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Note

Structure determination of three neutral oligosaccharides obtained from horse colostrum*

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Milk generally contains soluble oligosaccharides that are unique in the lacteal secretions. They have attracted considerable attention in regard to their antigenic properties[1], their enhancement of growth of *Bifidobacterium*[2], and their unique structures. Structural analysis of milk oligosaccharides is of interest for the study of their homology or heterogeneity among animal species, and of their biosynthesic pathway in lacteal cells. Research on the chemical structures of milk oligosaccharides has nevertheless been confined to humans[3,4], cows[5–8], tammar wallabies[9–12], grey kangaroos[9,10], echidnas[13,14], and platypuses[13].

More than 40 human milk oligosaccharides have been isolated and characterized[3,4]. Based on their chemical structures, they are divided into eight series; lactose, fucosyllactose, lacto-*N*-tetraose, lacto-*N*-neotetraose, lactodifucotetraose, lacto-*N*-hexaose, lacto-*N*-neohexaose, and mannooctaose. Bovine colostrum, but not milk, contains eight acidic oligosaccharides (one di-, six tri-, and one tetrasaccharide) having *N*-acetyl- or *N*-glycolyl-neuraminic acid residues[5,6]. Recently, the presence of five neutral oligosaccharides (two di- and three tri-saccharides) other than lactose in bovine colostrum has been elucidated[7,8]. On the other hand, eight neutral oligosaccharides (one tri-, two tetra-, two penta-, two hexa-, and one hepta-saccharide) and three oligosaccharides (fucosyllactose, difucosyllactose, and *N*-acetyl-4-*O*-acetyl-α-neuraminyllactose) other than lactose have been identified in marsupial[9–12] and monotreme[13,14] milk, respectively. These studies indicate both a homology and a heterogeneity in milk oligosaccharides of mammalian species.

Although some oligosaccharides other than lactose were detected in horse

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milk by paper chromatography[15], their chemical structures have not been elucidated. We describe herein the isolation and elucidation of three oligosaccharides other than lactose from the colostrum of three horses of thoroughbred breed, obtained by dialysis, and activated charcoal column and preparative paper chromatography. Five oligosaccharides, named HM-1–5 in order of the R_F values (Fig. 1), were observed by paper chromatography. There were no significant differences in chromatography patterns between individual horse colostra. The relative mobilities suggested that HM-1 and HM-2 were trisaccharides, and HM-3, HM-4, and HM-5 tetra-, penta-, and hexa-saccharide, respectively. The average amounts in defatted milk, proportions, and R_{Lac} values are given in Table I. The chemical structure of three of these oligosaccharides was determined mainly by ^{13}C-n.m.r. spectroscopy and methylation analysis. The ^{13}C-n.m.r. spectra were compared with those of lactose and *N*-acetyllactosamine (Table II).

The partially methylated alditol acetate derivatives obtained from HM-1 and HM-2 were identified by g.l.c.–m.s. as shown in Table III. These data indicated for HM-1 and HM-2 the sequences, Gal-(1→3)-Gal-(1→4)-Glc and Gal-(1→6)-Gal-(1→4)-Glc, respectively. The unequivocal structures of HM-1 and HM-2 were determined by comparison of their ^{13}C-n.m.r. spectra with those of 3′- and 6′-galactosyllactose obtained from bovine colostrum[8]. The chemical shifts and numbering of each sugar unit are given in Table II. The resonance at δ 84.54 of HM-1 was assigned to the linkage at O-3′ of the internal Gal unit, and that at δ 106.94 to the anomeric carbon atom of the nonreducing β-D-Gal-(1→3) group. The resonances at δ 71.67 and 106.01 of HM-2 were assigned to the linkage at O-6 of the internal Gal unit and the anomeric carbon atom of the nonreducing β-D-Gal-(1→6) group, respectively. The signal for C-6′ was shifted downfield (by 8 p.p.m.) from that of the nonlinked C-6″ of the Gal group. From these data, structure **1** was assigned to HM-1 and structure **2** to HM-2.

