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Bi-antennary oligo-(*N*-acetyllactosamino)glycans of I-type are galactosylated preferentially at the GlcNAcβ1-6Gal linked arms by α1,3-galactosyltransferase of bovine thymus

ANTTI SEPPO[1], LEENA PENTTILÄ[1], ANNE LEPPÄNEN[1], HANNU MAAHEIMO[1], RITVA NIEMELÄ[1], JARI HELIN[1], JEAN-MICHEL WIERUSZESKI[2] and OSSI RENKONEN[1]*

[1]*Institute of Biotechnology and Department of Biochemistry, University of Helsinki, Valimotie 7, 00380, Helsinki, Finland*

[2] *Laboratoire de Chimie Biologique and U.M.R. No. 111 du C.N.R.S., Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France*

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α1,3-Galactosylation of radiolabelled bi-antennary acceptors Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Gal-R (*R* = 1-OH, β1-4GlcNAc or β1-4Glc) with bovine thymus α1,3-galactosyltransferase was studied. At all stages of the reactions the three acceptors reacted faster at the 1 → 6 linked arm than at the 1 → 3 linked branch. Hence, in addition to the doubly α1,3-galactosylated products, practically pure Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Gal-R could be obtained from the three acceptors in reactions that had proceeded to near completion. The isomeric mono-α1,3-galactosylated products were identified by using exoglycosidases to remove the branches unprotected by α1,3-galactoses and by subsequently identifying the resulting linear glycans chromatographically.

Keywords: α1,3-Galactosyltransferase, branch specificity, oligo-(*N*-acetyllactosaminoglycans), wheat germ agglutinin

Abbreviations: Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Lac, lactose; LacNAc, Galβ1-4GlcNAc; MH, maltoheptaose; MP, maltopentaose; MT, maltotriose; MTet, maltotetraose; WGA, wheat germ agglutinin; 3′, position 3 of the galactose unit of LacNAc or Lac; 6′, position 6 of the galactose unit of LacNAc or Lac.

Introduction

Enzymatic *in vitro* synthesis of oligosaccharides in reactions of Leloir type [1], using sugar nucleotides and transferase enzymes [2–4], has been studied in the past mainly in the analytical tradition of biochemistry, while only occasional applications focusing on construction [5, 6] have been reported. More recently, the discovery of important biofunctions of oligosaccharides in cellular adhesion, for instance, has prompted an increasing interest in developing enzyme-aided synthesis of glycans [7–12].

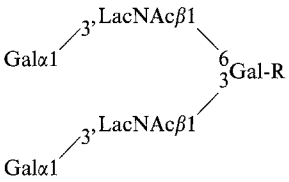
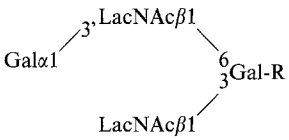
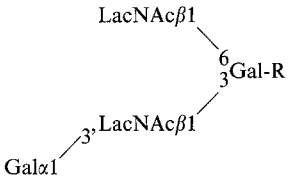
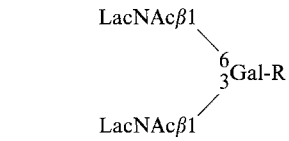
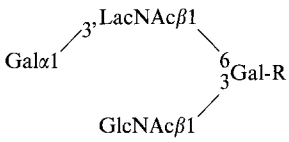
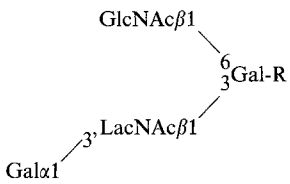
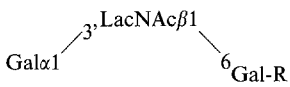
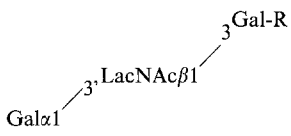
Here we describe reactions of bi-antennary oligo-(*N*-acetyllactosamino)-glycans **4**, **12** [13] and **20** [14] of I-type (see Table 1 for the structures of key saccharides) with UDP-galactose and α1,3-galactosyltransferase of bovine thymus [15, 16]. The α-galactosylation experiments show that conducting the reactions to near completion gives two kinds of saccharides, the doubly α1,3-galactosylated glycans, and the mono-α-galactosylated products carrying the single α1,3-linked galactose solely at the 1 → 6 bonded arm of the bi-antennary acceptors. Thus, the 'branch specificity' of the α1,3-galactosyltransferase is different from that of β1,3-*N*-acetylglucosaminyltransferase from human serum [13] or from α2,6-sialyltransferase from pork liver [8]. This suggests that 'branch specificity' is not only dictated by molecular properties of the acceptor saccharides but also by the binding properties of different glycosyltransferases.

Given the variety of different branch specificities among different glycosyltransferases it is now possible selectively to cap and/or elongate just one of the branches of I-type glycans, providing an *in vitro* biosynthetic way to unsymmetrical saccharide structures.

The synthesis products, representing mixtures of isomeric bi-antennary glycans, are analysed in the present experiments by converting them to linear isomers. These proved to be easier to separate than branched isomers.

* To whom correspondence should be addressed.

Table 1. Paper chromatographic mobilities of oligosaccharides in solvent A.

