2 K data matrix was obtained, which were zerofilled to 1 K × 2 K prior to Fourier-transformation, a sine-bell squared function was used in both dimensions.

The 2-D heteronuclear-correlated experiment was performed with simultaneous ^{1}H decoupling [14, 15] using the standard BRUKER pulse programme XHCORRD. In this experiment, the phase cycling of the refocusing pulse described by Wilde and Bolton [16] was additionally used. Refocusing delays were adjusted to an average $J_{C\text{-}H}$ coupling constant of 150 Hz [17]. Spectral windows of 9800 Hz, with 4096 data points, for ^{13}C; and 870 Hz, with 128 data points, for ^{1}H were employed. ^{1}H- and ^{13}C-90° pulse widths were 10.6 and 6 μsec respectively. A 128 W × 4 K data matrix was acquired which was zero-filled prior to Fourier-transformation to obtain a 512 W × 4 K spectral data matrix. The F_1 domain was multiplied by a sine-bell function and the F_2 domain by a line-broadening function (4 Hz) prior to processing.

Results and Discussion

Fraction VII obtained by paper chromatography was subfractionated by reverse phase HPLC into 18 peaks (Fig. 1). Peak VII-8 itself could be divided into four sub-fractions when a flow rate of 0.25 ml/min was applied to the column instead of 0.5 ml/min. In fact,

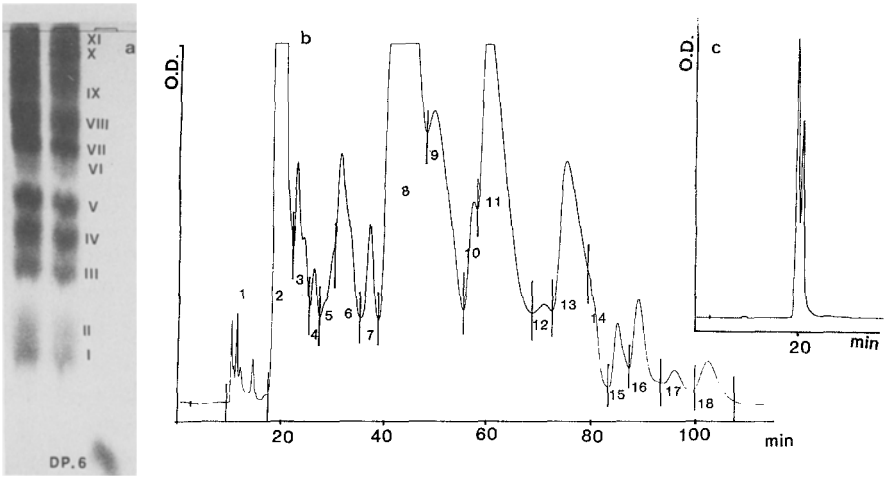

Figure 1. Fractionation of higher milk oligosaccharides.
(a) Paper chromatography of higher neutral oligosaccharides; migration, 12 days. (b) HPLC of fraction VII on an ODS column. (c) HPLC of fraction VII-2 on an ODS column.