In order to determine the molecular weight and carbohydrate sequence of HM-4, it was subjected to secondary-ion m.s. (see Fig. 2). The strong peaks at *m/z* 870 (M + 1) and 892 (M + Na) indicated a pentasaccharide containing one *N*-

TABLE I

YIELD, RATIO, R_{Lac} VALUES, AND REDUCING-END ANALYSIS OF THE OLIGOSACCHARIDES OBTAINED FROM HORSE COLOSTRUM

Oligo-saccharide	*Yield*[a] *(mg/L)*	*Ratio*[a] *(w/w, %)*	R_{Lac}	*Sugar residue in the reducting position*
HM-1	7.8	54	0.62	Glc
HM-2	4.8	33	0.52	Glc
HM-3	0.3	2	0.28	Glc
HM-4	1.1	8	0.18	Glc
HM-5	0.4	3	0.10	Glc

[a]Yields and ratios are mean values for the three horses.

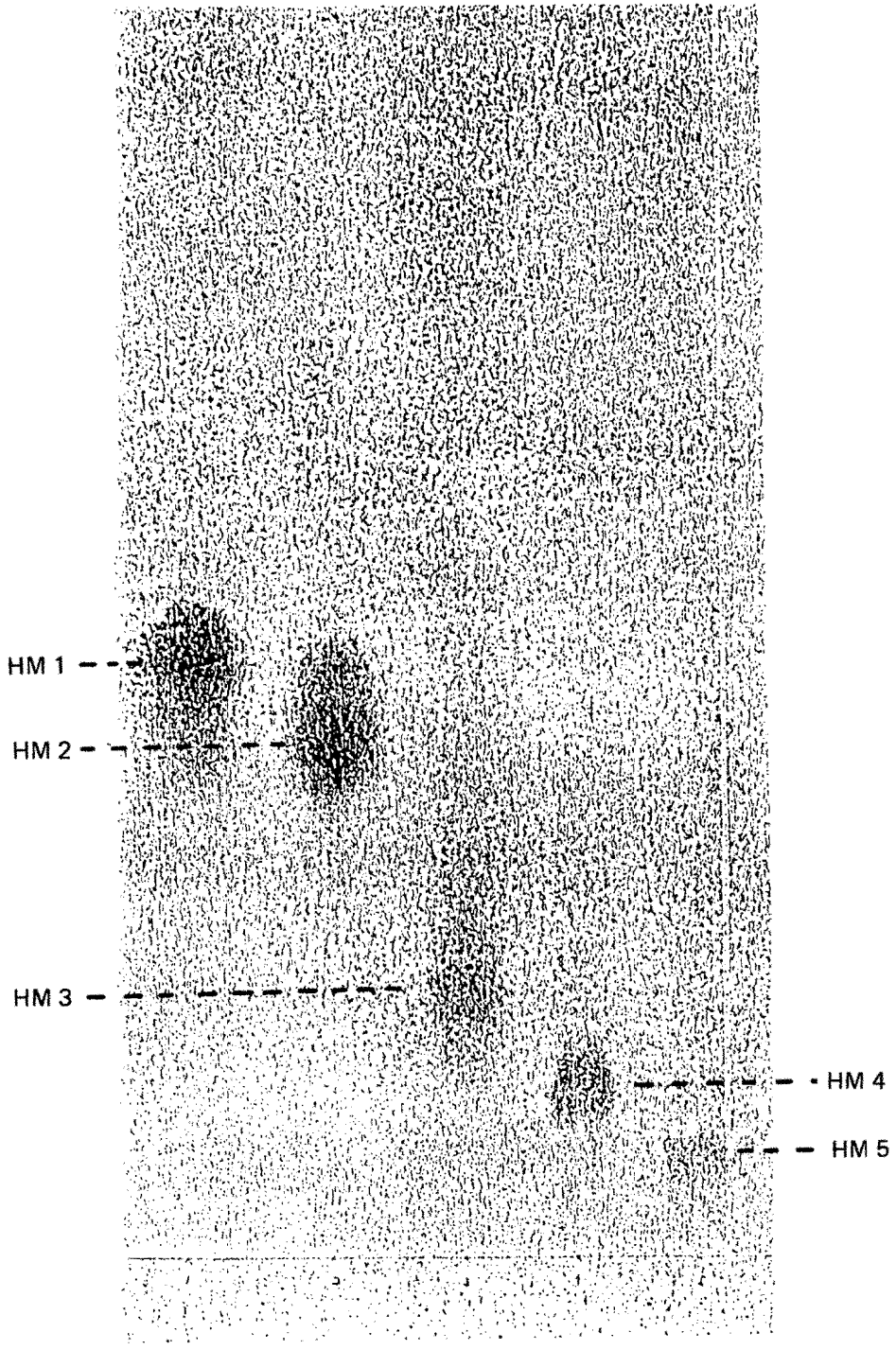


Fig. 1. Paper chromatogram of the neutral oligosaccharides isolated from horse colostrum.

TABLE II

^{13}C-CHEMICAL SHIFTS, OF OLIGOSACCHARIDES HM-1, HM-2, AND HM-4[a]

Atom	*HM-1*		*HM-2*		*HM-4*	
	α-D	β-D	α-D	β-D	α-D	β-D
D-*Glc*[1] *unit*						
C-1	94.44	98.46	94.98	98.34	94.54	98.40
C-2	74.06	76.52	73.71	76.46	73.92	76.70
C-3	74.19	77.05	74.30	77.22	74.09	77.11
C-4	80.91	80.91	81.90	82.14	81.12	81.12
C-5	72.89	77.46	72.68	77.34	72.72	77.11
C-6	62.7	62.7	62.7	62.7	62.72	62.68
β-D-*Gal*p[2] *unit*						
C-1	105.25		105.77		105.30	
C-2	72.89		73.48		72.72	
