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STRUCTURE OF A MARSUPIAL-MILK TRISACCHARIDE

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ABSTRACT

A trisaccharide, which is a major carbohydrate component of the milk of the tammar wallaby and the grey kangaroo, has been identified by chemical, enzymic, g.l.c.–m.s., and n.m.r. methods as *O*-β-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-glucose (3′-galactosyl-lactose).

INTRODUCTION

The milk of marsupials is unusual in that it contains a variety of carbohydrates of which lactose, the principal carbohydrate of eutherian milk[1], is a minor component[1–4]. The preponderant monosaccharide constituent of these carbohydrates is galactose, but their detailed structures are unexplored.

Milk of the grey kangaroo, *Macropus giganteus*, contains a mixture of neutral oligo- and poly-saccharides and sialyl saccharides, and a partial separation and characterisation of the neutral oligosaccharides has been achieved[5]. We now report on the structure of a neutral trisaccharide which is found in relatively high concentration in the milk of the grey kangaroo and the tammar wallaby, *Macropus eugenii*.

RESULTS AND DISCUSSION

The milk carbohydrate of the grey kangaroo was fractionated on Sephadex G-25, to give 10 peaks of neutral saccharides which were eluted after 2 or 3 indistinct peaks of material that contained sialic acid[5]. Fig. 1 shows similar results obtained with the milk carbohydrates of the tammar wallaby, which were fractionated into 8 peaks of neutral oligosaccharides of which three (2–4) had elution volumes corresponding to those of di-, tri-, and tetra-saccharides, respectively.

Paper chromatography (p.c.) showed that the trisaccharide peak (3) contained only one component, with a mobility identical to that of the trisaccharide from milk

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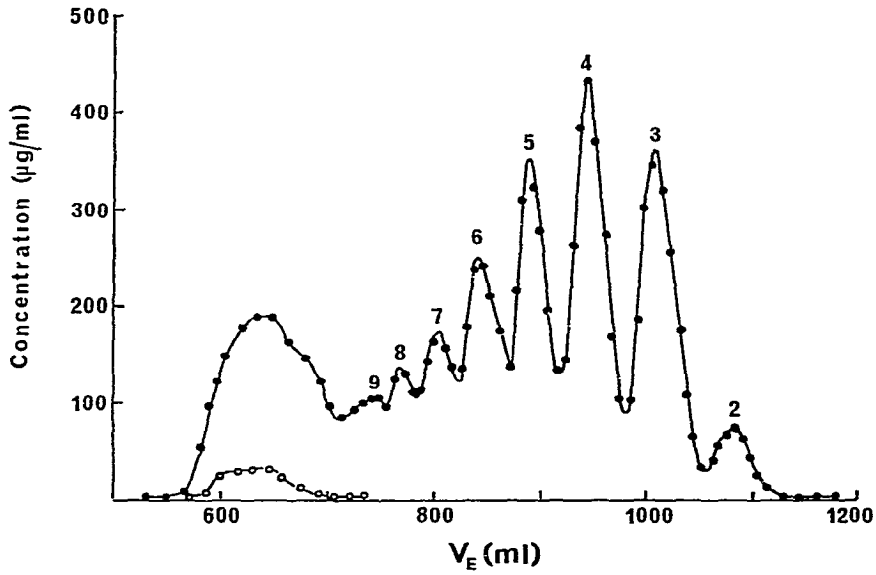

Fig 1 Gel filtration of milk carbohydrate of tammar wallaby After removal of milk fat and protein, the water-soluble fraction (100 mg, containing 87 mg of hexose) was passed through three columns (100 × 2 5 cm) of Sephadex G-25 (superfine), connected in series, which were eluted with water —●—, total hexose (lactose equivalents, phenol–sulphuric acid method), —○—, sialic acid (after acid hydrolysis, thiobarbiturate method), V_E, elution volume

