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Note

^{13}C-N.m.r. study of the structures of two branched oligosaccharides from marsupial milk

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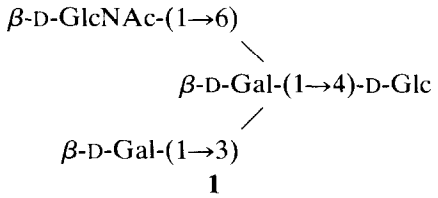
Marsupial milk contains several neutral oligosaccharides that are based on lactose, which itself occurs only as a minor component. In previous studies, we have characterised, mainly by ^{13}C-n.m.r. spectroscopy, an unbranched homologous series produced by the addition of successive (1→3)-linked β-D-galactosyl groups to the non-reducing end of lactose[1,2]. The first member of a second homologous series, lacto-*N*-novotetraose (**1**), is produced by substitution of galactose and 2-acetamido-2-deoxyglucose at positions 3 and 6, respectively, of the galactosyl group of lactose[3].



1

We now report on the structures of two higher members of this second series, namely, a pentasaccharide (**2**) and a hexasaccharide (**3**) that are produced by the addition of either one or two D-galactosyl groups at one or both of the non-reducing ends, respectively, of **1**. They were isolated by chromatographic techniques from the milk of the tammar wallaby, *Macropus eugenii*.

Acid hydrolysis of **2** yielded galactose, glucose, and 2-amino-2-deoxyglucose in the molar ratios 3:1:1. The ^{13}C-n.m.r. data for **2** are given in Table I. Because of the small amount of sample available, the smallest resonances from the α anomer of the reducing residue were just visible above noise.

The chemical shift data for three of the C-1 resonances agree closely with those for **1**, indicating that the basic structures are very similar. There is an addi-

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TABLE I

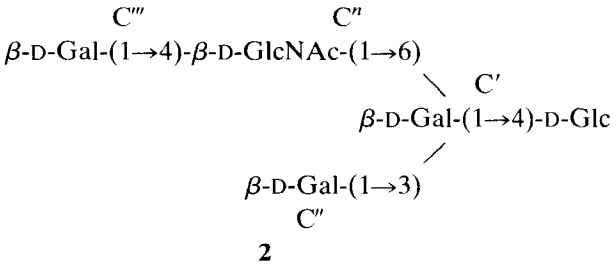
^{13}C-CHEMICAL SHIFT DATA[a]

Tetrasaccharide **1**[3]		*Pentasaccharide* **2**		*Hexasaccharide* **3**	
Chemical shift	*Assignment*	*Chemical shift*	*Assignment*	*Chemical shift*	*Assignment*
175.2	HNC=O	175.4	HNC=O	175.3	HNC=O
104.9	1″	105.1	1″	105.1	1‴
				104.8	1″
		103.8	1‴	103.7	1‴
103.3	1′	103.5	1′	103.5	1′
101.7	1^n	101.8	1^n	101.9	1^n
96.4	1β	96.6	1β	96.6	1β
92.7	1α	92.6	1α		
				82.8	3‴
82.2	3′	82.4	3′	82.3	3′
79.5	4α	79.7	4α,4^n	79.7	4β
79.4	4β	79.4	4β	79.4	4‴
76.5	5^n				
		76.2	5‴	76.2	5‴[b]
75.7	5″	75.9	5″	75.9	5″
75.4	5β	75.6	5^n,5β	75.6	5‴[b],5^n [b]
75.0	3β	75.2	3β	75.2	3β
74.5	5′,2β	74.7	5′,2β	74.7	5′
74.2	3^n	74.4	3^n	74.4	3^n
73.2	3″	73.4	3″,3‴	73.4	3″,3‴
72.1	3α	72.3	3α		
71.9	2α	72.1	2α		
71.7	2″	71.9	2″,2‴	71.9	2‴[c]
				71.8	2‴[c]
				71.1	2″
70.9	5α	70.9	2′,5α	70.9	2′
70.7	2′				
70.5	4^n				
69.2	4′,4″	69.4	4′,4″,4‴,6′	69.4	4′,4″,4‴,4‴′
69.1	6′			69.2	6′
61.6	6″	61.8	6″,6‴	61.8	6″,6‴,6‴′
61.4	6^n	61.0	6^n,6α,6β	60.9	6‴
60.7	6β				
60.6	6α				
56.2	2^n	55.9	2^n	55.9	2^n
23.0	COCH$_3$	23.3	COCH$_3$	23.3	COCH$_3$

[a]Spectra determined at 67.89 MHz for solutions in D_2O (internal 1,4-dioxane, δ 67.4) with accumulation times of 16–65 h. C, C′, C″, C‴, and C‴′ refer to carbon atoms in the reducing, second, third, fourth, and fifth residues, respectively, and C^n refers to those in GlcNAc (see structures **1**–**3**). [b,c]Assignments may be interchangeable.

tional resonance at δ 103.8 from the extra galactose residue, which therefore cannot be joined to the galactosyl group at the non-reducing end of **1**, since a chemical shift of ~105 p.p.m. would be expected[2]; hence, the extra galactose is linked *via* a β-linkage to the 2-acetamido-2-deoxy-D-glucosyl group; an α-linkage would cause the resonance to be further upfield. The linkage position (C-4) on the 2-acetamido-

2-deoxy-D-glucosyl group is obtained from the observation that the resonance at δ 70.5 for the 4^n carbon in **1** is not present in the spectrum of **2**. There is a new resonance for **2** at δ 79.7, which is assigned to the 4^n carbon linked β to the new galactose residue[4]. This result is consistent with the occurrence of a small upfield shift of the 5^n signal from δ 76.5 for **1** to δ 75.6 for **2**[4], no significant change in the position of the 3^n signal, and a small (0.4 p.p.m.) upfield shift of the 6^n resonance. The chemical shift data thus show that the pentasaccharide has structure **2** produced by the substitution of β-D-Gal at position 4 of GlcNAc of **1**.

