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Carbohydrate Research 262 (1994) 173–184

CARBOHYDRATE RESEARCH

Studies of the neutral trisaccharides of goat (*Capra hircus*) colostrum and of the one- and two-dimensional ^{1}H and ^{13}C NMR spectra of 6′-*N*-acetylglucosaminyllactose

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Received 18 October 1993; accepted 29 March 1994

Abstract

Four neutral trisaccharides were isolated from goat colostrum by dialysis, and ion-exchange, activated charcoal column, preparative paper, and Bio-Gel P-4 column chromatography. The following structures were elucidated by GC analysis of hydrolysis products and by 400-MHz ^{1}H NMR spectroscopy: α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 4)-D-Glc, α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc, β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc, and β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc. β-D-Glc*p*NAc-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc, previously reported to be present in goat milk, was not detected in this study. β-D-Glc*p*NAc-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc (6′-*N*-acetylglucosaminyllactose) was prepared by β-D-galactosidase digestion of β-D-Gal*p*-(1 → 3)[β-D-Glc*p*NAc-(1 → 6)]-β-D-Gal*p*-(1 → 4)-D-Glc (lacto-*N*-novotetraose) and characterized by one- and two-dimensional NMR spectroscopy at 600 MHz.

Key words: Goat colostrum; Oligosaccharides; Chemical structures; 6′-*N*-Acetylglucosaminyllactose; NMR

1. Introduction

Most milk oligosaccharides contain a lactose unit, which serves as a template from which larger structures are built by the addition of D-galactose, 2-acetamido-

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SSDI 0008-6215(94)00127-2

2-deoxy-D-glucose (*N*-acetylglucosamine), L-fucose, and/or sialic acid. These oligosaccharides are an important source of substrates for studies of glycosidases and glycosyltransferases [1,2].

The chemical structures of oligosaccharides isolated from the milk of humans [1,2], cows [3–7], sheep [8], horses [9,10], tammar wallabies [11–14], grey kangaroos [11,12], echidnas [15,16], platypuses [16], and goats [17,18] have been studied.

Chaturvedi and Sharma [17,18] have isolated five neutral oligosaccharides, other than lactose, from goat milk. In this report, we describe the structural determination of four trisaccharides isolated from goat colostrum. The trisaccharide β-D-Glc*p*NAc-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc (6′-*N*-acetylglucosaminyllactose) reported by Chaturvedi and Sharma [17], was not found in this study and we therefore prepared a sample, by enzymatic degradation, and characterized its structure by one- and two-dimensional NMR spectroscopy.

2. Results and discussion

The oligosaccharides were obtained from goat colostrum by dialysis followed by ion-exchange, activated charcoal, preparative paper, and Bio-Gel P-4 column chromatography. Fig. 1 shows the separation achieved after the initial preparative paper chromatography (see Experimental). Each fraction was further purified prior to characterization. Four trisaccharides named GM-1–GM-4 and two spots representing higher oligosaccharides were detected by paper chromatography (Fig. 1). The yields from 4 L of colostrum were: GM-1 (3.8 mg), GM-2 (6.4 mg), GM-3 (145 mg), and GM-4 (194 mg). Hydrolysis of GM-1 yielded one mole each of D-glucose, D-galactose, and L-fucose per mole. Hydrolysis of GM-2, GM-3, and GM-4 gave one mole of D-glucose and two of D-galactose per mole in each case. The data therefore showed the monosaccharide compositions to be (Glc) (Gal) (Fuc) for GM-1, and (Glc) $(Gal)_2$ for GM-2, GM-3, and GM-4. Preliminary studies suggest that the spots representing higher oligosaccharides contain several compounds; these have not been further investigated.

The chemical structures were determined by ^1H NMR spectroscopy (Table 1). The spectra of GM-1, GM-2, and GM-4, shown in Fig. 2, are in agreement with the structures of trisaccharides previously isolated from the milk of other species; these structures had originally been determined by ^{13}C NMR and methylation analysis [8,9,11,16]. The ^1H NMR spectrum of GM-3 agreed with the previously published data [19]. GM-1, α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 4)-D-Glc, has been found in human [20] and echidna [16] milk. GM-2, α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc, has previously been isolated from cow [7] and sheep [8] colostra. GM-3, β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc, has been detected in marsupial [11] and human [21] milk, and in cow [6], sheep [8], and horse [9] colostra. GM-3 has been found together with GM-4, β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc, in human milk [21,22] and in cow [6], sheep [8], and horse [9] colostra.

