



# $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\beta$ -D-Galp-containing oligosaccharides, novel products from lactose by the action of $\beta$ -galactosidase

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## Abstract

A mixture of oligosaccharides produced by  $\beta$ -galactosidase using lactose as a substrate was fractionated according to degree of polymerization using gel filtration, followed by high-pH anion-exchange chromatography. The fractions obtained were analyzed using monosaccharide analysis, methylation analysis, mass spectrometry, and NMR spectroscopy. Twelve novel non-reducing oligosaccharides were characterized, namely,  $[\beta$ -D-Galp-(1 $\rightarrow$ 4)]<sub>n</sub>- $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\beta$ -D-Galp[-(4 $\leftarrow$ 1)- $\beta$ -D-Galp]<sub>m</sub>, with  $n, m = (1, 2, 3, \text{ or } 4)$  and  $\beta$ -D-Galp-(1 $\rightarrow$ 2)- $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\beta$ -D-Galp. © 1998 Elsevier Science Ltd. All rights reserved.

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## 1. Introduction

Galacto-oligosaccharides, including the oligogalactosylated glucoses, are claimed to be beneficial for human health because they seem to promote the growth of bifidobacteria in the large intestine [1]. Several studies suggest that the volatile fatty acids resulting from the fermentation of galacto-oligosaccharides by the

intestinal microflora improve the absorption ability of the intestinal epithelium [2–4].

Oligogalactosylated glucoses can be prepared from lactose through the transgalactosylating activity of  $\beta$ -galactosidase (EC 3.2.1.23). Various parameters such as source of the enzyme, substrate concentration, pH, and temperature can influence the transgalactosylation and thereby the final yields of the different compounds formed [5]. The structures of oligogalactosylated glucoses reported so far range from dimers to pentamers [1,6–9]. Depending on the biological origin of the  $\beta$ -galactosidase applied, different linkages be-

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tween Gal and the reducing Glc unit have been identified, namely,  $\beta$ -D-Galp-(1 $\rightarrow$ 2)-D-Glcp,  $\beta$ -D-Galp-(1 $\rightarrow$ 3)-D-Glcp,  $\beta$ -D-Galp-(1 $\rightarrow$ 4)-D-Glcp, and  $\beta$ -D-Galp-(1 $\rightarrow$ 6)-D-Glcp. Branched Glc residues also occur, whereas the oligogalactose fragments contain mainly (1 $\rightarrow$ 4) or (1 $\rightarrow$ 6) linkages. So far oligogalactosylated glucose tetramers and pentamers have rarely been observed in large amounts.

Here, we report on a novel transgalactosylating activity of  $\beta$ -galactosidase leading to the formation of  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\beta$ -D-Galp elements in several oligogalactosylated glucoses.

## 2. Results

**Purification of oligosaccharides produced by  $\beta$ -galactosidase.**—The oligogalactosylated glucose sample supplied (Borculo Whey Products) was fractionated by gel-permeation chromatography using Fractogel TSK HW-40(S) followed by Bio-Gel P-2. Following this protocol, fractions of different degrees of polymerization were obtained. These pools of oligosaccharides were further fractionated by high-performance anion-exchange chromatography (HPAEC). In Fig. 1 HPAEC profiles of the starting oligogalactosylated glucose sample (upper panel) and of five Bio-Gel P-2 fractions (dimers to hexamers) are shown. The major components [ $(\beta$ -D-Galp-(1 $\rightarrow$ 4))<sub>n</sub>-D-Glcp, and  $(\beta$ -D-Galp-(1 $\rightarrow$ 6))<sub>n</sub>- $\beta$ -D-Galp-(1 $\rightarrow$ 4)-D-Glcp,  $n = 0$ –5] in each Bio-Gel P-2 fraction were characterized using HPAEC retention times and  $^1\text{H}$  NMR spectroscopy (data not shown). The oligogalactosylated glucose structures found were in agreement with those reported so far [5]. For the NMR analysis, use was made of the chemical shifts of linear  $\beta$ -(1 $\rightarrow$ 4)-linked D-galactobi- to pentaose (see Table 1). The  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of the latter compounds, which have not been reported so far in detail, are shown in Tables 1 and 2, respectively. Additionally to the known oligogalactosylated glucoses, each Bio-Gel P-2 fraction contained at least one less retarded component (Fig. 1, indicated by its fraction number) whose structure has not yet been reported. The structural characterization of these compounds is described in the following paragraphs.

**Characterization of Fractions 2.1 and 2.2.**—The pool of disaccharides contained, according to the HPAEC chromatogram, at least six components (Fig. 1). The  $^1\text{H}$  NMR spectrum of Fraction 2.2 indicated the presence of free Gal and Glc (data not shown). Fraction 2.1 contained a disaccharide consisting of Glc and Gal in a molar ratio of 1:1. Methylation analysis revealed the presence of equimolar amounts of terminal Galp and terminal Glcp. Electrospray ionization mass spectrometry (ESMS) analysis of the sodium borodeuteride-treated and permethylated Fraction 2.1 showed an ion at  $m/z$  477 (data not shown), corresponding to a sodium-cationized pseudo-molecular ion  $[\text{M} + \text{Na}]^+$  for nonreduced permethylated Hex<sub>2</sub>. The  $[\text{M} + \text{H}]^+$  ion at  $m/z$  455, observed in the FAB mass spectrum was used for a high energy collision experiment applying fast atom bombardment collision induced dissociation tandem mass spectrometry (FAB CID MS-MS). The most abundant and the only mass spectrometric sequence ion notable in the tandem mass spectrum was the B<sub>1</sub>

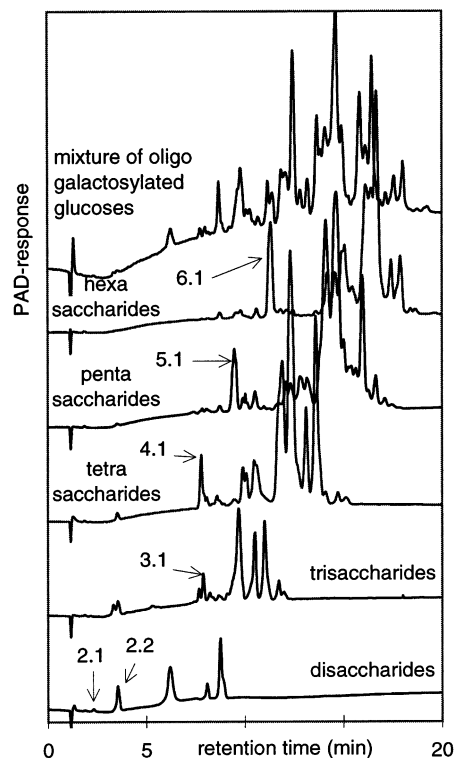


Fig. 1. HPAEC profiles of the starting galactosylated glucose sample (upper panel, as obtained from Borculo Whey Products) and of the different Bio-Gel P-2 fractions (disaccharides through hexasaccharides).

Table 1

<sup>1</sup>H chemical shifts of linear  $\beta$ -(1  $\rightarrow$  4)-linked galactobiose to pentaose (**1–4**; recorded at 300 K, at 500 or 600 MHz)<sup>a</sup>

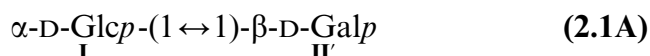
Compound	Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
Galactose <sup>b</sup>	$\alpha$ -D-Galp	5.265	3.825	3.875	4.005	4.105	3.805	3.725
	$\beta$ -D-Galp	4.585	3.502	3.665	3.945	3.715	3.805	3.725
<b>1</b> <sup>c</sup>	$\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\alpha$ -D-Galp	4.638	3.665	3.78	4.174	3.73	3.65–3.87	3.65–3.87
	4)- $\beta$ -D-Galp	5.271	3.88	3.95	4.23	4.13	3.81	3.72
<b>2</b> <sup>d</sup>	4)- $\beta$ -D-Galp	4.61	3.565	3.75	4.165	3.65–3.87	3.65–3.87	3.65–3.87
	$\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-Galp	4.595	3.591	3.665	3.905	3.77	3.65–3.87	3.65–3.87
	4)- $\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\alpha$ -D-Galp	4.638	3.665	3.78	4.174	3.73	3.65–3.87	3.65–3.87
	4)- $\alpha$ -D-Galp	5.271	3.88	3.95	4.23	4.13	3.81	3.72
<b>3</b> <sup>e</sup>	4)- $\beta$ -D-Galp	4.61	3.565	3.75	4.165	3.65–3.87	3.65–3.87	3.65–3.87
	$\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-Galp	4.597	3.592	3.665	3.906	3.767	3.65–3.87	3.65–3.87
	4)- $\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-Galp	4.645	3.68	3.78	4.175	3.65–3.87	3.65–3.87	3.65–3.87
	4)- $\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\alpha$ -D-Galp	4.634	3.661	3.78	4.167	3.65–3.87	3.65–3.87	3.65–3.87
	4)- $\alpha$ -D-Galp	5.271	3.883	3.949	4.227	4.126	3.65–3.87	3.65–3.87
<b>4</b> <sup>f</sup>	4)- $\beta$ -D-Galp	4.609	3.565	3.745	4.167	3.65–3.87	3.65–3.87	3.65–3.87
	$\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-Galp	4.599	3.592	3.66	3.907	3.65–3.87	3.65–3.87	3.65–3.87
	4)- $\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-Galp	4.64	3.65–3.70	3.78	4.17	3.65–3.87	3.65–3.87	3.65–3.87
	4)- $\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-Galp	4.64	3.65–3.70	3.78	4.17	3.65–3.87	3.65–3.87	3.65–3.87
	4)- $\beta$ -D-Galp-(1 $\rightarrow$ 4)- $\beta$ -D-Galp	4.64	3.65–3.70	3.78	4.17	3.65–3.87	3.65–3.87	3.65–3.87
	4)- $\alpha$ -D-Galp	5.273	3.885	3.95	4.228	3.65–3.87	3.65–3.87	3.65–3.87
	4)- $\beta$ -D-Galp	4.61	3.56	3.75	4.17	3.65–3.87	3.65–3.87	3.65–3.87