C-3	84.54		75.12		84.21	
C-4	71.08		71.14		71.20	
C-5	77.69		76.64		76.17	
C-6	63.65		71.67		71.20	
β-D-*Glc*p*NAc*[3] *unit*						
C-1	106.94		106.01		103.61	
C-2	73.71		73.48		57.68	
C-3	75.18		75.29		76.17	
C-4	71.26		71.31		81.50	
C-5	77.69		77.75		77.40	
C-6	63.65		63.65		63.65	
CH_3					25.04	
C–O					176.63	
β-D-*Gal*p[4] *unit*						
C-1					105.54	
C-2					73.66	
C-3					75.18	
C-4					71.20	
C-5					77.98	
C-6					63.65	
β-D-*Gal*p[5] *unit*						
C-1					106.94	
C-2					73.66	
C-3					75.18	
C-4					71.20	
C-5					77.75	
C-6					63.65	

[a] δ Values downfield from the signal of internal sodium 4,4-dimethyl-4-sila(2,3-$^2H_4$)pentanoate.

TABLE III

PARTIALLY METHYLATED ALDITOL ACETATES PREPARED FROM OLIGOSACCHARIDES HM-1, HM-2, AND HM-4

Partially methylated alditol acetate	*HM-1*	*HM-2*	*HM-4*
4-*O*-Acetyl-1,2,3,5,6-penta-*O*-methylglucitol	1	1	1
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol	1	1	2
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol	1		
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol		1	
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methylgalactitol			1
1,4,5-Tri-*O*-acetyl-3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol			1