Chaturvedi and Sharma [17,18] reported the isolation of a trisaccharide, a tetrasaccharide, and three pentasaccharides from goat milk. These authors did not

detect any of the trisaccharides reported here, whereas the structure β-D-Glc*p*NAc-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc, which was attributed to their trisaccharide, was not found in the current work. Chaturvedi and Sharma only examined the dominant fraction from their preliminary separation and, as noted above, we did not examine the higher oligosaccharide fractions in this work.

The 6′-*N*-acetylglucosaminyllactose unit has been found in some milk oligosaccharides, including lacto-*N*-novotetraose [13], lacto-*N*-hexaose, and related compounds [1]. The free 6′-*N*-acetylglucosaminyllactose, named lacto-*N*-novotriose, has been synthesized [23], but no NMR spectroscopic data were reported for the final product.

In this work, lacto-*N*-novotriose was prepared by exo-β-D-galactosidase digestion of lacto-*N*-novotetraose. Its 400-MHz ^{1}H NMR spectrum (not shown) was found to be distinctly different from that previously reported at the same field strength and temperature [17]. In particular, all of the anomeric proton resonances

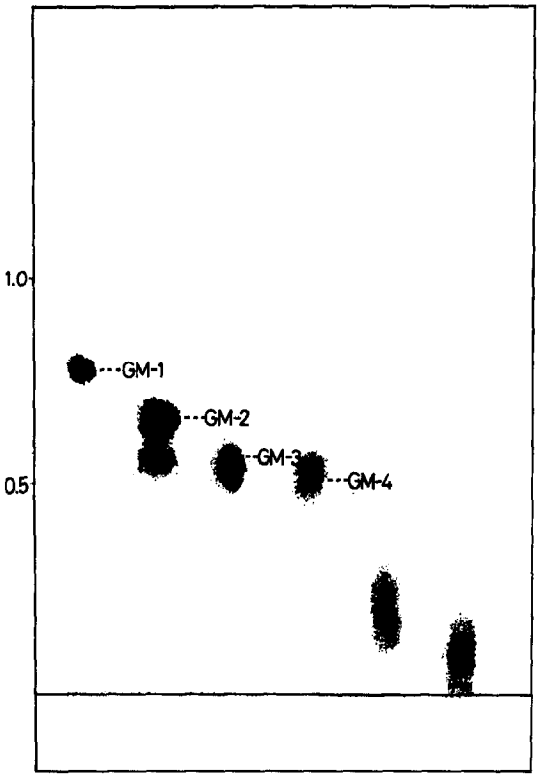

Fig. 1. Paper chromatogram following the initial fractionation of oligosaccharides obtained from goat colostrum. The saccharides were located with alkaline $AgNO_3$ reagent and the scale represents migration distance relative to lactose at 1.0. Other details of the separation are provided in the Experimental section. The trisaccharides in lanes 1–4 were further purified prior to characterization. The spots representing higher oligosaccharides (lanes 5 and 6) were not further investigated in this work.

were clearly resolved, compared with the earlier report where it was concluded that the β-Glc H-1 signal was coincident with that of β-GlcNAc. Because of these differences, we sought to confirm the structure of our digestion product by ^{1}H and ^{13}C NMR spectroscopy. This was undertaken at a proton frequency of 600 MHz, where there is significantly better dispersion within the envelope of non-anomeric protons.

