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STRUCTURE OF TWO NEW OLIGOSACCHARIDES ISOLATED FROM HUMAN MILK: SIALYLATED LACTO-*N*-FUCOPENTAOSES I AND II

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ABSTRACT

The structures of two minor oligosaccharides isolated from human milk have been investigated. By using f.a.b.-mass spectrometry, methylation analysis, and partial formolysis as the principal methods of structural investigation, these oligosaccharides were identified as IV^2-α-Fuc-III^6-α-NeuAc-LcOse$_4$ and IV^3-α-NeuAc-III^4-α-Fuc-LcOse$_4$. This latter oligosaccharide has been recently found to be the carbohydrate moiety of a ganglioside isolated from a colorectal carcinoma cell-line (see ref. 12).

INTRODUCTION

The oligosaccharides of human milk reflect the ability of the donor to synthetize glycan chains of cell surfaces and mucus secretions that are the structural determinants of blood type[1]. These oligosaccharides do not originate from the catabolism of glycolipids, but are products of the activity of mammary gland glycosyltransferases that use lactose as an acceptor[2]. Thus, the study of their structure may provide interesting information concerning the specificity of these glycosyltransferases.

We describe here the isolation and the identification of two minor oligosaccharides (<1 mg/L) having the structure of sialylated lacto-*N*-fucopentaoses I and II respectively (IV^2-α-Fuc-III^6-α-NeuAc-LcOse$_4$ and IV^3-α-NeuAc-III^4-α-Fuc-LcOse$_4$).

EXPERIMENTAL

Preparation of milk oligosaccharides. — The final supernatant solution resulting from the sequential $(NH_4)_2SO_4$ fractionation of 40 L of pooled milk

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proteins[3] was purified on charcoal–Celite. After washing the column with distilled water to eliminate ammonium salts, the oligosaccharides were eluted with 50% ethanol in water. The solution was evaporated under diminished pressure, and the oligosaccharides were dissolved in water and fractionated on an anion exchanger (Dowex 1 × 2, 200–400 mesh, HCO_3^-). Sialyloligosaccharides were eluted with a discontinuous gradient of pyridine–acetate buffer (pH 5.5) from 5–500mM.

Analytical procedures. — Descending paper chromatography was performed on Whatman No. 3 paper with 5:5:1:3 pyridine–ethyl acetate–acetic acid–water and detection by the aniline oxalate reagent[4].

Reduced oligosaccharides were isolated by high performance liquid chromatography (l.c.) on primary amine-bonded silica (5 μm Amino AS-5A column; 0.4 × 25 cm; Chromatem 33, Touzart et Matignon, Paris). A linear gradient to CH_3CN–15mM KH_2PO_4 buffer, pH 5.2 (3:1) was applied for 25 min, followed by a linear gradient to CH_3CN–15mM KH_2PO_4 buffer (11:9) for 65 min[5]. The flow rate was 1 mL/min. The oligosaccharides were detected at 200 nm.

The molar ratios of hexoses, *N*-acetylhexosamines, and *N*-acetylneuraminic acid were determined by g.l.c. of their trifluoroacetyl derivatives, obtained after methanolysis of oligosaccharides (methanol–0.5M HCl, 24 h, 80°)[6].

Methylation analysis. — Oligosaccharides, previously reduced with $NaB[^2H]_4$, were methylated[7] and then methanolysed (methanol–0.5M HCl, 24 h, 80°). The partially methylated methyl glycosides were acetylated[8] (1:1 pyridine–acetic anhydride, 0.2 mL) and the products were analysed by g.l.c.–m.s. using a capillary column (0.33 mm × 25 m) coated with fused silica CP-SIL 5CB (temperature programme, 100 → 240°, at 4°/min).

Desialylation. — Selective desialylation was achieved by heating the aqueous solution (pH 3.5) of sialyloligosaccharides for 15 min at 100°. Neutral oligosaccharides, constituting 10–15% of the starting material, were purified on an anion-exchanger (Dowex 1 × 2; 200–400 mesh; HCO_3^-).

Partial formolysis. — Reduced, permethylated oligosaccharides were partially formolyzed in 85% formic acid for 30 min at 80°[9]. After formolysis, the formic acid was evaporated under diminished pressure, and traces of acid were removed by two sequential additions and evaporations of methanol. The mixture of partially methylated oligosaccharides was then reduced with $NaB[^2H]_4$, and ethylated[9]. The resulting partially methylated, partially ethylated disaccharide-alditols were analyzed by g.l.c.–m.s. on a capillary column (0.35 mm × 50 m) coated with OV-101 (temperature programme: 130 → 330°, at 10°/min).