<sup>a</sup> Assignments were obtained from 2D DQF COSY, TOCSY and ROESY experiments.<sup>b</sup> Taken from Ref. [12].<sup>c</sup>  $\beta$ -D-Galp-(1  $\rightarrow$  4)-D-Galp.<sup>d</sup>  $\beta$ -D-Galp-(1  $\rightarrow$  4)- $\beta$ -D-Galp-(1  $\rightarrow$  4)-D-Galp.<sup>e</sup>  $\beta$ -D-Galp-(1  $\rightarrow$  4)- $\beta$ -D-Galp-(1  $\rightarrow$  4)- $\beta$ -D-Galp-(1  $\rightarrow$  4)-D-Galp.<sup>f</sup>  $\beta$ -D-Galp-(1  $\rightarrow$  4)- $\beta$ -D-Galp-(1  $\rightarrow$  4)- $\beta$ -D-Galp-(1  $\rightarrow$  4)- $\beta$ -D-Galp-(1  $\rightarrow$  4)-D-Galp.

ion at  $m/z$  219 [10]. This ion was accompanied by ions resulting from the loss of one and two methanol molecules from the  $B_1$  ion ( $m/z$  187 and 155) and the precursor ( $m/z$  423 and 391). Although the ion at  $m/z$  423 has the same mass as that expected for a  $B_2$  ion, its relatively low abundance is not consistent with its assignment as a  $B_2$  ion. All data are thus consistent with the presence of a (1  $\leftrightarrow$  1)-linked disaccharide.

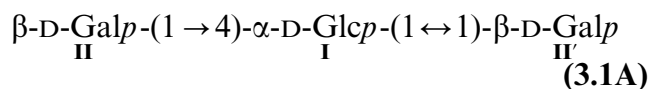
1D <sup>1</sup>H NMR analysis showed two anomeric signals at  $\delta$  5.230 and 4.571 (Fig. 2(A), Table 3) in the intensity ratio of 1:1. The signal at  $\delta$  5.230 (<sup>3</sup> $J_{1,2}$  3.7 Hz) had correlations to H-2,3,4,5,6a,6b in the TOCSY spectrum, identifying the  $\alpha$ -Glc residue. The signal at  $\delta$  4.571 (<sup>3</sup> $J_{1,2}$  7.6 Hz) only showed TOCSY cross-peaks to H-2,3,4; combined with the typical peak pattern of H-4 and the resemblance of the chemical shifts with those of the nonreducing residues in **1–4** (Table 1), the  $\beta$ -Galp residue could be assigned. Due to small <sup>3</sup> $J_{3,4}$  (2 Hz) and <sup>3</sup> $J_{4,5}$  (< 1 Hz) couplings no correla-

tions to H-5 could be seen. Consequently, Fraction 2.1 contains the following disaccharide:



In the ROESY spectrum an intense interresidual contact between the two anomeric protons was visible. The assignments of the carbon atoms as obtained from the seHSQC and HMBC spectra are given in Table 4. Notable are the chemical shift values for C-1 of Gal ( $\delta$  104.5) and Glc ( $\delta$  101.1), typical for a terminal  $\beta$ -Gal and a terminal  $\alpha$ -Glc residue. The HMBC spectrum showed two interresidual contacts (**I** H-1,**II'** C-1 and **II'** H-1,**I** C-1) and the H-1/C-1 coupling constants (Glc <sup>1</sup> $J_{C-1,H-1}$  174 Hz, Gal <sup>1</sup> $J_{C-1,H-1}$  163 Hz), confirming structure **2.1A**.

*Characterization of Fraction 3.1.*—HPAEC Fraction 3.1 (Fig. 1) contained as the major component compound **3.1A**:

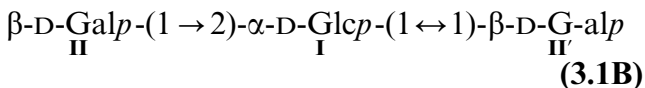


Quantitative monosaccharide analysis of Fraction 3.1 revealed Glc and Gal in a molar ratio of 1.0:1.9. Methylation analysis demonstrated the presence of terminal Galp and 4-substituted Glcp residues. In the ESMS spectrum of the sodium borodeuteride-treated and permethylated Fraction 3.1, an ion at *m/z* 681 was observed, corresponding to [M + Na]<sup>+</sup> for a nonreduced permethylated Hex<sub>3</sub>. The most abundant ions observed in the tandem mass spectrum obtained from the protonated derivatized oligosaccharide, *m/z* 659, were the B<sub>1</sub> and B<sub>2</sub> ions (*m/z* 219 and 423). The intense ion observed at *m/z* 455 corresponding in mass to fully methylated Hex<sub>2</sub> was assigned as resulting from ‘internal residue loss’ [11]. The very low abundance of the ion at *m/z* 627 corresponding to the loss of methanol from the precursor and having the same mass as a B<sub>3</sub> ion was consistent with the presence of a (1↔1) glycosidic linkage in Hex<sub>3</sub>.

1D <sup>1</sup>H NMR analysis of Fraction 3.1 (Fig. 2(B)) showed three, equally intense, anomeric signals at δ 5.228 (<sup>3</sup>*J*<sub>1,2</sub> 3.4 Hz, <sup>1</sup>*J*<sub>C-1,H-1</sub> 173 Hz), 4.576 (<sup>3</sup>*J*<sub>1,2</sub> 7.0 Hz, <sup>1</sup>*J*<sub>C-1,H-1</sub> 160 Hz), and 4.443 (<sup>3</sup>*J*<sub>1,2</sub> 7.9 Hz, <sup>1</sup>*J*<sub>C-1,H-1</sub> 163 Hz). Taking into account both the <sup>3</sup>*J*<sub>1,2</sub> and <sup>1</sup>*J*<sub>C-1,H-1</sub> (from HMBC) coupling constants, the first signal belonged to an α and the other two to β gluco/galactopyranosyl residues. 2D NMR spectroscopy (DQF COSY, TOCSY, and ROESY) together with the chemical shift values of 1–4 allowed the assignment of all proton resonances, as presented in Table 3. In this way, the α anomeric signal could be assigned to the Glcp residue and the β anomeric signals to Galp residues. The linkages between the different monosaccharide units were con-

firmed by the interresidual ROESY cross-peaks **II** H-1,**I** H-4 and **I** H-1,**II**′ H-1, and by the long range HMBC contacts (Fig. 3) **II** H-1,**I** C-4, **I** H-4,**II** C-1, **I** H-1,**II**′ C-1, and **II**′ H-1,**I** C-1. The assignments of the carbon chemical shifts as obtained from the HMBC spectrum are shown in Table 4. They are in agreement with terminal β-Galp (C-1, δ 103.8 and 104.4) and 4-substituted α-Glcp (C-1, δ 101.7; C-4, δ 79.0).

In the 1D <sup>1</sup>H NMR spectrum of Fraction 3.1 (Fig. 2(B)) some other anomeric signals with much lower intensity (ca. 10%) were visible (δ 5.45, <sup>3</sup>*J*<sub>1,2</sub> 3.4 Hz; 4.62, 7.2 Hz; 4.53, 7.0 Hz), suggesting the presence of compound **3.1B**:



A series of <sup>1</sup>H chemical shift values of low intensity peaks (Table 3) could be assigned from the different 2D spectra recorded of Fraction 3.1. Evidence for structure **3.1B** was found in the ROESY spectrum, showing a strong ROE **I** H-1,**II**′ H-1 and a weak ROE **II** H-1,**I** H-2. The substitution of Glc at O-2 is supported by the chemical shift of the anomeric proton of residue **I** (α H-1, 5.45 ppm) which is characteristic for a 2-substituted α-Glc residue [8]. No 2-substituted hexose was found in the methylation analysis, probably because of the small amount of this compound present in Fraction 3.1.

