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ISOLATION OF MONOSIALYLATED OLIGOSACCHARIDES FROM HUMAN MILK AND STRUCTURAL ANALYSIS OF THREE NEW COMPOUNDS*

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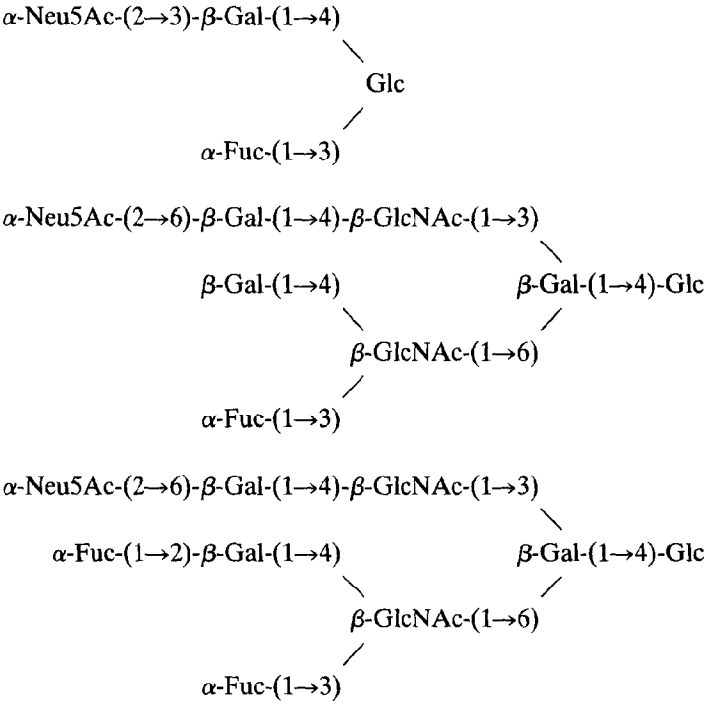
ABSTRACT

The monosialylated oligosaccharide fraction from combined samples of human milk was fractionated by gel filtration, ion-exchange chromatography, and h.p.l.c. with triethylamine as an ion-pairing reagent. Among the twelve oligosaccharides isolated, three were new compounds for which the following structures were established on the basis of chemical analyses, f.a.b.-m.s., and n.m.r. spectroscopy.



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INTRODUCTION

In addition to lactose, the major saccharide, human milk contains many neutral oligosaccharides[1,2]. Few sialic acid-containing oligosaccharides have been reported, probably due to the difficulty of purification. Traditionally, oligosaccharides have been isolated by gel filtration[3], ion-exchange chromatography[3,4], and paper chromatography[2]. The introduction of h.p.l.c. has made it possible to separate compounds with similar structures[5,6], and we now describe the isolation of monosialylated oligosaccharides and structural analysis of three new compounds.

EXPERIMENTAL

Materials. — The oligosaccharide fraction from combined samples of human milk was obtained as previously described[2]. Gel filtrations in water were carried out on columns of Sephadex G-15 (25 × 100 cm) and Bio-Gel P-2 (5 × 90 cm). Ion-exchange chromatography[3] (1 mL/min) was carried out on columns of DEAE-Sephadex A-25 (5 × 90 cm) and Dowex 1-X2 (AcO^-) resin (400 mesh) (2.5 × 100 cm). Eluates were monitored on the basis of refractive index. The h.p.l.c. instrument was equipped with an M-45 pump, a differential refractometer 410, an autoinjector 712, Wisp (Waters), and a reverse-phase column (10 × 300 mm) of Nucleosil C18 (5 μ, 100 A). Elution was with triethylamine–acetic acid buffer (5mM, pH 5.0) at 3 mL/min.

Analytical procedures. — Sialic acid was determined by the method of Jourdian *et al.*[8], and sugar and methylation analyses were carried out as previously described[9,10]. Methylation was performed as described[11] and each product was purified on a SepPak C18 reverse-phase cartridge[12]. For g.l.c., a Hewlett–Packard 5890 instrument was used, equipped with an SE-30 W.C.O.T. capillary column (0.25 mm × 30 m).

Mass spectrometry. — F.a.b.-m.s. (positive ion mode) was performed on a VG ZAB SE instrument. Solutions of samples in thioglycerol (1-thio-2,3-propanediol) were loaded onto the stainless steel target which was bombarded with xenon atoms with a kinetic energy of 8 keV. G.l.c.–e.i.-m.s. was carried out on a VG 12-250 quadrupole instrument fitted with the same column as for g.l.c. Spectra were recorded at 70 eV with an ion-source temperature of 200°.

N.m.r. spectroscopy. — A Bruker AM 500 spectrometer was used. Sialic acid was converted into the ammonium salt by adding NH_4OH. Samples were lyophilized repeatedly from D_2O and finally dissolved in high purity D_2O (2–10 mg/mL). ^{1}H-N.m.r. spectra were recorded at 27° with a trace of acetone (set to 2.225 p.p.m.) as internal standard. For ^{13}C-n.m.r. measurements, the spectrometer lock frequency was used as reference, which set 1,4-dioxane to 67.4 p.p.m. Assignments of the ^{1}H-n.m.r. spectra were based on 2D COSY[13] experiments, its one- and two-step RELAY[14] variants, and n.O.e.-difference spectroscopy[15]. ^{13}C-N.m.r. spectra were assigned by 2D ^{1}H,^{13}C correlation experiments (XHCORRD, Bruker

Spectrospin standard pulse program library), or CHORTLE[16] (^{1}H,^{13}C chemical shift correlation from 1D polarization transfer ^{13}C-n.m.r. spectra).

RESULTS AND DISCUSSION

Isolation and purification of monosialylated oligosaccharides. — From the carbohydrate fraction obtained from 20 L of combined samples of human milk, lactose was removed partially by precipitation with ethanol. Gel filtration on Sephadex G-15 gave fractions A–C according to Kobata[2]. Fractions B and C contained neutral oligosaccharides and were not studied further. Fraction A, which contained large neutral and sialylated oligosaccharides, was further fractionated on DEAE-Sephadex G-25 to give neutral, monosialylated, and disialylated oligosaccharides. The monosialylated fraction was desalted on Bio-Gel P-2 and fractionated on a Dowex (AcO^-) resin [3 g of monosialylated oligosaccharides were eluted at 4° with 18mM pyridine–acetic acid buffer (pH 5.0)]. The elution profile obtained is shown in Fig. 1. Fractions were combined as indicated, desalted on Bio-Gel P-2, and checked for purity by n.m.r. spectroscopy.

Four fractions S2:17, S2:19, S2:26, and S2:27 were pure compounds, and analysis by n.m.r. spectroscopy and comparison with reported data[17] revealed the known structures[18–20] shown in Table I. Compounds not pure after elution from the Dowex column were purified by h.p.l.c.