Enzymic digestion. — Oligosaccharide (100 μg) in 10 μL of McIlvaine buffer (pH 5.0) was added to 20 μL (6 μg of protein) of a mixture of exoglycosidases (free from α-D-galactosidase and α-D-hexosaminidase) from *Bifidobacterium bifidum* (unpublished results). Hydrolysis was performed for 48 h at 37°. The reaction was stopped by heating for 3 min at 70°.

Fast-atom bombardment (f.a.b.) mass spectrometry. — F.a.b. mass spectrometry was performed on a ZAB HF mass spectrometer (VG Analytical,

Manchester, U.K.) using xenon atoms having a kinetic energy equivalent to 9 keV. The mass marker was calibrated with CsI. The permethylated oligosaccharides were analyzed in the positive-ion mode. The target was first coated with sodium acetate and the permethylated oligosaccharides (3–5 μg), dissolved in 1 μL of methanol, were added to the thioglycerol matrix. Spectra were recorded in a linear mass-controlled scan of 1–2 min duration: they were evaluated by counting the spectral lines.

RESULTS

Isolation and purification of oligosaccharides. — Fig. 1 depicts the stained paper chromatogram of acidic oligosaccharides eluted from the anion-exchanger by a discontinuous gradient of pyridine–acetate buffer. The monosialyl-oligosaccharides were eluted by 2–20mM pyridine acetate buffer in order of decreasing molecular weights; the disialyl-oligosaccharides were fractionated with 20–200mM buffer. By preparative paper chromatography, 7 compounds were isolated from the fractions eluted with 10 and 20mM pyridine–acetate buffer.

Structure determination of **1–3**. — The structural analysis of oligosaccharides **1–3** shows their total structural identity with, respectively, sialyllacto-*N*-tetraose a[10], siallylacto-*N*-tetraose b[10], and sialyllacto-*N*-neotetraose[11] (Tables I and II).

F.a.b.-m.s. of **4**. — The f.a.b.-m.s. (positive ion) was performed on the reduced and permethylated fraction **4**. Fig. 2 shows the region of the quasimolecular ions M + Na^+ at 1303 and 1477 a.m.u. From this result, it may be concluded that fraction **4** contains at least two different oligosaccharides consisting respectively of 2 hexoses, 1 *N*-acetylhexosamine, 1 *N*-acetylneuraminic acid, and 1 hexitol; and 2 hexoses, 1 *N*-acetylhexosamine, 1 deoxyhexose, 1 *N*-acetylneuraminic acid, and 1 hexitol.

Following permethylation, the deoxyhexose-containing tetrasaccharide fraction was further purified by preparative, high-performance t.l.c. (E. Merck, Darmstadt, G.F.R.) in 2:2:1 chloroform–benzene–ethanol. Its f.a.b. spectrum is shown in Fig. 3. Such ions as *m/z* 1303, 1029, 825, 793, and 228 still indicate contamination of this fraction with IV^6-α-NeuAc-nLcOse$_4$-ol and possibly IV^3-α-NeuAc-LcOse$_4$-ol. The major component (**4b**), signaled by the quasimolecular ion M + Na^+ 1477 gives rise to a series of ions specific for the proposed structure. The intense ion of *m/z* 402 arising from the tetrasaccharide ion (*m/z* 999) is formed by elimination of the NeuAc-Gal residue linked to position 3 of GlcNAc. This result, together with the absence of an ion at *m/z* 967 (999 − 32) clearly points to a (1→4) linkage of the fucose residue. The ion having *m/z* 793 could possibly be formed by elimination of a deoxyhexose residue from *m/z* 999. The results of partial formolysis and linked-scan measurements preclude, however, the presence of a 3-linked deoxyhexose.

Isolation of **4a, 4b,** *and* **4c**. — Fraction **4** was subsequently subfractionated by l.c. into three components, **4a, 4b,** and **4c**, as shown in Fig. 4. Component **4a** was

