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Primary Structure of Human Milk Nona- and Decasaccharides Determined by a Combination of Fast Atom Bombardment Mass Spectrometry and ^{1}H-/^{13}C-Nuclear Magnetic Resonance Spectroscopy. Evidence for a New Core Structure, *Iso*-lacto-*N*-octaose

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Key words: ^{1}H-NMR spectroscopy; ^{13}C-NMR spectroscopy; FAB-MS; milk oligosaccharides

The structure of a nonasaccharide and of two decasaccharides isolated from human milk has been investigated by using methylation, fast atom bombardment mass spectrometry and ^{1}H-/^{13}C-nuclear magnetic resonance spectroscopy. The structures of these oligosaccharides were:
trifucosyllacto-*N*-hexaose;
Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3[Galβ1-4(Fucα1-3)GlcNAcβ1-6]Galβ1-4Glc,
difucosyllacto-*N*-octaoses;
Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-6[Galβ1-3GlcNAcβ1-3]Galβ1-4Glc
and
Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-6[Fucα1-2Galβ1-3GlcNAcβ1-3]Galβ1-4Glc.
The two decasaccharides possess a new type of core structure proposed to be named *iso*-lacto-*N*-octaose.

We previously described the fractionation of human milk oligosaccharides based on the combination of gel-filtration, paper chromatography and high performance liquid chromatography on a reverse phase octadecyl column [1]. More than 70 neutral oligosaccharides could be detected and despite the use of the HPLC column, many of these fractions remained heterogeneous. Nevertheless, the recycling of the material allowed the isolation of new pure components.

The present paper describes the structural studies of three oligosaccharides: a nonasaccharide and two decasaccharides.

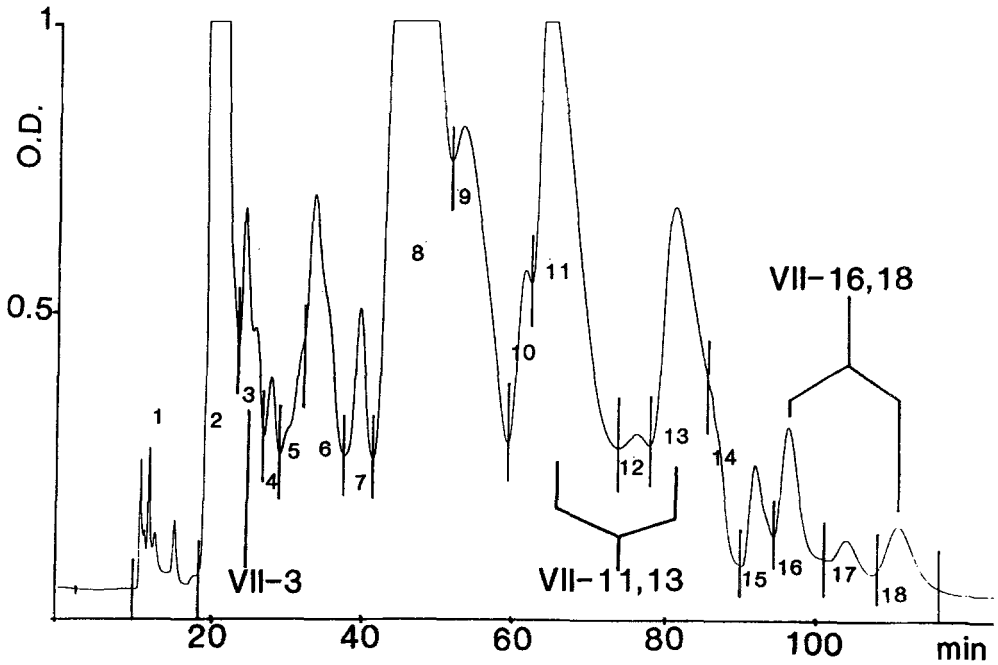


Figure 1. HPLC chromatogram of fraction VII using a 0.5 μ ODS Zorbax column (25 cm x 0.95 cm; Du Pont Instruments, Paris) with water as eluent. Flow rate: 0.5 ml/min for 1 h, then 2 ml/min up to the end of the analysis.

Materials and Methods

The fractionation of milk oligosaccharides which led to the isolation of the so-called "Fraction VII", has been previously described [1]. The analytical section, which included monosaccharide estimation, methylation analysis, fast-atom bombardment-mass spectrometry and NMR spectroscopy can also be consulted in the previous paper.

