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Note

Structures of four new oligosaccharides from marsupial milk, determined mainly by ^{13}C-n.m.r. spectroscopy

J. GRANT COLLINS, J. HOWARD BRADBURY,
Department of Chemistry, Australian National University, Canberra, A.C.T. 2600 (Australia)
EMIL TRIFONOFF, AND MICHAEL MESSER
Department of Biochemistry, University of Sydney, N.S.W. 2006 (Australia)

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A trisaccharide present as a major component of the milk carbohydrates of the grey kangaroo, *Macropus giganteus*, and the tammar wallaby, *Macropus eugenii*, was shown[1] by chemical, enzymic, g.l.c.–m.s., and n.m.r. methods to be β-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Glc (3′-galactosyl-lactose). Using mainly the n.m.r. techniques developed earlier[1,2], we have elucidated the structures of a novel series of related oligosaccharides isolated from milk of the tammar wallaby.

Chemical and enzymic studies. — The milk carbohydrates were fractionated on Sephadex G-25 into 8 peaks of neutral oligosaccharides[1]. Paper chromatography indicated that the tetra-, penta-, hexa-, and hepta-saccharide peaks (peaks 4–7, respectively, of Fig. 1 of ref. 1) each contained one major component and a minor component having greater chromatographic mobility. The components of each peak were fractionated by gel chromatography on Bio-Gel P-4, and we now report on the major components.

Hydrolysis (2M HCl, 1 h, 100°) of each purified oligosaccharide gave only galactose and glucose in the molar ratios 3:1, 4:1, 5:1, and 6:1, respectively. Hydrolysis of each borohydride-reduced oligosaccharide gave only galactose and glucitol (t.l.c.), indicating that glucose was the reducing residue of each parent saccharide. Hydrolysis following treatment with sodium periodate[1] showed that only the glucose and one of the galactose residues (presumably the one at the non-reducing end) had been oxidised, suggesting[3] that the galactose–galactose linkages were all (1→3).

Each oligosaccharide was resistant to the action of α-D-galactosidase, but was finally converted into a mixture of galactose and glucose by β-D-galactosidase, showing that all glycosidic linkages were β-D, and that glucose was at the reducing end. At an intermediate stage of the digestion with β-D-galactosidase, the tetrasaccharide (tetra) gave 3′-galactosyl-lactose (tri) and lactose (di); the pentasaccharide

TABLE I

CHEMICAL SHIFTS AND ASSIGNMENTS OF OLIGOSACCHARIDES[a]

Trisaccharide		*Tetrasaccharide*		*Chemical shifts of higher oligosaccharides*		
Chemical shift	*Assignment*[1]	*Chemical shift*	*Assignment*	*Penta*	*Hexa*	*Hepta*
105.2	C-1″	105.1	C-1‴	105.1	105.1	105.1
—		104.9	C-1″	104.8	104.8	104.8
103.4	C-1′	103.5	C-1′	103.4	103.4	103.4
96.6	C-1β	96.7	C-1β	96.6	96.6	96.6
92.7	C-1α	92.7	C-1α	92.7	92.7	92.7
—		82.9	C-3″	82.8	82.8	82.8
82.7	C-3′	82.7	C-3′	82.7	82.7	—
79.2	C-4α	79.2	C-4α	79.1	79.1	79.1
79.0	C-4β	79.1	C-4β	79.0	—	—
75.9	C-5′ + C-5″	75.9	C-5′ + C-5″ + C-5‴	75.9	75.9	75.9
75.6	C-5β	75.6	C-5β	75.5	75.5	75.5
75.2	C-3β	75.4	C-3β	75.2	75.2	75.3
74.7	C-2β	74.7	C-2β	74.6	74.6	74.6
73.4	C-3″	73.5	C-3‴	73.3	73.4	73.4
72.2	C-3α	72.2	C-3α	72.2	72.2	72.3
72.0	C-2α	72.1	C-2‴	72.0	72.0	71.9
71.9	C-2″	72.0	C-2α	71.9	71.9	71.8
71.0	C-2′	71.1	C-2′ + C-2″	71.1	71.1	71.1
70.9	C-5α	71.0	C-5α	71.0	—	—
—		69.5[c]	C-4″	—	—	—
69.4[b]	C-4″	69.4[c]	C-4‴	69.3	69.4	69.3
69.3[b]	C-4′	69.3[c]	C-4′	s[d]	69.2	s[d]
61.8	C-6′ + C-6″	61.9	C-6′ + C-6″ + C-6‴	61.8	61.7	61.7
61.1	C-6β	60.9	C-6β	60.9	60.9	60.9
60.9	C-6α	60.9	C-6α	60.8	60.8	60.9

[a] ^{13}C-Chemical shifts (p.p.m.) from internal 1,4-dioxane taken as 67.4 p.p.m. Carbon atoms in the reducing residue have no superscript, those in the second residue have one dash, those in the third residue two dashes, *etc.* [b] Assignments of these peaks may be reversed. [c] Assignments of these peaks may be interchanged. [d] s, Shoulder.