TABLE I

P C OF MARSUPIAL-MILK TRISACCHARIDES AND OF GALACTOSYL-LACTOSES[a]

Solvent system[b]	*Tammar-wallaby trisaccharide*	*Grey-kangaroo trisaccharide*	*3'-Galactosyl-lactose*[c]	*4'-Galactosyl-lactose*[d]	*6'-Galactosyl-lactose*[e]
A	0 54	0 55	0 54	0 64	0 53
B	0 70	0 69	0 69	0 82	0 74
C	0 61	0 60	0 61	0 73	0 65
D	0 75	0 74	0 74	0 79	0 70
E	0 55	0 56	0 54	0 64	0 42
F	0 47	0 47	0 46	0 59	0 46

[a]Results expressed as R_{Lac} (mobility relative to that of lactose) [b]See Experimental [c]*O*-*β*-D-Galactopyranosyl-(1→3)-*O*-*β*-D-galactopyranosyl-(1→4)-D-glucose [d]*O*-*β*-D-Galactopyranosyl-(1→4)-*O*-*β*-D-galactopyranosyl-(1→4)-)-D-glucose [e]*O*-*β*-D-Galactopyranosyl-(1→6)-*O*-*β*-D-galactopyranosyl-(1→4-)-D-glucose

of the grey kangaroo and 3'-galactosyl-lactose, but different from those of 4'- and 6'-galactosyl-lactose (Table I)

Hydrolysis (2M HCl, 1 h, 100°) of each trisaccharide gave only galactose and glucose (p c and t l c), in the molar ratio 2 1 Hydrolysis of each borohydride-

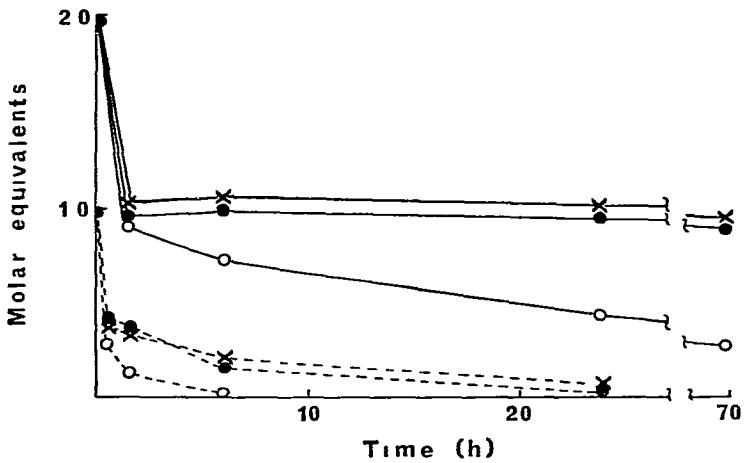

Fig 2 Degradation of galactose (——) and glucose (– – –) during periodate oxidation of trisaccharides ●, trisaccharide from tammar-wallaby milk, ×, 3′-D-galactosyl-lactose, ○, 4′-D-galactosyl-lactose

reduced trisaccharide gave only galactose and glucitol (t l c), indicating that glucose was the reducing residue of the parent trisaccharide

Each trisaccharide was resistant to the action of α-D-galactosidase, but not to that of β-D-galactosidase, which converted each into a mixture of galactose and glucose, thus establishing that both glycosidic linkages were β, and confirming that glucose was at the reducing end

When the trisaccharide from milk of the tammar wallaby was treated with sodium periodate and the product hydrolysed with acid, one galactose residue remained intact (Fig 2), suggesting[6] that the galactose–galactose linkage was (1→3) Authentic 3′-galactosyl-lactose showed similar behaviour, in contrast to 4′-galactosyl-lactose which suffered degradation of both residues

The tamar-wallaby trisaccharide was reduced with sodium borohydride and borodeuteride, and then methylated G l c revealed one major component, and Table II shows its partial mass-spectrum, which was interpreted on the basis of the g l c –m s data for permethylated trisaccharide alditols reported by Kärkkainen[7]