Results and Discussion

Isolation of Three Oligosaccharides

As previously reported [1], the higher oligosaccharides (DP-8 to DP-14) from human milk were fractionated by preparative paper chromatography into 11 sub-fractions, named I to XI. Fraction VII, in spite of its apparent homogeneity when checked by paper and thin layer chromatography, can be divided into 18 peaks by reverse phase HPLC (Fig. 1). After recycling on the same column, compounds VII-2 [1], VII-3, VII-11, 13 and VII-16, 18 were obtained in a pure state, while the other fractions remain to be fractionated with more suitable procedures. The doubling of the peaks VII-11, 13 and VII-16, 18 is due to the

Table 1. Methylation analysis of compounds VII-3, VII-11, 13 and VII-16, 18

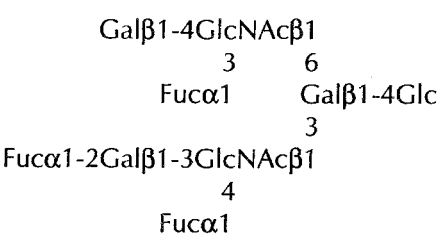
Methylated derivatives	Oligosaccharides		
	VII-3	VII-11, 13	VII 16, 18
2,3,4-Me_3-Fuc	2.5[b]	1.6[b]	1.5[b]
2,3,4,6-Me_4-Gal	0.9	0.9	1.5
2,4,6-Me_3-Gal	0	0.9	0.9
3,4,6-Me_3-Gal	1.1	1.0	0
2,4-Me_2-Gal[a]	1.0	1.0	1.0
4,6-Me_2-GlcNAc	0	1.7	0.8
6-Me-GlcNAc	1.8	0.9	1.8
1,2,3,5,6-Me_5-Glc-ol	0.8	0.8	0.8

[a] Values are given relative to 2,4-Me_2-Gal.
[b] The low values for the fucose derivative can be explained by the high volatility of the product.

anomerization effect. In both cases, the β-anomer eluted faster than the α-anomer. The amounts of material of compounds VII-3, VII-11, 13 and VII-16, 18 isolated from two litres of pooled human milk were 2, 27 and 8 mg, respectively.

Structure of Compound VII-3

Compound VII-3 contains three fucose residues, two galactose residues, two *N*-acetylglucosamine residues and one glucose residue. The methylation analysis (Table 1) shows the presence, *inter alia*, of the 2,4,-di-*O*-methylgalactose derivative, which indicates a branched structure. The FAB-MS analysis (Fig. 2) indicated for the pseudo-molecular ion ($M+Na^+$) a value of m/z 1913, and furnished two primary fragments at m/z 812 (Fuc_2Gal.GlcNAc) and 638 (Fuc.Gal.GlcNAc). The presence of the Le^b determinant was proved by the NMR analysis (see below), and, consequently, the daughter ions at m/z 402 and 432 can be easily interpreted as produced by the preferred elimination of Fuc.Gal(1-3) from m/z 812 and Fuc(1-3) from m/z 638, respectively (Fig. 2). On the basis of these results, the structure of oligosaccharide VII-3 was found to be identical to the trifucosylated lacto-*N*-hexaose recently isolated from faeces of a blood-group B, breast-fed infant [2]:



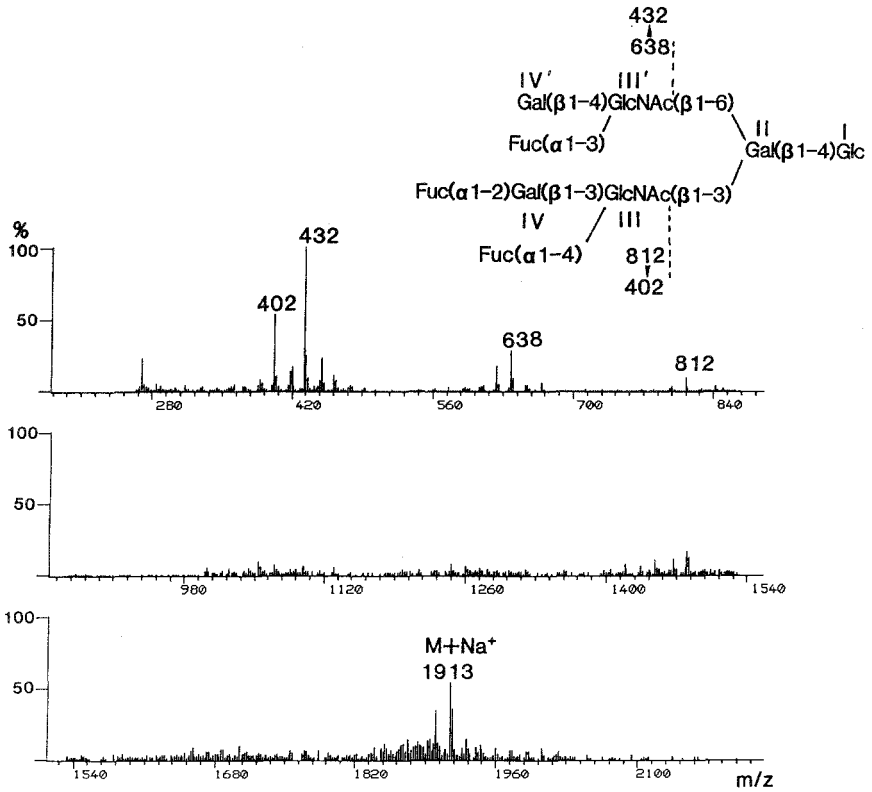


Figure 2. The FAB-MS spectrum of the reduced, methylated oligosaccharide VII-3.