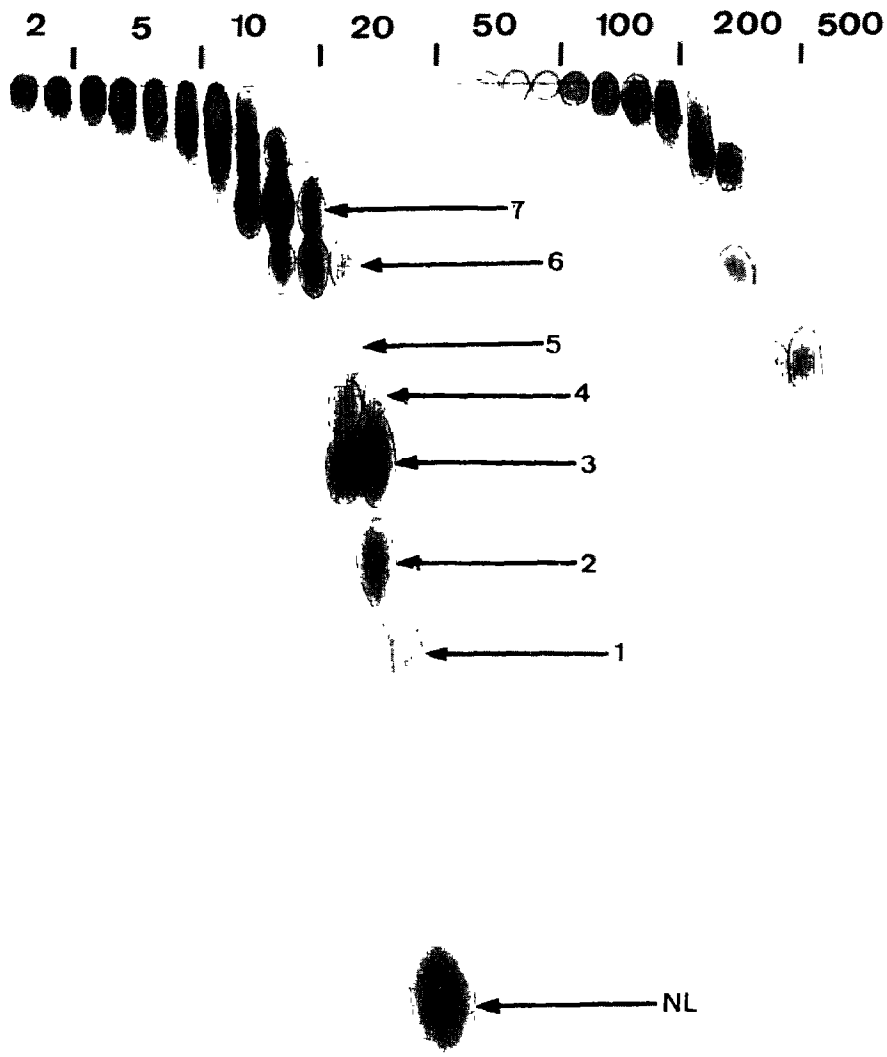

Fig 1. Paper chromatography of milk oligosaccharides eluted from an anion exchanger (Dowex 1 × 2; 200–400 mesh; HCO_3^-) with a discontinuous gradient of pyridine–acetate buffer (2–500mM). NL: α-D-NeuAc-(2→6)-β-D-Gal-(1→4)-D-Glc.

further identified as IV^6-α-NeuAc-nLcOse$_4$ (compound **3**), as shown in Tables I and II.

Structure of **4b**. — The sugar composition (Gal, 2; Fuc, 1; NeuAc, 1; GlcNAc, 1; Glc, 1) and the methylation analysis (2,4,6-tri-*O*-Me-Gal, 2; 2,3,4-tri-*O*-Me-Fuc, 1; 6-*O*-mono-Me-GlcNAc, 1; 1,2,3,5,6-penta-*O*-Me-Glc-ol, 1; 4,7,8,9-

TABLE I

MOLAR COMPOSITION OF OLIGOSACCHARIDES **1–4**

Oligosaccharide	*Molar ratios*[a]				
	Gal	*Glc*	*Fuc*	*GlcNAc*	*NeuAc*
1	2.03	1.00	—	1.05	1.04
2	2.00	1.00	—	1.04	1.10
3	1.93	1.00	—	1.15	1.13
4a	1.95	1.00	—	1.07	1.07
4b	2.05	1.00	0.95	1.12	1.12
4c	2.05	1.00	0.97	1.15	1.13

[a]On the basis of 1 Glc residue.

TABLE II

METHYLATION ANALYSIS OF OLIGOSACCHARIDES **1–4**[a]

	1	**2**	**3**	**4a**	**4b**	**4c**
1,2,3,5,6-Me_5-Glc-ol	1.0	1.0	1.0	1.0	1.0	1.0
2,3,4-Me_3-Fuc	—	—	—	—	0.8	0.9
2,3,4,6-Me_4-Gal	—	0.9	—	—	—	—
2,4,6-Me_3-Gal	1.9	0.9	0.9	0.9	1.8	0.9
2,3,4-Me_3-Gal	—	—	1.1	1.0	—	—
3,4,6-Me_3-Gal	—	—	—	—	—	1.1
4,6-Me_2-GlcNAc	0.8	—	—	—	—	—
3,6-Me_2-GlcNAc	—	—	0.8	0.8	—	—
6-Me-GlcNAc	—	—	—	—	0.8	—
4-Me-GlcNAc	—	0.8	—	—	—	0.9
4,7,8,9-Me_4-NeuAc	1.1	1.1	1.0	1.1	1.1	1.0

[a]Molar ratios are given on the basis of 1 residue of 1,2,3,5,6-Me_5-Glc-ol. The partially methylated, partially acetylated methyl glycosides were quantitated by comparison with the molar responses given by methylation analysis of oligosaccharide standards isolated from human milk (IV^2-α-Fuc-$LcOse_4$, IV^2-α-Fuc-III^4-α-Fuc-$LcOse_4$, and IV^3-α-NeuAc-III^6-α-NeuAc-$LcOse_4$).