TABLE II

PARTIAL MASS-SPECTRUM OF PERMETHYLATED ALDITOL OF TRISACCHARIDE FROM TAMMAR-WALLABY MILK

m/e
45 (100%), 71 (68), 75 (46), 88 (44), 89 (29), 101 (82), 103 (8), 111 (48), 115 (27), 133 (11[a]), 143 (9), 145 (9), 155 (10), 159 (20), 171 (10), 177 (0), 187 (42), 191 (8), 201 (0), 219 (10), 235 (15), 295 (0), 391 (0)

[a]In the deuterated derivative, > 50% of the ion is converted into the ion at *m/e* 134

The galactose–glucose linkage was not (1→6) or (1→2), because of the absence of the ion at *m/e* 177 (containing four carbons of the alditol chain) and the low intensity of the ion at *m/e* 145 (177 — MeOH) The ion at *m/e* 133 (containing three carbon atoms of the alditol chain) showed a distinct deuterium-effect, indicating that the linkage was (1→4) and not (1→3)

The galactose–galactose linkage was not (1→6) or (1→4), because the ion at *m/e* 88 was not the base peak and was low in intensity relative to the ion at *m/e* 101 The linkage was not (1→2), because of the absence of ions at *m/e* 201 and 391 The presence of a (1→3) linkage was confirmed by the relatively high intensity of the ion at *m/e* 159 and the absence of the ion at *m/e* 295

The mass spectra of the foregoing, methylated alditol and that derived from authentic 3′-galactosyl-lactose showed no significant differences

The ^{1}H-n m r spectrum of the trisaccharide from grey-kangaroo milk gave anomeric-proton doublets at δ 5 21 (H-1α) and 4 65 (H-1β) The glycosidic doublets had chemical shifts of δ 4 49 and 4 59, with coupling constants (J 7 5 and 7 3 Hz, respectively) characteristic of β linkages[8] The first doublet collapsed to a singlet on irradiation at δ 3 56 (H-2), and the second doublet collapsed on irradiation at δ 3 64 The chemical shifts of the H-2 resonances were consistent with galactose rather than glucose residues[8], confirming that glucose was the reducing residue

The trisaccharide was treated with sodium cyanide followed by hydrolysis[8] The ^{1}H-n m r spectrum (D_2O, pH-meter reading ~6) of the resulting carboxylic acid was determined in the presence of increasing amounts of $GdCl_3$ The glycosidic-proton resonance at δ 4 49 broadened before that at 4 59, the former resonance was assigned to H-1′ (see Table III) Its chemical shift was consistent with a (1→4)-β-D linkage as in lactose[8], the chemical-shift data for the trisaccharide [δ 5 21 (H-1α), 4.65 (H-1β), 4 49 (H-1′), 3 56 (H-2′), 4 59 (H-1″), and 3 64 (H-2″)] agreed reasonably well with the corresponding values for lactose [δ 5 26 (H-1α), 4 68 (H-1β), 4 47 (H-1′), and 3 52 (H-2′)] 6′-Galactosyl-lactose gave signals at δ 4.44 and 4 47 for the glycosidic protons, the latter was consistent with that of the glycosidic proton of the lactose part of the marsupial trisaccharide, but the former did not agree with the value of δ 4 59 (see above), hence the galactose–galactose linkage was not (1→6)-β-D

Table III contains ^{13}C-n m r data for various model saccharides and the grey-kangaroo trisaccharide, which were identical with those for the tammar-wallaby trisaccharide The separation and assignment of every carbon atom in the molecule shows the advantage of ^{13}C- as compared with ^{1}H-n m r spectroscopy[8] The data for lactose agreed closely with literature values, the assignments made previously[9], and modified recently by Pfeffer *et al*[10], have been used as a basis for assignment of the trisaccharides The carbon atoms of the non-reducing residues of the model disaccharides β-D-Gal-(1→4)-D-Gal and β-D-Gal-(1→3)-D-Gal were assigned on the basis of the lactose data, and those of the reducing residues were assigned by comparison with the data for D-galactose[10]