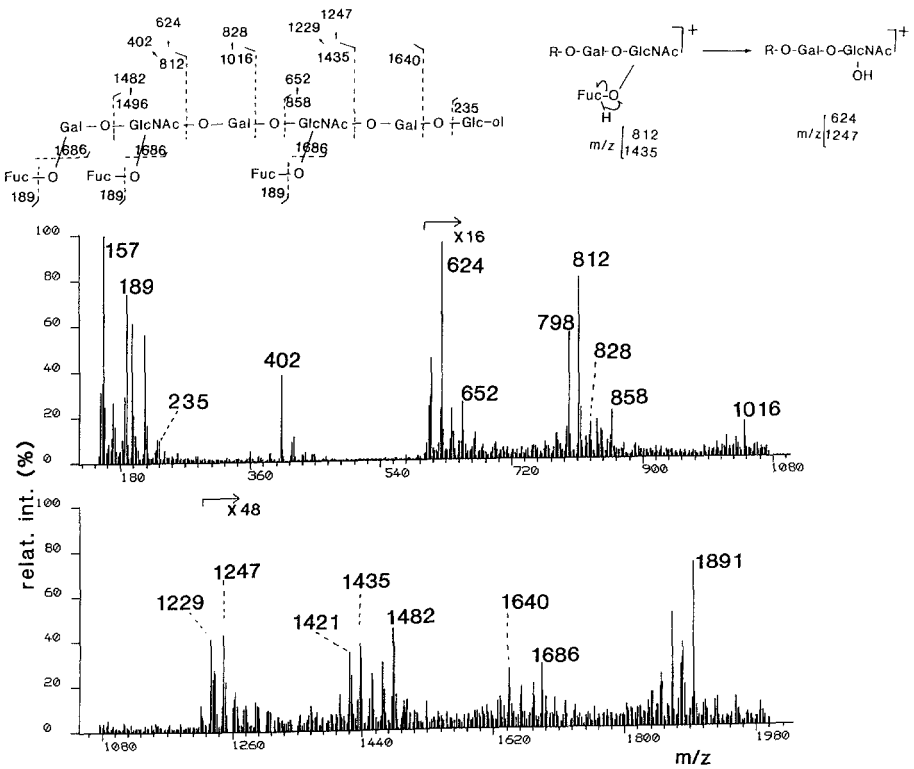

Figure 2. FAB-mass spectrum obtained from 10 μg of permethylated oligosaccharide VII-2.

Table 1. Carbohydrate composition and relative intensity of major ions of diagnostic importance observed in the FAB-MS spectra of reduced and permethylated nonasaccharide.

m/z	Relative intensity (% of base peak)	Carbohydrate composition
1891	1.6	$Fuc_3Gal_3GlcNAc_2Glc\text{-}ol + H$
1686	0.6	$Fuc_2Gal_3GlcNAc_2Glc\text{-}ol$
1640	0.6	$Fuc_3Gal_3GlcNAc_2$
1496	0.2	$Fuc_2Gal_2GlcNAc_2Glc\text{-}ol$
1482	1.1	$Fuc_2Gal_2GlcNAc_2Glc\text{-}ol\ -14$
1435	0.9	$Fuc_3Gal_2GlcNAc_2$
1421	0.8	$Fuc_3Gal_2GlcNAc_2\ -14$
1247	1	$Fuc_2Gal_2GlcNAc_2 + H_2O$[a]
1229	1	$Fuc_2Gal_2GlcNAc_2$[b]
858	1.2	Fuc.GlcNAc.Gal.Glc-ol
812	5	$Fuc_2Gal.GlcNAc$
652	1.5	GlcNAc.Gal.Glc-ol[b]
624	6	$Fuc.Gal.GlcNAc + H_2O$[a]
402	42	Fuc.GlcNAc[b]
235	12	Glc-ol
189	72	Fuc

[a] These fragments are formed as illustrated in Fig. 2.
[b] Secondary fragments.

many of these peaks correspond to the α and β anomers of the same compound (VII-11 = VII-13; VII-16 = VII-18). After recycling on the same column, fraction VII-2 was obtained in a pure state (Fig. 1).

Structure Determination of Oligosaccharide VII-2

The molar ratio of the monosaccharides Fuc:Gal:GlcNAc:Glc was found to be 3:3:2:1 by GLC of their trifluoroacetyl derivatives. The methylation analysis indicated the following methylated derivatives: 3,4,6-tri-*O*-methyl galactose (1 residue); 2,4,6-tri-*O*-methyl galactose (1.8 residues); 2-deoxy-2-methylacetamido-6-mono-*O*-methyl glucose (1.8 residues); 1,2,3,5,6-penta-*O*-methyl glucitol (0.8 residues); and 2,3,4-tri-*O*-methyl fucose (2.4 residues). The absence of 2,4-di-*O*-methyl galactose indicates that the oligosaccharide possesses a linear structure related to *p*-lacto-*N*-hexaose or to *p*-lacto-*N*-neohexaose, according to the trivial names given by Kobata [4].