The anomeric proton resonances in the ^{1}H NMR spectrum of the β-D-galactosidase digestion product could be assigned by comparison with the spectra of lactose and lacto-*N*-novotetraose (Fig. 3 and Table 2). The ^{1}H NMR signal at δ 4.611, assigned to H-1 of Gal″ β3 in lacto-*N*-novotetraose, is absent in the digestion product, consistent with the removal of the terminal β-(1 → 3)-galactosyl group. Moreover, the shift in the H-1 signal for Gal′β4 from δ 4.452 in lactose to δ 4.441 in the product is similar to that observed previously [19,24] upon introduction of GlcNAc to Gal′β4 of β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc. Two doublets at δ 4.618 and 4.613 were shown by integration to be equivalent to a single proton and were assigned to H-1 of GlcNAc, there being slightly different chemical shifts for each anomer of the trisaccharide. The GlcNAc H-1 protons are similarly resolved in lacto-*N*-novotetraose [19,24]. Interestingly, the *N*-acetyl resonances in the trisaccharide studied here are also resolved for each anomer (δ 2.060 and 2.062).

Most of the remainder of the ^{1}H NMR spectrum was assigned from a double-quantum filtered correlation (DQF-COSY) experiment [25], with one-dimensional correlation experiments (1D TOCSY) [26], initiated by a $\pi/2$ Gaussian pulse [27], being used to determine approximate chemical shifts for strongly coupled resonances. Practical considerations for the use of 1D TOCSY to facilitate the assignment of ^{1}H spectra of oligosaccharides have been published [28]. The assignments are further clarified with the additional dispersion provided by the ^{13}C

Table 1
^{1}H Chemical shifts of the oligosaccharides GM-1–GM-4 [a] obtained from goat colostrum

Reporter group	Residue	Chemical shifts, δ (coupling constants, Hz)			
		GM-1	GM-2	GM-3	GM-4
H-1	β-Gal′(1 → 4)	4.527 (7.7)	4.524 (7.7)	4.511 (7.7)	4.461 (8.1)
	α-Fuc″(1 → 2)	5.315			
	α-Gal″(1 → 3)		5.145 (4.0)		
	β-Gal″(1 → 3)			4.614 (7.7)	
	β-Gal″(1 → 6)				4.483 (9.5)
	α-Glc	5.226 (3.7)	5.225 (4.0)	5.224 (3.7)	5.222 (3.7)
	β-Glc	4.638 (7.7)	4.668 (8.1)	4.666 (8.1)	4.668 (8.1)
H-4	β-Gal′(1 → 4)			4.197 (3.3)	
CH_3	α-Fuc″(1 → 2)	1.226 (6.6)			

[a] GM-1: α-D-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 4)-D-Glc.
GM-2: α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc.
GM-3: β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc.
GM-4: β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc.

dimension in a (^{1}H, ^{13}C) shift correlation experiment.

^{13}C NMR data for the β-D-galactosidase digestion product are presented in Fig. 4 and Table 3. The CH_3 and CO resonances of the *N*-acetyl group are unequivocally assigned from the one-dimensional spectrum. The remaining ^{13}C assignments were obtained from a ^{1}H-detected (^{1}H,^{13}C) shift correlation experiment (Figs. 5 and 6a) [29] and are consistent with data for lactose [6] and β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc (6′-galactosyllactose) [8], as reported in Table 3. The interglycosidic linkages are identified from a (^{1}H,^{13}C) correlation experiment, optimized for long-range couplings (Fig. 6b) [30].