Methylation of hexopyranose hydroxyl-groups causes[11] upfield shifts of ~4 5

TABLE III

^{13}C-CHEMICAL SHIFTS (P P M DOWNFIELD FROM SIGNAL FOR Me_4Si) AND ASSIGNMENTS OF DISACCHARIDES AND TRISACCHARIDES[a]

β-D-Gal-(1→4)-D-Gal		*β-D-Gal-(1→3)-D-Gal*		*β-D-Gal-(1→4)-D-Glc (Lactose)*		*3'-Galacto-syl-lactose*	*Grey-kangaroo trisaccharide*	
Chemical shift	*Assign-ment*	*Chemical shift*	*Assign-ment*	*Chemical shift*	*Assign-ment*	*Chemical shift*	*Chemical shift*	*Assign-ment*
105 1	1′	105 2	1′	—	—	105 1	105 2	1″
—	—	—	—	103 7	1′	103 4	103 4	1′
97 3	1β	97 0	1β	96 6	1β	96 6	96 6	1β
93 2	1α	93 0	1α	92 7	1α	92 7	92 7	1α
—	—	83 3	3β	—	—	82 7	82 7	3′
—	—	80 2	3α	—	—	—	—	—
79 2	4α	—	—	79 4	4α	79 2	79 2	4α
78 1	4β	—	—	79 3	4β	79 0	79 0	4β
76 0	5′	75 9	5′	76 2	5′	75 9	75 9	5′ + 5″
—	—	—	—	75 6	5β	75 6	75 6	5β
75 1	5β[c]	75 6	5β	75 2	3β	75 2	75 2	3β
—	—	—	—	74 7	2β	74 6	74 7	2β
74 1	3β[c]	—	—	—	—	—	—	—
73 6	3′	73 4	3′	73 4	3′	73 3	73 4	3″
73 1	2β	—	—	—	—	—	—	—
—	—	—	—	72 2	3α	72 2	72 2	3α
72 2	2′	71 9	2	71 9	2′	71 9	71 9	2″
—	—	—	—	72 0	2α	72 0	72 0	2α
—	—	71 8	2β	—	—	71 0	71 0	2
70 5	5α[d]	70 9	5α	70 9	5α	70 9	70 9	5α
70 6	3α[d]	—	—	—	—	—	—	—
—	—	69 9	4α	—	—	—	—	—
69 7	2α	—	—	—	—	—	—	—
69 5	4′	69 4	4β,4′	69 4	4′	69 4	69 4	4″[b]
—	—	—	—	—	—	69 3	69 3	4′[b]
—	—	68 3	2α	—	—	—	—	—
61 8	6′	61 8	6′	61 9	6′	61 8	61 8	6′,6″
61 6	6α	62 0	6α	61 0	6β	60 9	61 1	6β
61 4	6β	61 8	6β	60 9	6α	60 8	60 9	6α

[a]Unprimed numbers refer to reducing residue, primed numbers to next residue, *etc* Error in chemical shifts, ±0 1 p p m [b–d]Pairs of peaks, assignments of which may be reversed

p p m on β-carbon atoms having axial hydroxyl groups This rule was extended to apply to glycosylation and has been illustrated[12] for α-D-Gal-(1→3)-D-Gal and two other cases However, our results for β-D-Gal-(1→3)-D-Gal and the trisaccharide 3′-galactosyl-lactose showed no such shift of the C-4′ resonance, relative to that of C-4′ of lactose Thus, the stereochemistry produced by the glycosylation is an important factor in determining the chemical shift of the axially substituted β-carbon atom