The FAB-MS spectrum of VII-2 (Fig. 2) confirms that the compound is a nonasaccharide (pseudo-molecular ion at m/z 1891). Key ions are m/z 1435 together with the daughter ion at m/z 1229, which was produced by the preferred elimination of the fucose residue linked to C-3 of GlcNAc-III; and m/z 812, (Fuc_2+Gal+GlcNAc). An intense ion at m/z 402 is indicative of the elimination of the (1-3)-linked Fuc-Gal sequence from fragment m/z 812, that shows the fucose to be α(1-4)-linked to GlcNAc-V. The non-specific loss of (Fuc + OH) is also observed from m/z 812 and 1435, which may arise from the fragmentation of fucose (Fig. 2). The other significant fragments are depicted in Table 1. On the basis of

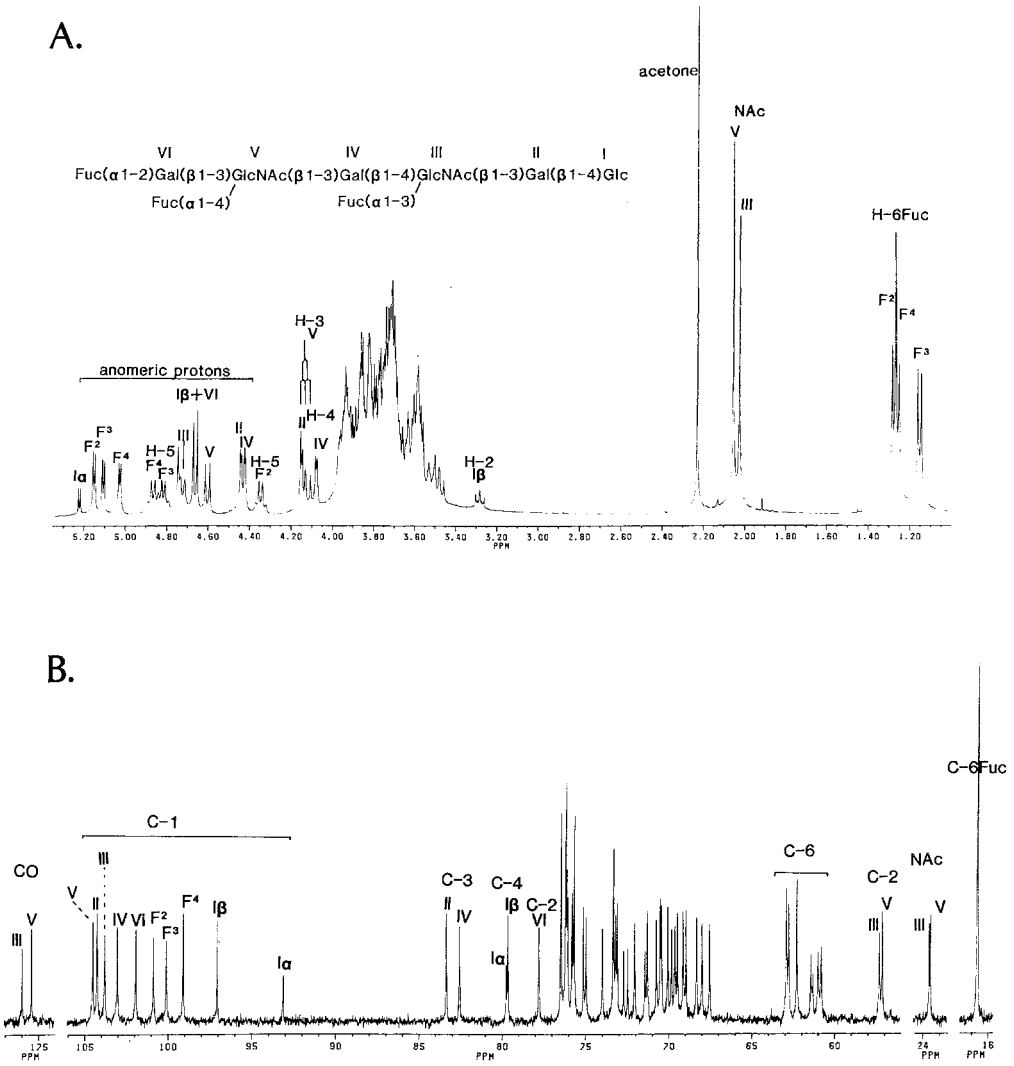

Figure 3. (A) ^{1}H- and (B) ^{13}C-NMR spectra of oligosaccharide VII-2.

these results, the structure of VII-2 may be established as a trifucosyl *p*-lacto-*N*-hexaose, substituted on monosaccharides III, V and VI with α(1-3)-, α(1-4)- and α(1-2)-linked fucose residues, respectively.