Correlations to the anomeric protons, whose signals are close to the relatively

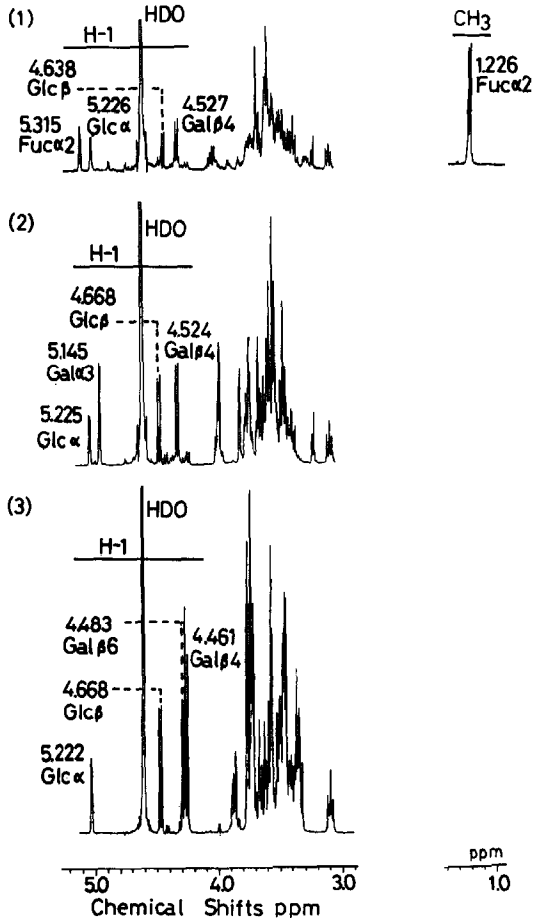

Fig. 2. ^{1}H NMR spectra of the oligosaccharides (1) GM-1, (2) GM-2, and (3) GM-4 isolated from goat colostrum. The ^{1}H NMR spectra of the saccharides were obtained in D_2O at 400 MHz with a Jeol JNM-GSX-400 NMR spectrometer operated at a probe temperature of 300 K. The ^{1}H NMR spectrum of GM-3 has been published elsewhere [19].

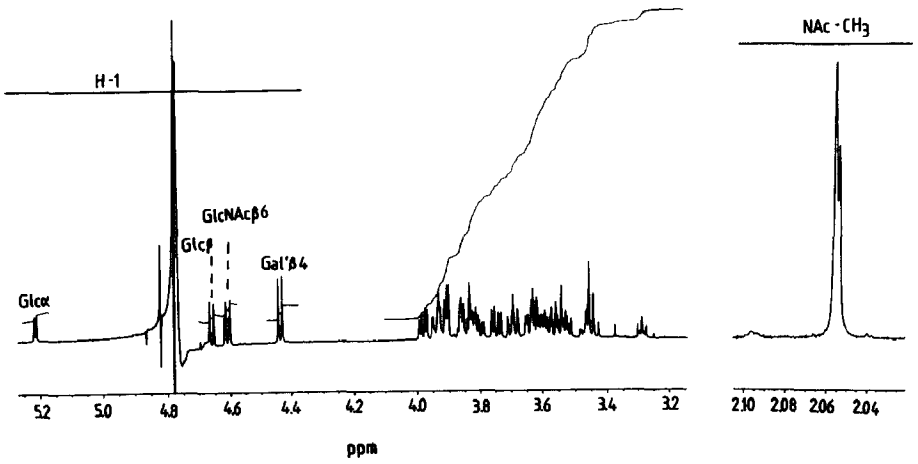

Fig. 3. 600-MHz ^{1}H NMR spectrum of 6′-*N*-acetylglucosaminyllactose (lacto-*N*-novotriose) obtained from lacto-*N*-novotetraose by digestion with β-D-galactosidase, *Aspergillus oryzae*. For details, see the Experimental section.

intense HOD signal, were significantly enhanced by modification of the presaturation procedure in the standard pulse program. On our spectrometer, presaturation with a single low-power pulse produces sufficient t_1 noise to obscure C–H correlations close to the solvent resonance [31,32]. This noise is significantly reduced by presaturation with a sequence of square pulses of shorter duration, controlled by the pulse shaping routine in the software.

Table 2
Identification of oligosaccharides [a] by ^{1}H NMR data

Reporter group	Residue	Anomer of compound	Chemical shifts, δ (coupling constants, Hz)		
			Lactose [b]	Lacto-*N*-novotriose	Lacto-*N*-novotetraose [b]
H-1	β-Gal′(1 → 4)		4.452 (7.8)	4.441 (7.8)	4.498 (7.8)
	β-Gal″(1 → 3)				4.611 (7.4)
	β-GlcNAc″(1 → 6)	α		4.618 (8.4)	4.626 (8.5)
		β		4.613 (8.5)	4.621 (8.5)
	Glc	α	5.223 (3.8)	5.222 (3.8)	5.223 (3.9)
		β	4.665 (8.0)	4.667 (8.0)	4.669 (8.0)
H-4	β-Gal′(1 → 4)				4.178 (3.3)
NAc		α		2.060	2.066
		β		2.062	2.066

[a] Lactose [β-D-Gal*p*-(1 → 4)-D-Glc], lacto-*N*-novotetraose {β-D-Gal*p*-(1 → 3)[β-D-Glc*p*NAc-(1 → 6)]-β-D-Gal*p*-(1 → 4)-D-Glc}, and lacto-*N*-novotriose [β-D-Glc*p*NAc-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc] obtained from lacto-*N*-novotetraose by digestion with β-D-galactosidase (*Aspergillus oryzae*).
[b] Data from ref 24.