tetra-*O*-Me-NeuAc, 1) of **4b** (Tables I and II) indicate that fucose and *N*-acetylneuraminic acid probably occur on the same Gal→GlcNAc disaccharide unit. In order to specify the relative position of these two sugars, permethylated **4b** was submitted to partial formolysis. After reduction with $NaB[^2H]_4$ and ethylation, the peralkylated disaccharide-alditols were analyzed by g.l.c.–m.s. (Fig. 5). Two components, A and B, were characterized. Spectrum A corresponds to the disaccharide-alditol 3-mono-*O*-Et-2,4,6-tri-*O*-Me-Gal-(1→4)-1,2,3,5,6-penta-*O*-Me-Glc-ol-1-[^{2}H], as is clearly indicated by the occurrence of fragments *ald* (*m/z* 236), aA_1 (*m/z* 233), and $aaldJ_1$ (*m/z* 310). This last fragment shows that the ethyl group in this compound is attached at O-3 of galactose. Disaccharide-alditol B was identified as 3-mono-*O*-Et-2,4,6-tri-*O*-Me-Gal-(1→3)-1,4,5-tri-*O*-Et-6-mono-*O*-Me-GlcNAc-ol-1-[^{2}H]. The fragment *m/z* 319 indicates that the GlcNAc-ol residue carries three

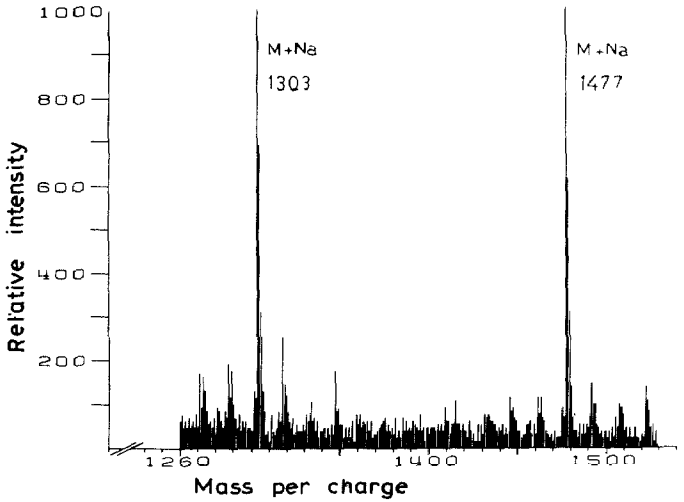

Fig. 2. The quasimolecular-ion region of reduced and permethylated compounds **4a** ($M + Na^+ = 1303$) and **4b,c** ($M + Na^+ = 1477$) in f.a.b.-m.s.

ethyl groups, located at O-1, O-4, and O-5 (*m/z* 465, 508, and 523). The (1→3) linkage is confirmed by the presence of an intense ion at *m/z* 407. The position of the ethyl group at O-3 of the galactose residue is demonstrated by the occurrence of the fragment $aaldJ_1$ at *m/z* 393.

The same experiment was performed on desialylated oligosaccharide **4b**. A new disaccharide-alditol (C) was identified as 2,3,4,6-tetra-*O*-Me-Gal-(1→3)-1,4,5-tri-*O*-Et-6-mono-*O*-Me-GlcNAc-ol-1-[^{2}H] (Fig. 6). This result shows that the NeuAc residue was attached at O-3 of galactose. On the basis of these results, the structure of **4b** was established as IV^3-α-NeuAc-III^4-α-Fuc-$LcOse_4$.

Structure of **4c**. — The sugar composition (Gal, 2; Fuc, 1; GlcNAc, 1; NeuAc, 1; Glc, 1) and the methylation analysis (3,4,6-tri-*O*-Me-Gal, 1; 2,4,6-tri-*O*-Me-Gal, 1; 2,3,4-tri-*O*-Me-Fuc, 1; 1,2,3,5,6-penta-*O*-Me-Glc-ol, 1; 4,7,8,9-tetra-*O*-Me-NeuAc, 1; 4-mono-*O*-Me-GlcNAc, 1) of **4c** (Tables I and II) show that fucose and *N*-acetylneuraminic acid are also here located on the same Gal→GlcNAc residue. Partial formolysis of permethylated **4c** and ethylation of the new free hydroxyl groups permitted the peralkylated oligosaccharide-alditols A and D to be characterized (Fig. 7). The spectrum D shows fragments at *m/z* 319 (tri-*O*-ethylated GlcNAc-ol), 233 (mono-*O*-ethylated Gal), and 379 ($aaldJ_1$). This last fragment (*ald* + 60) indicates that a methyl group occurs at O-3 of galactose. The same experiment performed on asialo-**4c** (Fig. 7) leads to the loss of an ethyl group of the GlcNAc-ol residue (spectrum E). In this case, the presence of fragments at *m/z* 451 and 509 confirms that NeuAc was linked at O-6 of GlcNAc and, consequently, shows that fucose was linked at O-2 of Gal. These results allow the conclusion that **4c** is IV^2-α-Fuc-III^6-α-NeuAc-$LcOse_4$.