The ^{1}H- and ^{13}C-NMR spectra of the nonasaccharide are depicted in Fig. 3. The assignments of the protons were made according to the results of the COSY 45, simple and double Relayed COSY spectra (Fig. 4 and 5). For the fucose residues, the signals of Fuc^{2} H-5 and Fuc^{3} H-6 were easily recognized, according to previous data [18, 19]. The

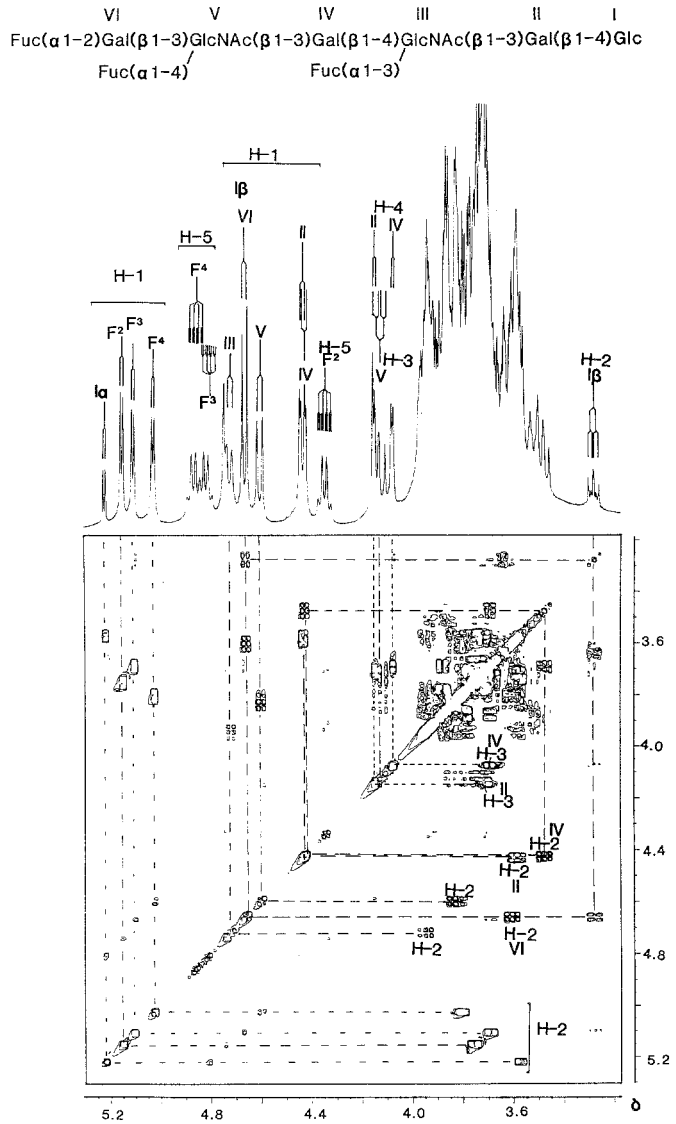

Figure 4. Homonuclear COSY 45 spectrum of VII-2.

cross-peak observed in the COSY 45 spectrum between H-5 and H-6 of Fuc^2 indicates that the methyl group of Fuc^2 resonates at δ = 1.270 ppm. The correlations between H-5 and H-6 of the two other fucose residues furnish their respective NMR parameters. Starting from the H-5 proton of each fucose residue, and by examining the double Relayed-COSY spectrum, the resonances of the anomeric protons were deduced from the correlation H-5 → H-4 → H-1 → H-2 → H-3 (Fig. 4). The anomeric proton of Glcα was identified through its $J_{1,2}$ value (3.75 Hz) and its relative low intensity, while H-1 of Glcβ was deduced from the COSY 45 spectrum where it is correlated with an H-2 signal which possesses

