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Note

Structure of a branched tetrasaccharide from marsupial milk

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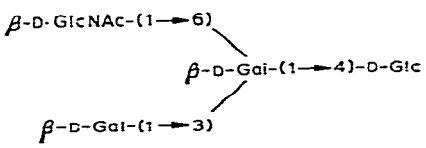
The milk of marsupials contains a variety of neutral and acidic oligosaccharides of which lactose is a minor component[1–3]. The major, neutral oligosaccharides comprise a trisaccharide, *O*-β-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-glucose (3′-galactosyl-lactose)[4], and its homologues produced by the addition of successive (1→3)-linked β-D-galactosyl groups to the non-reducing end of lactose. Members of the series up to the heptasaccharide have been separated, and their structures determined mainly by ^{13}C-n.m.r. spectroscopy[5]. We now report on a novel tetrasaccharide found in milk of the tammar wallaby, *Macropus eugenii*, at a relatively late stage of lactation. It was characterised by enzymic and ^{13}C-n.m.r. methods as **1**.



1

The tetrasaccharide **1** was detected by t.l.c. of the oligosaccharides in milk obtained at 32 weeks *post partum*; it gave a prominent spot of mobility intermediate between those of 3′-galactosyl-lactose and the unbranched tetrasaccharide 3′,3″-digalactosyl-lactose (*cf.* Fig. 5 of ref. 3). It was isolated, by chromatography on Sephadex G-25 and Bio-Gel P-4, from the carbohydrate fraction of milk obtained from several animals at 28–36 weeks *post partum*.

Acid hydrolysis of **1** {$[\alpha]_D^{20}$ +15° (*c* 1, water)} yielded galactose, glucose, and 2-amino-2-deoxyglucose in the molar ratios 2:1:1, so that **1** had the same monosaccharide composition as lacto-*N*-tetraose ($[\alpha]_D$ +25.5°)[6] or lacto-*N*-neotetraose

($[\alpha]_D$ +27°)[7] of human milk. The mobility of **1** in t.l.c. was significantly lower than those of lacto-*N*-tetraose and lacto-*N*-neotetraose.

The presence of a GlcNAc residue in **1** is reflected in the behaviour during gel chromatography, since one such residue behaves[8] as do two residues of hexose on Bio-Gel P-4, resulting in a higher rate of elution.

TABLE I

^{13}C-CHEMICAL SHIFTS[a] AND ASSIGNMENTS FOR TETRASACCHARIDE **1** AND RELATED COMPOUNDS[a]

Tetrasaccharide **1**		*3'-Galactosyl-lactose*[4]		*β-GlcNAc*[10]	*Derivative of β-GlcNAc*[b]	*Assignment for β-GlcNAc derivatives*
Chemical shift	*Assignment*	*Chemical shift*	*Assignment*			
175.2	HNC=O			174.9	174.6	HNC=O
104.9	1″	105.1	1″			
103.3	1′	103.4	1′			
101.7	1^n			95.3	101.4	1^n
96.4	1β	96.6	1β			
92.7	1α	92.7	1α			
82.2	3′	82.7	3′			
79.5	4α	79.2	4α			
79.4	4β	79.0	4β			
76.5	5^n			76.3	76.1	5^n
75.7	5″	75.9	5′ + 5″			
75.4	5β	75.6	5β			
75.0	3β	75.2	3β			
74.5	5′					
74.5	2β	74.6	2β			
74.2	3^n			74.3	74.1	3^n
73.2	3″	73.3	3″			
72.1	3α	72.2	3α			
71.9	2α	72.0	2α			
71.7	2″	71.9	2″			
70.9	5α	70.9	5α			
70.7	2′	71.0	2′			
70.5	4^n			70.3	70.3	4^n
69.2	4′	69.3	4′			
69.2	4″	69.4	4″			
69.1	6′					
61.6	6″	61.8	6′ + 6″			
61.4	6^n			61.0	61.1	6^n
60.7	6β	60.9	6β			
60.6	6α	60.8	6α			
56.2	2^n			57.1	55.9	2^n
23.0	COCH$_3$			22.3	22.6	COCH$_3$

[a] D_2O, internal 1,4-dioxane (δ 67.4). C, C′, and C″ connote carbon atoms in the reducing, second, and third residues, respectively, and C^n connotes those in GlcNAc. [b] 8-Carboxyoctyl 2-acetamido-2-deoxy-β-D-glucopyranoside[11].

When **1** was incubated with purified β-*N*-acetylglucosaminidase, the only products (t.l.c. and p.c.) were 2-acetamido-2-deoxyglucose and 3′-galactosyl-lactose, showing that **1** is 3′-galactosyl-lactose carrying a β-GlcNAc residue. Treatment of **1** with a purified exo-β-D-galactosidase[9] yielded galactose and an unknown oligosaccharide (*X*); hence, β-Gal is a non-reducing, terminal unit. The t.l.c. mobility of *X* was intermediate between those of lactose and 3′-galactosyl-lactose, consistent with its structure being GlcNAc-Gal-Glc, but not GlcNAc-Glc, since the latter would move more rapidly than lactose; the GlcNAc residue is therefore not linked to the Glc residue. Thus, **1** (lacto-*N*-novotetraose) is branched, with a GlcNAc residue attached to the middle (Gal) residue of 3′-galactosyl-lactose.

^{13}C-N.m.r. data for **1** and some related compounds are given in Table I. Each of the twelve resonances for the reducing residue had an intensity less than that for a single carbon. Several resonances from the α anomer were only marginally more intense than the noise level, but these and all the other resonances from the reducing residue had the same chemical shifts as those of lactose and 3′-galactosyl-lactose (Table I), indicating that glucose is at the reducing end of **1** and that it is linked only at C-4.