Determination of the anomeric configurations. — Oligosaccharides **4b** and **4c**

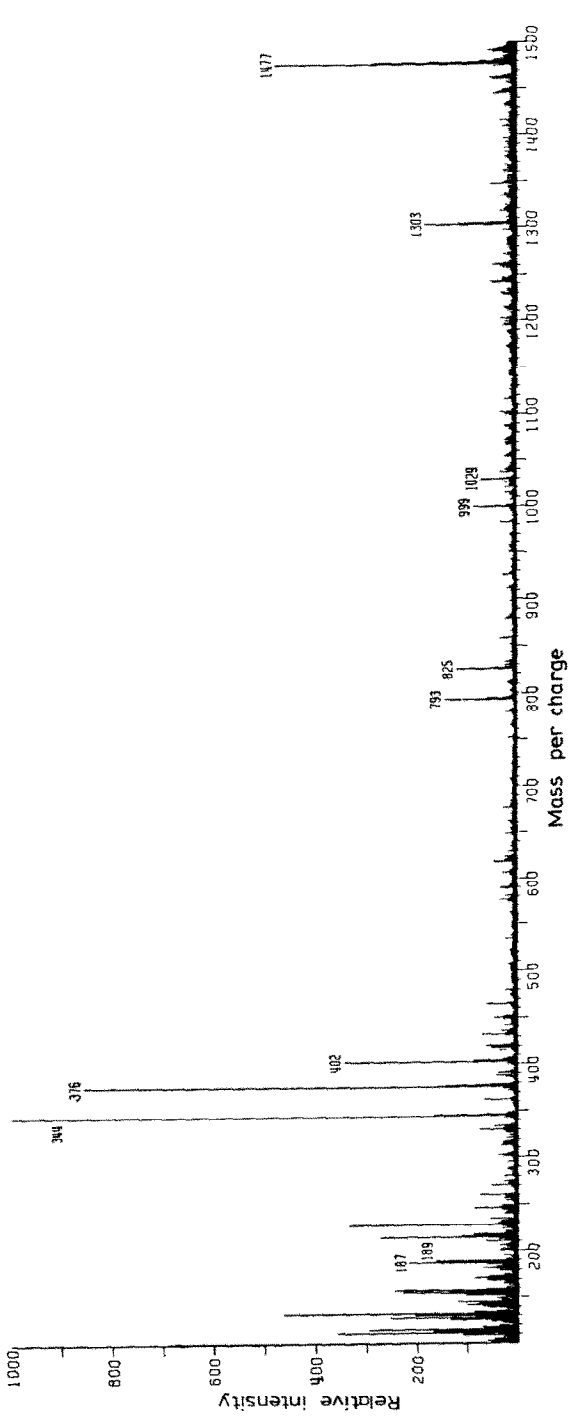

Fig. 3. F.a.b.-m.s. of enriched reduced and permethylated compound **4b** after purification on high-performance t.l.c. silica gel plates. Signals produced by the thioglycerol matrix are omitted.

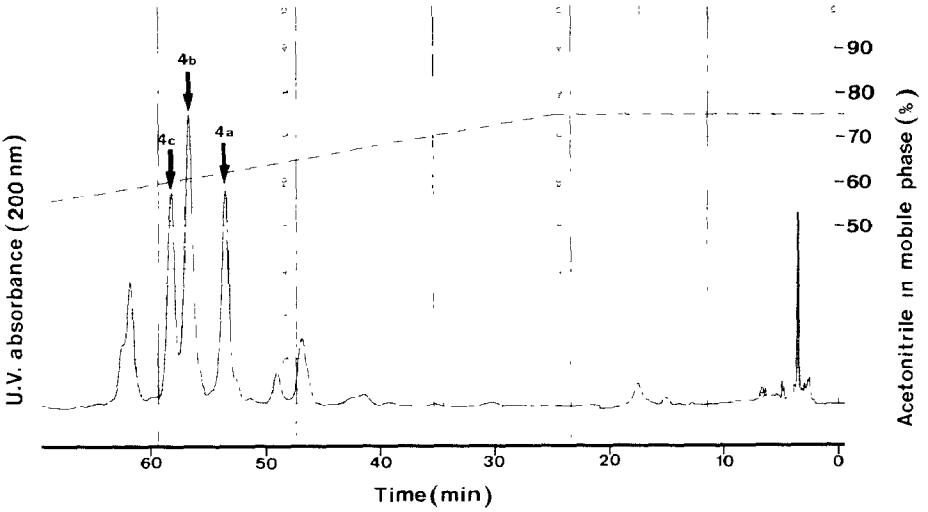

Fig. 4. Analysis of fraction **4** by semipreparative chromatography on 5-μm Amino AS-5A. For chromatographic conditions, see Experimental section.