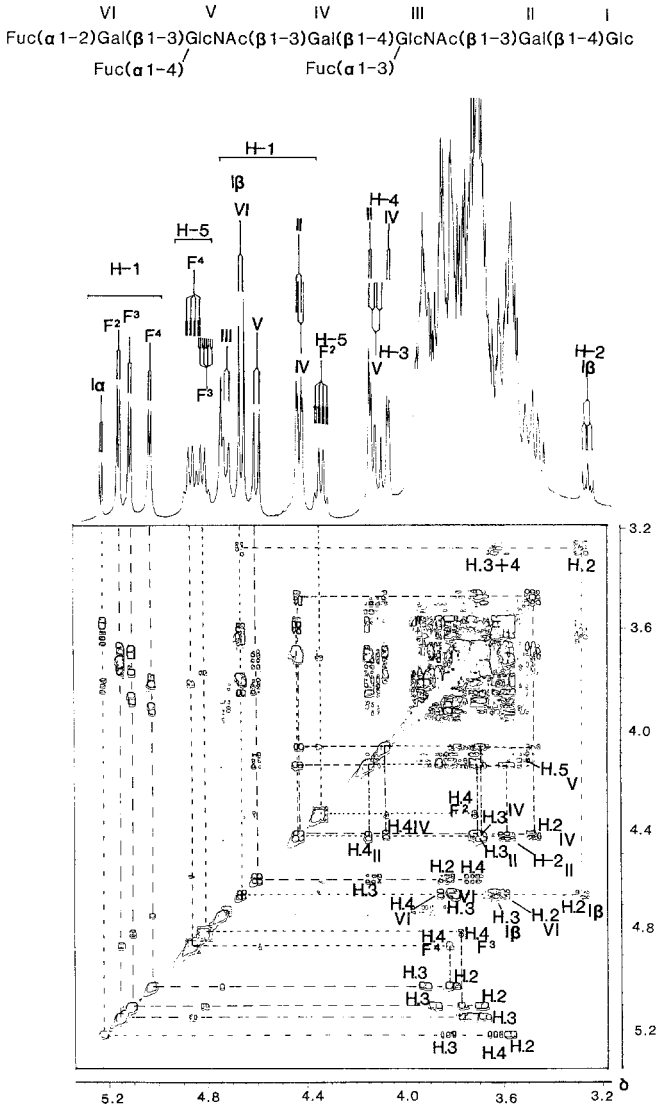

Figure 5. Homonuclear double relayed-COSY spectrum of VII-2.

a characteristic value (δ = 3.276 ppm) [20]. The anomeric protons of the *N*-acetylglucosamine residues were assigned according to their downfield shifted H-2 and H-3 signals, in comparison with those of galactose [19]. At δ = 4.127 ppm is observed the H-3 signal of an *N*-acetylglucosamine residue involved in the Le^b determinant structure [19]. Consequently, the H-1 and H-2 signals of GlcNAc-III and GlcNAc-V were clearly assigned (Fig. 5 and Table 2). In a series of fucosylated and sialylated lacto-*N*-tetraoses [19, 20], the H-4 of Gal-II was always found to resonate at δ = 4.130-4.171 ppm. On the other hand, the H-1 signal of the Fucα1-2Gal residue is shifted significantly downfield [19, 20]. Based

Table 2. ^{1}H-NMR Chemical shifts of milk nonasaccharide.

Group	H-1	H-2	H-3	H-4	H-5	H-6	$COCH_3$
Fucα1-2	5.147	3.756	3.728	3.704	4.341	1.270	—
Galβ1-3	4.658	3.602	3.800	3.858	3.58	3.73	—
Fucα1-4	5.022	3.802	3.919	3.818	4.860	1.254	—
GlcNAcβ1-3	4.598	3.828	4.127	3.726	3.52	3.87 3.92	2.050
Galβ1-4	4.424	3.472	3.691	4.074	3.67	3.74	—
Fucα1-3	5.103	3.688	3.876	3.773	4.811	1.147	—
GlcNAcβ1-3	4.721(α) 4.717(β)	3.958	3.87	3.94	3.56	3.85 3.93	2.020
Galβ1-4	4.430	3.580	3.708	4.146	3.71	3.76	—
Glcα	5.216	3.572	3.826	3.634	3.94	3.86	—
Glcβ	4.658	3.276	3.628	3.64	3.64	n.d.[a]	—

[a] n.d. = not detected.

Table 3. ^{13}C-NMR Chemical shifts of milk nonasaccharide.