Oligosaccharide core	**R** = β1-4GlcNAc	R_{MTet}	R_{MP}	R_{MH}	**R** = 1-OH	R_{MT}	R_{MTet}	R_{MP}	R_{MH}	**R** = β1-4Glc	R_{MTet}	R_{MP}	R_{MH}
	**1**		0.28	0.60	**9**			0.34	0.70	**17**		0.22	0.46
	**2**		0.43	0.91	**10**			0.53	1.10	**18**		0.34	0.72
	**3**		0.43	0.91	**11**			0.53	1.10	**19**		0.34	0.72
	**4**	0.46	0.72	1.48	**12**			0.81	1.66	**20**		0.57	1.18
	**5**		0.81	1.68	**13**			0.88	1.80	**21**		0.59	1.19
	**6**		0.81	1.68	**14**			0.88	1.80	**22**		0.59	1.19
	**7**	0.67	1.03	2.06	**15**	0.52	0.80	1.21		**23**	0.53	0.83	1.72
	**8**	0.82	1.22	2.50	**16**	0.64	1.02	1.51		**24**	0.57	0.89	1.88

Note: R_{MT}, R_{MTet}, R_{MP} and R_{MH} give 'standard' chromatographic mobilities in relation to maltotriose, -tetraose, -pentaose and heptaose, respectively. The 'standard' values represent means of several independent observations; sometimes even from different kinds of experiments. In contrast, the mobilities of marker saccharides constructed by enzyme aided synthesis and given in the text, mainly in the materials and methods section, mostly derive from a single experiment.

Materials and methods

α1,3-Galactosyltransferase reaction

Bovine thymus α1,3-galactosyltransferase was isolated as described by Blanken and van den Eijnden [17] up to the α-lactalbumin agarose step. Starting with 270 g of fresh bovine thymus (obtained from a local slaughterhouse) 125 mU of α1,3-galactosyltransferase was obtained in a volume of 10 ml. Enzyme activity measurement and unit definition is according to Blanken and van den Eijnden [17]. The α1,3-galactosyltransferase-reaction was carried out essentially according to [17]. Typical reaction mixtures contained in 50 µl, 0.1 M sodium cacodylate pH 6.0, 0.05 M $MnCl_2$, 0.8% TX-100, 50 µg bovine serum albumin, 100 mM UDP-Gal and 1.1 mU of α1,3-galactosyltransferase, together with specified amounts of the radiolabelled acceptor. The reactions were terminated by adding a 10-fold volume of 0.02 M EDTA, and by boiling for 3 min. Buffer salts and the detergent were removed by passing the mixture in water through a bed of AG 1 (AcO^-) and AG 50 W (H^+) ion exchange resins*. The eluent was lyophilized and subjected to paper chromatography. The yield of labelled Galα1-3[^{14}C]Galβ1-4GlcNAc by using this procedure was greater than 90%.

Oligosaccharides

Unlabelled saccharides. D-Galactose, lactose, and the malto-oligosaccharides were from Sigma (St Louis, MO, USA). Lacto-*N*-neotetraose and lacto-*N*-neohexaose were from BioCarb (Lund, Sweden). Galα1-3Gal was a gift from Professor Winifred Watkins (Royal Postgraduate Medical School, Hammersmith Hospital, London, UK).

Radiolabelled markers and acceptors for the experiments of Table 1. The bi-antennary hexasaccharide Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**4**) was constructed as described earlier [18]. A mixture of equal amounts of the isomeric bi-antennary hexasaccharides GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**5**) and Galα1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**6**) was constructed by cleaving **4** partially with *E. coli* β-galactosidase (EC 3.2.1.23), isolating the resulting pentasaccharide fraction that is known to contain equal amounts of the isomeric GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc and Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc [18], and by α1,3-galactosylating this mixture with UDP-galactose and α1,3-galactosyltransferase from bovine thymus essentially as described above. Paper chromatography revealed only one product peak (R_{MP} = 0.83; R_{MH} = 1.67, solvent A) and no remaining acceptor, which implies that both pentasaccharide isomers had reacted completely.

* In early experiments the detergent was removed by passing the reaction mixture in water through a 100 mg-column of Bond-Elut C-18 (Analyti-Chem International, Harbor City, CA, USA).

The linear pentasaccharide Galα1-3[^{14}C]Galβ1-4GlcNAcβ1-6[^{14}C]Galβ1-4GlcNAc (**7**) (R_{MTet} = 0.68; R_{MP} = 1.05, solvent A) was constructed by α1,3-galactosylating [^{14}C]Galβ1-4GlcNAcβ1-6[^{14}C]Galβ1-4GlcNAc derived from murine embryonal carcinoma cells (PC 13) [19]. The linear pentasaccharide Galα1-3[^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc (**8**) (R_{MTet} = 0.81; R_{MP} = 1.26, solvent A) was constructed by α1,3-galactosylating [^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc [20]; isotopic isomers of radiolabelled **8** having the same chromatographic mobility were also isolated and characterized from partial acid hydrolysates of metabolically labelled poly-(*N*-acetyllactosamino)glycans of murine embryonal carcinoma cells.