The amount of material VII-3 available was too small to permit the recording of ^{13}C-NMR spectra, and therefore only the ^{1}H-NMR analysis was performed (Fig. 3). In Table 2 are reported the ^{1}H-resonances observed on the 1D spectrum (anomeric protons) and on the relayed COSY spectra. The H-1 and H-2 resonances of GlcNAc-III′ were very broad, but their assignment was confirmed after observing the same phenomenon for the same structural unit in VII-11, 13 and VII-16, 18. The set of chemical shifts of the Fuc^{2} and the Fuc^{4} atoms (δ = 1.273 ppm and 1.257 ppm) and GlcNAc-III, Gal-IV H-1 atoms (δ = 4.599 ppm and 4.654 ppm) is typical of the Le^{b} determinant [3]. The NMR parameters of the carbohydrates of the (1-6)-branch are also in good agreement ($\Delta\delta \pm 0.004$ ppm) with those found in III^{3} αFuc-nLcOse$_4$ [3], except for the H-5 atom of the α(1-3)-linked fucose residue which is shifted upfield by -0.042 ppm in VII-3. Our assignments are in good agreement with those previously reported [2], except for the NAc signal of the GlcNAc-III and -III′ residues that we interchanged on the basis of the absence of an anomeric effect for the GlcNAc-III residue, as observed for a series of fucosylated lacto-*N*-tetraoses [3].