The charge on the sialic acid residues causes problems in reverse-phase h.p.l.c., which can be solved by adding tertiary amines to the eluant[21]. The formation of an ion-pair will make a sialylated oligosaccharide behave as a neutral compound and interact with the hydrophobic phase. Of several amines tested[7], the best result was obtained with 5mM triethylamine adjusted to pH 5.0 with acetic acid. For larger oligosaccharides, the retention times could be reduced by adding methanol.

The peak eluted from the Dowex column at ~80 h (Fig. 1) was divided into S2:21 and S2:22, and each was analysed by h.p.l.c. (Fig. 2A) to give S2:21:3 and

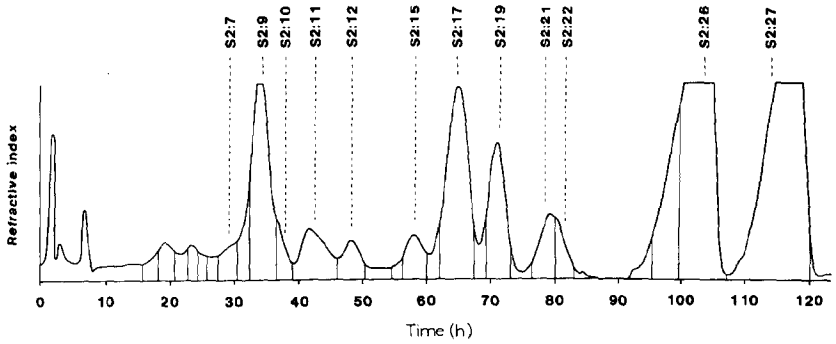


Fig. 1. Elution profile of monosialylated oligosaccharides from a Dowex 1-X2 (AcO^-) resin.

TABLE I

RETENTION TIME AND YIELD OF SIALYLATED COMPOUNDS ISOLATED BY H.P.L.C.

Compound	*Structure*	*Retention time (min)*		*Yield (mg)*	*Ref.*
		β Anomer	*α Anomer*		
S2:7:3	α-Neu5Ac-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→3)-{α-Fuc-(1→2)-β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-(1→6)}-β-Gal-(1→4)-Glc	29.2		15	
S2:9:1	α-Neu5Ac-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→3)-{β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-(1→6)}-β-Gal-(1→4)-Glc	19.9		154	
S2:10:6	α-Neu5Ac-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→6)-[α-Fuc-(1→2)-β-Gal-(1→3)-β-GlcNAc-(1→3)]-β-Gal-(1→4)-Glc	47.6	61.5	22	24
S2:11:1	α-Neu5Ac-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→3)-[β-Gal-(1→4)-β-GlcNAc-(1→6)]-β-Gal-(1→4)-Glc	21.7	24.8	10	23
S2:11:4	α-Neu5Ac-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→6)-[β-Gal-(1→3)-β-GlcNAc-(1→3)]-β-Gal-(1→4)-Glc	34.2	37.4	5	23
S2:12:1	α-Neu5Ac-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-[α-Fuc-(1→3)]-Glc	12.5	15.1	25	22
S2:17	α-Neu5Ac-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc	11.9	13.0	197	18
S2:19	α-Neu5Ac-(2→6)-[β-Gal-(1→3)]-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc	11.4	12.5	109	18
S2:21:3	α-Neu5Ac-(2→3)-β-Gal-(1→4)-[α-Fuc-(1→3)]-Glc	10.2	10.7	37	
S2:22:1	α-Neu5Ac-(2→3)-β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc	14.8	16.9	45	18
S2:26	α-Neu5Ac-(2→6)-β-Gal-(1→4)-Glc	8.7	9.0	280	19
S2:27	α-Neu5Ac-(2→3)-β-Gal-(1→4)-Glc	9.7	9.9	160	20

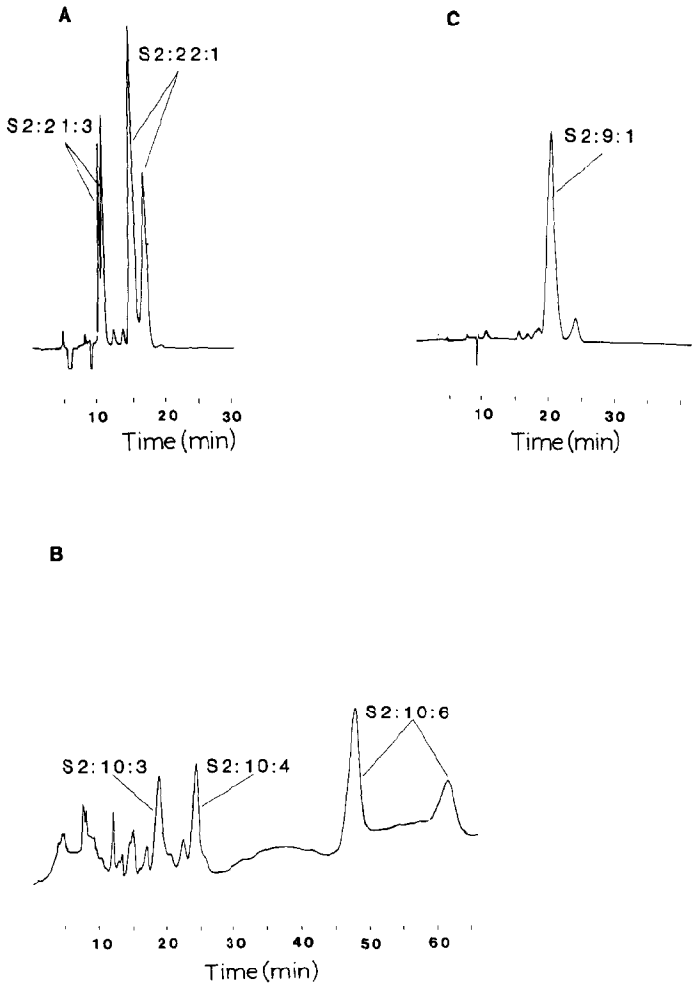


Fig. 2. H.p.l.c. of S2:21:3 (A), S2:10:6 (B), and S2:9:1 (C) (see Fig. 1).

S2:22:1. Two peaks were obtained for each compound representing the α and β anomers of the reducing glucose residue. Analyses of S2:21:3 and S2:22:1 by n.m.r. spectroscopy and comparison with reference compounds[17] revealed the structure for S2:22:1 shown in Table I. S2:21:3 was a new compound.