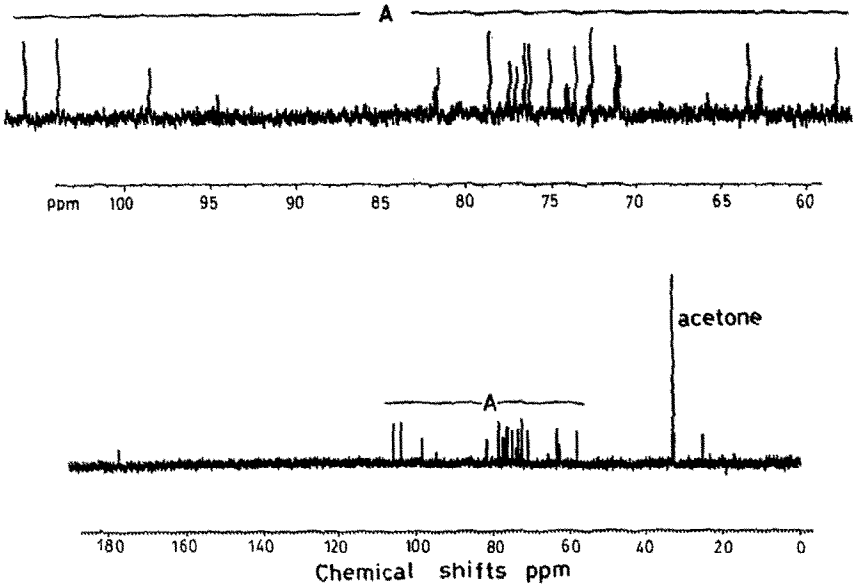

Fig. 4. 150-MHz ^{13}C NMR spectrum of lacto-*N*-novotriose. For details, see the Experimental section.