The bi-antennary pentasaccharide Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)[^{14}C]Gal (**12**) was constructed as described earlier [18]. The linear tetrasaccharide Galα1-3[^{14}C]Galβ1-4GlcNAcβ1-6Gal (R_{MTet} = 0.79; R_{MP} = 1.22, solvent A) was constructed by α1,3-galactosylating [^{14}C]-Galβ1-4GlcNAcβ1-6Gal [19]. The linear tetrasaccharide Galα1-3Galβ1-4[^{14}C]GlcNAcβ1-3Gal (R_{MT} = 0.65; R_{MTet} = 1.01; R_{MP} = 1.56, solvent A) was constructed by α1,3-galactosylating the trisaccharide Galβ1-4[^{14}C]GlcNAcβ1-3Gal, that had been prepared as described in [19].

The bi-antennary hexasaccharide lacto-*N*-neohexaose (**20**) was purchased from BioCarb (Lund, Sweden); it was converted to the isotopic isomer [^{3}H]Galβ1-4GlcNAcβ1-3([^{3}H]Galβ1-4GlcNAcβ1-6)Galβ1-4Glc (R_{MP} = 0.57; R_{MH} = 1.19, solvent A) as described in [21]. The conversion was effected by a cleavage with jack bean β-galactosidase (see below) to GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glc and subsequent β1,4-[^{3}H]galactosylation with UDP-[^{3}H]Gal and β1,4-galactosyltransferase (EC 2.4.1.90) from bovine milk (Sigma). The branched hexasaccharide Galα1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glc (**22**) (R_{MP} = 0.60, solvent A) was constructed from the linear pentasaccharide Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc with UDP-GlcNAc and the midchain β1,6-GlcNAc-transferase [22]. The linear pentasaccharide Galα1-3[^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc (**23**) (R_{MP} = 0.82; R_{MH} = 1.72, solvent A) was constructed by α1,3-galactosylating the linear tetrasaccharide [^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc [20]. The linear pentasaccharide Galα1-3[^{3}H]Galβ1-4GlcNAcβ1-3Galβ1-4Glc (**24**) (R_{MP} = 0.88; R_{MH} = 1.81, solvent A) was constructed by α1,3-galactosylating the linear tetrasaccharide [^{3}H]Galβ1-4GlcNAcβ1-3Galβ1-4Glc that had been obtained from lacto-*N*-neotetraose (BioCarb) by a treatment with jack bean β-galactosidase followed by β1,4-[^{3}H]galactosylation.

Glycosidase digestions

Digestions with jack bean β-*N*-acetylhexosaminidase (EC 3.2.1.30) (Sigma) were carried out in 45 µl reaction mixtures containing the radiolabelled substrate, 150 mU of the enzyme, 0.05 M sodium citrate, pH 4.0, and 10.5 mg ml^{-1} of γ-galactonolactone. The mixtures were incubated at 37 °C

for 6–16 h. Under these conditions the enzyme cleaved GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc completely, but [U-^{14}C]Galβ1-4GlcNAc remained intact.

Hydrolysis with β-galactosidases (EC 3.2.1.23) from jack bean (Sigma), and from *D. pneumoniae* (Boehringer) were carried out as described [19], the *E. coli* enzyme was used as described in [18].

Hydrolysis with α-galactosidase (EC 3.2.1.22) from green coffee beans (Sigma) was conducted by incubating the saccharides with 1 U of the enzyme for 7 h at 37 °C in 0.1 ml of citrate-phosphate buffer, pH 6.6 [23, 24]. Under these conditions [^{14}C]Galβ1-4GlcNAc was not degraded.

Hydrolysis with endo-β-galactosidase (EC 3.2.1.103) of *Escherichia freundii* was carried out as described in [19].

Chromatographic methods

Paper chromatography of radiolabelled oligosaccharides was carried out in the descending mode using Whatman No III Chr paper with the upper phase of n-butanol:acetic acid:water, 4:1:5 by volume (Solvent A), with n-butanol: ethanol:water, 10:1:2 by volume (Solvent E), or with n-butanol:acetic acid:water, 10:3:7 by volume (Solvent F), as described earlier [19], but the counting was carried out in OptiScint 'Hisafe' from LKB, Uppsala, Sweden. Each sample lane was flanked on both sides by marker lanes.

WGA-agarose chromatography was carried out as described [25]. Having observed that the sample size, and also the age of the lectin column exert an influence on the mobility of saccharides, samples of approximately equal size were used and the experiments were performed at not too distant dates.

Gel filtration on Bio-Gel P-10 was performed as in [26].

[^{1}H]-NMR spectroscopy

Prior to NMR-measurements the oligosaccharide samples were repeatedly dissolved in 99.9% D_2O (C.E.A., France) and lyophilized. Finally the samples were dissolved in 0.45 ml of 99.96% D_2O (C.E.A., France) and transferred into a $\varnothing$ 5 mm NMR-tube. Spectra were recorded with a Bruker AM-400 spectrometer at 298 K.

Partial acid hydrolysis

Partial acid hydrolysis of the oligosaccharides with 0.1 M trifluoroacetic acid (100 °C, 40 min) was carried out as described earlier [27].

