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A direct enzymatic synthesis of β-D-galactopyranosyl-D-xylopyranosides and their use to evaluate rat intestinal lactase activity in vivo

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Abstract

By enzymatic β-D-galactosylation of D-xylose a mixture of 4-, 3-, and 2-*O*-β-D-galactopyranosyl-D-xyloses (**1**, **4**, and **7**, respectively) was obtained in 50% isolated yield. Disaccharides **1**, **4**, and **7** are substrates of intestinal lactase isolated from lamb small intestine with K_m values of 250.0, 4.5, and 14.0 mM, respectively. The mixture was used to monitor the normal decline in lactase activity in rats that takes place after weaning. The data obtained by this method correlated with the levels of intestinal lactase activity in the same animals after being sacrificed. © 1996 Elsevier Science Ltd.

Keywords: Intestinal lactase evaluation; β-D-Galactopyranosyl-D-xylopyranosides; Enzymatic glycosylation; *E. coli* β-D-galactosidase

1. Introduction

We have developed a new non-invasive method to evaluate the activity of intestinal lactase [1,2] based on oral administration of 4-*O*-β-D-galactopyranosyl-D-xylose (**1**), a disaccharide very similar to lactose but lacking the hydroxymethyl group at position 5.

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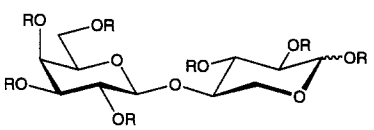
This disaccharide was found to be a substrate of the enzyme, yielding D-galactose and D-xylose. The latter is passively absorbed from the small intestine, is not phosphorylated, and is eliminated in the urine where it can be estimated by a simple colorimetric procedure.

Compound **1** was obtained from acetobromoxylose in a classical chemical approach that involved seven reaction steps with 8.8% overall yield [1]. When we considered the practical application of the diagnostic method, this synthesis of **1** turned out to be too inefficient and we sought a more direct preparation of **1**. Enzymes offer the opportunity of one-step preparations under mild conditions in a regio- and stereo-selective manner. Glycosidases are commercially available and have frequently been used for the synthesis of disaccharides [3,4]. We have recently reported [5,6] a systematic study of the β-D-galactosylation of differently substituted β-D-xylopyranosides catalyzed by the β-D-galactosidase from *E. coli*. Yield and regioselectivity of disaccharide products were dependent on the substitution at the anomeric position of the xylose. Controlled syntheses of β-D-galactopyranosyl-β-D-xylopyranoside derivatives were obtained [6].

We now report the synthesis of the 2-, 3-, and 4-*O*-β-D-galactopyranosyl-D-xylose disaccharides (**7**, **4**, and **1**, respectively) in one reaction step by enzymatic β-D-galactosylation using *o*-nitrophenyl β-D-galactopyranoside (**10**) or lactose as substrate donors, unprotected D-xylose as acceptor, and β-D-galactosidase from *E. coli* as the enzyme. Compounds **4** and **7** are also substrates of intestinal lactase, giving galactose and xylose. The disaccharide mixture obtained by this enzymatic procedure was therefore used to monitor the normal decline in lactase activity in rats that takes place after weaning. The data obtained by this method correlated with the levels of intestinal lactase activity in the same animals after being sacrificed.



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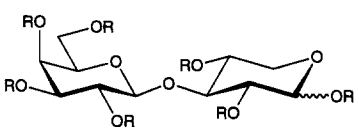
2 R = Ac (α)
3 R = Ac (β)



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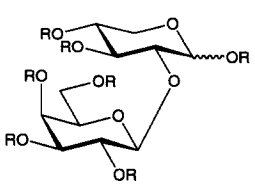
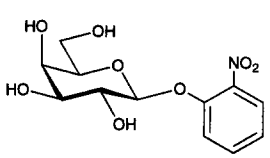
5 R = Ac (α)
6 R = Ac (β)



7 R = H
8 R = Ac (α)
9 R = Ac (β)



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2. Results and discussion

The reaction of D-xylose with **10** as galactosyl donor in the presence of β-D-galactosidase from *E. coli* was followed by gas–liquid chromatography (GLC) which showed the formation of 2-, 3-, and 4-*O*-β-D-galactopyranosyl-D-xyloses (**7**, **4**, and **1**, respectively). The identities of the peaks assigned to **1**, **4**, and **7** were determined as follows: the reaction was allowed to proceed until the galactosyl donor was consumed, then the mixture was chromatographed on an activated carbon column. Fractions containing disaccharides were acetylated and subsequently purified by normal phase HPLC. In this way acetylated galactosyl-xylose disaccharides **2**, **3**, **5**, **6**, **8**, and **9** were obtained and characterized by their ^{1}H NMR spectra (Table 1). Deacetylation of **2**–**3**, **5**–**6**, and **8**–**9** afforded **1**, **4**, and **7**, respectively. The pure disaccharides **1**, **4**, and **7** were analyzed by GLC as trimethylsilyl ethers.