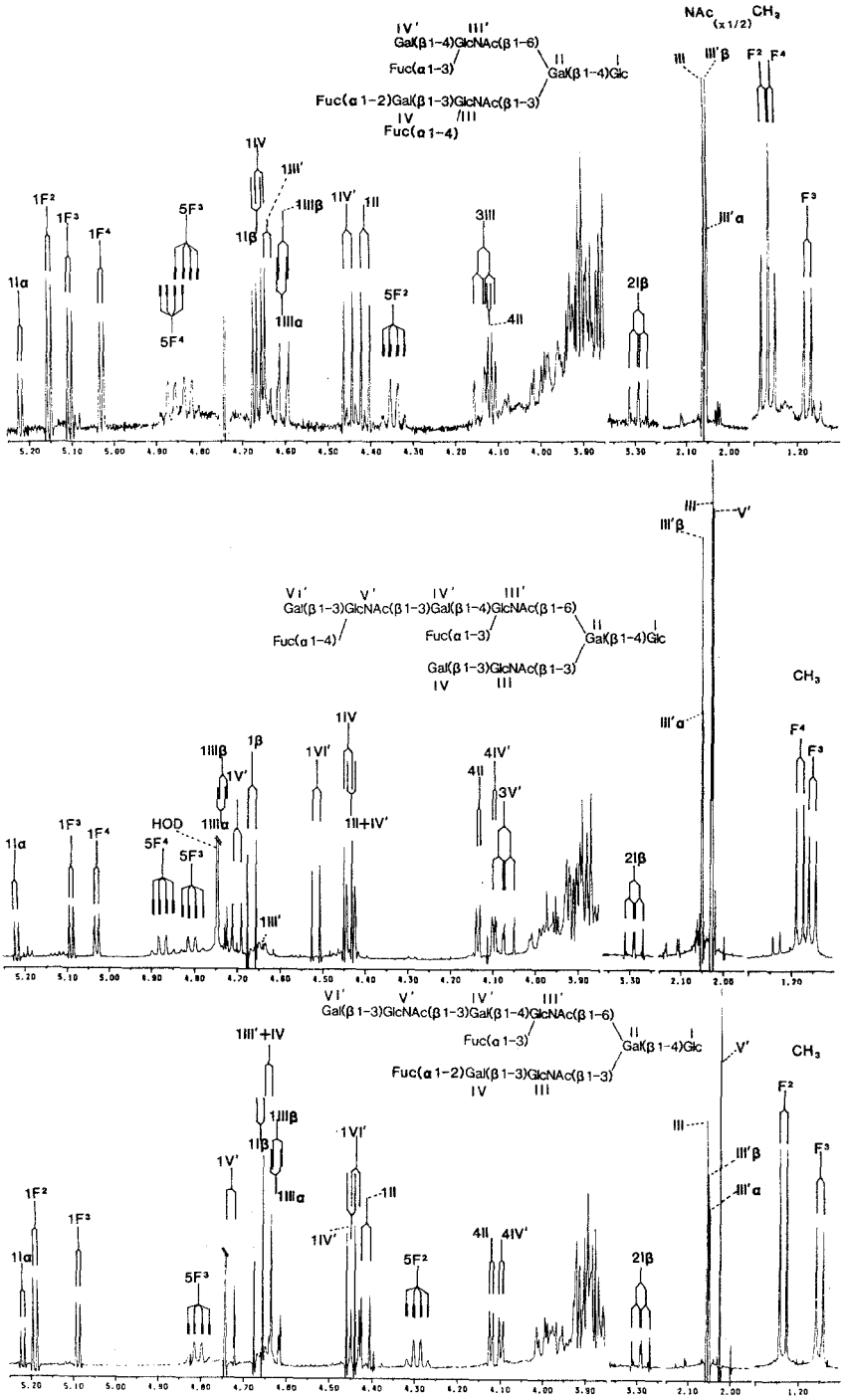


Figure 3. The 400 MHz ^{1}H-NMR spectra of VII-3, VII-11, 13 and VII-16, 18.

Table 2. The ^{1}H-NMR chemical shifts of compound VII-3.

	H-1	H-2	H-3	H-4	H-5	H-6	NAc
Fucα(1-2)-	5.149	3.754	3.697	3.740	4.341	1.273	-
Galβ(1-3)-	4.654	3.606	3.806	3.84	n.d.[a]	n.d.	-
IV							
Fucα(1-4)-	5.026	3.806	3.923	n.d.	4.861	1.257	-
GlcNAcβ(1-3)-	4.602(α)	3.832	4.130	3.731	3.52	3.86[b]	2.058
III	4.599(β)					3.92	
Galβ(1-4)-	4.450	3.493	3.654	3.90	n.d.	n.d.	-
IV							
Fucα(1-3)-	5.101	3.692	3.901	n.d.	4.823	1.174	-
GlcNAcβ(1-6)-	4.642	3.98	n.d.	n.d.	n.d.	n.d.	2.049(α)
III′							2.051(β)
Galβ(1-4)-	4.409	3.550	3.697	4.128	n.d.	n.d.	-
II							
Glcα	5.217	3.584	3.823	n.d.	n.d.	n.d.	-
Glcβ	4.663	3.288	3.640	n.d.	n.d.	n.d.	-

[a]n.d. = not determined.
[b]Values relative to H-6 and H-6′.

Structure of Compound VII-11, 13

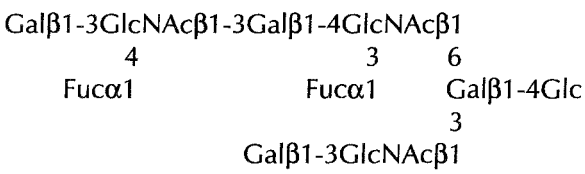
Compound VII-11, 13 is a decasaccharide consisting of fucose, galactose, *N*-acetylglucosamine and glucose in the ratio 2:4:3:1, as confirmed by sugar analysis, and FAB-MS of the reduced, methylated oligosaccharide, which furnished a pseudo-molecular ion, M+Na$^+$, at m/z 2156. The branched structure is shown by the presence of 2,4-di-*O*-methylgalactose among the methyl ether derivatives (Table 1). One of the two branches is characterized by the primary fragment at m/z 1261 ($Fuc_2Gal_2GlcNAc_2$) and its daughter ion at m/z 1055, which results from the preferred elimination of an α(1-3)-linked fucose residue. A second primary fragment at m/z 638 (Fuc.Gal.GlcNAc), accompanied by the daughter ion at m/z 402 is derived from the same branch, since the second one is necessarily devoid of fucose. The structure of the oligosaccharide VII-11, 13 was confirmed by NMR analysis, which showed the characteristic resonances of α(1-3)-linked and α(1-4)-linked fucose residues (Fig. 3 and Tables 3 and 4). The complete ^{1}H- and ^{13}C-NMR assignment of VII-11, 13 was achieved by 2D homonuclear and heteronuclear spectroscopy. When compared with those of III^{4}αFuc-$LcOse_4$, IV^{3}Fuc-n$LcOse_4$ and $LcOse_4$ [3] the ^{1}H-resonances found in the Galβ1-3(Fucα1-4)GlcNAc, Galβ1-4(Fucα1-3)GlcNAc and Galβ1-3GlcNAc units of the decasaccharide remain practically unchanged, except for the protons located around the glycosidic bound of the GlcNAcβ1-3Gal unit. As observed for VII-3, the signals related to the GlcNAc-III′ residue are strongly broadened, but are perfectly assignable on the ^{13}C-^{1}H COSY

Table 3. The ^{1}H-NMR chemical shifts of compound VII-11, 13.