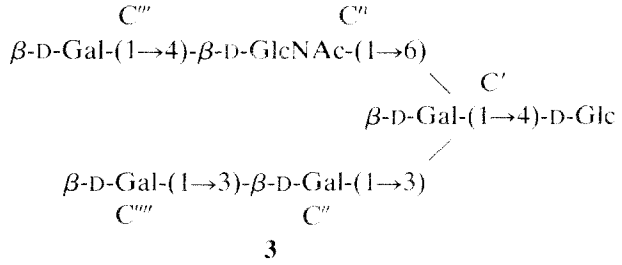


2

Acid hydrolysis of **3** yielded galactose, glucose, and 2-amino-2-deoxyglucose in the molar ratios 4:1:1. The ^{13}C-n.m.r. data (Table I) show that the signal level of the spectrum of **3** was not sufficient to observe resonances from all of the carbons of the α and β forms of the reducing sugar residue.

The hexasaccharide has six separated resonances from the six C-1 atoms, which agree closely in chemical shift with those of **2**, with one additional resonance located at δ 104.8. This has been assigned to C-1″, which undergoes a slight upfield shift from δ 105.1 when an additional galactosyl group is added[2] at position 3. The resonance at δ 105.1 is then assigned to C-1‴ of the new terminal galactosyl group, which is concluded to be β-linked to position 3″ of **2**. The alternative possibility, which would involve β-linkage to position 3‴ of **2**, would be expected to shift the signal for C-1‴ and leave that for C-1″ unchanged, hence giving two resonances of δ 105.1. This was not observed. There are two resonances at δ 82.8 and 82.3 (Table I) which are assigned to the C-3 atoms involved in (1→3)-linkages to β-D-Gal on the basis of the pentasaccharide assignment. The C-2 atoms adjacent to the linkage position give resonances that undergo a small (0.2 p.p.m.) upfield shift (observed in **2**), and thus the signals at δ 71.1 and 70.9 are assigned to C-2″ and C-2′, respectively. Similarly, there are two single resonances at 71.9 and 71.8, which arise from C-2 atoms of terminal galactosyl groups, and hence are assigned to C-2‴ and C-2⁗. As with **2**, the chemical shifts of all the C-4 galactose resonances coincide. The observation of resonances at δ 79.7, 79.4, and 69.2 agrees with the data for **2** and shows the retention of the (1→4)-linkage to Glc, the (1→4)-linkage to GlcNAc, and the (1→6)-linkage to Gal, respectively. Furthermore, the close correspond-

ence of the other resonances for **2** and **3** show that the hexasaccharide has the same structure as the pentasaccharide, with the addition of β-D-Gal at position 3″ as shown in **3**.



3

EXPERIMENTAL

Isolation of oligosaccharides. — Extraction of the milk carbohydrate and fractionation of the saccharides on Sephadex G-25 were carried out as described previously[5].

For the isolation of **2**, the contents of the pentasaccharide peak (peak 5 of Fig. 1, ref. 1) were eluted with water from two columns (each 150 × 1.1 cm) of Bio-Gel P-4 (Bio-Rad, –400 mesh) connected in series. Two peaks were obtained: 5A (V_t 198 mL, 10%) and 5B (V_t 206 mL, 90%). The contents of each peak were isolated by freeze-drying. T.l.c., using silica gel 60 (Merck 5553) with 2-propanol–acetone–0.1M acetic acid, showed that 5B contained a trigalactosyl-lactose[2], whereas 5A contained **2** (R_F 0.17) plus another minor oligosaccharide (R_F 0.20), presumed to be a pentasaccharide, which migrated just in front of **2**. Preparative t.l.c. of 5A and chromatography on Bio-Gel P-4 then gave 5 mg of **2** and 2 mg of the other pentasaccharide.

For the isolation of **3**, the contents of the hexasaccharide peak (peak 6 of Fig. 1, ref. 1) were eluted with water from two columns (each 150 × 1.63 cm) of Bio-Gel P-4 connected in series, yielding two peaks, 6A(V_t 386 mL, 20%) and 6B(V_t 401 mL, 80%). The contents of each peak were isolated by freeze-drying. T.l.c. showed that 6B contained a tetragalactosyl-lactose[2], whereas 6A contained **3** (R_F 0.10) plus two other oligosaccharides (R_F 0.12, 0.07), presumed to be hexasaccharides, one of which migrated just in front of, and the other just behind, **3**. Preparative t.l.c. of 6A and chromatography on Bio-Gel P-4 gave sufficient material for an acceptable ^{13}C-n.m.r. spectrum to be obtained for only one (**3**) of the hexasaccharides; this spectrum contained several peaks due to impurities and which were readily identified as such by their large intensities compared with those of the peaks for **3**.

Preparative t.l.c. — Silica gel 60 (Merck 5553) was used with 2-propanol–acetone–0.1M acetic acid (2:1:1), and ~1 mg of 5A or 6A was applied to each plate as an 18-cm streak. The solvent was allowed to run into a pad of Whatman No. 3

paper when it reached the top of the plate. For the isolation of **2**, 15 plates, each run for 16 h, were used. For the isolation of **3**, 30 plates, each run for 22 h, were used. Detection of saccharides on test strips was done with aniline–diphenylamine. Zones containing the saccharides were scraped off and the scrapings extracted with water. After centrifugation, the supernatant solution was filtered through a Millipore filter and the saccharides were isolated by freeze-drying.

ACKNOWLEDGMENTS

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