Results

The Galα1-3Gal linkage is generated by bovine thymus α-galactosyltransferase even in the reaction with Galβ1-4GlcNAcβ1-6Galβ1-4Glc

The linkage generated by the bovine thymus α1,3-galactosyltransferase [16, 17] has been studied previously in detail by methylation analysis, exoglycosidase digestions, and ^{1}H-NMR spectroscopy. These methods have revealed the formation of a Galα1-3Gal sequence at the non-reducing termini of Galβ1-4GlcNAc, Galβ1-4Glc, paragloboside and asialo-α_1-acid glycoprotein [16, 28, 29]. In the present experiments confirmatory product identification was needed, because we wanted to study acceptors that contain the non-reducing sequence LacNAcβ1-6Gal, not previously analysed. To this end, the characterization of the product obtained in the α1,3-galactosyltransferase reaction with [^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc was undertaken. In paper chromatography the pentasaccharide product migrated at a distinct position ($R_{MP} = 0.79$; $R_{MH} = 1.60$, solvent A) more slowly than the tetrasaccharide acceptor ($R_{MP} = 1.26$, solvent A). The newly generated pentasaccharide was stable against the action of jack bean β-galactosidase, and completely cleaved by α-galactosidase of green coffee beans (not shown). Partial acid hydrolysis of the pentasaccharide product gave a mixture of [^{14}C]-labelled saccharides that were resolved in seven clearly separated peaks by paper chromatography in solvent A (Fig. 1A). The disaccharide in Peak 5 chromatographing like Galα1-3Gal marker, was oxidized with periodate and subsequently hydrolysed with acid to give [^{14}C]lyxose in 30% yield (data not shown). This established that the disaccharide was indeed Galα1-3[U-^{14}C]Gal [31], proving that the pentasaccharide was Galα1-3[U-^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc.

Partial α-galactosylation of Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**4**)

The structures of key saccharides of this experiment, as well as their chromatographic mobilities are collected in the left section of Table 1 (structures **1**–**8**).

A sample of Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**4**) (20 000 cpm, 37 pmol) was incubated with UDP-galactose (1.6 µmol) and α1,3-galactosyltransferase of bovine thymus (1.1 mU) for 5 min. Paper chromatography of the resulting mixture separated into three peaks representing the unreacted hexasaccharide acceptor (**4**), the mono-α-galactosylated heptasaccharide products (**2** and **3**) and the doubly α-galactosylated octasaccharide product (**1**) (Fig. 1B). Quantitation of the three peaks revealed that 27% of available acceptor sites of **4** had reacted.

The relative amounts of the two isomeric heptasaccharide components were determined by cleaving the mixture sequentially with jack bean β-galactosidase and β-*N*-acetylhexosaminidase, whereafter the resulting pair of isomeric pentasaccharides could be separated chromatographically. Fig. 1C shows the paper chromatogram obtained from the β-galactosidase digest – a complete cleavage had been effected, yielding a hexasaccharide fraction that migrated like a constructed marker mixture containing equal amounts of radiolabelled **5** and **6**. The hexasaccharide fraction was cleaved further with β-*N*-

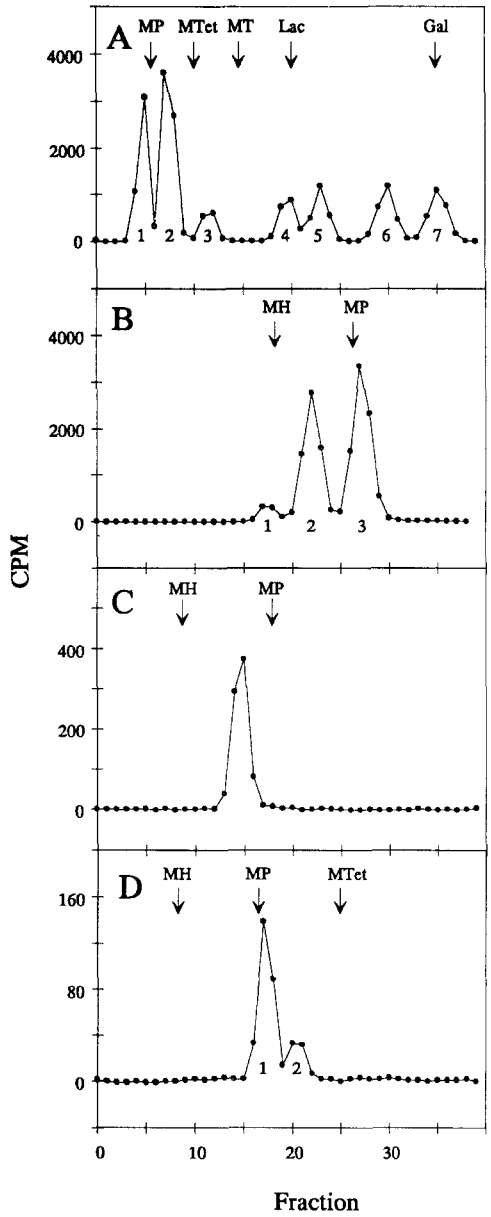


Figure 1. Paper chromatograms related to oligosaccharide constructs.

(A) A partial acid hydrolysate of enzymatically constructed Galα1-3[^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc run for 65 h with solvent A. Peak 1 represents the original pentasaccharide, peak 2 is a mixture of tetrasaccharides [^{14}C]Galα1-3[^{14}C]Galβ1-4GlcNAcβ1-6Gal and [^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc, peak 3 migrates like [^{14}C]Galβ1-4GlcNAcβ1-6Gal, peak 4 behaves like Galα1-3[^{14}C]Galβ1-4GlcNAc, peak 5 represents Galα1-3[^{14}C]Gal, peak 6 is [^{14}C]Galβ1-4GlcNAc, and peak 7 represents [^{14}C]Gal. The arrows marked Gal, Lac, MT and MP show the positions of galactose, lactose, maltotriose and maltopentaose.