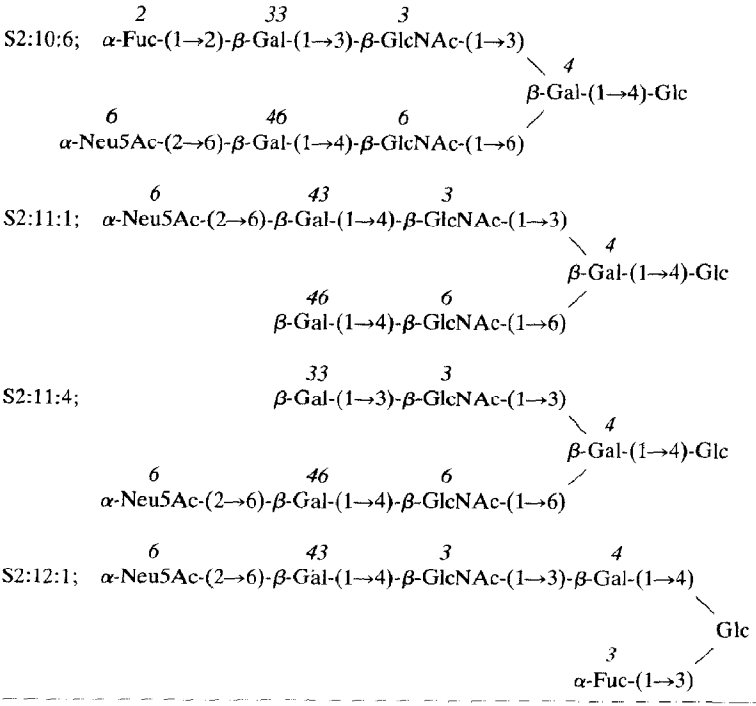
H.p.l.c. of fraction S2:15 gave two major, incompletely separated, compounds. Analysis by n.m.r. spectroscopy indicated a mixture of fucosylated oligosaccharides which were not characterized further. S2:12 contained a mixture of three compounds, of which only one, S2:12:1, could be prepared in a pure state[22] (Table I). The ^{1}H-n.m.r. data on S2:12:1 are presented in Table II. The purification and structural analysis of the other two components will be reported elsewhere.

S2:11 contained two major components S2:11:1 and S2:11:4. Analysis by

TABLE II

^{1}H-CHEMICAL SHIFTS (P.P.M.) FOR THE STRUCTURAL REPORTER GROUPS

Residue	*Reporter group*	*Compound*			
		S2:10:6	*S2:11:1*	*S2:11:4*	*S2:12:1*
α,β-Glc	H-1	5.219/4.657	5.218/4.666	5.219/4.655	5.179/4.652
	H-2	n.d.[a]/3.308	n.d./3.292	n.d./3.306	n.d./3.461
β-Gal[4]	H-1	4.416	4.434	4.434	4.419
	H-4	4.131	4.145	4.147	4.098
GlcNAc[3]	H-1	4.622/4.618	4.730/4.727	4.729/4.723	4.726/4.722
	CH_3	2.053	2.051	2.025	2.047
β-Gal[33]	H-1	4.643		4.439	
β-Gal[43]	H-1		4.454		4.455
β-GlcNAc[6]	H-1	4.67	4.646/4.638	4.66	
	CH_3	2.082/2.086	2.059/2.062	2.083/2.086	
β-Gal[46]	H-1	4.442	4.470	4.442	
α-Fuc[2]	H-1	5.187			
	H-5	4.293			
	CH_3	1.236			
α-Fuc[3]	H-1				5.370/5.427
	H-5				4.819/4.806
	CH_3				1.168/1.163
α-Neu5Ac[6]	H-3*a*	1.715	1.720	1.714	1.722
	H-3*e*	2.666	2.670	2.669	2.668
	CH_3	2.028	2.028	2.028	2.028



[a]Not determined.

n.m.r. spectroscopy (Table II) revealed isomeric branched structures previously reported[23] (Table I). The only structural differences in these oligosaccharides are the position of substitution of the GlcNAc residue and the location position of the sialic acid.

S2:10 was a complex mixture (Fig. 2B). Three major compounds S2:10:3, S2:10:4, and S2:10:6 could be isolated. S2:10:6 was analysed by n.m.r. spectroscopy (Table II) and a previously reported structure was found[24] (Table I). Structures S2:10:3 and S2:10:4 were new compounds and their structures will be reported elsewhere.

S2:9 contained (Fig. 2C) one major compound, S2:9:1, and S2:7 gave one pure compound, S2:7:3, not previously reported. Where the α and β anomers were separated, the β anomer always had the shorter retention time, sometimes by as much as 10 min.

Structure of the new oligosaccharides. — Each of the oligosaccharides discussed contained one Glc residue, which was the reducing terminal. The reducing terminal was identified by m.s. of the alditol acetates, prepared by reduction with $NaBD_4$ before, and with $NaBH_4$ after, hydrolysis, and by n.m.r. spectroscopy of the parent compounds.

The monosaccharide sequence and some linkage positions were determined by f.a.b.-m.s. As model compounds, the common human-milk oligosaccharides β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc (lacto-*N*-tetraose, LNT), β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc (lacto-*N*-neotetraose, LNnT), β-Gal-(1→3)-[α-Fuc-(1→4)]-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc (lacto-*N*-fucopentaose II, LNF II), and β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc (lacto *N*-fucopentaose III, LNF III) were methylated and analysed by f.a.b.-m.s. Parts of the fragmentation patterns of LNT and LNnT containing monosubstituted GlcNAc residues are shown in Fig. 3. The primary sequence ion at *m/z* 464 for LNT gives a major secondary fragment of *m/z* 228 by elimination of the Gal residue, whereas LNnT gives *m/z* 432, by elimination of methanol. The fragment of *m/z* 196 is formed by elimination of both the Gal residue and methanol.

The fragments formed from LNF II and LNF III containing a 3,4-substituted GlcNAc residue are shown in Fig. 4. After a primary cleavage of the GlcNAc linkage to give *m/z* 638, the major secondary fragments are *m/z* 402 for LNF II and *m/z* 432 for LNF III. These examples show that the secondary fragments, formed after a primary cleavage of a HexNAc linkage, arise by preferential elimination of the 3-substituent of the HexNAc residue.

In contrast to e.i.-mass spectra, there are no sequence ions containing the reduced terminal (*i.e.*, the J-series of ions[25]) in the f.a.b.-mass spectra, as the energy is not sufficient for cleavage of C–C bonds.