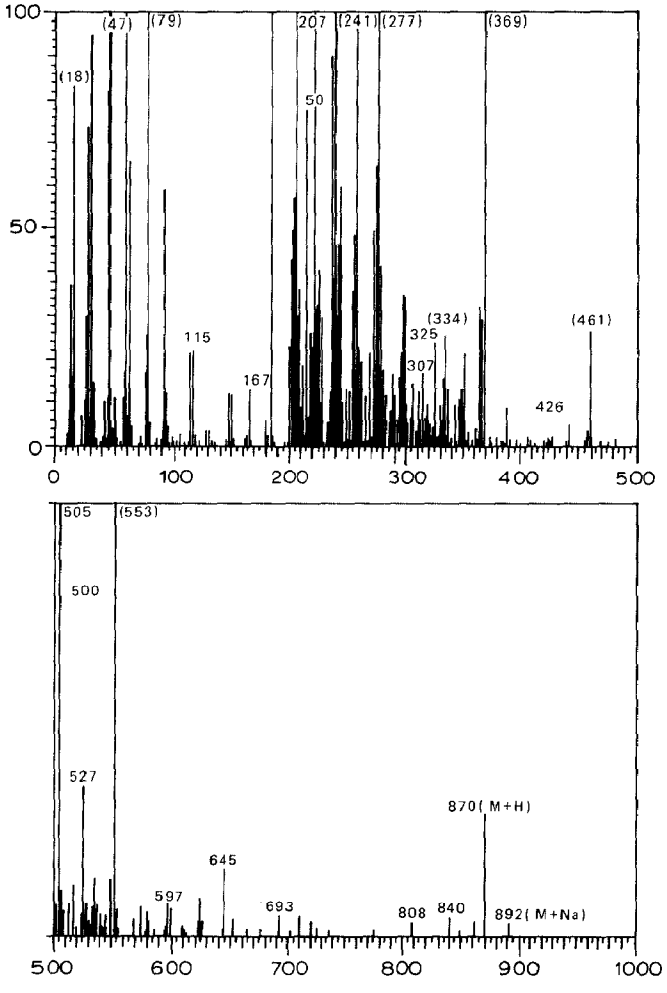
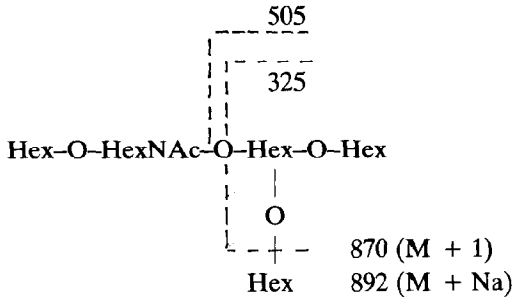


Fig. 2. Secondary-ion mass spectrum of HM-4.

acetylhexosamine unit and four aldohexose units. The characteristic fragment at *m/z* 325 denoted a Hex→Hex sequence, and the ions at *m/z* 505 and 527 a Hex→Hex→Hex sequence (see Scheme 1). These attributions were supported by the observation that a partial hydrolyzate of HM-4 yielded spots, on the paper chromatogram, having R_F values identical to those of lactose and HM-1. The data indicate that HM-4 contains a 3′-GL unit.



Scheme 1.