(B) Products from a short α1,3-galactosylation reaction of Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**4**). Peak 1 represents the di-α1,3-galactosylated product **1**; peak 2 is a mixture of two isomeric mono-α1,3-galactosylated products (**2** and **3**), while peak 3 is the unreacted hexasaccharide (**4**) (R_{MP} = 1.00; R_{MH} = 1.47, solvent F). The arrows MP and MH represent maltopentaose and maltoheptaose markers, respectively. Solvent F; 60.5 h. (See Table 1 for the structural formulae.)

(C) β-Galactosidase digest of an aliquot (1030 cpm) of the heptasaccharide fraction from Fig. 1B. The peak at fraction 15 represents a mixture of the isomeric hexasaccharides GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**5**) and Galα1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**6**). The peak of the intact substrate would be located at position 7.7, if present. Markers are as above; Solvent A; 143 h.

(D) β-*N*-Acetylhexosaminidase digest of an aliquot (399 cpm) of the hexasaccharide fraction from Fig. 1C. Markers are as above; Solvent A; 137.5 h.

acetylhexosaminidase. Fig. 1D shows that two major components were obtained: Peak 1, representing 75% of total radioactivity, migrated like the marker Galα1-3Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc (**7**), while Peak 2 (25% of the label) chromatographed like Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc marker (**8**). These data show that Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**2**) was the major component in the original heptasaccharide fraction of Fig. 1B.

α1,3-Galactosylation experiments of 30 min and 120 min filled 72% and 96%, respectively, of the acceptor sites of **4** (not shown). At these later stages of the reaction, mainly the two heptasaccharides **2** and **3** served as acceptors being converted into the octasaccharide **1**. The two advanced reaction mixtures yielded heptasaccharide fractions consisting of 90% and 97%, respectively, of the isomer Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**2**) (data not shown). Hence, the heptasaccharide isomer **3** was *consumed* faster than **2** in the well advanced α1,3-galactosylation reactions. The hallmark of **3** is the reactive 1 → 6 linked LacNAc-branch.

The above data were confirmed by deriving the linear pentasaccharides (**7**/**8**) as above from an α1,3-galactosyltransferase reaction of **4** that had proceeded to 90% completion and subjecting it to chromatography on immobilized wheat germ agglutinin (WGA). As shown in Fig. 2A, the product chromatographed as a pure component that was bound to the lectin as the synthetic marker **7** requiring 0.2 M GlcNAc for elution as opposed to the isomeric **8** that migrated very close to the void volume (data not shown). Hence, the WGA experiments confirmed the paper chromatography data, establishing that the heptasaccharide fraction obtained from the well advanced α-galactosylation reactions of **4** represented pure Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4GlcNAc (**2**).

Partial α-galactosylation of Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)[^{14}C]Gal (**12**)

The structures and chromatographic mobilities of key saccharides of this experiment are collected in the middle section of Table 1 (structures **9**–**16**).

The pentasaccharide acceptor Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)[^{14}C]Gal (**12**) was partially α1,3-galactosylated by controlling the extent of the reactions kinetically as above. Three reaction mixtures were prepared, in which the extent of the α-galactosylation was 5%, 28%, and 87%, respectively, as judged from paper chromatography (not shown). The hexasaccharide fractions from each reaction, representing one or both of the mono-α1,3-galactosylated products **10** and **11**, were subjected to β-galactosidase treatments yielding pentasaccharides **13** and/or **14**, and finally cleaved with β-*N*-acetylhexosaminidase. The resulting tetrasaccharide fractions contained mostly Galα1-3Galβ1-4GlcNAcβ1-6[^{14}C]Gal (**15**) as judged by paper chromatography (not shown). The identity of the tetrasaccharide derived from the hexasaccharide fraction from the most advanced α-galactosylation reaction of **12** was confirmed as **15** also by WGA-agarose chromatography (Fig. 2B), which separates **15** and **16** convincingly.