(penta) gave tetra, tri, and di; the hexasaccharide (hexa) gave penta, tetra, tri, and di; and the heptasaccharide gave hexa, penta, tetra, tri, and di (t.l.c.). This suggested that the oligosaccharides were members of a homologous series.

N.m.r. spectroscopy. — The ^{13}C-n.m.r., chemical-shift data for the trisaccharide 3′-galactosyl-lactose and the higher oligosaccharides are given in Table I; the chemical shifts of the tetrasaccharide resonances are very close to those of the trisaccharide.

The additional resonances in the tetrasaccharide spectrum are assigned to the extra, internal galactosyl residue 3, the carbon atoms of which are labelled C-1″, C-2″, *etc.*, in Table I. The assignment of these additional resonances are δ 104.9 (C-1″), 82.9 (C-3″), and 69.5 (C-4″). There are three resonances that have increased intensities in the tetrasaccharide spectrum, as compared with the trisaccharide spectrum, at δ 75.9, 71.1, and 61.9, due to the additional resonances for C-5″, C-2″,

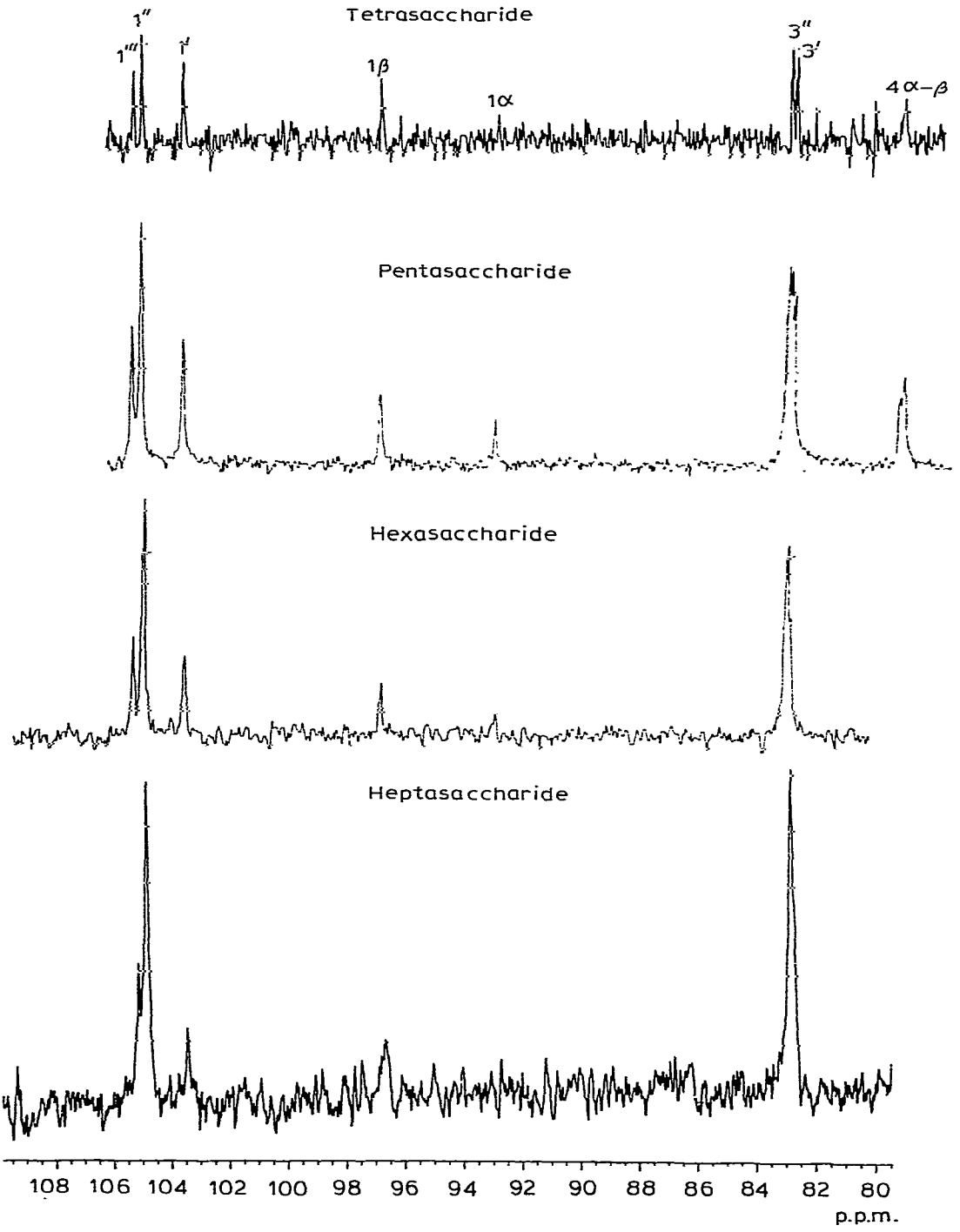

Fig. 1. ^{13}C-N.m.r. spectra at 67.5 MHz (downfield region) of oligosaccharides of the structure [β-D-Gal-(1→3)]$_n$-β-D-Gal-(1→4)-D-Glc, where n = 2–5.

and C-6″, respectively, from the third sugar residue. There is a small upfield shift from δ 105.1 to 104.9 of the C-1 resonance of a terminal galactose residue when it becomes an internal residue with a link at position 3.