The chemical shifts of the carbon resonances of the D-glucose residues of the α and β anomers of lactose and of 3′-galactosyl-lactose were similar The signals for

the corresponding carbon atoms of each marsupial trisaccharide showed similar chemical shifts, which established that each trisaccharide had a (1→4)-linked β-D-glucose residue at the reducing end Similarly, the chemical shifts of the ^{13}C-resonances assigned to the D-galactosyl groups at the non-reducing ends of β-D-Gal-(1→4)-D-Gal, β-D-Gal-(1→3)-D-Gal, lactose, and 3′-galactosyl-lactose agreed with each other and with those assigned to the residues at the non-reducing ends of both marsupial trisaccharides Thus, these trisaccharides each had a β-D-galactosyl group at the non-reducing end

The chemical shifts assigned to the 2′, 3′, and 4′ carbon atoms of the marsupial trisaccharides (Table III) differed considerably from those assigned to the corresponding carbon atoms of the β anomer of the disaccharide β-D-Gal-(1→4)-D-Gal, hence, the galactose–galactose linkage of the trisaccharides was not (1→4)-β-D The shifts assigned to the 2′, 3′, 4′, 5′, and 6′ carbon atoms of the trisaccharides were close to those assigned to the corresponding carbon atoms of the β anomer of β-D-Gal-(1→3)-D-Gal, which showed that the galactose–galactose linkage was (1→3)-β-D Finally, the agreement between the ^{13}C spectra of the two marsupial trisaccharides and that of 3′-galactosyl-lactose showed that the three molecules were identical

3′-Galactosyl-lactose has been isolated from culture filtrates of various species of the fungus *Chaetomium*[13], but has not previously been found in milk or in any other mammalian source Human milk contains[14] the trisaccharide 6′-galactosyl-lactose, but at a concentration of <4 mg/litre Peak 3 (Fig 1) contributes ~16% of the total milk hexose, since the mean hexose-content[15] of tammar-wallaby milk, at 18 weeks *post partum*, is 10 3%, the concentration of 3′-galactosyl-lactose in the original milk was ~16 g/litre

EXPERIMENTAL

Isolation of milk trisaccharides — The milk from 6 tamar wallabies at 18 weeks *post partum* was pooled Extraction of the carbohydrate and separation of the saccharides by gel filtration were performed as described previously[5] The contents of peak 3 (Fig 1) were freeze-dried and then passed through a column (100 × 1 1 cm) of Bio-Gel P-2 (200–400 mesh) to remove contaminants The trisaccharide from milk of the grey kangaroo was similarly isolated from peak 3 of Fig 1*b* of ref 5 Each freeze-dried product showed $[\alpha]_D^{20}$ +49° (*c* 1, water)

6′-Galactosyl-lactose was isolated[14] from human colostrum Samples of 3′-galactosyl-lactose and 4′-galactosyl-lactose were obtained from Dr P A J Gorin (Saskatoon, Canada) β-D-Gal-(1→3)-D-Gal and β-D-Gal(1→4)-D-Gal were obtained from Dr M Martin-Lomas (Madrid, Spain)

Chromatography — P c was performed on Whatman No 1 paper by the descending method for 24–48 h, using *A*, ethyl acetate–pyridine–water (12 5 4), *B*, ethyl acetate–pyridine–acetic acid–water (5 5 1 3), *C*, ethyl acetate–acetic acid–water (3 1 1), *D*, ethyl acetate–1-propanol–water (1 6 3), *E*, 2-propanol–water

(4 1), *F*, 1-butanol–1-propanol–acetic acid–water (1 2 1 1), and detection with alkaline silver nitrate

T l c was performed on Silica Gel 60 (Merck 5553) Monosaccharides were separated by the procedure of Hansen[16] Mixtures of monosaccharides and their alditols were separated by using 1-butanol–2-propanol–0 5% boric acid (2 1 1), which was run for 30 h into a sheet of Whatman No 3M paper stapled to the top of the plate, and detected with benzidine–periodate The R_{Glc} values for galactose, galactitol, and glucitol were 0 89, 0 74, and 0 65, respectively