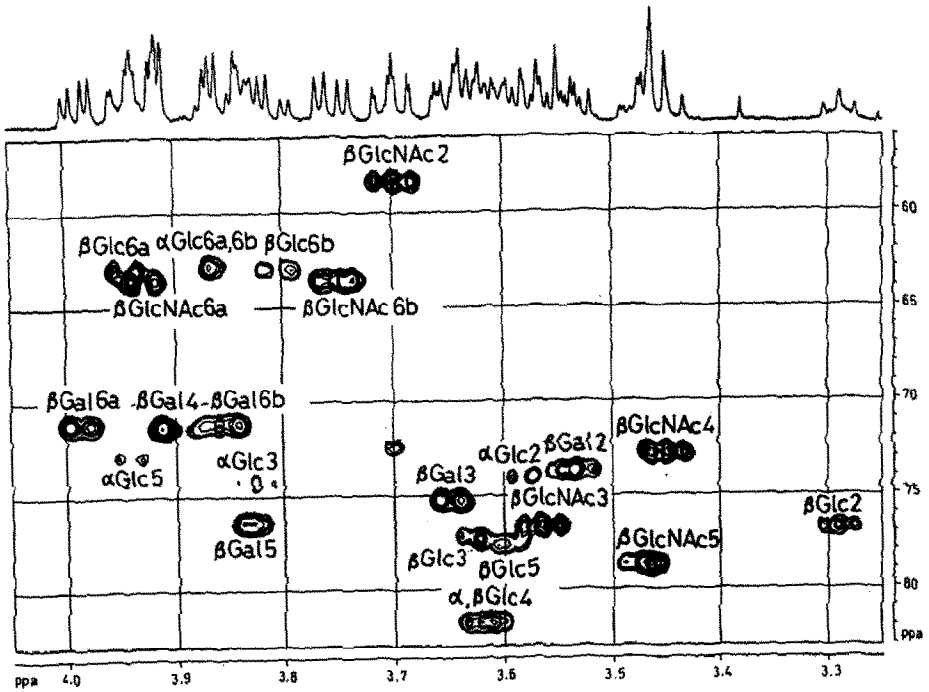

Fig. 5. Part of the phase-sensitive (TPPI) $^{1}H-^{13}C$ correlation spectrum of lacto-*N*-novotriose. The total measuring time was 16.5 h. The corresponding section of the ^{1}H NMR spectrum obtained from single pulse acquisition is shown at the top of the plot. Assignments for the trisaccharide are indicated next to the relevant contours; the peak at (δ 3.7, δ 72.4) is assumed to be due to a contaminant.

The β-D-Glc*p*NAc-(1 → 6)-D-Gal*p* linkage is established from the long-range correlation between GlcNAc H-1 and Gal C-6 and from the 8 ppm shift to high frequency for C-6 of lactose [33]. The linkage between Gal H-1 and Glc C-4 is also clearly identified in Fig. 6b and supported by comparison with chemical shifts of C-4 for the Gal residue in related compounds (Table 3). Correlations (not shown) supportive of these assignments are obtained between Gal H-6a,6b and GlcNAc C-1, and also between Glc H-4 and Gal C-1.

In summary, two-dimensional NMR methods can be employed for full characterization of relatively small amounts of oligosaccharides. The structure of the trisaccharide obtained from β-D-galactosidase digestion of lacto-*N*-novotetraose has been unequivocally established as β-D-Glc*p*NAc-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc. As the ^{1}H NMR spectrum of this compound is significantly different from that of a compound obtained from goat milk and previously assigned the same structure by one-dimensional NMR techniques [17], we conclude that the occurrence of free lacto-*N*-novotriose in goat milk has not yet been demonstrated. Four other trisaccharides were, however, isolated from goat milk and their structures confirmed by comparison of their ^{1}H NMR spectra with those of the authentic samples.

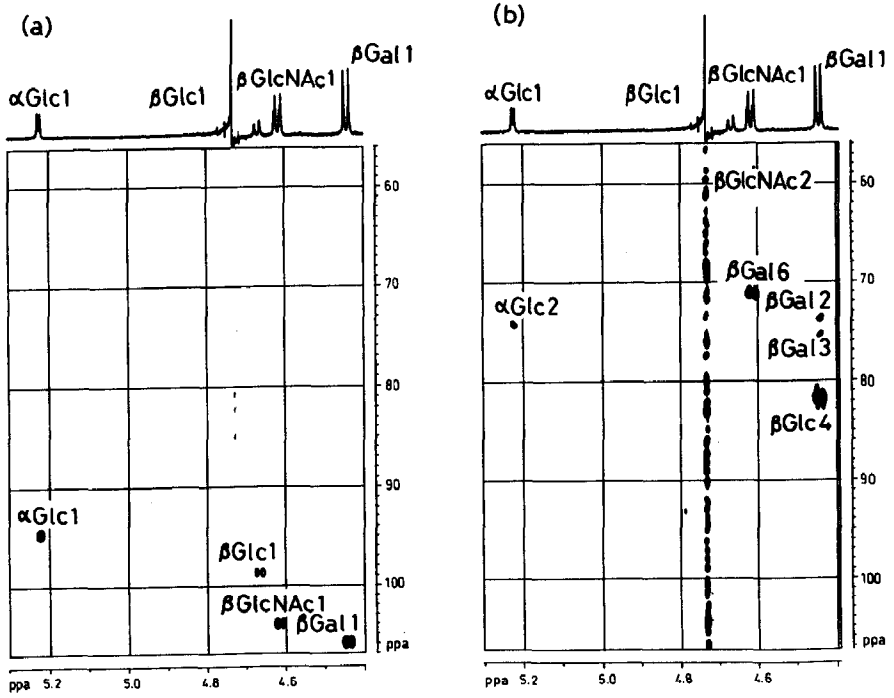

Fig. 6. ^{1}H–^{13}C correlations of anomeric protons for lacto-*N*-novotriose. The appropriate part of the 1D ^{1}H NMR spectrum, with proton assignments, is shown at the top of each plot. Correlated carbons are indicated next to the relevant contours. (a) Part of the one-bond correlation spectrum shown in Fig. 5. (b) Absolute value display of the same region for a heteronuclear multiple bond experiment, for which the total measuring time was 33.7 h.