F.a.b.-m.s. of methylated oligosaccharide-alditols also gave $[M + 1]^+$ ions which could be discriminated from fragment ions by adding sodium iodide to give $[M + 23]^+$; fragment ions were unaffected. For ions with *m/z* >1000, the decimals of the masses are important. The contribution of the isotopes affects ions with *m/z*

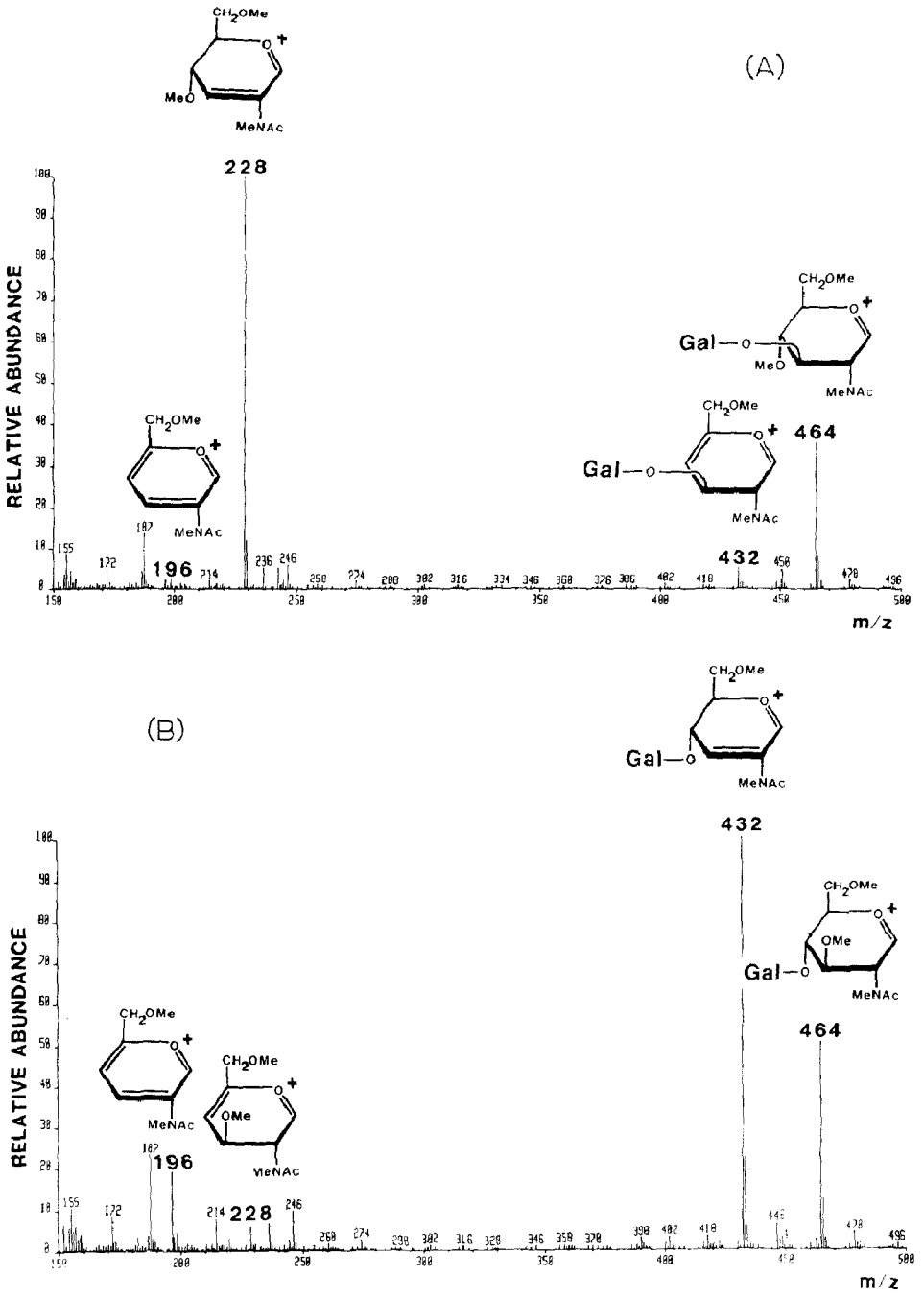


Fig. 3. Part of the f.a.b.-mass spectra of methylated LNT (A) and LNnT (B).