	H-1	H-2	H-3	H-4	H-5	H-6	$COCH_3$
Galβ(1-3)- VI′	4.512	3.480	3.619	3.884	3.57	3.77	-
Fucα(1-4)- GlcNAcβ(1-3)- V′	5.024 4.696	3.798 3.944	3.888 4.075	3.788 3.754	4.869 3.54	1.177 3.90[a] 4.04	- 2.023
Galβ(1-4)- IV′	4.430	3.523	3.720	4.093	3.59	3.72	-
Fucα(1-3)- GlcNAcβ(1-6)- III′	5.085 4.642	3.688 3.884	3.882 3.818	3.771 3.93	4.801 3.61	1.147 3.88[a] 3.97	- 2.047(α) 2.049(β)
Galβ(1-3)- IV	4.437	3.523	3.64	3.910	3.70	3.77	-
GlcNAcβ(1-3)- III	4.735(α) 4.730(β)	3.898	3.825	3.566	3.48	3.81[a] 3.88	2.027
Galβ(1-4)- II	4.430	3.588	3.706	4.131	3.89	3.73	-
Glcα Glcβ	5.216 4.662	3.580 3.280	3.827 3.619	3.59 3.61	3.94 3.59	3.87 3.84[a] 3.92	- -

[a] Values relative to H-6 and H-6′.

spectrum (Fig. 4). The C-5/H-5 signals of the galactose residues were identified on the ^{13}C-^{1}H COSY spectrum by comparison with those of reference products, except for Gal-II. Indeed, this latter residue appeared to possess a normal C-5 chemical shift value (δ = 76.10 ppm), but the corresponding H-5 signal is downfield shifted at δ = 3.89 ppm. Combination of the aforementioned structural elements indicates that the structure of compound VII-11, 13 is a difucosyllacto-*N*-octaose:



Structure of Compound VII-16, 18

The compound VII-16, 18 is a decasaccharide consisting of fucose, galactose, *N*-acetylglucosamine and glucose in the ratio 2:4:3:1, as shown by sugar analysis and by the observation of a pseudo-molecular ion, M+Na$^+$, at m/z 2156. The methylation analysis indicated the

Table 4. The ^{13}C-NMR chemical shifts of compound VII-11, 13.

	C-1	C-2	C-3	C-4	C-5	C-6	C=O	CH_3
Galβ(1-3)-VI′	104.09	71.80	73.77	69.75	76.10	62.91	-	-
Fucα(1-4)-	99.27	69.10	70.43	73.15	68.12	16.67	-	-
GlcNAcβ(1-3)-V′	103.80	57.16	77.24	73.43	76.56	60.93	175.94	23.59
Galβ(1-4)-IV′	103.08	71.97	82.97[a]	69.63	75.72	62.72	-	-
Fucα(1-3)-	99.97	68.95	70.50	73.24	68.00	16.58	-	-
GlcNAcβ(1-6)-III′	102.04	57.00	74.66	74.40	76.63	61.09	175.55	28.81
Galβ(1-3)-IV	104.75	71.97	73.77	69.82	76.49	62.31	-	-
GlcNAcβ(1-3)-III	103.75	56.01	83.38	69.82	76.49	61.83	176.18	23.55
Galβ(1-4)-II	104.31	71.17	82.92[a]	69.75	76.10	62.31	-	-
Glcα	93.07	72.48	72.72	80.40	71.28	61.25	-	-
Glcβ	96.99	75.14	75.69	80.31	75.99	61.38	-	-

[a] Values given for C-3 Gal-II and -IV′ may have to be interchanged.

presence of a 2,4-di-*O*-methylgalactose derivative, due to the branched structure of the compound. The two branches are monofucosylated, one consisting of one fucose, one galactose and one *N*-acetylglucosamine (primary fragment at m/z 638), and the second composed of one fucose, two galactoses and two *N*-acetylglucosamines (m/z 1087) (Fig. 5). The daughter ion observed at m/z 881 shows that the GlcNAc-III′ residue of the largest branch (m/z 1087) is substituted by one α(1-3)-linked fucose residue. The fragments at m/z 638 and 464 together give a secondary fragment of m/z 228, which indicates the corresponding *N*-acetylglucosamine residue to be substituted at O-3. Moreover, the structure of the second branch can be established as Fucα1-2Galβ1-3GlcNAc, according to the methylation results which indicate the presence of 3,4,6-Me_3Gal.

The NMR analysis (Table 5 and 6) confirms the presence of the Fucα1-2Galβ1-3GlcNAc unit which is evident from the resonance of Gal-IV H-1 and GlcNAc-III H-1 at δ = 4.641 and 4.621 ppm, respectively. The parameters, which are identical to those observed for lacto-*N*-fucopentaose I [3] lead to the conclusion that oligosaccharide VII-16, 18 results from an elongation of the pentaose by a branch linked to the O-6 position of Gal-II. The resonances attributable to Gal-VI′ and GlcNAc-V′ are practically identical to those observed for lacto-*N*-tetraose. The exact assignment of the H-1 to H-4 resonances of the galactose residues is