Group	C-1	C-2	C-3	C-4	C-5	C-6	$CO\mathbf{C}H_3$	$CO\mathbf{C}H_3$
Fucα1-2	100.80	69.43	70.69	73.28	67.49	16.64	—	—
Galβ1-3	101.86	77.73	74.91	70.01	76.00	62.85	—	—
Fucα1-4	99.02	69.09	70.39	73.28	68.26	16.66	—	—
GlcNAcβ1-3	104.44	57.09	75.74	73.04	76.40	60.76	175.36	23.48
Galβ1-4	102.98	72.01	82.51	69.78	75.62	62.72	—	—
Fucα1-3	100.03	68.91	70.47	73.15	67.94	16.63	—	—
GlcNAcβ1-3	103.72	57.26	76.12	73.94	76.40	60.96	175.92	23.55
Galβ1-4	104.15(α) 104.19(β)	71.26(α) 71.24(β)	83.29	69.58	76.12	62.22	—	—
Glcα	99.07	72.41	72.67	79.70	71.38	61.26	—	—
Glcβ	96.99	75.07	75.62	79.59	76.05	61.40	—	—

upon these observations, the signal at δ = 4.146 ppm was assigned to Gal-II H-4, and the double Relayed-COSY spectrum allowed us to distinguish Gal-II H-1 from Gal-IV H-1, found at δ = 4.430 and 4.424 ppm, respectively. Gal-VI H-1 resonates with the anomeric proton of Glcβ at δ = 4.658 ppm, as confirmed by the COSY 45 spectrum. The other protons were assigned *via* the ^{1}H-^{13}C COSY spectrum, and by comparison of the ^{1}H- and ^{13}C-NMR data observed for identical structural elements present in Le^{a} and le^{b} determinants [19] (Tables 3 and 4). Indeed, although the comparison of the ^{13}C-NMR spectra already provides good similarities of chemical shifts for identical residues, the consideration of the couples of values ^{1}H-^{13}C eliminates the risk of confusion. As shown in Table 4, the only discrepancy concerns Gal-IV C-5, which is C-3 substituted in the nonasaccharide, that introduces a significant shift effect of -0.53 ppm. The comparison of the ^{13}C-NMR chemical shifts of VII-2 with those of fucosylated lacto-*N*-tetraose [19]

Table 4. Comparison of some ^{1}H- and ^{13}C-NMR data of the milk nonasaccharide with those of IV^{2}Fuc,III^{4}Fuc-LcOse$_4$ and III^{3}Fuc-nLcOse$_4$ [19].

	Fucα1-2Galβ1-3 [Fucα1-4]GlcNAcβ1-3R[a,b]		Galβ1-4[Fucα1-3]GlcNAc β1-3R[a,c]	
H-3	—	4.130	—	3.876
C-3	—	75.79	—	76.41
H-5	3.576	3.517	?	3.548
C-5	76.06	76.49	75.15	75.15
H-6	3.733	3.861; 3.924	3.730	3.918; ?
C-6	62.89	60.80	62.75	60.92
C=O	—	175.41	—	175.89
	VI	V	IV	III
	Fucα1-2Galβ1-3	[Fucα1-4]GlcNAcβ1	-3Galβ1-4	[Fucα1-3]GlcNAc β1-3R[a,d]
H-3	—	4.127	—	3.87
C-3	—	75.74	—	76.12
H-5	3.58	3.52	3.67	3.548
C-5	76.00	76.40	75.62	76.40
H-6	3.73	3.87; 3.92	3.74	3.85; 3.93
C-6	62.85	60.76	62.72	60.96
C=O	—	175.36	—	175.92

[a] R = lactose.
[b] IV^{2}Fuc, III^{4}Fuc-LcOse$_4$.
[c] III^{3}Fuc-nLcOse$_4$.
[d] VI^{2}Fuc,V^{4}Fuc,III^{3}Fuc-*p*-LcOse$_6$.

does not shown significant alterations of the signal relevant from Glc-β and Fuc-α residues. These values may be directly and unambiguously assigned for similar carbohydrate structures, and the complete assignment of the NMR parameters of more than 20 higher oligosaccharides is presently being performed in our laboratory. The most important shift effects observed involve the C-3 atom of galactose and *N*-acetylglucosamine, with regard to the nature of the substitution and the presence of additional α(1-6)-linked *N*-acetyllactosamine residues.