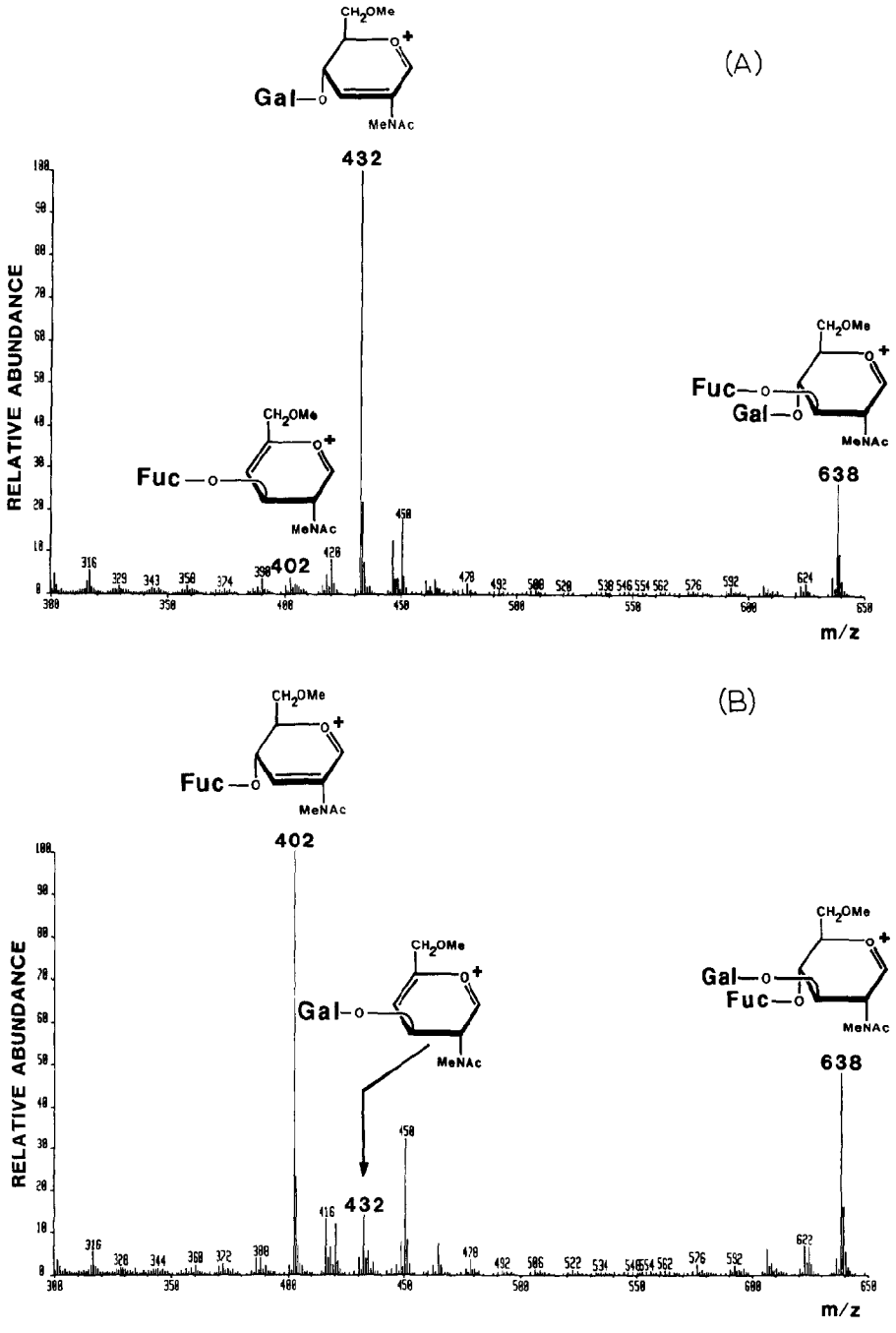


Fig. 4. Part of the f.a.b.-mass spectra of methylated LNF III (A) and LNF II (B).

>1500 and gives rise to several clusters of ions where two ions, separated by one mass unit, have about the same intensity. A characteristic feature of f.a.b.-mass spectra of methylated acetamido-containing oligosaccharides is elimination of ketene from the $[M + 1]^+$ ion. A mechanism for this elimination has been proposed for methylated glycosphingolipids[26].

Structural information not furnished by chemical analyses and f.a.b.-m.s. was obtained by n.m.r. spectroscopy. ^{1}H- and ^{13}C-n.m.r. data on sialylated oligosaccharides[17] and fucosylated derivatives of the β-Gal-(1→4)-β-GlcNAc-(1→ sequence[27] have been reported. α-Neu5Ac-(2→3)-β-Gal-(1→4)-Glc (3′-sialyl-lactose, 3-SL), α-Neu5Ac-(2→6)-β-Gal(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc (sialyl-lacto-*N*-neotetraose, LST c), and β-Gal-(1→4)-[α-Fuc-(1→3)]-Glc (3-fucosyl-lactose, 3-FL) were used as model compounds. Signals for the structural reporter groups are well separated from those of the bulk of the ring protons (3.5–4 p.p.m.). The location of a Neu5Ac residue can be deduced as follows. (*a*) The signals for H-3*a* and H-3*e* of the Neu5Ac residue can be used to discriminate between (2→3)- and (2→6)-α-linkages of Neu5Ac to Gal. (*b*) For an α-Neu5Ac-(2→3)-β-Gal-(1→ sequence, the signal for H-3 of the Gal residue is shifted downfield of the ring protons by ~0.6 p.p.m. Also, in a β-GlcNAc-(1→3)-β-Gal-(1→ sequence, the signal for H-4 of the Gal residue appears at ~4.15 p.p.m.

In ^{13}C-n.m.r. spectra, substitution at a primary or secondary carbon by α-Neu5Ac will deshield the carbon and shift the signal by 2–3.5 p.p.m., which is less than half the value obtained by substitution with a neutral monosaccharide residue.

COSY experiments and its RELAY extensions not only give shift information but also coupling patterns, which allow each monosaccharide residue to be identified and designated α or β. Assignments of ^{13}C resonances were made with H–C correlated experiments.

TABLE III

PARTIALLY METHYLATED ALDITOL ACETATES OBTAINED FROM THE NEW COMPOUNDS

Sugar derivative	*Compound*		
	S2:21:3	*S2:9:1*	*S2:7:3*
1,2,3,5,6-Me_5-Glc	0[a]	0.5	0.4
2,3,4-Me_3-Fuc	0.5	0.5	1.0
1,2,5,6-Me_4-Glc	0.7	0	0
2,3,4,6-Me_4-Gal	0	0.9	0
2,4,6-Me_3-Gal	1.0	0	0
3,4,6-Me_3-Gal	0	0	0.9
2,3,4-Me_3-Gal	0	0.9	0.7
2,4-Me_2-Gal	0	1.0	1.0
3,6-Me_2-GlcN(Me)Ac	0	[b]	[b]
6-Me-GlcN(Me)Ac	0	[b]	[b]

[a]Values calculated from peak areas in g.l.c. without considering response factors. [b]Present but not quantified due to unknown response factor in g.l.c.

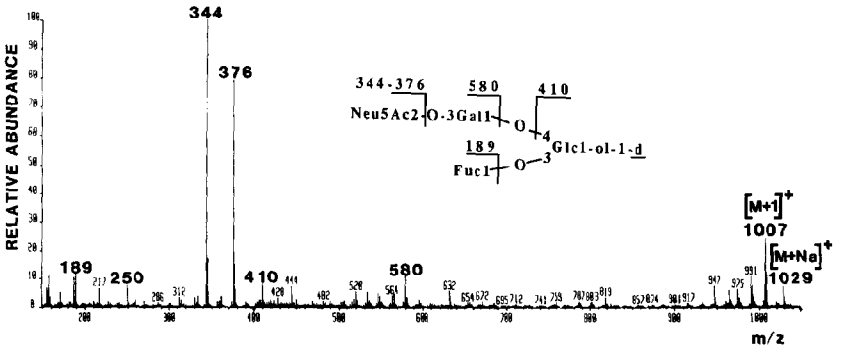


Fig. 5. F.a.b.-mass spectrum of the methylated alditol of S2:21:3.

Structure of S2:21:3. — Sugar analysis of S2:21:3 revealed approximately equal molar proportions of Fuc, Gal, Glc, and Neu5Ac in the ratios 0.9:0.9:1.0:1.2. Methylation analysis revealed a 3,4-substituted reducing Glc unit, a non-reducing terminal Fuc, and a 3-substituted Gal (Table III).

F.a.b.-m.s. of the methylated alditol-*1-d* of S2:21:3 (Fig. 5) gave an $[M + 1]^+$ ion with *m/z* 1007, consistent with the above monosaccharide composition. The sequence ions of *m/z* 376 and 580 indicated Neu5Ac-Gal and the ion *m/z* 189 indicated non-reducing terminal Fuc. Therefore, Neu5Ac-Gal and Fuc must be linked to positions 3 and 4 of the reducing Glc unit. The exact position was determined by n.m.r. spectroscopy.