*Characterization of Fraction 4.1.*— Monosaccharide analysis of Fraction 4.1 revealed the presence of Glc and Gal in a molar ratio of 1.3:2.7. In the ESMS spectrum of the sodium borodeuteride-treated and permethyl-

Table 2  
<sup>13</sup>C chemical shifts of β-D-Galp-(1→4)-β-D-Galp-(1→4)-β-D-Galp-(1→4)-D-Galp (**3**)<sup>a</sup>

Compound	Residue	C-1	C-2	C-3	C-4	C-5	C-6
<b>3</b>	β-D-Galp-(1→4)-β-D-Galp-(1→4)-β-D-Galp-(1→4)-D-Galp	105.18	72.26	73.64	69.51	76.04	61.87 <sup>d</sup>
	4)-β-D-Galp-(1→4)-β-D-Galp-(1→4)-β-D-Galp-(1→4)-D-Galp	105.23	72.69 <sup>b</sup>	74.13	78.01	75.39 <sup>c</sup>	61.61 <sup>d</sup>
	4)-β-D-Galp-(1→4)-β-D-Galp-(1→4)-β-D-Galp-(1→4)-D-Galp	105.23	72.73 <sup>b</sup>	74.17	78.45	75.34 <sup>c</sup>	61.61 <sup>d</sup>
	4)-α-D-Galp	93.20	70.74	70.59	79.72	69.71	61.77 <sup>d</sup>
	4)-β-D-Galp	97.29	72.78	73.13	78.75	75.20	69.44 <sup>d</sup>

<sup>a</sup> Assignments were obtained from a 1D <sup>13</sup>C and a HMBC experiment recorded at 75 and 500 MHz, respectively.  
<sup>b</sup> Assignments may have to be interchanged.  
<sup>c</sup> See footnote b.  
<sup>d</sup> See footnote b.

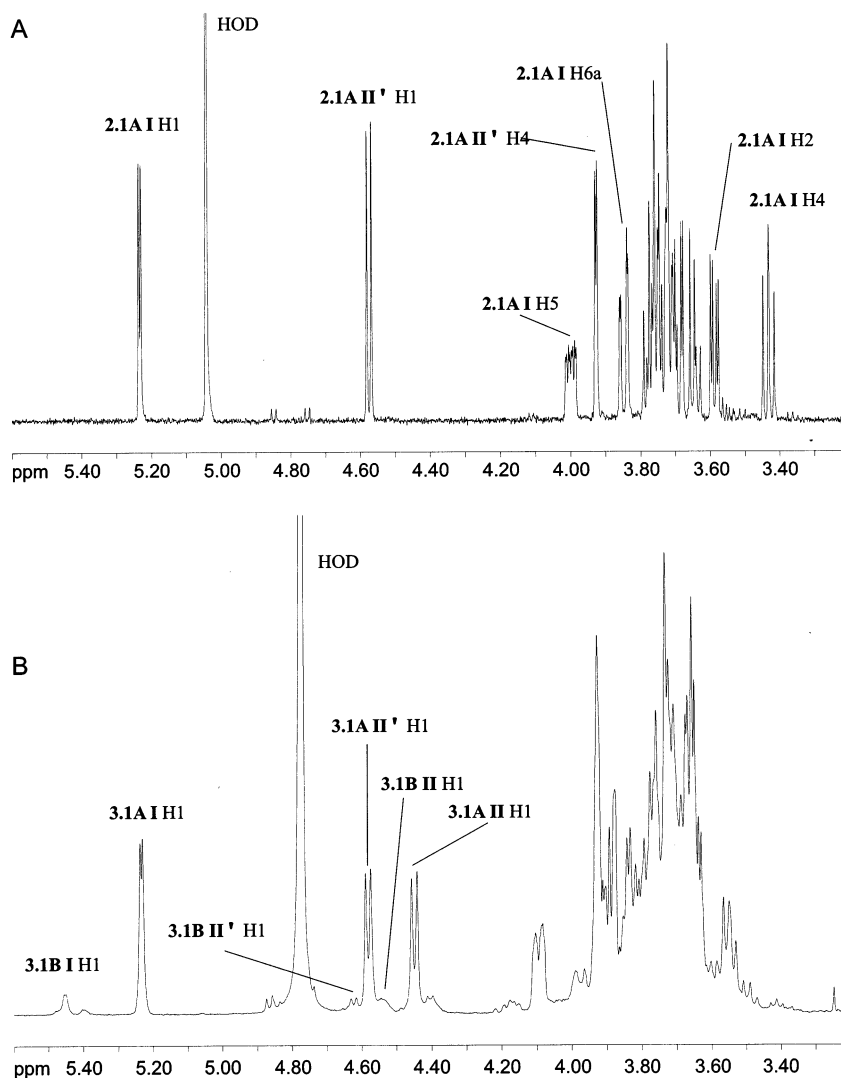


Fig. 2. 1D  $^1\text{H}$  NMR spectrum of (A) Fraction 2.1 (600 MHz, 278 K) and (B) Fraction 3.1 (500 MHz, 300 K). Assignments are as follows: **3.1A I H1** means H-1 of residue I of compound **3.1A**.

ated Fraction 4.1, an ion at  $m/z$  885 was observed, corresponding to  $[\text{M} + \text{Na}]^+$  for a nonreduced permethylated  $\text{Hex}_4$ .

In the 1D  $^1\text{H}$  NMR spectrum of Fraction 4.1 (Fig. 4(A)) two almost equally intense H-1 $\alpha$  signals around 5.22 ppm are present. The TOCSY spectrum showed that both signals belong to Glcp residues. Because these oligosaccharides were synthesized by  $\beta$ -galactosidase action, each oligosaccharide can only contain one Glc unit, and, consequently, this fraction contains at least two components. The H-1 $\beta$  signals were assigned to Galp residues based on their TOCSY spin system. The ROESY spectrum showed for each H-1 $\alpha$  signal an interresidual contact to a H-1 $\beta$  signal. One of these  $\beta$ -Galp residues ( $\delta$  4.571)

was not substituted whereas the other ( $\delta$  4.60) was substituted at O-4 (H-4 at  $\delta$  4.18 instead of  $\sim$  3.90; see Table 1). In total, two 4-substituted Galp residues were found (H-1,  $\delta$  4.478 with H-4,  $\delta$  4.18; H-1,  $\delta$  4.60 with H-4,  $\delta$  4.18). The typical chemical shifts of two H-1 $\beta$  signals below  $\delta$  4.50 ( $\delta$  4.478 and 4.439) indicated the presence of two Galp residues linked to O-4 of a Glcp residue [8,12] (structure **3.1A**).

On the basis of the results above, the following two structures, **4.1A** and **4.1B**, can be established:

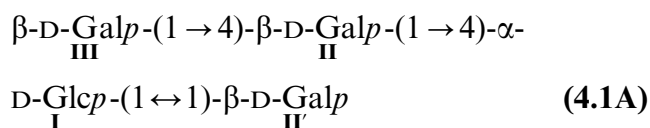


Table 3

<sup>1</sup>H NMR chemical shifts of analyzed oligogalactosylated glucoses (recorded at 300 K, at 500 or 600 MHz)