Monosaccharide analysis — Trisaccharide (~1 mg) was hydrolysed with 2M HCl (1 ml) at 100° for 1 h in a sealed tube A part of the hydrolysate (0 1 ml) was dried *in vacuo* over KOH, and the residue subjected to chromatography The remainder was neutralised with M NaOH and diluted to 5 ml with water Samples (0 5 ml) were analysed[5] for D-glucose with D-glucose oxidase and for D-galactose with D-galactose dehydrogenase

Borohydride reduction — Trisaccharide (~2 mg) and sodium borohydride or borodeuteride (~1 mg) were dissolved in water (0 4 ml) After 24 h at 20°, the solution was treated with Dowex-50W X8 (H^+) resin (200–400 mesh) (40 mg), filtered, and concentrated to dryness *in vacuo* To remove boric acid, methanol (5 × 3 ml) was evaporated from the residue, which was then either hydrolysed with acid and subjected to t l c , or permethylated as described below

Action of galactosidases — Solutions of the trisaccharide (1 mg) in water (50 μl) and ~0 08 U of either α-D-galactosidase or β-D-galactosidase (Boehringer Mannheim) in water (5 μl) were mixed and incubated at 20° Samples (1 μl) were subjected to t l c After 24 h, the trisaccharide treated with α-D-galactosidase had remained unchanged, whereas that treated with β-D-galactosidase had been converted into galactose and glucose At intermediate times, a spot having the same mobility as lactose was observed

Periodate oxidation — A solution of the trisaccharide (1 0 μmol, 50 4 μg) in water (0 1 ml) was treated with 8 0 μmol of $NaIO_4$ (0 1 ml) at ~0°, and the mixture was kept in the dark at 2° The reaction was stopped by the addition of 20 μmol (0 1 ml) of aqueous lead acetate, and the mixture was filtered through a column (0 4 × 5 cm) of mixed-bed ion-exchange resin (AG 501-X8, 20–50 mesh), which had been pretreated with CO_2 to prevent retention and degradation of carbohydrate[17] The eluate (2 ml) was freeze-dried, and a solution of the residue in 2M HCl (0 6 ml) was heated at 100° for 1 h and then neutralised with M NaOH and diluted to 5 0 ml Samples of the diluted solution were analysed for glucose and galactose (see above)

Methylation and g l c–m s — For methylation, the following modification of the Hakomori procedure[18] was used The residue from the borohydride or borodeuteride reduction was treated with 1 ml of a solution of sodium methylsulphinylmethanide in dimethyl sulphoxide[19], under nitrogen The mixture was agitated in a sonication bath for 15 min, and then treated with methyl iodide (0 2 ml) and subjected to further sonication under nitrogen until it became clear (~15 min) Water (5 ml) was added and the mixture was extracted with chloroform (5 × 3 ml) The combined

extracts were washed with water (3 × 5 ml) and concentrated to dryness, and the residue was subjected to g l c using a Pye 104 chromatograph (5% SE-30 or 3% OV-1 as stationary phases, and a 2-m column at 260°) Mass spectrometry was performed on an AEI MS902 instrument at 70 eV The stationary phase used in g l c–m s was 5% SE-30

N m r spectroscopy — Spectra were measured on solutions in D_2O (>99 7%) at 20°, using a Brucker HX-270 spectrometer (National NMR Centre, Canberra) For ^{1}H-n m r spectra, the HDO signal (4 83 p p m) was used as the reference for chemical shifts (error ±0 02 p p m) ^{13}C-N m r spectra (internal 1,4-dioxane, 67 4 p p m) were normally obtained on 3–10 mg samples An accumulation time of up to 16 h was adequate

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