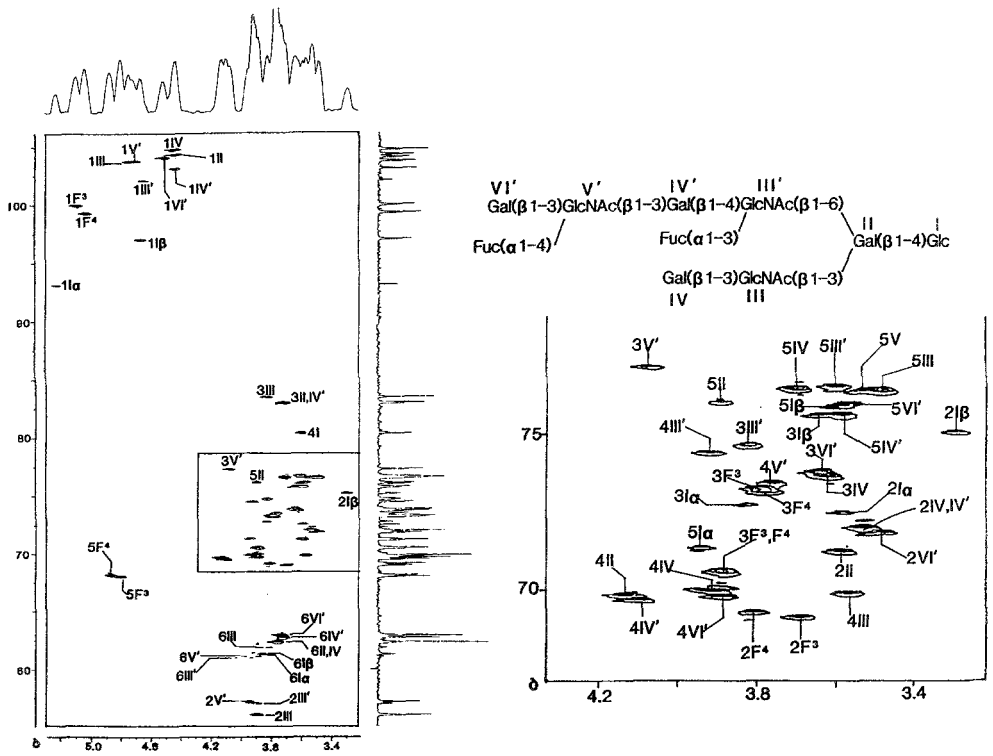


Figure 4. The heteronuclear-correlated NMR spectrum of VII-11, 13.

described in Fig. 6, which presents an extension of the double relayed COSY spectrum, in the range 3.2-4.8 ppm/4.1-4.8 ppm. More significant is the ^{13}C-NMR spectrum (Table 6) which confirms the identity of the chemical shift values found for each Gal-GlcNAc unit, by comparison with those measured for the reference fucosylated lacto-*N*-tetraose [3]. In combination with the FAB-MS analysis, the NMR data indicate the following structure for compound VII-16, 18:

Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1
3 6
Fucα1 Galβ1-4Glc
3
Fucα1-2Galβ1-3GlcNAcβ1