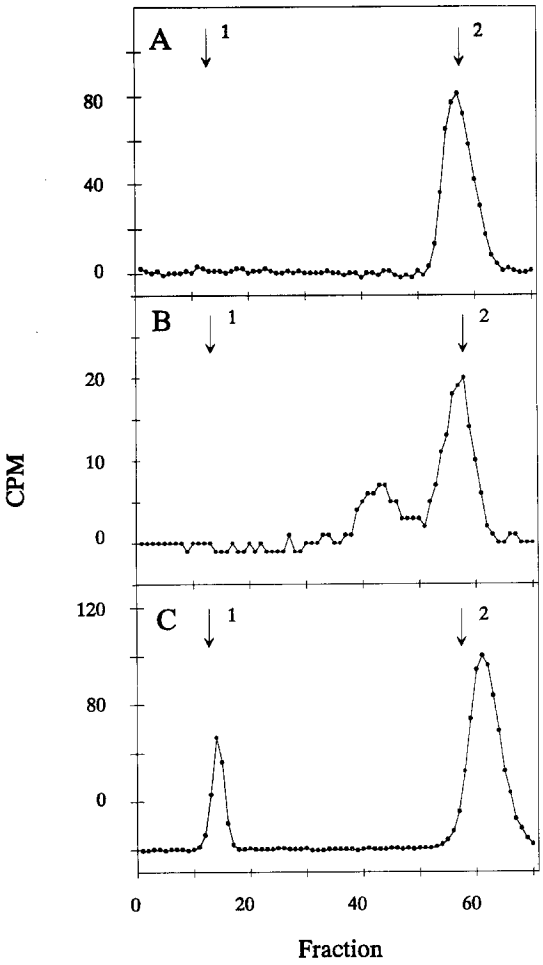


Figure 2. Affinity chromatograms on WGA-agarose.

(A) The linear pentasaccharide Galα1-3[^{3}H]Galβ1-4GlcNAc-β1-6Galβ1-4GlcNAc derived from a heptasaccharide fraction of a well advanced α-galactosylation reaction of [^{3}H]Galβ1-4GlcNAc-β1-3([^{3}H]Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc (**4**). Elution of

Figure 2 (*cont.*)

fractions 1–40 was carried out with sugar-free buffer, 0.2 M *N*-acetylglucosamine was added to the eluent at fraction 41. The peak positions of calibration markers, galactose and reduced *N,N',N''*-triacetylchitotriose are indicated by arrows marked 1 and 2, respectively.

(B) The tetrasaccharide Galα1-3Galβ1-4GlcNAcβ1-6[^{14}C]Gal (**15**) derived from the hexasaccharide Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)[^{14}C]Gal (**10**) by sequential treatments with β-galactosidase and β-*N*-acetylhexosaminidase, and isolated by paper chromatography. An identical elution profile was obtained from the marker Galα1-3[^{14}C]Galβ1-4GlcNAcβ1-6Gal, prepared by direct enzyme-aided synthesis (not shown). The double peak profile is characteristic to WGA-agarose chromatograms of several pure oligosaccharides possessing the reducing end sequence GlcNAcβ1-6Gal [27]. It is believed to be caused by different retardation of the mutarotational isomers of the saccharide by the lectin. The isomeric tetrasaccharide Galα1-3Galβ1-4GlcNAcβ1-3Gal marker (**16**) gave a WGA-chromatogram revealing a peak at Fraction 14, right after the void volume of the column (not shown). Elution conditions and markers are as above.

(C) The [^{3}H]pentasaccharide fraction obtained by sequential β-galactosidase and β-*N*-acetylhexosaminidase treatments from the [^{3}H]heptasaccharide mixture containing [^{3}H]Galβ1-4GlcNAcβ1-3(Galα1-3[^{3}H]Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4Glc (**18**) and Galα1-3Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)[^{14}C]Galβ1-4Glc (**19**). Peak 1 represents Galα1-3Galβ1-4GlcNAcβ1-3[^{14}C]-Galβ1-4Glc while peak 2 is Galα1-3Galβ1-4GlcNAcβ1-6[^{14}C]-Galβ1-4Glc as shown by WGA agarose chromatography of the appropriate marker saccharides. Elution conditions and markers are as above.

When combined, our data show that the hexasaccharide **10** represented 74%, 79% and 95% of the total hexasaccharides at the early, middle and late stage of the α1,3-galactosylation reaction of **12**, respectively. Hence, the α1,3-galactosyltransferase preferred as acceptor the 1,6-linked branch of **12**, rather then the 1,3-linked arm. The data show that even among the mono-α-galactosylated glycans **10** and **11** the enzyme preferred **11**, the substrate with an 'unused' acceptor site at the 1,6-linked arm.

Partial α-galactosylation of [^{3}H]Galβ1-4GlcNAcβ1-3([^{3}H]Galβ1-4GlcNAcβ1-6)Galβ1-4Glc (**20**)

The structures and the chromatographic mobilities of key saccharides of this experiment are collected in the right section of Table 1 (structures **17**–**24**).

The radiolabelled lacto-*N*-neohexaose [^{3}H]Galβ1-4GlcNAcβ1-3([^{3}H]Galβ1-4GlcNAcβ1-6)Galβ1-4Glc (**20**) (770 000 dpm, 9 pmol) was mixed with 233 nmol of the unlabelled **20**, and incubated overnight at 37 °C with 1900 nmol UDP-Gal and 310 μU of bovine thymus α1,3-galactosyltransferase. Only 8.5% of the available acceptor sites of **20** reacted under these conditions, yielding a [^{3}H]heptasaccharide fraction representing **18** and/or **19**.