The chemical shifts of the higher oligosaccharides are also given in Table I, and all of the resonances fit the scheme in which a β-D-Gal residue is added successively at position 3 of the non-reducing end of the chain. As this occurs, there is a progressive change in the relative intensities of resonances, which is best observed with the C-1 and the C-3 resonances and is shown in Fig. 1.

In the C-1 region of the spectrum (δ 103.4–105.1), three resonances are observed for the tetrasaccharide and higher oligomers. The downfield resonance at δ 105.1 is assigned to C-1 of the non-reducing, terminal residue, the middle resonance to C-1 of the internal galactosyl residues, and the peak at δ 103.4 to C-1 at the linkage between galactose and glucose. In the tetrasaccharide, height and area measurements indicate averaged ratios of 0.94 : 1.24 : 1 for these three resonances compared with the theoretical expectation of 1 : 1 : 1. As shown in Fig. 1, the height and area of the central resonance increases progressively as compared with the two outside resonances, as follows: 1.24 (tetra), 2.05 (penta), 2.44 (hexa), and 3.30 (hepta). Theoretically, the series 1 (tetra), 2 (penta), 3 (hexa), and 4 (hepta) would have been expected. The progressive reduction in the experimental values, as compared with those expected theoretically, requires some explanation.

The terminal galactose residue in stachyose has a longer T_1 value than the interior residues[4]. If the time between pulses were insufficient to allow complete relaxation of the nuclei, it would be expected that the signal from the C-1 atoms of the internal galactosyl residues would be enhanced as compared with the two signals from the terminal sugar residues. Just the opposite effect is observed, and hence this is not the explanation. The likely explanation is that, as the molecules increase in size, there is an increase in the correlation time τ_C of the nuclei, coupled with a progressive decrease in their n.O.e.[5]. As the internal galactosyl residues have the least mobility, they would experience the decrease in n.O.e. to a greater degree than the terminal residues, and thus give rise to the observed effect.

The effect of increased line-width is shown clearly in Fig. 1, in the C-3 series of resonances. As shown previously[1], the chemical shift of the C-3 resonance of a galactose residue moves 9–10 p.p.m. downfield when it becomes involved in a glycosidic linkage to another galactose residue. Thus, in the trisaccharide, C-3″ resonates at δ 73.4, and C-3′ at δ 82.7; the values for the tetrasaccharide are δ 82.7 (C-3′) and 82.7 (C-3″), and for the pentasaccharide δ 82.7 (C-3′), 82.74 (C-3″), and 82.86 (C-3‴). However, with the hexasaccharide, there are only two distinct resonances, and only one broad peak is observed for the heptasaccharide, due to line broadening.

Thus, the ^{13}C-n.m.r. data allow complete identification of the higher oligosaccharides up to the heptasaccharide [β-D-Gal-(1→3)]$_5$-β-D-Gal-(1→4)-D-Glc. The limit of the ability of the ^{13}C-n.m.r. technique to distinguish between higher oligosaccharides of the same series has not yet been reached, because it is still possible to distinguish the peaks at δ 104.8 and 105.1, and to measure the relative heights of the

peaks in the C-1 region. However, because of the progressive increase in τ_C values, with consequent decrease in T_2 values and increase in line widths of resonance resultant on increase of molecular size, the limit may perhaps be reached at about a decasaccharide.

EXPERIMENTAL

Extraction of the carbohydrates from tammar-wallaby milk and gel chromatography on Sephadex G-25 were done as described previously[6]. The contents of peaks 4–7 (Fig. 1 of ref. 1) were isolated by freeze-drying and then passed through 2 columns (1.1 × 150 cm) of Bio-Gel P-4 (minus 400 mesh), connected in series, with water as the eluant. Fractions (1.5 ml) were analysed for hexose by a modified[7] phenol–sulphuric acid procedure. Two peaks were obtained in each fractionation. The contents of the major peak (eluted after a minor peak) were isolated by freeze-drying. The dried products gave $[\alpha]_D^{23}$ +48°, +42°, +39°, and +37° (*c* 1, water) for the tetra-, penta-, hexa-, and hepta-saccharides, respectively.

Paper chromatography (descending) was performed on Whatman No. 1 paper with ethyl acetate–pyridine–water (12:5:4) and detection with alkaline silver nitrate. ^{13}C-N.m.r. spectroscopy, t.l.c., monosaccharide analysis, borohydride reduction, periodate oxidation, and the experiments with galactosidases were performed as described previously[1].

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