The alditol acetate derivatives obtained from the permethylated alditol of HM-4 were identified by g.l.c.–m.s. as shown in Table III. The existence of di-substituted (at O-3 and O-6) galactitol derivatives suggest for HM-4 a branching structure with the sequence Gal-(1→4)-GlcNAc-(1→6)-[Gal-(1→3)]-Gal-(1→4)-Glc. The unequivocal structure of HM-4 was elucidated by comparing the ^{13}C-n.m.r. spectrum of HM-1 (**1**) with that of lacto-*N*-novopentaose obtained from marsupial milk[12]. The resonances at δ 106.94 and 105.30, which were assigned to C-1^5 of the β-D-galactosyl group linked (1→3), and to C-1^2 of the β-D-galactosyl residue linked (1→4) to the reducing D-glucose residue, respectively, showed the presence of a lactosyl unit and a nonreducing β-D-galactosyl group linked (1→3). The signal at δ 84.21 is thought to correspond to the linkage at O-3^2 of the galactosyl residue of the lactose unit, indicating the existence of unit **1** in HM-4. The signal at δ 81.50 was assigned to the linkage at O-4 of the 2-acetamido-2-deoxyglucosyl residue. The resonance at δ 105.54, which was assigned to C-1^4 of the β-D-galactosyl group linked to the 2-acetamido-2-deoxyglucosyl residue, corresponds to the C-1′ signal in free *N*-acetyllactosamine[7]. Because of the resonance at δ 103.61, the 2-acetamido-2-deoxyglucosyl residue is thought to be linked *via* a β-D-linkage. The signal lies slightly upfield from the signal of the corresponding carbon atom (δ 104.4) of β-D-Gal*p*-(1→4)-D-Glc*p*NAc-(1→6)-[β-D-Gal*p*-(1→3)]-D-GalNAcol, which has been isolated from bovine κ-casein[16]. A small upfield shift (by 1.5 p.p.m.) of the C-5^2 signal, from δ 77.69 for **1** to 76.17 for HM-4, is due to the linkage between O-6^2 and C-1^3 of the 2-acetamido-2-deoxyglucosyl residue. Thus, the chemical shift data showed that HM-4 has the structure of **1** with O-6^2 of the D-Gal^2 residue substituted with an *N*-acetyl-β-lactosamine group (**3**).

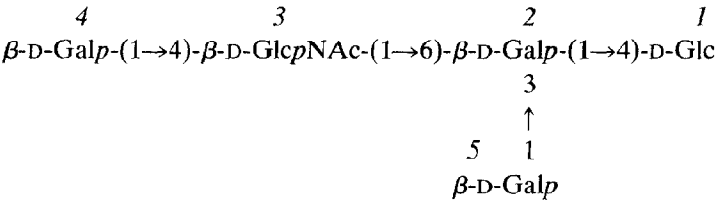
Oligosaccharide **1** is a major carbohydrate component of marsupial milk: the concentration in the original milk is ~16 g/L (ref. 9). Human milk contains[7] a small quantity of **2**. The dual presence of both 3′- and 6′-isomers of galactosyllactose was reported for bovine colostrum[8]. HM-4 (**3**) has been identified in marsupial milk and named lacto-*N*-novopentaose[12], but it has not been identified previously in either human or cow milk.

β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc

1

β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc

2



3

EXPERIMENTAL

Methods. — ^{13}C-N.m.r. spectra were recorded with a Jeol-FX-100 spectrometer operating at 25 MHz with 3-(trimethylsilyl) propionic acid-d_4 sodium salt as an internal standard, mass spectra with a Jeol-DX-300 mass spectrometer (ionization current 100 μA, and voltage 70 eV), and secondary ion-mass spectra with a Hitachi 80 B mass spectrometer. G.l.c. was performed on a Hitachi model 163 gas chromatograph, equipped with a flame-ionization detector and a glass column (0.2 × 200 cm) packed with 2% OV-17 or 3% SE-30 Chromosolv W, and operated[16] at a temperature gradient of 3° min^{-1} from 150 to 250°.

Materials. — Colostrum, collected from three thoroughbred horses (A, 1.4 L; B, 2.5 L; C, 2 L) within 10 h after parturition, was defatted by centrifugation (5000 r.p.m. for 10 min) an stored at −30°. Milk sugars were prepared by dialysis from defatted colostrum after thawing. Lactose was removed by crystallization by dissolving the lyophilized dialyzate in 50% ethanol solution and keeping the solution for 2 days at 4°. After filtration, the solution was passed through a column (2 × 25 cm) of Dowex 1-X2 (200–400 mesh, Cl^-) ion-exchange resin and fractionated by chromatography in a column (7 × 50 cm) of activated charcoal and stepwise elution with 5, 15, 30, and 50% (v/v) ethanol. The 30% ethanol fraction was further