A β-galactosidase treatment of the [^{3}H]heptasaccharide fraction released 51% of total label as [^{3}H]galactose (not shown). The remaining [^{3}H]hexasaccharide fraction was treated with β-*N*-acetylhexosaminidase. The product migrated in paper chromatography as a relatively broad peak (R_{MP} = 0.83; R_{MP} = 1.74, solvent A) at the same position as the Galα1-3[^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc marker (**23**). In order to distinguish this from the isomeric Galα1-3[^{3}H]Galβ1-4GlcNAcβ1-3Galβ1-4Glc (**24**) (R_{MP} = 0.89; R_{MH} = 1.88, solvent A), the [^{3}H]pentasaccharide fraction was subjected to WGA-agarose chromatography, where it separated into two distinct peaks (Fig. 2C). The minor component (19% of total label) behaved like Galα1-3[^{3}H]Galβ1-4GlcNAcβ1-3Galβ1-4Glc (**24**), while the major component (81% of the label) chromatographed like Galα1-3Galβ1-4GlcNAcβ1-6Galβ1-4Glc (**23**). Put together, our data show that in the beginning of the reaction, the enzymic α1,3-galactosylation of the hexasaccharide **20** generated about 81% of **18** and 19% of **19**.

A one dimensional ^{1}H-NMR spectrum of the above mixture of the heptasaccharides **18** and **19** was recorded at 400 MHz in D_2O at 298 K. The β-galactose anomeric proton region (4.40–5.55 ppm) of the spectrum revealed two sets of signals in 8:2 intensity ratio (see Table 2 for chemical shift values) while the rest of the anomeric proton region showed signals of intensity 10. By comparison with a ^{1}H-NMR spectrum of **20** from [30], the major and minor components could be assigned to **18** and **19**, respectively: The major component shows two 8 Hz doublets at 4.544 and 4.480 ppm, respectively. The latter can be assigned to H-1 of a nonsubstituted terminal β-galactose at the 3-branch as in **20** while the former corresponds to H-1 of a 3-substituted subterminal β-galactose at the 6-branch. The minor component again shows two 8 Hz doublets at 4.553 and 4.470 ppm, respectively. These can be assigned to H-1 of a 3-substituted subterminal β-galactose at the 3-branch and H-1 of a nonsubstituted terminal β-galactose at the 3-branch, respectively. The glycosylation induced shifts of H-1 at subterminal galactoses in substituted branches of both components (+0.069 and +0.070 ppm) are in good agreement with experiments of van Halbeek *et al.* [28] suggesting α1-3 substitution. This notion is also supported by signals at 5.145 ppm (4 Hz doublet) and 4.192 ppm assigned to H-1 and H-5, respectively of αGal:s of both components, and also the appearance of a new Gal H-4 signal at 4.181 ppm corresponding to 3-substituted, subterminal galactoses of both components. The anomeric proton signals of the βGlcNAc:s and the lactose unit of both components coincide and are identical to signals of **20**.

An aliquot of an isolated radiolabelled heptasaccharide fraction containing about 80% of **18** and 20% of **19** (160 pmol) was subjected to a second round of partial α1,3-galactosylation using unlabelled UDP-Gal (5 μmol) as the donor. The reaction mixture revealed in paper chromatography 64% of the label as the octasaccharide (**17**), the rest behaving like the unreacted heptasaccharide fraction (data not shown).

Table 2. ^{1}H chemical shifts (δ) of oligosaccharides **18**, **19** and **20**.

Residue	*Proton*[a]	*Oligosaccharide*		
		18	**19**	**20**[b]
Glc	H-1α	5.220	5.220	5.220
	H-1β	4.665	4.665	4.664
	H-2β	3.292	3.292	3.246
Galβ	H-1	4.429	4.429	4.431
	H-4	4.145	4.145	4.145
^{3}GlcNAcβ	H-1(α)	4.707	4.707	4.706
	H-1(β)	4.703	4.703	
^{6}GlcNAcβ	H-1(α)	4.639	4.639	4.645
	H-1(β)	4.637	4.637	4.639
^{3}Galβ	H-1	4.480	4.552	4.483
	H-4	3.926	4.181	3.929
^{6}Galβ	H-1	4.543	4.471	4.475
	H-4	4.181	3.926	3.929
^{4,3}Galα	H-1	–	5.145	–
	H-5	–	4.192	–
^{4,6}Galα	H-1	5.145	–	–
	H-5	4.192	–	–

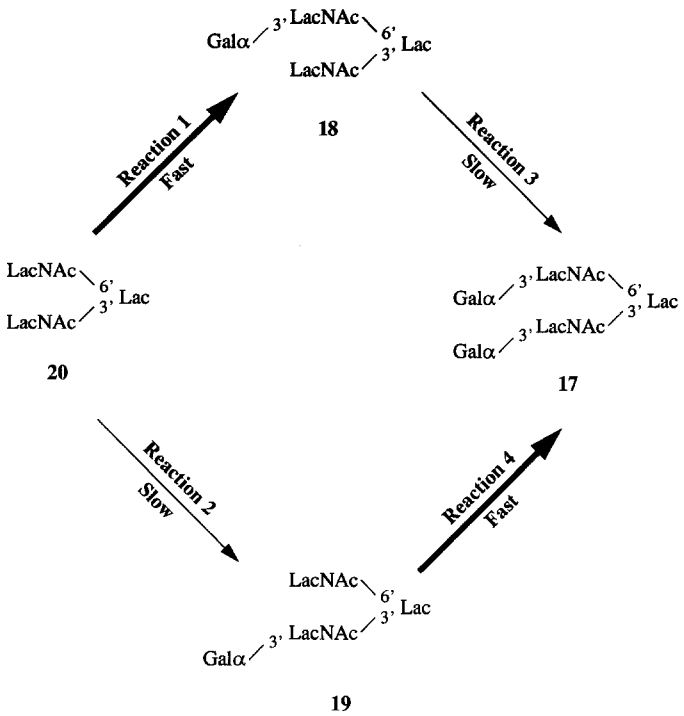
Note: Chemical shifts are expressed in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone set to 2.225 ppm.
[a] α and β refer to α- and β-anomers of the reducing end Glc.
[b] Data from [30].