Table 3
Identification of oligosaccharides by ^{13}C NMR data

Reporter group	Chemical shifts (ppm)						
	Lactose [a] [β-D-Gal*p*-(1 → 4)-D-Glc] II I		6′-Galactosyllactose [b] [β-D-Gal*p*-(1 → 6)-β-D-Gal*p*- III II (1 → 4)-D-Glc] I		Lacto-*N*-novotriose [β-D-Glc*p*NAc-(1 → 6)-β-D-Gal*p*- III II (1 → 4)-D-Glc] I		
C-1	α 94.7	β 98.6	α 94.98	β 98.34	α 94.492	β 98.416	I
C-2	α 74.0	β 76.7	α 73.71	β 76.46	α 73.910	β 76.563	
C-3	α 74.3	β 77.2	α 74.30	β 77.22	α 74.115	β 77.049	
C-4	α 81.3	β 81.4	α 81.90	β 82.14	α 81.512	β 81.651	
C-5	α 73.0	β 77.7	α 72.68	β 77.34	α 72.699	β 77.408	
C-6	α 63.9	β 63.9	α 62.70	β 62.70	α 62.566	β 62.749	
C-1	105.8		105.77		105.697		II
C-2	73.8		73.48		73.515		
C-3	75.4		75.12		75.059		
C-4	71.4		71.14		70.940		
C-5	78.2		76.64		76.492		
C-6	63.0		71.67		71.136		
C-1			106.01		103.752		III
C-2			73.48		58.196		
C-3			75.29		76.231		
C-4			71.31		72.564		
C-5			77.75		78.544		
C-6			63.65		63.382		
CH_3					25.019		
CO					177.356		

[a] Data from Saito et al. [6].
[b] Data from Urashima et al. [8].

3. Experimental

Materials.—Colostrum (4 L) was taken within 2 days after parturition from goats (Japanese Saanen) bred at a private goat farm in Obihiro City. Standard samples of β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc, β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc, and α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc were obtained from sheep and horse colostrum and tammar wallaby milk, respectively, as previously described [7–11].

α-L-Fuc*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc was purchased from Sigma, St. Louis, MO, USA. L-Fucose was from Kishida Chemical Co, Osaka, Japan.

Lacto-*N*-novotetraose was synthesized from β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc and UDP-GlcNAc, using the *N*-acetylglucosaminyltransferase activity of a homogenate of lactating tammar wallaby mammary gland [24].

Preparation of neutral oligosaccharides from goat colostrum.—The extraction of the carbohydrates from colostrum was performed as previously described [7–11]. The defatted colostrum was dialysed against distilled water (10 L), using dialysis tubing (Viskose Sales Corp, Chicago, USA) of 43 mm width, 0.0203 mm thickness, with a molecular weight cut-off of ca. 12000. The dialysates were concentrated to 1 L by rotary evaporation, and lactose was then crystallized by adding an equal volume of ethanol and keeping the solution for 1 day at 4°C. After the removal of crystallized lactose by centrifugation, ethanol was removed by rotary evaporation and the concentrated solution was passed through Dowex 1-X2 (200–400 mesh, Cl^- form) ion-exchange resin (2 L), followed by fractionation on a charcoal column (7 × 50 cm), using stepwise elution with 2 L each of 5, 15, 30, and 50% (v/v) aq ethanol. The 30% ethanol fraction was concentrated to 10 mL and further purified by preparative paper chromatography in an ascending system of 6:4:3 butan-1-ol–pyridine–water, using five successive developments. The sugars were located with alkaline $AgNO_3$ reagent. Each of the oligosaccharides was further purified by re-chromatography with the same solvent, and passage through a column (2.5 × 100 cm) of Bio-Gel P-4 (−400 mesh, extra fine) [2]. The fractions containing oligosaccharides were monitored by the phenol–H_2SO_4 method [34].