In Fig. 1 is shown the total yield of galactosyl-xylose disaccharides **1**, **4**, and **7** as a function of time at different concentrations of D-xylose. It can be observed that maximum yields and rates of formation of the disaccharides increased when the D-xylose concentration was increased from 0.2 to 1.0 M. The highest yield (80%) was obtained at 2.0 M D-xylose, but some enzyme inhibition at this concentration must occur as the formation rate of disaccharides decreased. Eventually, 0.5 M D-xylose was chosen to prepare galactosyl-xylose disaccharides on a multigram scale since at this concentration the maximum yield of disaccharides is still high, while the use of a large excess of D-xylose that could make the purification process difficult is avoided. After column

Table 1
^{1}H NMR data for the peracetylated disaccharides dissolved in $CDCl_3$

Xylose moiety													
Compound	Chemical shifts (δ) [a]						Coupling constants (Hz)						
	H-1	H-2	H-3	H-4	H-5ax	H-5eq	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5ax}$	$J_{4,5eq}$	$J_{5ax,5eq}$	
2	6.21	4.98	5.19	3.87	3.66	3.79	3.5	7.6	7.7	11.0	5.6	11.2	
3	5.69	4.95	5.19	3.86	3.52	4.03	6.6	7.8	7.7	8.3	4.6	12.0	
5	6.12	4.94	4.03	4.95	3.63	3.89	3.6	9.2	9.1	10.2	5.7	11.4	
6	5.73	4.88	3.88	4.95	3.56	4.17	5.1	6.3	6.3	5.8	3.9	12.4	
8	6.12	3.19	5.41	4.95	3.61	3.80	3.7	9.9	9.8	10.9	5.7	11.3	
9	5.75	3.74	5.13	4.87	3.52	4.06	6.2	8.0	8.0	7.9	4.8	12.1	

Galactose moiety														
Compound	Chemical shifts (δ) [a]							Coupling constants (Hz)						
	H-1′	H-2′	H-3′	H-4′	H-5′	H-6a′	H-6b′	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{4',5'}$	$J_{5',6a'}$	$J_{5',6b'}$	$J_{6a',6b'}$
2	4.50	5.11	4.98	5.37	3.91	4.12		7.8	10.5	3.5	1.4		6.7	—
3	4.53	5.13	4.99	5.37	3.91	4.12		8.0	10.5	3.3	1.4		6.7	—
5	4.60	5.10	4.93	5.34	3.90	4.01	4.22	7.9	10.5	3.5	1.2	7.5	5.9	11.0
6	4.57	5.13	4.95	5.35	3.89	4.06	4.16	7.9	10.4	3.5	1.2	6.6	6.7	11.1
8	4.53	5.09	4.92	5.32	3.80	4.08		7.8	10.5	3.4	1.1		6.6	—
9	4.57	5.19	4.94	5.34	3.88	4.06	4.15	8.0	10.2	3.4	1.2	6.6	6.8	11.0

[a] Relative to $CDCl_3$ = 7.23 ppm.