For the compositional analysis, the isolated heptasaccharide fraction was degraded by β-galactosidase and further by β-*N*-acetylhexosaminidase into a pentasaccharide fraction. WGA-agarose chromatography confirmed that the pentasaccharide fraction consisted of pure **23**, yielding a single, highly retarded peak at the same position as the synthetic marker **23** (data not shown).

These data establish that the fraction of the heptasaccharide acceptors that reacted slowly with the α1,3-galactosyltransferase, surviving in the conditions of the partial reaction, represented a pure sample of **18**.

Discussion

The present experiments reveal the branch specificity of bovine thymus α1,3-galactosyltransferase towards branched oligo-(*N*-acetyllactosaminoglycans). A summary of the present data is presented in Scheme 1, showing that α1,3-galactosylation of the hexasaccharide acceptor **20** proceeds *via* two pathways to the octasaccharide **17**. As catalysed by bovine thymus α1,3-galactosyltransferase, the conversion of **20** to the heptasaccharide **18** (reaction 1) proceeds about four times faster than the conversion of **20** to the isomeric heptasaccharide **19** (reaction 2). Our data show further that the heptasaccharide **19** reacts intrinsically faster (reaction 4) than the isomeric heptasaccharide **18**



Scheme 1. The two pathways of α1,3-galactosylation of lacto-*N*-neohexaose (**20**) by bovine α1,3-galactosyltransferase. The glycans **4** and **12** react in the same way as **20**.

(reaction 3) to yield the octasaccharide **17**. This implies that the acceptor site residing in the more flexible 1 → 6 linked branch is the preferred one in the hexasaccharide **20** as well as in the heptasaccharide mixture of **18** and **19**.

Even the acceptors **4** and **12** were α1,3-galactosylated by the bovine thymus enzyme about three times faster at the 1 → 6 linked branches than at the 1 → 3 linked arms. The findings with the acceptor **12** are noteworthy because, using a completely different analytical approach based on selective acetolysis, Blanken *et al.* [31] reported in 1984 that in **12** the calf thymus enzyme prefers the 1 → 6 branch five times more than the 1 → 3 branch. Elices and Goldstein [32] found that in *N*-linked complex-type glycans the LacNAc units of the 1,6-linked Man arm react particularly fast.

The preferential reactivity of the bovine thymus α1,3-galactosyltransferase with the 1 → 6 branches of the I-type acceptors **4**, **12** and **20** contrasts strikingly with the equal reactivity of the 1 → 6 and 1 → 3 branches of the acceptors **4** and **12** in the reaction with β1,3-*N*-acetylglucosaminyltransferase of human serum [13]. The difference between the two enzymes is remarkable because they act at precisely the same site of the acceptors. The two enzymes must somehow differ in their recognition of the difference between glycosidic linkages to the branching galactose: the branching glycosidic linkages themselves may be included in the binding site of the α1,3-galactosyltransferase but perhaps not in that of the β1,3-GlcNAc transferase.

Immobilized α2,6-sialyltransferase from pork liver reacts with high preference at the 1 → 3 linked branch of Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-OMe [8]. The examples provided by the α1,3-galactosyl-, the β1,3-*N*-acetylglucosaminyl-, and the α2,6-sialyltransferases imply that all possible types of branch selectivities, ranging from high preference for the 3-branch to high preference for the 6-branch can be found in glycosyltransferases acting on bi-antennary oligo-(*N*-acetyllactosamino)glycans. The present data allow the preparation of pure bi-antennary glycans of the type represented by the saccharides **2**, **10** and **18**, each bearing an α1,3-linked galactose 'cap' at the 1 → 6 linked branch. Our previous work has provided a route to pure molecules of the type of the glycan **6** bearing an α1,3-galactose 'cap' at the 1 → 3 linked branch [22]. To generate pure oligosaccharides bearing different branches of increasing size, the pure isomers now obtainable can be easily elongated, branched and β1,4-galactosylated at the branches that are not 'capped' by the α1,3-linked galactose group.

Affinity chromatography in a small column of agarose-bound wheat germ agglutinin (WGA) was of considerable importance in establishing the identity of the linear α-galactosylated saccharides **7**, **8**, **15**, **16**, **23**, and **24** in the present experiments. The data show that the distal α1,3-linked galactose units acts as an enhancer of the oligosaccharide affinity to WGA-agarose, so that *Galα1-3*Galβ1-4GlcNAcβ1-6Galβ1-R (**7**, **15** or **23**) bound always more tightly in the present experiments than corresponding Galβ1-4GlcNAcβ1-6Galβ1R in our early experiments [19, 25]. Indeed, the saccharides **15** and **23** elute from WGA-agarose a little behind the alditol of *N*,*N*′,*N*″-triacetylchitotriose, showing strikingly the impact of the hexose residues on binding to the lectin column, that has been generally considered specific for *N*-acetylglucosamine and sialic acid.

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