There was a close correspondence between the chemical shifts of the 8 resonances of a 2-acetamido-2-deoxy-β-D-glucose derivative[11] substituted at C-1 and those for **1**. These chemical shifts are virtually identical with those for 2-acetamido-2-deoxy-β-D-glucose, except for the C-1 and C-2 resonances which were moved, on the average, 6.3 p.p.m. downfield and 1.0 p.p.m. upfield, respectively, as a result of substitution at C-1. Thus, **1** contains a β-GlcNAc residue.

The remaining 12 resonances of **1** had chemical shifts which were virtually identical with those of 3′-galactosyl-lactose (except for the resonances located at δ 69.1 and 74.5), indicating **1** to be a derivative of 3′-galactosyl-lactose. The excellent agreement in chemical shifts assigned to C-2′,2″,3″,4′,4″ in **1** compared with those of the corresponding carbons in 3′-galactosyl-lactose shows that the β-D-GlcNAc residue is not linked to any of these positions. The peaks at δ 69.1 and 74.5 have been assigned to C-6 and C-5, respectively, implying a downfield shift of 7.3 p.p.m. at C-6 and an upfield shift of 1.4 p.p.m. at C-5 as a result of the attachment of GlcNAc at C-6′ or C-6″ of 3′-galactosyl-lactose. These shifts are of similar magnitude to those found at C-3′ (9.3 p.p.m. downfield) and C-2′ (0.9 p.p.m. upfield) as a result of attachment of Gal at C-3′ of lactose[4]. The alternative assignment of the new peaks to C-5 and C-6, respectively, would involve a downfield shift of 12.7 p.p.m. for the C-6 resonance and an upfield shift of 6.8 p.p.m. for the C-5 resonance, both of which are considered to be too large[12,13].

Thus, the n.m.r. data show that β-GlcNAc is attached to C-6 of one of the Gal residues of 3′-galactosyl-lactose. That C-6′, but not C-6″, is involved is supported by the upfield shift of 0.5 p.p.m. of the C-3′ resonance in **1** compared with that of 3′-galactosyl-lactose (there was no shift in the resonance due to C-3″), whereas, in

the unbranched tetrasaccharide 3′,3″-digalactosyl-lactose, there was no change in the C-3′ chemical shift as a result of attachment of an additional Gal to the terminal Gal of 3′-galactosyl-lactose[5]. Also, in a branched oligosaccharide, the correlation time for C-1′ of the Gal residue involved in the branch would be expected to be longer than that of the outlying C-1″ and C-1‴ atoms. This could cause some reduction of the n.O.e. of C-1′ (observed earlier in a homologous series of oligosaccharides[5]) as compared with those of C-1″ and C-1‴, and a reduction in the intensity of the C-1′ resonance. The intensities C-1′ < C-1″ ⩽ C-1‴ were observed for **1**, whereas C-1′ ~ C-1″ < C-1‴ would be expected for the alternative, unbranched structure.

Thus, the structure shown above for **1** is established.

EXPERIMENTAL

General. — T.l.c. was performed on Silica gel 60 (Merck, 5553), using 2-propanol–acetone–0.1M lactic acid (2:2:1) and detection with aniline–diphenylamine[4]. Preparative p.c. was performed on Whatman No. 3 paper for 96 h, using ethyl acetate–pyridine–water (12:5:4) and detection with alkaline silver nitrate. Monosaccharide analysis was carried out as described previously[4], except that acid hydrolysis involved 2M HCl, and 2-amino-2-deoxyglucose was determined with an amino acid analyser[3].

Isolation of the tetrasaccharide **1**. — Extraction of the milk carbohydrate and separation of the saccharides on Sephadex G-25 were carried out as described previously[3]. The contents (20 mg) of the tetrasaccharide peak (peak 4 of Fig. 3, ref. 3) isolated by freeze-drying were eluted with water from 2 columns (each 150 × 1.1 cm) of Bio-Gel P-4 (Bio-Rad, −400 mesh), connected in series. Two peaks were obtained: 4A (V_E 206 mL, 40%) and 4B (V_E 215 mL, 60%). The contents of each peak were isolated by freeze-drying. T.l.c. showed that 4A contained **1** contaminated with a small proportion of an unknown, higher oligosaccharide. The contaminant was removed by preparative p.c. and the product was then re-chromatographed on Bio-Gel P-4 and isolated by freeze-drying.

Action of enzymes. — (*a*) A solution of **1** (250 μg) in water (25 μL) was mixed with a solution (25 μL) containing 0.5 U of β-*N*-acetylglucosaminidase (Boehringer, Mannheim), which had been dialysed to remove ammonium sulphate and adjusted to pH 5 with dilute citric acid. The mixture was incubated at 37°. Samples (1 μL) were subjected to t.l.c. After 1 h of incubation, **1** had disappeared, and 2-acetamido-2-deoxyglucose and 3′-galactosyl-lactose were detected. There were no further changes during the next 5 h.

(*b*) A solution of **1** (500 μg) in water (50 μL) was mixed with a solution (5 μL) containing β-D-galactosidase (25 μg, 12 U) from *E. coli* (Sigma Chemical Co., grade IV). The mixture was incubated at 25°. T.l.c. showed that, after 24 h, **1** had disappeared and had been replaced by galactose and an oligosaccharide whose mobility

(R_{Lac} 0.88) was intermediate between that of lactose and 3'-galactosyl-lactose (R_{Lac} 0.81).

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