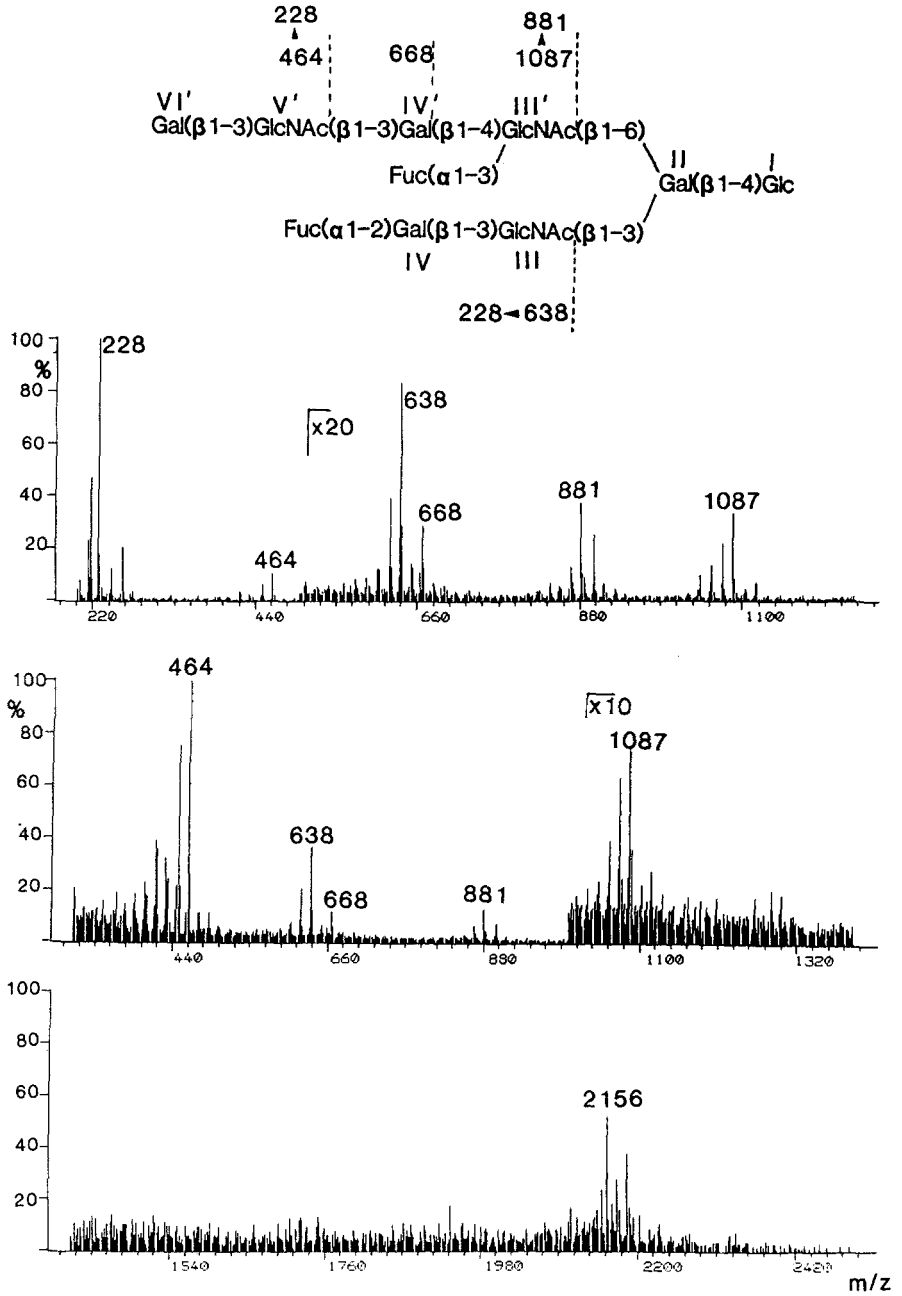


Figure 5. The FAB-MS spectrum of the reduced, methylated compound VII-16, 18.

Table 5. The ^{1}H-NMR chemical shifts of compound VII-16, 18.

	H-1	H-2	H-3	H-4	H-5	H-6	$COCH_3$
Galβ(1-3)- VI′	4.436	3.522	3.645	3.914	n.d.[a]	n.d.	-
GlcNAcβ(1-3)- V′	4.727	3.896	3.818	3.576	3.475	3.77[b] 3.92	2.021
Galβ(1-4)- IV′	4.446	3.525	3.728	4.095	n.d.	n.d.	-
Fucα(1-3)- GlcNAcβ(1-6)- III′	5.085 4.641	3.688 3.896	3.884 3.82	3.775 n.d.	4.801 n.d.	1.147 n.d.	- 2.046(α) 2.049(β)
Fucα(1-2)- Galβ(1-3)- IV	5.187 4.641	3.771 3.588	3.666 3.832	3.740 3.888	4.290 n.d.	1.232 n.d.	- -
GlcNAcβ(1-3)- III	4.624(α) 4.621(β)	3.812	3.992	3.536	3.50	3.82[b] 3.92	2.052
Galβ(1-4)- II	4.411	3.562	3.706	4.118	n.d.	n.d.	-
Glcα	5.215	3.584	3.824	3.63	n.d.	n.d.	-
Glcβ	4.661	3.286	3.63	n.d.	n.d.	n.d.	-

[a] n.d. = not determined.
[b] Values relative to H-6 and H-6′.

Conclusion

The combination of an HPLC reverse phase octadecylsilyl column, for the purification of fucosylated oligosaccharides, and NMR or MS techniques, for the elucidation of the structures, provide a renewed interest in studying human milk oligosaccharides. Up to now, more than fifty different compounds have been characterized [4, 5], but the structures were often studied starting from mixtures of isomers. Trifucosyllacto-*N*-hexaose has been recently isolated from faeces of a breast-fed infant [2], but was not until now characterized in human milk. The compounds VII-11, 13 and VII-16, 18 possess a new type of core structure having terminal β(1-3)-linked galactose residues on both branches. By comparison with the previously described lacto-*N*-octaose and lacto *N*-neooctaose [4], we propose to name it *iso*-lacto-*N*-octaose.

Table 6. The ^{13}C-NMR chemical shifts of compound VII-16, 18.