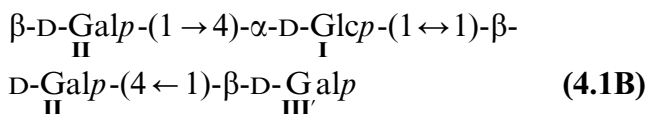
Compound		Residue	H-1	H-2	H-3	H-4	H-5	H-6a, 6b
<b>2.1A</b>	<b>I</b>	α-D-Glcp-(1 ↔	5.230	3.585	3.76	3.429	3.992	3.84, 3.74
	<b>II'</b>	1)-β-D-Galp	4.571	3.63	3.68	3.924	3.72	3.75–3.72
<b>3.1A</b>	<b>II</b>	β-D-Galp-(1 →	4.443	3.55	3.65	3.92	3.70	3.73–3.78
	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.228	3.64	3.88	3.69	4.09	3.82–3.88
<b>3.1B</b>	<b>II'</b>	1)-β-D-Galp	4.576	3.64	3.67	3.92	3.71	3.71–3.76
	<b>II</b>	β-D-Galp-(1 →	4.53	3.63	3.63	3.92		
<b>4.1A</b>	<b>I</b>	2)-α-D-Glcp-(1 ↔	5.45	3.72	3.92	3.65	4.16	
	<b>II'</b>	1)-β-D-Galp	4.62	3.64	3.67	3.91		
<b>4.1B</b>	<b>III</b>	β-D-Galp-(1 →	4.59	3.57 <sup>b</sup>	3.65	3.89	3.67	3.67–3.80
	<b>II</b>	4)-β-D-Galp-(1 →	4.478	3.61	3.77	4.18	3.68	3.67–3.80
<b>5.1A</b>	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.216	3.64	3.85	3.70	4.04	3.83–3.89
	<b>II'</b>	1)-β-D-Galp	4.571	3.64	3.67	3.92	3.71	3.67–3.80
<b>5.1B</b>	<b>II</b>	β-D-Galp-(1 →	4.439	3.53	3.66	3.91	3.65	3.67–3.80
	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.223	3.63	3.88	3.69	4.08	3.82–3.88
<b>5.1C</b>	<b>II'</b>	1)-β-D-Galp-(4	4.60	3.71	3.78	4.18	3.68	3.83–3.89
	<b>III'</b>	← 1)-β-D-Galp	4.59	3.60 <sup>b</sup>	3.65	3.89	3.67	3.67–3.80
<b>6.1A</b>	<b>IV</b>	β-D-Galp-(1 →	4.60	3.58	3.66	3.89	3.68	3.72–3.87
	<b>III</b>	4)-β-D-Galp-(1 →	4.646	3.65	3.77	4.19	3.72	3.72–3.87
<b>6.1B</b>	<b>II</b>	4)-β-D-Galp-(1 →	4.484	3.62	3.78	4.20	3.69	3.72–3.87
	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.216	3.64	3.89	3.71	4.04	3.81–3.87
<b>6.1C</b>	<b>II'</b>	1)-β-D-Galp	4.58	3.63	3.68	3.90	3.72	3.72–3.87
	<b>III</b>	β-D-Galp-(1 →	4.60	3.58	3.66	3.89	3.76	3.72–3.87
<b>6.1D</b>	<b>II</b>	4)-β-D-Galp-(1 →	4.484	3.62	3.78	4.20	3.69	3.72–3.87
	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.221 <sup>a</sup>	3.64	3.89	3.71	4.10	3.81–3.87
<b>6.1E</b>	<b>II'</b>	1)-β-D-Galp-(4	4.61	3.71	3.79	4.21	3.76	3.72–3.87
	<b>III'</b>	← 1)-β-D-Galp	4.60	3.58	3.66	3.90	3.76	3.72–3.87
<b>6.1F</b>	<b>II</b>	β-D-Galp-(1 →	4.445	3.56	3.65	3.92	3.71	3.72–3.87
	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.227 <sup>a</sup>	3.64	3.89	3.70	4.10	3.81–3.87
<b>6.1G</b>	<b>II'</b>	1)-β-D-Galp-(4	4.61	3.71	3.79	4.21	3.76	3.72–3.87
	<b>III'</b>	← 1)-β-D-Galp-(4	4.651	3.65	3.77	4.19	3.72	3.72–3.87
<b>6.1H</b>	<b>IV'</b>	← 1)-β-D-Galp	4.60	3.58	3.66	3.90	3.76	3.72–3.87
	<b>V</b>	β-D-Galp-(1 →	4.605	3.59	3.67	3.91	3.77	3.69–3.86
<b>6.1I</b>	<b>IV</b>	4)-β-D-Galp-(1 →	4.65–4.67	3.66–3.69	3.74	4.18–4.19	3.72–3.73	3.69–3.86
	<b>III</b>	4)-β-D-Galp-(1 →	4.65–4.67	3.66–3.69	3.74	4.18–4.19	3.72–3.73	3.69–3.86
<b>6.1J</b>	<b>II</b>	4)-β-D-Galp-(1 →	4.487	3.64	3.79	4.20	3.70	3.69–3.86
	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.238	3.64	3.90	3.71	4.11	3.81–3.90
<b>6.1K</b>	<b>II'</b>	1)-β-D-Galp	4.582	3.64	3.68	3.94		3.69–3.86
	<b>IV</b>	β-D-Galp-(1 →	4.605	3.59	3.67	3.91	3.77	3.69–3.86
<b>6.1L</b>	<b>III</b>	4)-β-D-Galp-(1 →	4.65–4.67	3.66–3.69	3.74	4.18–4.19	3.72–3.73	3.69–3.86
	<b>II</b>	4)-β-D-Galp-(1 →	4.487	3.64	3.79	4.20	3.70	3.69–3.86
<b>6.1M</b>	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.229 <sup>c</sup>	3.64	3.88	3.71	4.06	3.81–3.90
	<b>II'</b>	1)-β-D-Galp-(4	4.610	3.72–3.74	3.81	4.21	3.77–3.78	3.69–3.86
<b>6.1N</b>	<b>III'</b>	← 1)-β-D-Galp	4.605	3.59	3.67	3.91	3.77	3.69–3.86
	<b>III</b>	β-D-Galp-(1 →	4.605	3.59	3.67	3.91	3.77	3.69–3.86
<b>6.1O</b>	<b>II</b>	4)-β-D-Galp-(1 →	4.487	3.64	3.79	4.20	3.70	3.69–3.86
	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.232 <sup>c</sup>	3.64	3.88	3.71	4.06	3.81–3.90
<b>6.1P</b>	<b>II'</b>	1)-β-D-Galp-(4	4.610	3.72–3.74	3.81	4.21	3.77–3.78	3.69–3.86
	<b>III'</b>	← 1)-β-D-Galp-(4	4.65–4.67	3.66–3.69	3.74	4.18–4.19	3.72–3.73	3.69–3.86
<b>6.1Q</b>	<b>IV'</b>	← 1)-β-D-Galp	4.605	3.59	3.67	3.91	3.77	3.69–3.86
	<b>II</b>	β-D-Galp-(1 →	4.429	3.56	3.67	3.93		3.69–3.86
<b>6.1R</b>	<b>I</b>	4)-α-D-Glcp-(1 ↔	5.23	3.64	3.88	3.71	4.06	3.81–3.90
	<b>II'</b>	1)-β-D-Galp-(4	4.610	3.72–3.74	3.81	4.21	3.77–3.78	3.69–3.86
<b>6.1S</b>	<b>III'</b>	← 1)-β-D-Galp-(4	4.65–4.67	3.66–3.69	3.74	4.18–4.19	3.72–3.73	3.69–3.86
	<b>IV'</b>	← 1)-β-D-Galp-(4	4.65–4.67	3.66–3.69	3.74	4.18–4.19	3.72–3.73	3.69–3.86
<b>6.1T</b>	<b>V'</b>	← 1)-β-D-Galp	4.605	3.59	3.67	3.91	3.77	3.69–3.86

<sup>a</sup> Assignments may have to be interchanged.<sup>b</sup> See footnote a.<sup>c</sup> See footnote a.

Table 4

<sup>13</sup>C NMR chemical shifts of analyzed oligogalactosylated glucoses (recorded at 300 K, at 125 or 150 MHz)