were submitted to the action of the exoglycosidases extracted from *Bifidobacterium bifidum*, which contain α-L-fucosidase, *N*-acetyl-α-D-neuraminidase, β-D-galactosidase, and β-D-hexosaminidase, but are completely devoid of α-D-galactosidase and α-D-hexosaminidase (unpublished results). Quantitative release of *N*-acetylneuraminic acid, fucose, galactose, and *N*-acetylglucosamine was observed, confirming the anomeric configurations and the enantiomeric (D or L) identities of the proposed structures.

DISCUSSION

Human milk is a rich source of oligosaccharides, and many compounds remain to be characterized, despite the already long list described in the literature. Although the procedure followed for the fractionation of milk oligosaccharides does not permit exact determination of the amounts of **4b** and **4c**, we may estimate that they do not exceed 0.5–2 mg/L of pooled milk. Sialylated lacto-*N*-fucopentaose II (IV^3-α-NeuAc-III^4-α-Fuc-LcOse$_4$) has been recently found to be the carbohydrate moiety of a ganglioside isolated from a colorectal carcinoma cell-line[12]. Milk oligosaccharides do not constitute products derived from ganglioside catabolism, but result from the sequential addition of monosaccharide residues to lactose[2]. Nevertheless, it is interesting to observe that fucose and *N*-acetylneuraminic acid may be added to the same β-D-Gal-(1→3)-GlcNAc disaccharide unit, although purified fucosyl- and sialyl-transferases isolated from human milk[13] and porcine submaxillary glands[14] respectively do not add fucose or *N*-acetylneuraminic acid to IV^3-α-NeuAc-LcOse$_4$ or III^4-α-Fuc-LcOse$_4$. Their appearance in human milk could signify that the corresponding gastrointestinal cancer-associated ganglioside re-