Analysis of monosaccharide composition.—The monosaccharide composition of each oligosaccharide was determined by GC analysis, after hydrolysis with 2 M CF_3CO_2H at 100°C for 5 h. The hydrolysate was evaporated to dryness and kept in a vacuum desiccator with KOH for 1 day, followed by trimethylsilylation with a TMS–HT kit (Tokyo Kasei Co, Japan).

GC analyses were run on a Shimadzu 6 A gas chromatograph equipped with a flame-ionization detector and a glass column (0.2 × 300 cm) packed with 3% SE-30 on Chromosolv W. The chromatograph was operated at a temperature gradient of 3°C/min from 150 to 250°C. The monosaccharide compositions were determined by comparison of retention times with those of standard samples in GC.

*Preparation of lacto-*N*-novotriose from lacto-*N*-novotetraose by β-D-galactosidase digestion.*—A solution of lacto-*N*-novotetraose (~ 2 mg) in water (600 μL) was incubated for 1 h at 37°C with 160 μL of 20 mM NaOAc buffer, pH 4.5, containing 160 units of exo-β-D-galactosidase from *Aspergillus oryzae* (Calbiochem, San

Diego, CA, USA). The incubate was then filtered through two connected columns (2.5 × 100 cm) of Bio-Gel P-4, extra fine. Elution with water at 3.8 mL h^{-1} was monitored by scintillation counting. Fractions containing the product were freeze-dried, repeatedly dissolved in D_2O (99.8 atom %D), then freeze-dried and redissolved in D_2O, and examined by 600-MHz ^1H and 150-MHz ^{13}C NMR.

NMR spectroscopy.—Chemical shifts are expressed relative to internal 2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt (DSS), but were actually measured by reference to internal acetone (^1H, δ 2.225; ^{13}C, δ 32.910). ^1H NMR spectra of GM-1, GM-2, GM-3, and GM-4 were recorded in D_2O (99.95 atom %D, Merck, Germany) at 399.78 MHz with a Jeol JNM-GSX-400 spectrometer, with the probe temperature set at 300 K. The 400.13-MHz spectrum of lacto-*N*-novotriose in D_2O (99.96 atom %D, Sigma, USA) was recorded at 295 K on a Bruker AMX-400 wide-bore spectrometer, with a 5-mm proton probe. All other spectra of this trisaccharide were obtained, at 600.14 MHz for ^1H or 150.92 MHz for ^{13}C, with a Bruker AMX-600 spectrometer, employing a 5-mm broadband inverse detection probe set at 303 K. Standard Bruker pulse programs (UXNMR 930301) were used, with conditions and modifications noted below.

For all two-dimensional spectra, the water resonance was presaturated during a relaxation delay of 2 s. For the ^1H–^{13}C correlation experiments, the standard Bruker pulse programs were modified to control this suppression, through the pulse shaping utility, by repetition of 20 square pulses of 100-ms duration at 3-μs intervals. All data matrices employed 2048 points in F_2, with 512 t_1 increments of 16 scans for the COSY experiment, 405 t_1 increments of 64 scans for the one-bond ^1H–^{13}C correlation, and 320 t_1 increments of 160 scans for the multiple-bond ^1H–^{13}C correlation. Pulse lengths (90°) were routinely 13 μs for ^1H and 10 μs for ^{13}C. Fixed delays were 3.125 ms for evolution of one-bond C–H couplings and 80 ms for long-range C–H couplings. For all spectra, the F_1 dimension was zero-filled to 1024 points and a $\pi/2$ shifted sine-bell was used for processing in each dimension.

The ID TOCSY subspectra were initiated by selective excitation of α-Glc H-1, β-Gal H-1, or β-Glc H-2 protons with a Gaussian pulse (35 ms); a delay of 115 ms was used to allow the magnetization to evolve after excitation, the 90° pulse for the spin lock was 28 μs, and a fixed delay of 4 ms was used for the z-filter.

Acknowledgments

We thank Mr. Michihiko Koshiyama and Mr. Hideaki Hirabayashi for the goat colostrum, and Dr. Tadao Saito and Professor Takatoshi Ito of Tohoku University for ^1H NMR measurements.

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