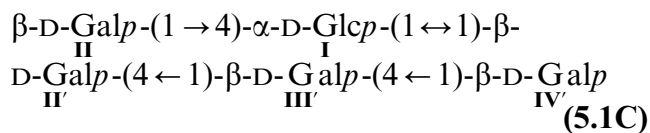
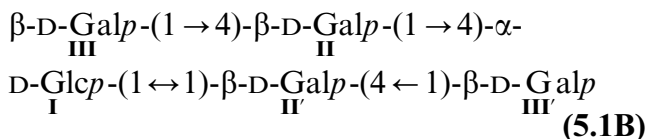
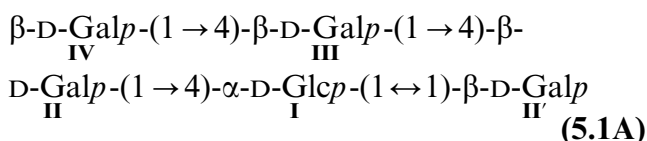
Compound		Residue	C-1	C-2	C-3	C-4	C-5	C-6
<b>2.1A</b>	<b>I</b>	α-D-Glcp-(1 ↔	101.1	72.1	73.4	70.2	73.2	61.1
	<b>II'</b>	1)-β-D-Galp	104.5	71.4	72.9	69.1	76.1	61.7
<b>3.1A</b>	<b>II</b>	β-D-Galp-(1 →	103.8	71.9	73.4	69.4	76.4	61.8
	<b>I</b>	4)-α-D-Glcp-(1 ↔	101.7	72.1	72.6	79.0	72.2	60.8
	<b>II'</b>	1)-β-D-Galp	104.4	71.6	73.2	69.2	76.4	61.8
<b>4.1A</b>	<b>III</b>	β-D-Galp-(1 →	105.4	72.7	73.9	70.0	76.4	62–63
	<b>II</b>	4)-β-D-Galp-(1 →	104.0	72.6	74.1	78.3	76.4	62–63
	<b>I</b>	4)-α-D-Glcp-(1 ↔	101.5	72.4	72.7	79.1	72.7	61.0
	<b>II'</b>	1)-β-D-Galp	104.7	72.9	73.6	69.8	76.7	62–63
<b>4.1B</b>	<b>II</b>	β-D-Galp-(1 →	104.0	72.1	73.8	69.9	76.3	62–63
	<b>I</b>	4)-α-D-Glcp-(1 ↔	101.1	72.4	72.7	79.3	72.6	61.0
	<b>II'</b>	1)-β-D-Galp-(4	104.8	72.4	74.0	78.3	76.4	62–63
	<b>III'</b>	← 1)-β-D-Galp	105.4	72.7	73.9	70.0	76.4	62–63
<b>5.1A</b>	<b>IV</b>	β-D-Galp-(1 →	105.0	72.2	73.5	69.5	75.4	61.5–61.9
	<b>III</b>	4)-β-D-Galp-(1 →	105.0	72.0	73.9	78.0	76.2	61.5–61.9
	<b>II</b>	4)-β-D-Galp-(1 →	103.6	72.1	73.7	78.3	76.1	61.5–61.9
	<b>I</b>	4)-α-D-Glcp-(1 ↔	100.8	72.6	72.2	79.1	72.2	60.9
	<b>II'</b>	1)-β-D-Galp	104.2	72.2	73.4	69.4	75.2	61.5–61.9
<b>5.1B</b>	<b>III</b>	β-D-Galp-(1 →	105.0	72.2	73.5	69.5	75.4	61.5–61.9
	<b>II</b>	4)-β-D-Galp-(1 →	103.6	72.1	73.7	78.3	76.1	61.5–61.9
	<b>I</b>	4)-α-D-Glcp-(1 ↔	100.6	72.6	72.2	79.1	72.1	60.9
	<b>II'</b>	1)-β-D-Galp-(4	104.2	71.9	73.6	77.8	76.0	61.5–61.9
	<b>III'</b>	← 1)-β-D-Galp	105.0	72.2	73.5	69.5	75.4	61.5–61.9
<b>5.1C</b>	<b>II</b>	β-D-Galp-(1 →	103.6	72.2	73.5	69.2	75.2	61.5–61.9
	<b>I</b>	4)-α-D-Glcp-(1 ↔	100.6	72.6	72.2	79.1	72.1	60.9
	<b>II'</b>	1)-β-D-Galp-(4	104.2	71.9	73.6	77.8	76.0	61.5–61.9
	<b>III'</b>	← 1)-β-D-Galp-(4	105.0	72.0	73.9	78.0	76.2	61.5–61.9
	<b>IV'</b>	← 1)-β-D-Galp	105.0	72.2	73.5	69.5	75.4	61.5–61.9
<b>6.1A</b>	<b>V</b>	β-D-Galp-(1 →	105.2	71.5–74	71.5–74	69.6	75.6	61.5–62
	<b>IV</b>	4)-β-D-Galp-(1 →	105.2	71.5–74	71.5–74	78.1–78.5	75.4	61.5–62
	<b>III</b>	4)-β-D-Galp-(1 →	105.2	71.5–74	71.5–74	78.1–78.5	75.4	61.5–62
	<b>II</b>	4)-β-D-Galp-(1 →	103.8	71.5–74	71.5–74	78.1–78.5	76.2	61.5–62
	<b>I</b>	4)-α-D-Glcp-(1 ↔	100.8	72.8	72.4	79.2	72.5	60.8
	<b>II'</b>	1)-β-D-Galp	104.5	71.5–74	71.5–74	69.4		61.5–62
<b>6.1B</b>	<b>IV</b>	β-D-Galp-(1 →	105.2	71.5–74	71.5–74	69.6	75.6	61.5–62
	<b>III</b>	4)-β-D-Galp-(1 →	105.2	71.5–74	71.5–74	78.1–78.5	75.4	61.5–62
	<b>II</b>	4)-β-D-Galp-(1 →	103.8	71.5–74	71.5–74	78.1–78.5	76.2	61.5–62
	<b>I</b>	4)-α-D-Glcp-(1 ↔	101.0	72.8	72.4	79.2	72.5	60.8
	<b>II'</b>	1)-β-D-Galp-(4	104.5	71.5–74	71.5–74	77.9	75.6	61.5–62
	<b>III'</b>	← 1)-β-D-Galp	105.2	71.5–74	71.5–74	69.6	75.6	61.5–62
<b>6.1C</b>	<b>III</b>	β-D-Galp-(1 →	105.2	71.5–74	71.5–74	69.6	75.6	61.5–62
	<b>II</b>	4)-β-D-Galp-(1 →	103.8	71.5–74	71.5–74	78.1–78.5	76.2	61.5–62
	<b>I</b>	4)-α-D-Glcp-(1 ↔	101.0	72.8	72.4	79.2	72.5	60.8
	<b>II'</b>	1)-β-D-Galp-(4	104.5	71.5–74	71.5–74	77.9	75.6	61.5–62
	<b>III'</b>	← 1)-β-D-Galp-(4	105.2	71.5–74	71.5–74	78.1–78.5	75.4	61.5–62
	<b>IV'</b>	← 1)-β-D-Galp	105.2	71.5–74	71.5–74	69.6	75.6	61.5–62
<b>6.1D</b>	<b>II</b>	β-D-Galp-(1 →	104.3	71.5–74	71.5–74	69.4		61.5–62
	<b>I</b>	4)-α-D-Glcp-(1 ↔	101.0	72.8	72.4	79.2	72.5	60.8
	<b>II'</b>	1)-β-D-Galp-(4	104.5	71.5–74	71.5–74	77.9	75.6	61.5–62
	<b>III'</b>	← 1)-β-D-Galp-(4	105.2	71.5–74	71.5–74	78.1–78.5	75.4	61.5–62
	<b>IV'</b>	← 1)-β-D-Galp-(4	105.2	71.5–74	71.5–74	78.1–78.5	75.4	61.5–62
	<b>V'</b>	← 1)-β-D-Galp	105.2	71.5–74	71.5–74	69.6	75.6	61.5–62



Specific assignments of the  $^1\text{H}$  and  $^{13}\text{C}$  resonances of these two compounds, as obtained from TOCSY, ROESY, and seHSQC spectra, are shown in Tables 3 and 4, respectively.

1D  $^1\text{H}$  NMR analysis of Fraction 4.1 shows anomeric signals ( $\delta$  5.26, 5.25, 4.68, 4.64, 4.56, and 4.52) of some minor components (ca. 10%) that could not be assigned due to the low amounts of the individual components.

**Characterization of Fraction 5.1.**—In view of the foregoing, the presence of different compounds with a  $(1\leftrightarrow 1)$  glycosidic linkage in Fraction 5.1 can be expected, and evidence is provided for the occurrence of compounds **5.1A**, **5.1B**, and **5.1C**.



In the ESMS spectrum of the sodium borodeuteride-treated and permethylated Fraction 5.1, an ion at  $m/z$  1089 was observed, corresponding to  $[\text{M} + \text{Na}]^+$  for a nonreduced permethylated Hex<sub>5</sub>.

In the 1D  $^1\text{H}$  NMR spectrum (Fig. 4(B)) several groups of anomeric signals are visible. In the H-1 $\alpha$  region  $\delta$  5.21–5.23, three overlapping doublets ( $^3J_{1,2}$  3–4 Hz) at  $\delta$  5.216, 5.221, and 5.227 are present in a relative abundance of 1:0.7:1. Based on TOCSY spectra these three doublets were assigned to  $\alpha\text{-Glcp}$  residues. The resonances in the H-1 $\beta$  region  $\delta$  4.4–4.5 are indicative of  $\beta\text{-Galp}$  residues linked to O-4 of  $\alpha\text{-Glcp}$  residues (see above). In the 1D 600 and 750 MHz spectra two doublets are visible at  $\delta$  4.445 and  $\delta$  4.484, respectively, in the relative intensity of 1:3. Comparing the relative intensities of these two anomeric signals with those of the three Glc H-1 $\alpha$  resonances, it can be concluded that two signals overlap at  $\delta$  4.484, since at least three compounds are present in this fraction. By comparing the chemical shift values of Frac-