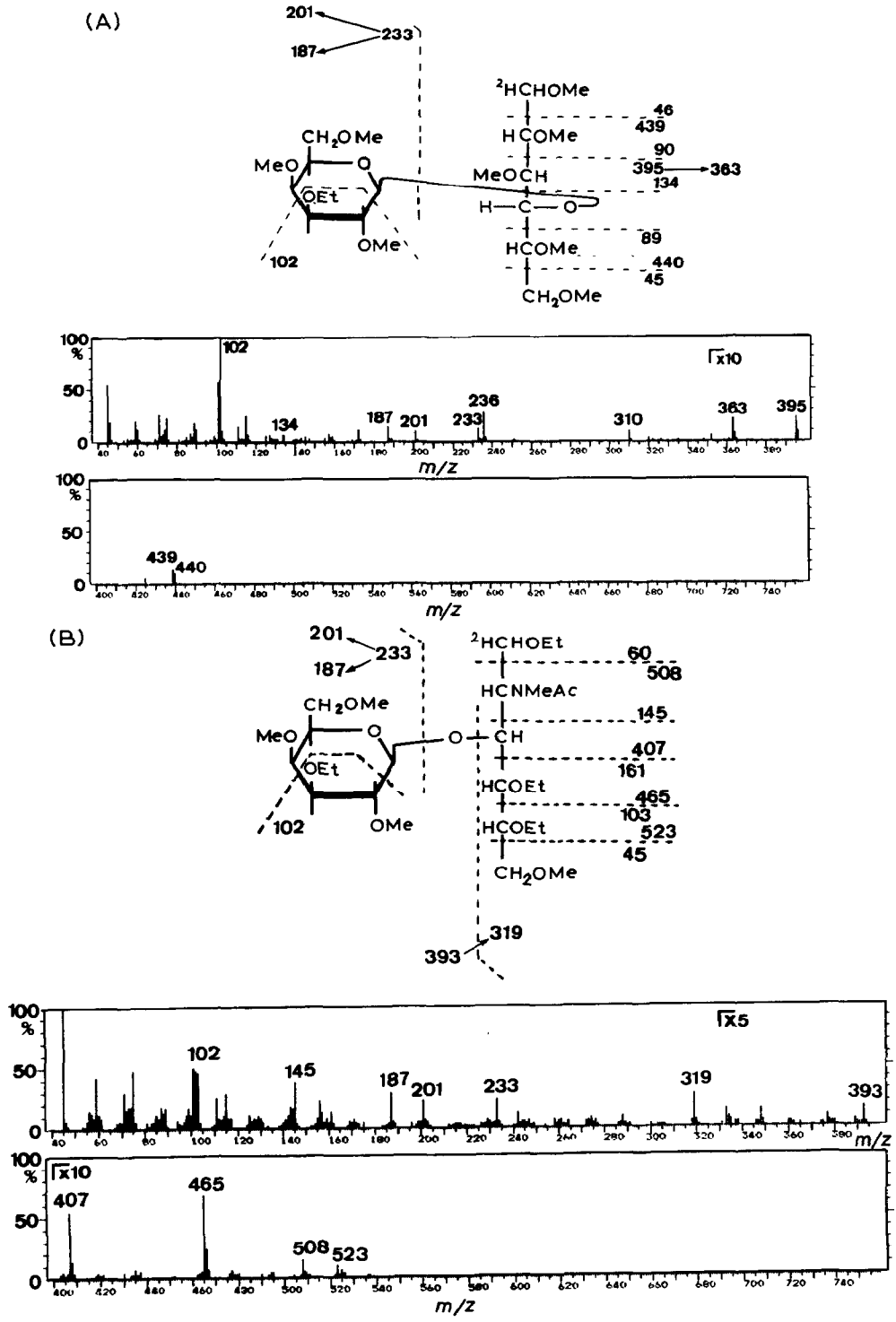

Fig. 5. Mass spectra A and B resulting from the partial formolysis of **4b**.

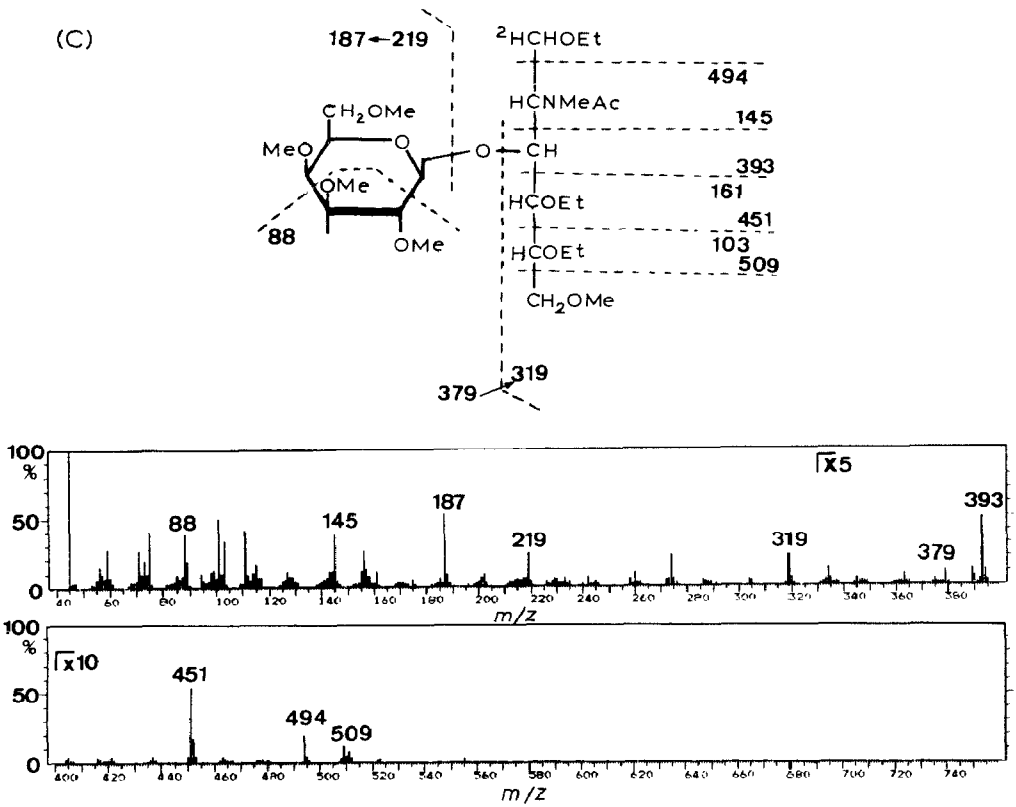

Fig. 6. Mass spectrum C resulting from the partial formolysis of asialo **4b**.

1 α-D-NeupAc-(2→3)-β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-D-Glcp

IV^{3}-α-NeuAc-LcOse$_4$

α-D-NeupAc

1

↓

6

2 β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-D-Glcp

III^{6}-α-NeuAc-LcOse$_4$

3,4a α-D-NeupAc-(2→6)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-D-Glcp

IV^{6}-α-NeuAc-nLcOse$_4$

α-L-Fucp

1

↓

4

4b α-D-NeupAc-(2→3)-β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-D-Glcp