The ^{1}H- and ^{13}C-n.m.r. data for S2:21:3 are listed in Table IV. Five signals could be assigned to anomeric protons. DOUBLE RELAY COSY confirmed the signals at 5.176 ($J_{1,2}$ 3.6 Hz) and 4.651 p.p.m. ($J_{1,2}$ 7.9 Hz) to belong to H-1α and H-1β, respectively, of Glc. H-1 of α-Fuc gave two signals at 5.374 ($J_{1,2}$ 3.6 Hz) and 5.432 p.p.m. ($J_{1,2}$ 3.6 Hz) due to the $\alpha\beta$-Glc residue. The signal at 4.497 p.p.m. ($J_{1,2}$ 7.8 Hz) is assigned to H-1 of β-Gal with a shift for H-3 to 4.084/4.077 p.p.m. (α/β), suggesting substitution of this residue at position 3 by Neu5Ac. The signals for H-3*e* and H-3*a* for Neu5Ac at 1.795 and 2.762 p.p.m., respectively, are typical for an α-Neu5Ac-α-(2→3)-β-Gal sequence.

Comparison of the ^{13}C-chemical shifts for S2:21:3 and 3-FL showed a downfield shift of 3.2 p.p.m. for the resonance of C-3 of the Gal residue and upfield shifts for the signals of C-4 (1.0 p.p.m.) and C-2 (1.7 p.p.m.). This result is consistent with substitution at position 3 by Neu5Ac. These results are in agreement with previously reported data on α-Neu5Ac-(2→3)-β-Gal linkages[17]. Thus, S2:21:3 has the structure

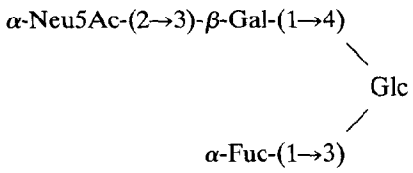


TABLE IV

^1H- AND ^{13}C-N.M.R. DATA FOR S2:21:3

α-Neu5Ac-(2→3)-β-Gal-(1→4)
\
Glc
/
α-Fuc-(1→3)

Residue	*Reporter group*	*Chemical shift (p.p.m.)*[a]		*Residue*	*Reporter group*	*Chemical shift (p.p.m.)*	
		^{13}C	^1H			^{13}C	^1H
α-Glc	1	92.9	5.176	α-Fuc	1	99.3/99.2	5.374/5.432
	2	73.6	3.76		2	68.9	3.78
	3	75.4	3.94		3	70.0	3.96
	4	73.4	3.88		4	72.8	3.78
	5	71.6	3.96		5	67.3	4.82/4.81
	6	60.4	3.89		CH_3	16.1	1.18/1.17
	6′		3.89				
β-Glc	1	96.6	4.651	α-Neu5Ac	1	174.7	
	2	76.4	3.46		2	100.5	
	3	77.7	3.77		3	40.6	1.795(*a*)
	4	73.5	3.89				2.762(*e*)
	5	76.1	3.58		4	69.1	3.68
	6	60.5	3.83		5	52.5	3.85
	6′		3.98		6	73.7	3.65
					7	68.9	3.62
β-Gal	1	102.4	4.497		8	72.7	3.88
	2	70.2	3.50/3.52		9	63.4	3.64/3.88
	3	76.4	4.084/4.077		C=O	175.8	
	4	68.1	3.93/3.94		CH_3	22.9	2.030
	5	75.7	3.57				
	6	62.3	3.69				
	6″		3.69				

[a]Accurate to ±0.01 p.p.m. or 0.002 p.p.m. (when three decimals) for ^1H (acetone 2.225 p.p.m.) and ±0.1 p.p.m. for ^{13}C (1,4-dioxane 67.4 p.p.m.).

Structure of S2:9:1. — Sugar analysis of S2:9:1 revealed Glc, Gal, Fuc, GlcNAc, and Neu5Ac in the molar proportions of 1.0:2.8:0.9:2.0:0.8. Methylation analysis revealed 4-substituted reducing Glc, non-reducing terminals of Gal and Fuc (Table III), 6- and 3,6-substituted Gal, and 4- and 3,4-substituted GlcNAc.

F.a.b.-m.s. of reduced ($NaBD_4$) and methylated S2:9:1 derivative (Fig. 6) gave an $[M + 1]^+$ ion of *m/z* 1906 in agreement with an octasaccharide structure. The ion at *m/z* 1864 was formed by elimination of ketene from the $[M + 1]^+$ ion. The primary sequence ion with *m/z* 638 and the secondary ion with *m/z* 432 indicate the sequence Gal-(1→4)-[Fuc-(1→3)]-GlcNAc as discussed above (Fig. 4). Another sequence of Neu5Ac-Gal-GlcNAc is indicated by the primary sequence ions of *m/z* 376 and 825. The secondary fragments (*m/z* 793, 228, and 196) formed

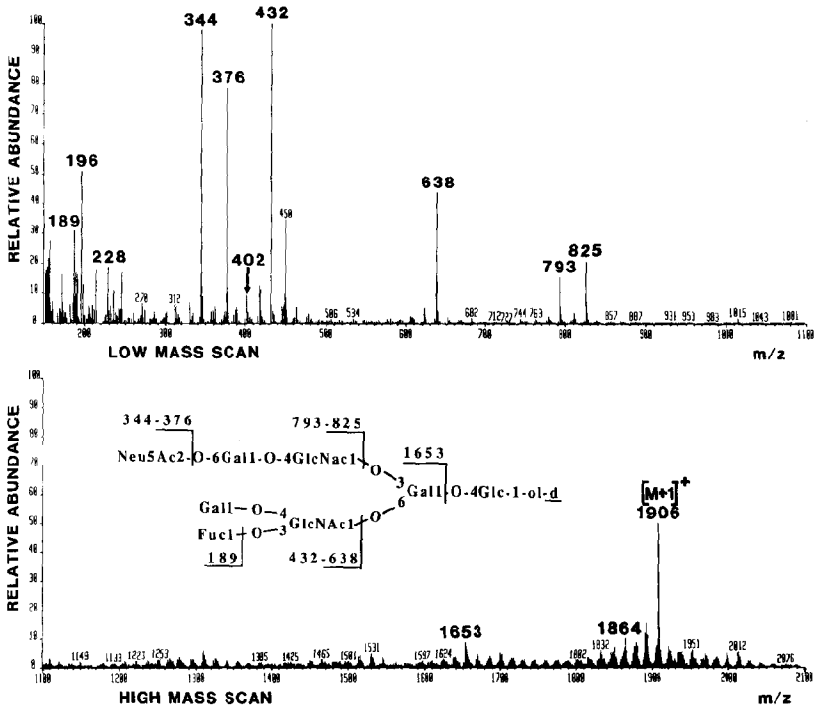


Fig. 6. F.a.b.-mass spectrum of the methylated alditol of S2:9:1.