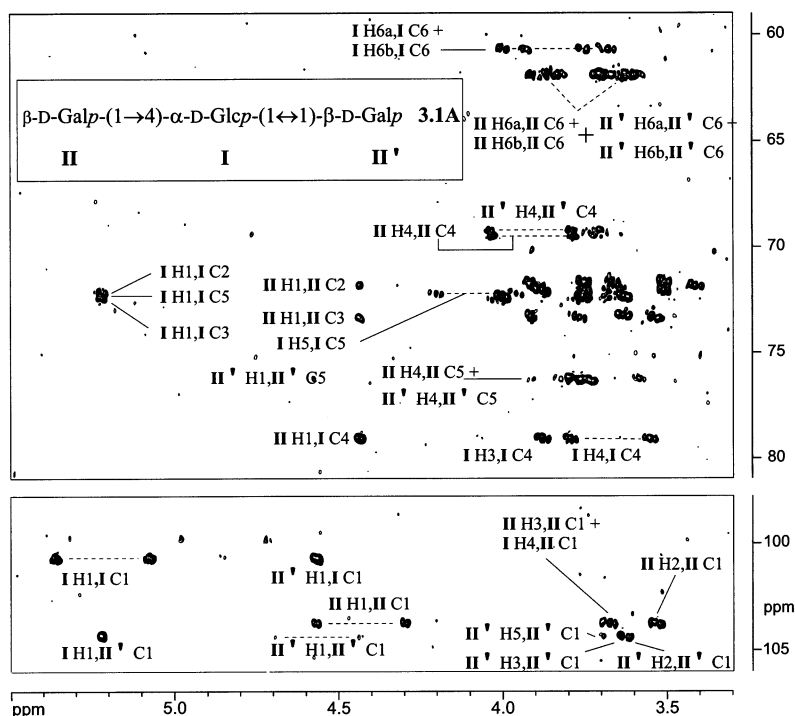


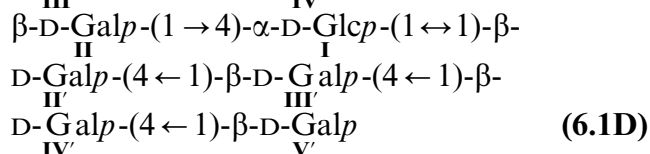
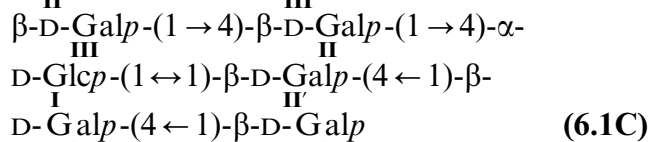
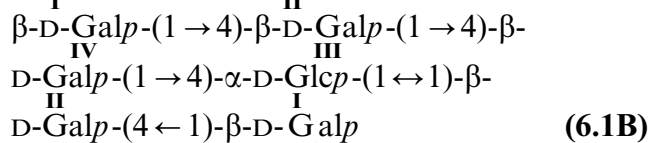
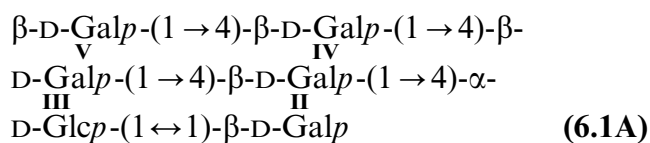
Fig. 3. HMBC spectrum (600 MHz, 300 K) of Fraction 3.1. Assignments are as follows: I H1, II C1 means a cross-peak between H-1 of residue I and C-1 of residue II' of compound **3.1A**; signals of **3.1B** are not visible.

tion 5.1 with those obtained for Fractions 3.1 and 4.1, the signal at  $\delta$  4.445 was assigned to terminal  $\beta$ -Galp (compound **5.1C**) and that at  $\delta$  4.484 to 4-substituted  $\beta$ -Galp linked to O-4 of  $\alpha$ -Glc p (compounds **5.1A** and **5.1B**). According to the ROESY and TOCSY data only terminal and 4-substituted  $\beta$ -Galp residues occur in this fraction (terminal  $\beta$ -Galp H-4,  $\delta \sim 3.90$ ; 4-substituted  $\beta$ -Galp H-4,  $\delta \sim 4.20$ ), and each  $\alpha$ -Glc H-1 signal ( $\delta$  5.216, 5.221, 5.227; Table 3) has an interresidual ROESY cross-peak to a  $\beta$ -Galp H-1 resonance. The chemical shift of C-1 ( $\delta$  100.6–100.8; Table 4) gives supporting evidence for the substitution of the anomeric centers of the  $\alpha$ -Glc p residues.

Based on the reasoning presented above, the structure with the  $\beta$ -Gal H-1 signal at  $\delta$  4.445 is **5.1C**. The  $\alpha$ -Glc H-1 doublet at  $\delta$  5.216 has a ROESY cross-peak with a  $\beta$ -Galp H-1 signal at  $\delta$  4.58. According to the TOCSY

spectrum this Galp residue occurs in a terminal position (H-4,  $\delta$  3.90), thereby indicating structure **5.1A**. The third component has structural similarities with **5.1A** and **5.1C** (i.e., the presence of the following structural elements:  $\beta$ -Galp-(1 $\rightarrow$ 4)- $\beta$ -Galp and  $\beta$ -Galp-(1 $\rightarrow$ 4)- $\alpha$ -Glc p-(1 $\leftrightarrow$ 1)- $\beta$ -Galp) giving rise to overlap in the NMR spectra (compare Table 1). These similarities lead to propose the presence of structure **5.1B**.

*Characterization of Fraction 6.1.*—Structural analysis of Fraction 6.1 gave evidence for the occurrence of compounds **6.1A**, **6.1B**, **6.1C**, and **6.1D**.



Monosaccharide analysis revealed the presence of Gal and Glc in a molar ratio of 4.9:1.1. Methylation analysis showed the occurrence of terminal Gal, 4-substituted Gal, and 4-substituted Glc. In the ESMS spectrum of the sodium borodeuteride-treated and permethylated Fraction 6.1, an ion at  $m/z$  1293 was observed, corresponding to  $[M + Na]^+$  for a nonreduced permethylated Hex<sub>6</sub>.

TOCSY, ROESY, and seHSQC experiments revealed the presence of the structural elements  $\beta$ -Galp-(1 $\rightarrow$ ,  $\rightarrow$ 4)- $\beta$ -Galp-(1 $\rightarrow$ , and  $\rightarrow$ 4)- $\alpha$ -Glc p-(1 $\rightarrow$ . In the 1D  $^1\text{H}$  NMR spectrum of Fraction 6.1 (Fig. 5) the doublet at  $\delta$  4.429 is characteristic for a terminal  $\beta$ -Galp residue (1 $\rightarrow$ 4)-linked to  $\alpha$ -Glc p (see compounds **5.1C** and **4.1B**) and the doublet with the same intensity at  $\delta$  3.93 is typical for H-4 of a terminal  $\beta$ -Galp residue, giving rise to structure **6.1D**. The H-1 $\alpha$  region ( $\delta \sim 5.23$ ) is

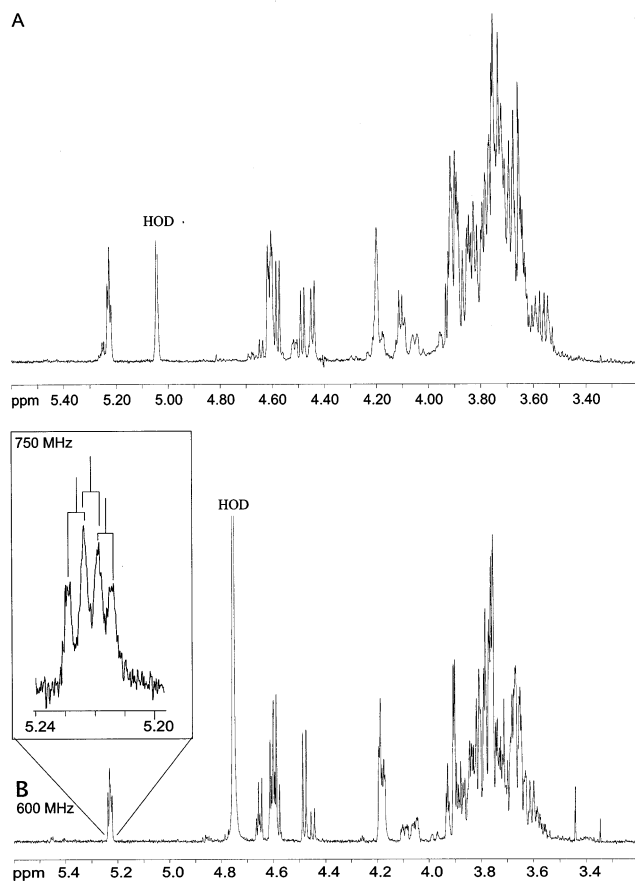


Fig. 4. 1D  $^1\text{H}$  NMR spectrum of (A) Fraction 4.1 (600 MHz, 278 K) and (B) Fraction 5.1 (600 MHz, 300 K). The enlargement in the spectrum of Fraction 5.1 contains the corresponding region in the 750 MHz 1D  $^1\text{H}$  spectrum of this fraction. The three  $\alpha$  anomeric signals of compounds **5.1A**, **5.1B**, and **5.1C** are indicated.