	C-1	C-2	C-3	C-4	C-5	C-6	CO	CH_3
Galβ(1-3)- VI′	104.73	71.98	73.77	69.76	76.57	62.32	-	-
GlcNAcβ(1-3)- V′	103.75	55.98	83.34	69.72	76.46	61.81	176.17	23.54
Galβ(1-4)- IV′	103.07	71.84	82.60	69.52	75.75	62.72	-	-
Fucα(1-3)-	99.99	68.97	70.52	73.15	68.00	16.58	-	-
GlcNAcβ(1-6)- III′	102.06	56.99	74.64	74.41	76.65	61.09	175.55	23.82
Fucα(1-2)-	100.79	69.36	70.74	73.15	67.78	16.58	-	-
Galβ(1-3)- IV	101.53	77.94	74.80	70.43	76.38	62.44	-	-
GlcNAcβ(1-3)- III	104.47	56.28	78.49	69.83	76.57	61.75	175.45	23.48
Galβ(1-4)- II	104.34	71.37	82.83	69.76	76.15	62.32	-	-
Glcα	93.1	72.50	72.70	80.26	71.32	61.24	-	-
Glcβ	96.99	75.15	75.68	80.19	76.03	61.38	-	-

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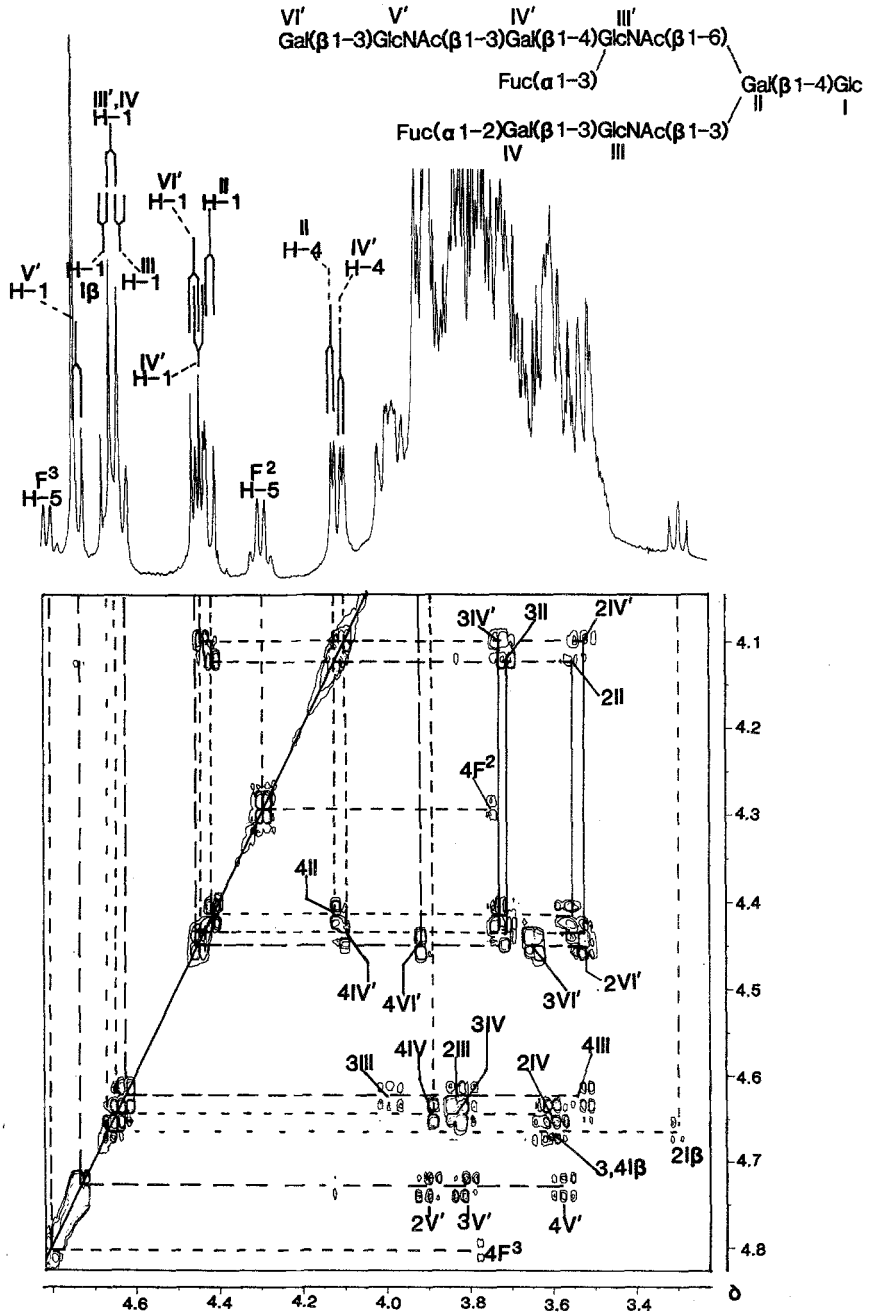

Figure 6. The 3.2-4.8 ppm and 4.1-4.8 ppm regions of the homonuclear double-relayed-COSY spectrum of VII-16, 18.

Note Added in Proof

After submitting this paper for publication, a report entitled "Oligosaccharides from faeces of preterm infants fed on breast milk", by Sabharwal H, Nilsson B, Grönberg G, Chester MA, Dakour J, Sjöblad S and Lundblad A [Arch Biochem Biophys (1988) 265:390-406] described a difucosyl-*iso*-lacto-*N*-octaose (compound VII-11, 13 in this paper).

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