IV^{3}-α-NeuAc- III^{4}-α-Fuc -LcOse$_4$

α-D-NeupAc

2

↓

6

4c α-L-Fucp-(1→2)-β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-D-Glcp

IV^{2}-α-Fuc-III^{6}-α-NeuAc-LcOse$_4$

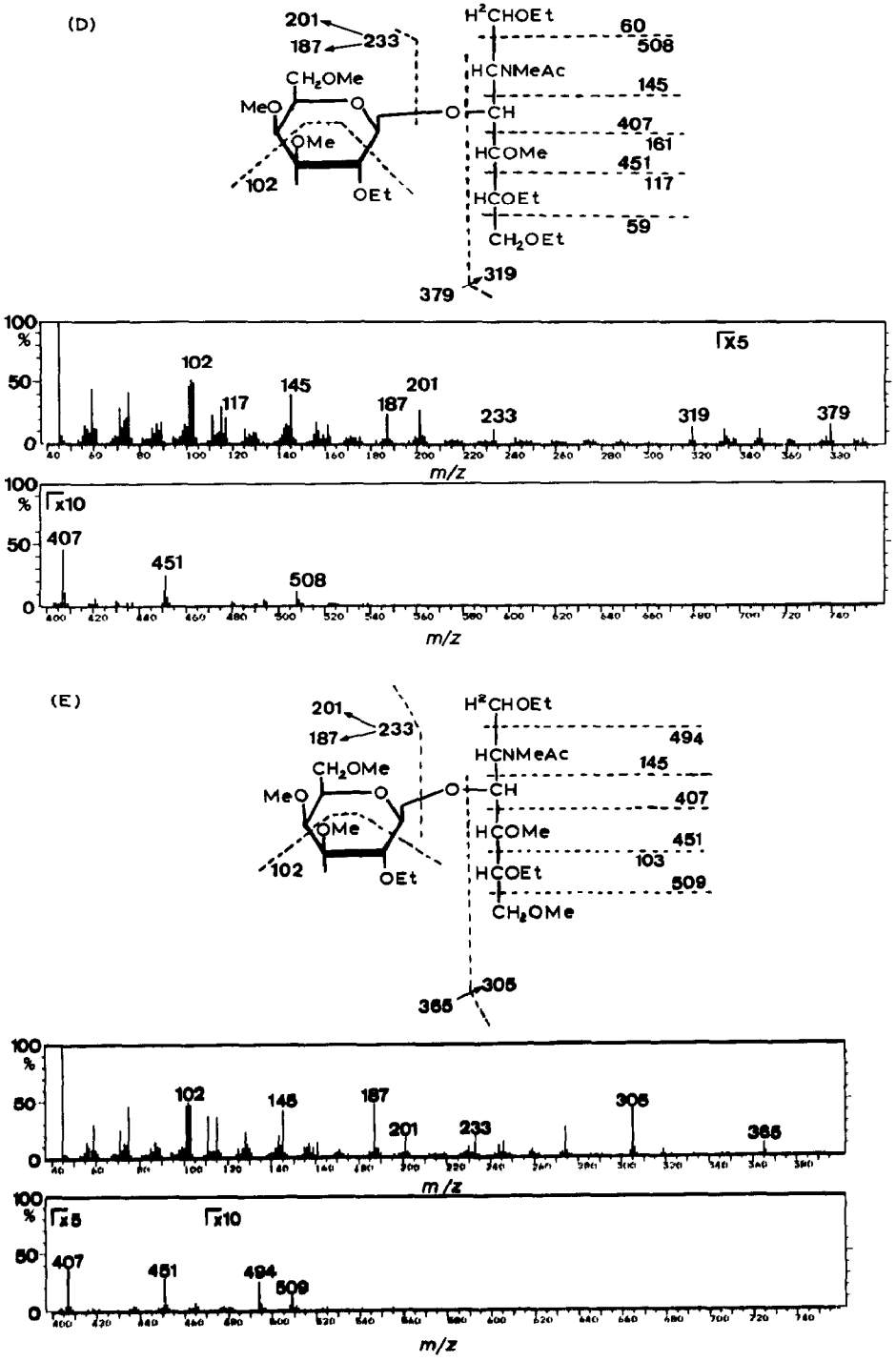

Fig. 7. Mass spectra resulting from the partial formolysis of **4c** (spectrum D) and asialo **4c** (spectrum E).

cently reported[12] is more a quantitative than a qualitative phenomenon. Another explanation might be that the glycosyltransferase activities of mammary gland have, during lactation, the characteristics of those of fetal tissue, mainly due to the high concentration of nucleotide sugars and acceptors.

ACKNOWLEDGMENTS

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NOTE ADDED IN PROOF

After submitting this paper for publication, a report entitled "Isolation of a new sialyloligosaccharide from human milk using anti-oligosaccharide antibodies", by P. A. Prieto and D. F. Smith, was presented at the Annual Meeting of the Society for Complex Carbohydrates (Oct. 9–12, 1983). Their report describes the characterization of IV^2-α-Fuc-III^6-α-NeuAc-$LcOse_4$.

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