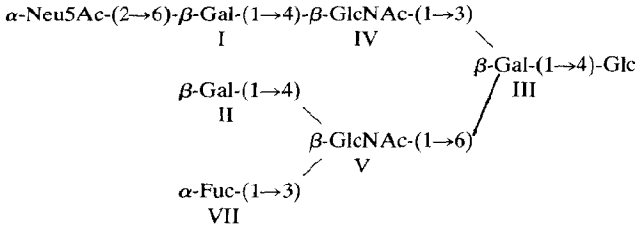
from *m/z* 825 by eliminations determine the position of substitution of the GlcNAc residue. The relative intensities of the ions with *m/z* 196 and 228 showed one GlcNAc residue to be 4-substituted (Fig. 3). The ion at *m/z* 1653 combined with the methylation analysis data shows that the 3,6-substituted Gal is substituted by the fucosylated and the sialylated sequences.

The positions of substitution of the branched Gal cannot be inferred from the f.a.b.-mass spectrum.

The ^{1}H- and ^{13}C-n.m.r. data for S2:9:1 are given in Table V. Besides the signals from 4-substituted reducing Glc, at 5.217 ($J_{1,2}$ 3.6 Hz, H-1α), 4.664 ($J_{1,2}$ 7.8 Hz, H-1β), and 3.289 p.p.m. (H-2β), there were six additional signals for anomeric protons, namely, for 3,6-substituted Gal at 4.431 p.p.m. (H-4 at 4.138 p.p.m.), two Gal residues at 4.455 ($J_{1,2}$ 7.9 Hz) and 4.452 p.p.m. ($J_{1,2}$ 7.9 Hz), α-Fuc 3-linked to GlcNAc at 5.101 p.p.m. ($J_{1,2}$ 3.6 Hz), and two β-linked GlcNAc residues at 4.65 and 4.73 p.p.m., respectively. These signals appeared as multiplets due to the α- and β-forms of the reducing Glc unit and strong coupling between H-2 and H-3. The signals at 4.73 p.p.m. is assigned to a GlcNAc in the sialylated branch. The shifts for the H-1 resonance and the ^{13}C signals of this residue agree well with those of LST c. On irradiation of this signal, an n.O.e. effect was seen at H-3 of the 3,6-substituted Gal residue (3.72 p.p.m.). The signal at 4.65 p.p.m. could now be

TABLE V

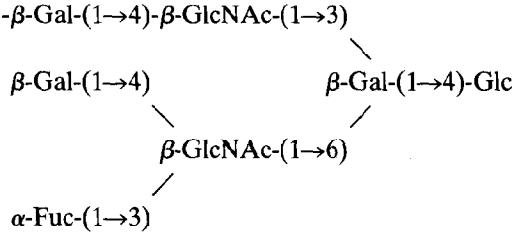
^1H- AND ^{13}C-N.M.R. DATA FOR S2:9:1



Residue	*Reporter group*	*Chemical shift (p.p.m.)*[a]		*Residue*	*Reporter group*	*Chemical shift (p.p.m.)*	
		^{13}C	^1H			^{13}C	^1H
α-Glc	1	92.6	5.217	β-GlcNAc (V)	1	101.6	4.65
	2	72.0	3.58		2	56.7	3.90
	3	72.3	3.83		3	75.6	3.90
	4	79.9	3.61		4	74.1	3.92
	5	70.8	3.94		5	7.57	3.60
	6	60.7	3.86		6	60.6	3.85
	6′		3.86		6′		4.01
					C=O	175.1	
					CH_3	23.3	2.051
β-Glc	1	96.5	4.664	β-Gal (II)	1	102.6	4.455
	2	74.6	3.289		2	71.8	3.49
	3	75.2	3.64		3	73.2	3.66
	4	79.8	3.61		4	69.2	3.89
	5	75.5	3.60		5	76.2	3.60
	6	60.9	3.78		6	62.3	~3.72
	6′		3.96		6′		~3.72
β-Gal (III)	1	103.9	4.431	α-Fuc (VII)	1	99.4	5.103
	2	70.7	3.58		2	68.5	3.69
	3	82.5	3.72		3	70.0	3.90
	4	69.2	4.138		4	72.7	3.78
	5	74.2	3.83		5	67.5	4.829
	6	69.6	3.83		CH_3	16.3	1.173
	6′		3.99				
β-GlcNAc	1	103.3	4.73	α-Neu5Ac	1	174.0	
(IV)	2	55.8	3.80		2	101.0	
	3	73.1	3.78		3	40.8	1.725(*a*)
	4	81.3	3.66				2.669(*e*)
	5	75.1	3.60		4	69.2	3.56
	6	61.0	3.85		5	52.7	3.80
	6′		3.97		6	73.4	3.70
	C=O	175.7			7	69.0	3.65
	CH_3	23.1	2.051		8	72.5	3.88
					9	63.5	3.64/3.87
					C=O	175.7	
β-Gal (I)	1	104.3	4.452		CH_3	22.9	2.027
	2	71.5	3.53				
	3	73.3	3.67				
	4	69.2	3.93				
	5	74.5	3.82				
	6	64.2	3.54				
	6′		3.98				

[a]See footnote to Table IV.

assigned to the GlcNAc in the branch 6-linked to the branched Gal residue. Irradiation of the α-Fuc signal at 5.101 p.p.m. gave an n.O.e. effect at H-3 for this GlcNAc (3.90 p.p.m.). This result confirms that the fucosylated branch is 6-linked in the 3,6-substituted Gal residue. The signals of H-1 and C-1 of the GlcNAc in the 6-position are shifted upfield compared to those of the GlcNAc in the 3-position of the 3,6-substituted Gal. Therefore, S2:9:1 has the structure



Structure of S2:7:3. — Sugar analysis of S2:7:3 revealed Glc, Gal, Fuc, GlcNAc, and Neu5Ac in the molar proportions of 1.0:2.7:1.8:2.3:0.8. Methylation analysis showed the reducing terminal Glc to be 4-substituted and Fuc to be the