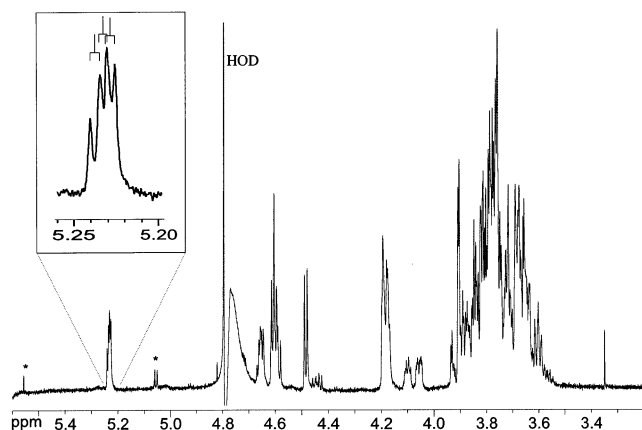


Fig. 5. 1D  $^1\text{H}$  NMR spectrum of Fraction 6.1 (750 MHz, 298 K). The  $\alpha$  anomeric signals are enlarged. \* Peaks belong to non-carbohydrate impurities resonating between 1 and 3 ppm. They are folded to these positions due to the small spectral width used for the acquisition of this spectrum.

composed of three doublets with relative intensities 1:2:2, as indicated in Fig. 5. According to TOCSY and seHSQC data these doublets belong to  $\alpha$ -Glc p H-1 atoms. Since each compound can only contain one Glc residue, Fraction 6.1 contains at least three compounds (see above). The doublet of **6.1D** H-1 is hidden in the multiplet around  $\delta \sim 5.23$ . Therefore, the presence of four instead of three compounds is suggested. The H-1 $\beta$  signal at  $\delta$  4.582 is characteristic for terminal  $\beta$ -Galp, (1 $\leftrightarrow$ 1)-linked to  $\alpha$ -Glc p (H-1,  $\delta$  5.238) leading to structure **6.1A**. Compounds **6.1B** and **6.1C** have structural similarities with parts of **6.1A** (and the minor component **6.1D**) and with each other (i.e., the occurrence of the following structural elements: ( $\beta$ -Galp-(1 $\rightarrow$ 4)) $_n$ - $\beta$ -Galp  $n=0-2$  and  $\beta$ -Galp-(1 $\rightarrow$ 4)- $\alpha$ -Glc p-(1 $\leftrightarrow$ 1)- $\beta$ -Galp) giving rise to overlap in the NMR spectra. By consequence, no complete assignment could be established. Exclusive evidence for their presence is the number of Glc H-1 $\alpha$  signals.

### 3. Discussion

This structural study of oligosaccharides produced by the transgalactosylation activity of  $\beta$ -galactosidase using lactose as a substrate, revealed the formation of a series of nonreducing oligosaccharides containing one  $\alpha$ -Glc p unit and a variable number of  $\beta$ -Galp units

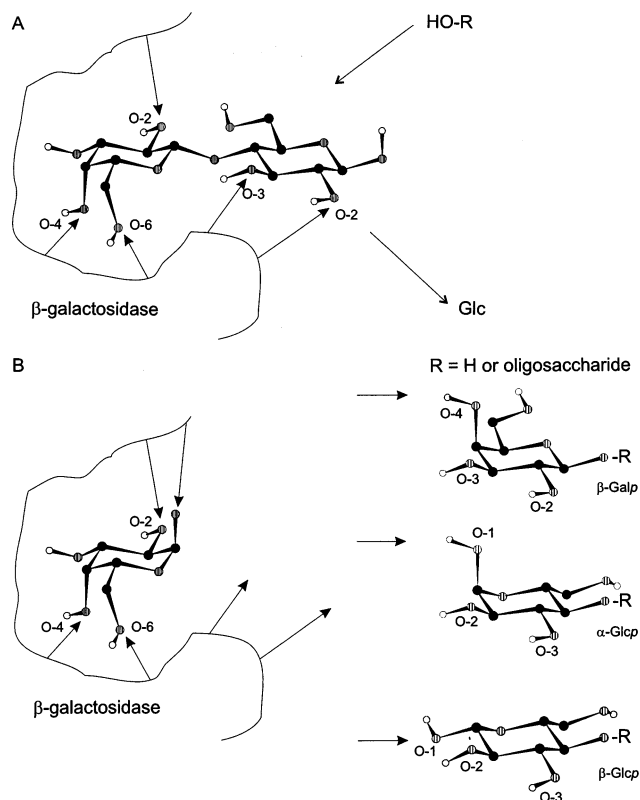


Fig. 6. (A) Proposed hydrogen bonding between  $\beta$ -galactosidase and lactose [13]. During the transgalactosylation process (B), the terminal residue of the galactosyl acceptor will be located in the same part of the pocket as Glc in a galactosidase reaction. Presumably, the orientation of the hydroxyl groups of the acceptor determine the ability of the enzyme to link Gal to a certain position.

having the structural motif  $\alpha$ -D-Glc p-(1 $\leftrightarrow$ 1)- $\beta$ -D-Galp in common. It would be interesting to investigate the gastrointestinal effect of galactosylated glucoses with respect to the Glc unit being reducing or not.

Interestingly, no  $\beta$ -Glc p was found in the nonreducing oligosaccharides described above. This can be explained by the way the enzyme binds to lactose [13]. According to [13] lactose can be positioned on the enzyme via several hydrophobic contacts and intermolecular hydrogen bonds (Fig. 6(A)) to Gal (O-2, O-4, and O-6) and Glc (O-2 and O-3). During the transgalactosylation process, the terminal Gal residue of the galactosyl acceptor is located in the same part of the pocket as Glc in a galactosidase reaction (Fig. 6(B)). Presumably the same intermolecular hydrogen bonds are present, as Gal is the C-4 epimer of Glc having a similar orientation of O-2 and O-3. This might explain the preference of the

enzyme for Gal O-4 in transgalactosylation reactions. When the anomeric center of Glc enters the active site as galactosyl acceptor, the orientation of Glc O-1,2,3 (Fig. 6(B)) depends on the anomeric configuration: for  $\alpha$ -Glc the orientation is the same as for Gal O-4,3,2. This is probably the reason for the absence of  $\beta$ -Glc-containing nonreducing oligosaccharides.

#### 4. Experimental

**Model compounds.**—Galactobiose to pentase (1–4) were generated by a controlled digestion of soy arabinogalactan using endogalactanase from *A. niger* [14], then isolated via gel-permeation chromatography on Bio-Gel P-2, when necessary combined with HPAEC on CarboPac PA-100 [15].

**Trans-galacto-oligosaccharides.**—A mixture of galactosylated glucoses, produced by a pure  $\beta$ -galactosidase using lactose as substrate, was a gift of Borculo Whey Products (Borculo, The Netherlands). The sample supplied stemmed from an incubation mixture from which most of the free Gal and Glc and some di- and trisaccharide material had been removed by charcoal column chromatography. Inspection of the sample using gel-permeation chromatography on Bio-Gel P-2 showed that the material consisted of di- through hexasaccharides in a relative abundance of 3, 6, 17, 37, and 26%, respectively, whereas 11% of the material consisted of larger oligomers. The non-reducing oligosaccharide-containing fractions comprised 0.3, 0.3, 0.5, 4.6, 2.6% of di-through hexasaccharides, respectively, in the total mixture of oligosaccharides.

**Purification of the galactosylated glucoses sample.**—The galactosylated glucoses sample was fractionated on two connected columns (each 600  $\times$  26 mm) packed with Fractogel TSK HW-40(S) (25–40  $\mu$ m, E. Merck, Darmstadt, Germany), thermostated at 60 °C and eluted with water (2 mL/min), using a Pharmacia Hiload system equipped with a Pharmacia P50 pump. A Shodex RI-72 detector was used to monitor the refractive index. The various fractions were subfractionated on a Bio-Gel P-2 column (100  $\times$  2.8 cm) at 60 °C,

eluted with water; the elution profile was established via an auto-analyser (Skalar Analytical BV, Breda, The Netherlands) using the orcinol–H<sub>2</sub>SO<sub>4</sub> assay for neutral sugars [16]. Further fractionation of Bio-Gel P-2 fractions was performed by HPAEC on a preparative (Fractions 2, 3, and 6) or a semi-analytical (Fractions 4 and 5) scale.

Preparative HPAEC was performed using a Spectra Physics P4000 pump equipped with a CarboPac PA-100 column (250  $\times$  22 mm) and coupled with a Spectra Physics AS3000 autosampler (900  $\mu$ L samples; flow rate, 20 mL/min) and a pulsed electrochemical detector (PED). Gradients were optimized for each sample using 0.2 M NaOH, 2 M NaOAc in 0.2 M NaOH and Millipore water as eluents. The effluent actually passing the detector was reduced to 1 mL/min by splitting the effluent post-column, and 10 mL fractions were collected and immediately neutralized by on-line addition of 1 M HOAc. Relevant fractions were concentrated and desalted on a Sephadex G-10 column (600  $\times$  50 mm) using distilled water as eluent (RI detection). The purity of the oligosaccharides was checked by analytical HPAEC.