purified by preparative paper chromatography in an ascending system of 6:4:3 (v/v) butanol–pyridine–water with five developments. The sugars were located on the paper chromatograms with alkaline $AgNO_3$ reagent. HM-1 and HM-2 were further purified by a second paper chromatography with the same solvent. The oligosaccharides were finally purified by passage through columns (0.5 × 3 cm) of AG 50W-X8 (200–400 mesh, H^+) and AG 3-X4A (200–400 mesh, OH^-) ion-exchange resins.

Sugar analysis. — Reduction of oligosaccharides was performed in M $NaBD_4$ (Merck, F.R.G.) solution at 4° for 12 h in the dark. Methanolysis was performed in 0.5M HCl in methanol at 80° for 24 h, followed by per(trimethyl)silylation and g.l.c. analysis. Partial hydrolysis was performed by heating for 3 h with 0.2M trifluoroacetic acid at 100°. Oligosaccharide alcohols were methylated by the method of Hakomori[18]. Alditol acetate derivatives of partially methylated sugars, prepared from permethylated oligosaccharidritols by the method of Stellner *et al.*[19], were identified by g.l.c.–m.s.

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REFERENCES

1 J. R. E. Hoover, G. A. Brown, and P. György, *Arch. Biochem. Biophys.*, 47 (1953) 216–217.
2 P. György, R. F. Norris, and C. S. Rose, *Arch. Biochem. Biophys.*, 48 (1954) 193–201.
3 A. Kobata, in M. I. Horowitz and W. Pigman (Ed.), *Glycoconjugates*, Vol. I, Academic Press, New York, 1977, pp. 423–440.
4 A. Kobata, K. Yamashita, and Y. Tachibana, *Methods Enzymol.*, 50 (1978) 216–220.
5 R. Kuhn and R. Gauhe, *Chem. Ber.*, 98 (1965) 395–413.
6 R. Kuhn and R. Brossmer, *Chem. Ber.*, 89 (1956) 2013–2025.
7 T. Saito, T. Ito, and S. Adachi, *Biochim. Biophys. Acta*, 801 (1984) 147–150.
8 T. Saito, T. Ito, and S. Adachi, *Carbohydr. Res.*, 165 (1987) 43–51.
9 M. Messer, E. Trifonoff, W. Stern, J. G. Collins, and J. H. Bradbury, *Carbohydr. Res.*, 83 (1980) 327–334.
10 J. G. Collins, J. H. Bradbury, E. Trifonoff, and M. Messer, *Carbohydr. Res.*, 92 (1981) 136–140.
11 M. Messer, E. Trifonoff, J. G. Collins, and J. H. Bradbury, *Carbohydr. Res.*, 102 (1982) 316–320.
12 J. H. Bradbury, J. G. Collins, G. A. Jenkins, E. Trifonoff, and M. Messer, *Carbohydr. Res.*, 122 (1983) 327–331.
13 G. A. Jenkins, H. Bradbury, M. Messer, and E. Trifonoff, *Carbohydr. Res.*, 126 (1984) 157–161.
14 J. P. Kamerling, L. Dorland, H. van Halbeek, J. F. G. Vliegenthart, M. Messer, and R. Schauer, *Carbohydr. Res.*, 100 (1982) 331–340.
15 R. Jennes, E. A. Regehr, and R. E. Sloan, *Comp. Biochem. Physiol.*, 13 (1964) 339–352.
16 T. Saito, T. Ito, S. Adachi, T. Suzuki, and T. Usui, *Biochim. Biophys. Acta*, 678 (1981) 257–267.
17 K. Yamashita and A. Kobata, *Arch. Biochem. Biophys.*, 161 (1974) 164–170.
18 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
19 K. Stellner, H. Saito, and S. Hakomori, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.