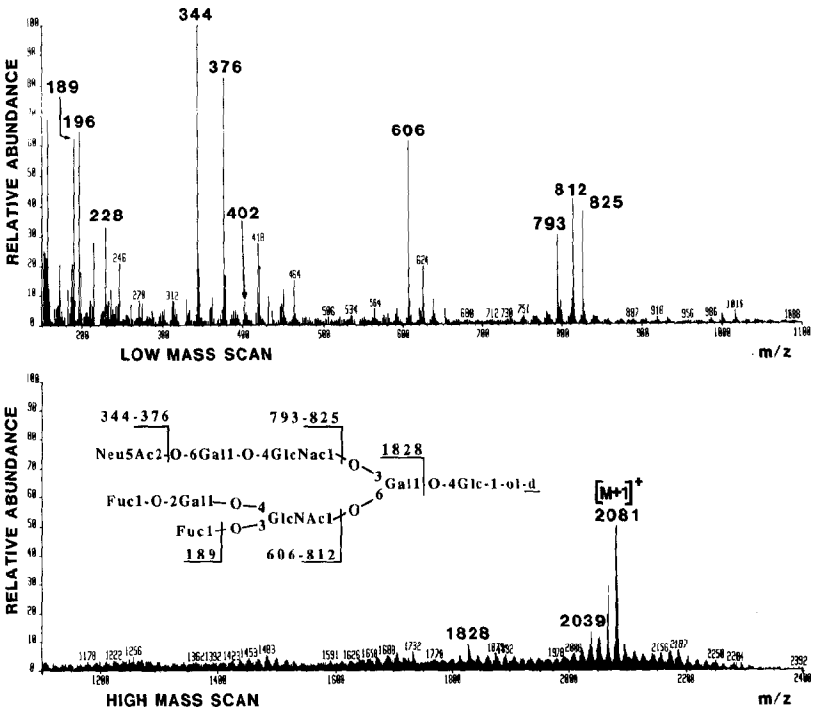
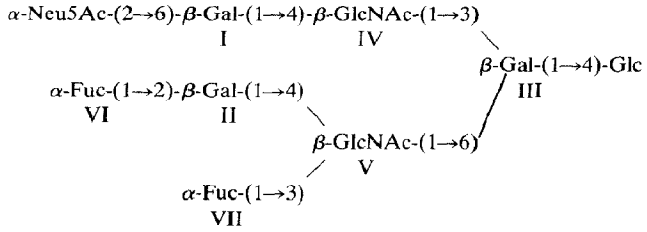


Fig. 7. F.a.b.-mass spectrum of the methylated alditol of S2:7:3.

TABLE VI

^1H- AND ^{13}C-N.M.R. DATA FOR S2:7:3



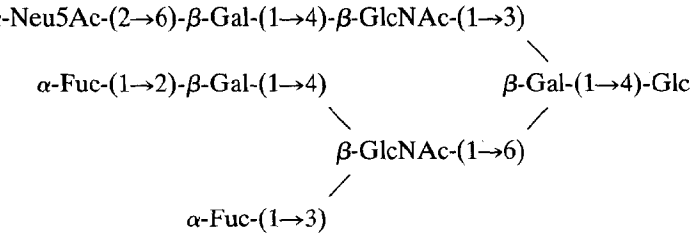
Residue	*Reporter group*	*Chemical shift (p.p.m.)*[a]		*Residue*	*Reporter group*	*Chemical shift (p.p.m.)*	
		^{13}C	^1H			^{13}C	^1H
α-Glc	1	92.6	5.217	β-GlcNAc (V)	1	101.6	4.62
	2	72.0	3.58		2	56.7	3.88
	3	72.3	3.83		3	75.6	3.88
	4	80.1	3.59		4	74.1	3.92
	5	70.8	n.d.[b]		5	76.4	3.46
	6	60.9	n.d.		6	60.5	n.d.
	6′		n.d.		6′		n.d.
					C=O	175.2	
					CH_3	23.3	2.055
β-Glc	1	96.5	4.664	β-Gal (II)	1	101.1	4.501
	2	74.6	3.289		2	77.1	3.64
	3	75.2	3.64		3	74.4	3.84
	4	80.0	3.59		4	69.6	3.87
	5	75.5	3.60		5	76.2	3.60
	6	60.9	n.d.		6	62.3	3.72
	6′		n.d.		6′		3.72
β-Gal (III)	1	103.9	4.433	α-Fuc (VII)	1	99.4	5.090
	2	70.7	3.58		2	68.5	3.69
	3	82.5	3.72		3	70.0	3.92
	4	69.1	4.143		4	72.8	3.81
	5	74.1	3.83		5	67.7	4.88
	6	69.6	n.d.		CH_3	16.3	1.234
	6′		n.d.				
β-GlcNAc	1	103.4	4.727	α-Fuc (VI)	1	100.2	5.275
(IV)	2	55.8	3.79		2	69.1	3.79
	3	73.1	3.78		3	70.5	3.78
	4	81.3	3.66		4	72.5	3.82
	5	75.1	3.61		5	67.7	4.26
	6	61.0	n.d.		CH_3	16.3	1.268
	6′		n.d.				
	C=O	175.7					
	CH_3	23.1	2.051				
β-Gal (I)	1	104.3	4.453	α-Neu5Ac	1	174.3	
	2	71.6	3.54		2	(101)	
	3	73.2	3.67		3	40.9	1.721(*a*)
	4	69.2	3.93				2.670(*e*)
	5	74.5	3.82		4	69.3	3.56
	6	64.2	n.d.		5	52.7	3.81
	6′		n.d.		6	73.4	3.69
					7	69.0	3.65
					8	72.5	3.90
					9	63.5	3.64/3.87
					C=O	175.7	
					CH_3	22.9	2.027

[a]See footnote to Table IV. [b]Not determined.

only non-reducing terminal (Table III). The Gal was 2-, 6-, and 3,6-substituted, and the GlcNAc was 4- and 3,4-substituted.

F.a.b.-m.s. of the methylated alditol-*1-d* of S2:7:3 (Fig. 7) gave an $[M + 1]^+$ ion of *m/z* 2081 which confirms the monosaccharide composition. A secondary fragment of *m/z* 2039 formed from the $[M + 1]^+$ ion by elimination of ketene is characteristic for acetamido-containing compounds as discussed above. Two sets of primary sequence ions were seen at *m/z* 189 and 812, representing a fucosylated branch, and at *m/z* 376 and 825, representing a sialylated branch. A secondary fragment *m/z* 606 formed from *m/z* 812 showed the 3,4-substituted GlcNAc residue to be 3-substituted by a Fuc residue. The 4-substitution of the GlcNAc in the sialylated sequence is determined by the secondary fragments *m/z* 196, 228, and *m/z* 793 as discussed for S2:9:1. The fragment at *m/z* 1828 showed the branched Gal residue to be substituted by the fucosylated and the sialylated sequences, but the positions of substitution could not be deduced.

The ^{1}H- and ^{13}C-n.m.r. data for S2:7:3 are given in Table VI. S2:7:3 has one α-Fuc more than S2:9:1, as seen from the H-1 signals at 5.275 p.p.m. ($J_{1,2}$ 3.0 Hz). Comparison of the chemical shift data for S2:7:3, S2:9:1, and LST c, showed that the sialylated branch has the same structure and was 3-linked to the branched Gal residue. The additional Fuc residue is 2-linked to the Gal residue in the fucosylated sequence, which is 6-linked to the branched Gal residue, since the signal of C-2 of the Gal residue is shifted 5.3 p.p.m. to 77.1 p.p.m. The unusual shift (5.275 p.p.m.) of the signal of H-1 of the Fuc anomer agrees with the findings of Hindsgaul *et al.*[27] for the Le^y determinant, α-Fuc-(1→2)-β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-(1→. Therefore, S2:7:3 has the structure



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