The trifucosyl *p*-lacto-*N*-hexaose was isolated from milk pooled from different donors. Nevertheless, we may assume that it only occurs in the milk of donors with Le/Se phenotype, according to the enzymatic and genetic basis for the expression of the Lewis blood group antigens [20].

The large number of human milk polyfucosylated oligosaccharides (mainl y nonaose to dodecaose) has not been up to now successfully separated by conventional chromatographic methods. The use of high performance reverse phase columns represents a powerful tool for separation of these components. Moreover, as previously stated by Bruntz *et al.* [7], the combination of both NMR and MS techniques appears the ideal procedure for the structural elucidation of these complex carbohydrates.

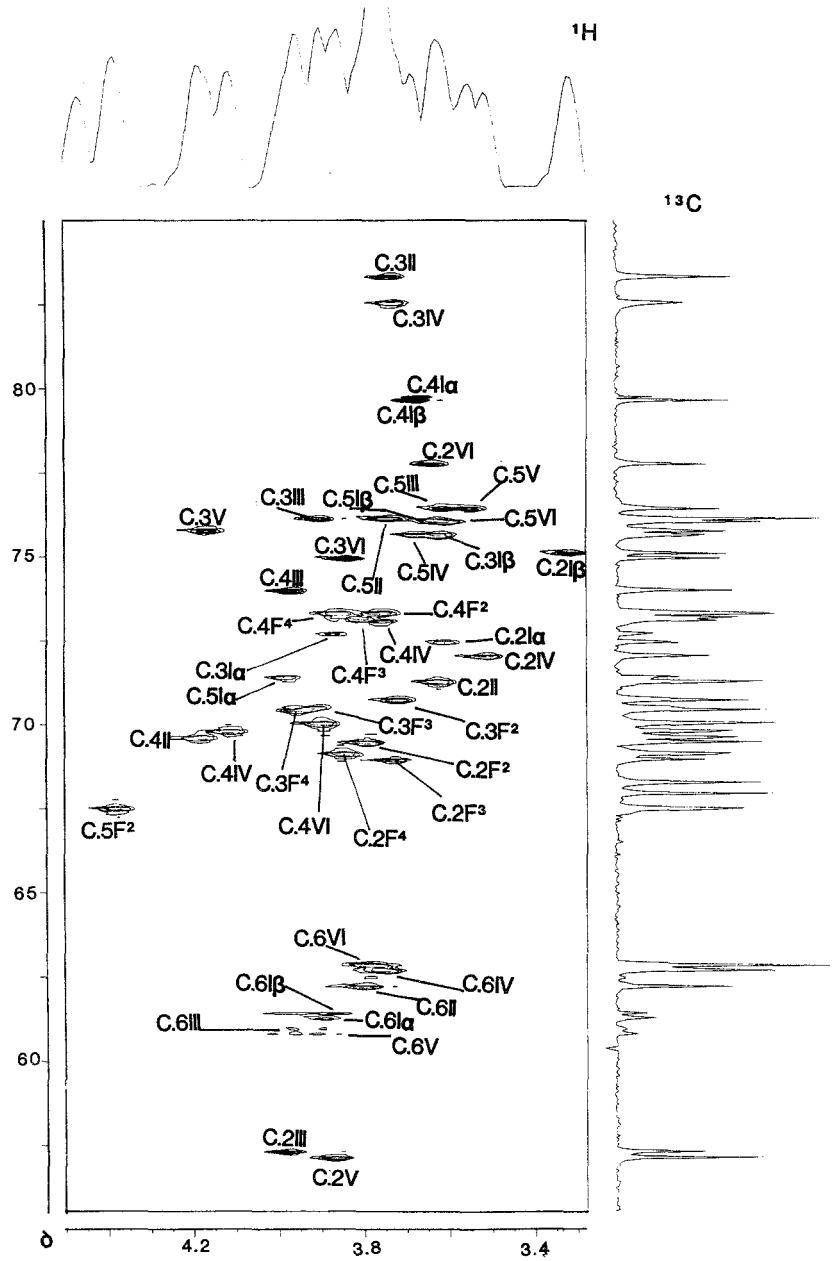

Figure 6. The 3.2-4.6 ppm and 55-85 ppm regions of the 2D heteronuclear-correlated NMR spectrum of VII-2.

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