Semi-preparative HPAEC was performed using a Dionex LC system with pulsed amperometric detection (PAD), equipped with a CarboPac PA-1 column (250 mm  $\times$  9 mm), eluted with a gradient of NaOAc in 0.1 M NaOH at a flow rate of 4 mL/min. PAD-detection was carried out with a gold working electrode and triple-pulse amperometry was used. Immediately after collection, fractions were neutralized manually with aq 99% HOAc, followed by desalting on a Dowex AG 50W-X12 (100–200 mesh, H<sup>+</sup>-form, Bio-Rad) column (RI detection) using distilled water as eluent, and subsequent lyophilization.

**Gas-liquid chromatography.**—GLC analyses were performed on a Chrompack CP9002 gas chromatograph, equipped with a CP-Sil 5 CB DFc.25 (Chrompack) capillary column (25 m  $\times$  0.32 mm) using a temperature program of 130–230 °C at 4 °C/min.

**Monosaccharide analysis.**—Oligosaccharides were subjected to methanolysis (methanolic 1 M HCl, 18 h, 85 °C), and the resulting mix-

tures of methyl glycosides were trimethylsilylated with 1:1:5 hexamethyldisilazane–trimethylchlorosilane–pyridine, and quantitatively analyzed by GLC [17].

**Methylation analysis.**—Samples were dissolved in 0.5 M  $\text{NH}_4\text{OH}$  (250  $\mu\text{L}$ ) containing  $\text{NaBD}_4$  (10 mg/mL). After 1 h, the mixture was neutralized with aq 99% HOAc, then concentrated. Boric acid was removed by repetitive co-evaporation with 9:1 MeOH–HOAc and MeOH. Permethylation was carried out essentially as described [18]. Briefly, freshly ground NaOH pellets (250 mg) were added to solutions of samples in  $\text{Me}_2\text{SO}$  (200  $\mu\text{L}$ ), and portions of MeI (250  $\mu\text{L}$ ) were added after 5, 15, and 25 min. The reaction was stopped after 20 min by adding aq  $\text{Na}_2\text{S}_2\text{O}_3$  (1 mL, 100 mg/mL) and  $\text{CHCl}_3$  (1 mL). The chloroform layer was extracted with water ( $3 \times 0.5$  mL), then concentrated. After hydrolysis with 2 M  $\text{CF}_3\text{COOH}$  (0.3 mL; 120 °C, 1 h), reduction (see above), acetylation with  $\text{Ac}_2\text{O}$  (0.5 mL; 120 °C, 3 h), quenching with water (0.5 mL), and neutralization with  $\text{NaHCO}_3$ , the partially methylated alditol acetates were extracted with  $\text{CH}_2\text{Cl}_2$  ( $3 \times 0.7$  mL). After concentration to about 20  $\mu\text{L}$  ( $\text{N}_2$ ), samples were analyzed by GLC and GLC–EIMS.

**Gas–liquid chromatography–electron impact mass spectrometry (GLC–EIMS).**—GLC–EIMS analyses were carried out on a Fisons MD800/8060 system (electron energy, 70 eV; carrier gas, He) equipped with a DB-1 fused-silica capillary column (30 m  $\times$  0.32 mm, J&W Scientific) using a temperature program of 110–240 °C at 4 °C/min followed by isothermal elution for 3 min.

**Mass spectrometry.**—Approximately 10–25% of the total amount of each fraction was used for mass spectrometric analysis.  $\text{NaBD}_4$ -treated and permethylated carbohydrates were dissolved in 1:1 MeOH–aq 1% HCOOH (100  $\mu\text{L}$ ) for ESMS. Positive-ion mode ES mass spectra were obtained using a VG Platform II single quadrupole mass spectrometer. Aliquots of 10  $\mu\text{L}$  of the samples were infused into a mobile phase of 1:1 MeOH–aq 1% HCOOH and introduced into the electrospray source at a flow rate of 5  $\mu\text{L}/\text{min}$ . Spectra were scanned

at a speed of 8 s for  $m/z$  200–2000, with a cone voltage of 95 V, recorded and processed using the MassLynx software, version 2.0. Mass calibration was performed by multiple-ion monitoring of peracetylated malto-oligosaccharides.

FABMS was carried out using a Jeol JMS-SX/SX102A tandem mass spectrometer (BEBO geometry) at 10 kV accelerating voltage. A Xe beam of about 6 kV translational energy (gun current 10 mA) was used for FAB ionization. The CID tandem mass spectra were obtained by using the collision cell in the third field free region of the mass spectrometer with air as collision gas at a pressure sufficient to reduce the intensity of the selected ion beam by 50%. As the collision cell was held at ground potential, the collision energy in the MS-MS experiments was 10 keV. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, recorded and averaged using a Hewlett–Packard HP9000 data system running Jeol complement software.  $\text{NaBD}_4$ -treated and permethylated carbohydrates were dissolved in 10  $\mu\text{L}$  MeOH and about 1  $\mu\text{L}$  of the solution was mixed with 0.8  $\mu\text{L}$  thioglycerol matrix on the probe tip.

**NMR spectroscopy.**—Prior to NMR analysis samples were exchanged twice in  $\text{D}_2\text{O}$  (99.9 atom% D, Cambridge Isotope Laboratories, MA, USA) and then dissolved in  $\text{D}_2\text{O}$  (99.96 atom% D, Isotec, USA). NMR spectra were recorded on Bruker AC-300 (Department of Organic Chemistry, Utrecht University), Bruker AMX-500 (Bijvoet Center, Utrecht University), Bruker AMX-600 (Bijvoet Center, Utrecht University or NSR Center, University of Nijmegen) or Varian UNITY-plus 750 (Bijvoet Center, Utrecht University) instruments at probe temperatures of 27 or 5 °C. Chemical shifts for  $^1\text{H}$  are expressed in ppm relative to internal acetone ( $\delta$  2.225) or acetate ( $\delta$  1.908), and for  $^{13}\text{C}$  to external glucose ( $\delta$  Glcp C-1 $\alpha$  92.9, Glcp C-1 $\beta$  96.7 [12]).

1D  $^1\text{H}$  NMR spectra were recorded with a sweep width of 5000 Hz at 500 MHz or 6000 Hz at 600 MHz in data sets of 16,384 points. Suppression of the HOD signal was achieved by applying the WEFT pulse as described [19]. Proton decoupled  $^{13}\text{C}$  NMR spectra were ac-

quired at 75.469 MHz. Typically, 50,000 transients of 16,384 data points were recorded. All 2D NMR spectra employed the time-proportional phase increment (TPPI) method [20]. Only seHSQC used the STATES-TPPI method [21]. In the 2D homonuclear proton NMR spectra the HOD signal was suppressed using presaturation during a relaxation delay for 1 s. 2D TOCSY spectra were acquired using MLEV 17 mixing sequences of 20–100 ms preceded by a trim pulse of 2.5 ms. The spin-lock field strength corresponded with a 90° pulse of 25–30  $\mu$ s and the spectral width was between 4 and 6 ppm in each dimension. Typically, 400–512 experiments of 2048 points were acquired with 4–32 scans per increment. 2D DQF-COSY spectra were recorded with a spectral width as in the 2D TOCSY spectra. Typically, 400–512 free induction decays, each acquired as 8 or 16 transients of 2048 data points were recorded. 2D ROESY spectra were obtained with a mixing time of 200–250 ms. The spin-lock field strength was in accordance with a 90° pulse of 100–120  $\mu$ s. The spectral width corresponded with 5–7 ppm in each dimension. Typically, 400–512 experiments of 2048 points were acquired with 32–64 scans per increment. The frequency offset was initially placed on the HOD resonance and switched to about 5.7 ppm just before application of the spin-lock pulse thereby reducing the Hartmann–Hahn transfer during the ROE mixing time [22]. 2D sensitivity enhanced HSQC spectra were proton detected and two gradients were applied for  $^{13}\text{C}$  coherence selection. These spectra were acquired with the proton offset at about 4.6 ppm and a sweepwidth of about 6 ppm. In the  $^{13}\text{C}$  dimension the offset was placed around 80 ppm and a sweep width of 60 ppm was used. Typically, 300–350 free induction decays of 1024 data points were acquired using 128–256 scans per decay. 2D HMBC spectra were recorded essentially as the 2D seHSQC spectra. The delay for the evolution of the long range couplings was 40–50 ms. NMR data sets were processed using Bruker UXNMR software or the Triton NMR software package (Bijvoet Center, Utrecht University). Briefly, time domain data were multiplied by phase-shifted (squared-)sine-bell

functions or with a Lorentzian-to-Gaussian multiplication. After zerofilling and Fourier transformation datasets of  $1024 \times 1024$  or  $2048 \times 1024$  points were obtained, which were baseline corrected with a fourth-order polynomial function when necessary. Accurate chemical shift values obtained from 1D spectra are represented with three (protons) or two (carbons) decimals, whereas values obtained from 2D spectra are depicted with two (protons) or one (carbon) decimals.

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