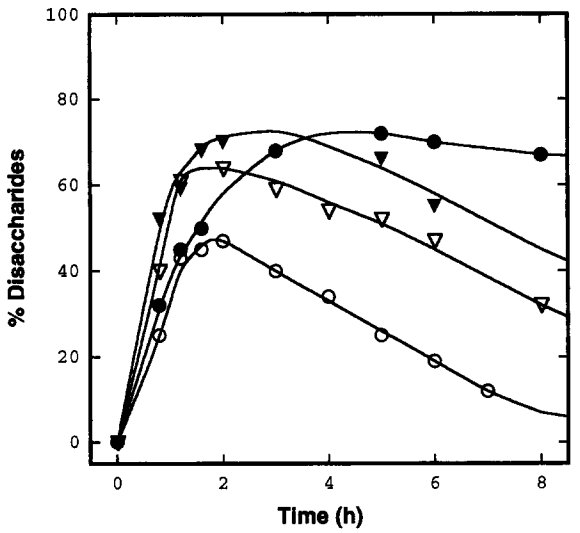

Fig. 1. Time course of the enzymatic production of disaccharide mixture **1**, **4**, **7** depending on the xylose concentration: ○, 0.2 M; ▽, 0.5 M; ▼, 1 M; ●, 2 M. Values are percentages relative to initial concentration of the donor **10** (50 mM). At the different times, aliquots of the reaction mixture were withdrawn and analyzed by GLC as described in Experimental.

chromatography, the β-D-galactosidase-catalyzed reaction of **10** (4 g, 50 mM) and D-xylose (20 g, 0.5 M) gave a mixture of regioisomers **1**, **4**, and **7** in 8.6:1.4:1.0 ratios (determined by GLC) in 50% yield.

We have also studied the galactosidation using lactose as galactosyl donor. Lactose (0.1 M) and D-xylose (0.5 M) were incubated at 25°C in the presence of β-D-galactosidase from *E. coli*. The reaction was stopped once the maximum formation of galactosyl-xylose disaccharides was reached. After chromatography, compounds **1**, **4**, and **7** were isolated in 25% yield (estimated by GLC) containing lactose and its β-(1 → 6) regioisomer, allolactose, which could not be removed. GLC analysis indicated that the ratios of **1**, **4**, and **7** were 5.5:3.1:1.0. In this case the regioselectivity of the galactosylation leading to **1** decreased as compared to the reaction using **10** as donor. This result can be explained by the fact that lactose is a less efficient galactosyl donor than **10**. Under these circumstances, a certain amount of the galactosyl-xylose products was hydrolyzed while the lactose concentration was still high; compound **1** was hydrolyzed in a higher proportion. Although for economical reasons lactose is the most convenient donor, further work must be done to improve the yield and to separate galactosyl-xylose from allolactose and remaining lactose.

In our previous report of the β-D-galactosylation of substituted β-D-xylopyranosides [5,6], it was observed that the regioselectivity of β-D-galactopyranosyl-β-D-xylopyranoside products varied drastically depending on whether methyl or benzyl β-D-xylopyranoside was used as acceptor. While the use of the methyl glycoside as acceptor gave exclusively the β-(1 → 4) disaccharide, the reaction of the benzyl derivative provided the β-(1 → 3) regioisomer as the major product. In the present work, the selective

Table 2
Kinetic parameters, K_m (Michaelis constant) and V_{max} (maximum velocity) for intestinal lactase substrates (see Experimental section for details)

Compound	K_m (mM)	V_{max} (μM min^{-1} mg^{-1})	V_{max}/K_m (%)
Lactose	7.3±2	14.5	100.0
1	250.0±50	6.8	1.4
4	4.5±1	21.2	237.0
7	14.0±2	11.6	41.2

formation of the β-(1 → 4) disaccharide using free D-xylose is in agreement with the result obtained with the methyl glycoside. However, the fact that D-xylose exists in the reaction medium as a mixture of α and β anomers must be taken into account when interpreting regioselectivity, since this can be influenced by the anomeric configuration of the acceptor [3].

In the above enzymatic disaccharide preparations, together with compound **1**, significant amounts of the regioisomers **4** and **7** were also obtained. Previously it has been observed that calf intestinal lactase [7] and lamb intestinal lactase [1] are able to hydrolyze efficiently other β-D-galactosyl-D-glucoses. It was important to check if compounds **4** and **7** were also substrates of intestinal lactase. Enzymatic assays were performed using intestinal lactase isolated from lamb small intestine as reported previously [8]. In Table 2 are presented the kinetic parameters for lactose and compounds **1**, **4**, and **7**. It should be noted that intestinal lactase hydrolyses more efficiently the regioisomer **4**, with a β-(1 → 3)-glycosidic bond, than compound **1**, with a β-(1 → 4) bond. In fact, the V_{max}/K_m ratio for compound **4** is at least of the same order as that for lactose. This result is in agreement with previous studies of substrate specificity of intestinal lactase, which showed the importance of the 5-hydroxymethyl group of lactose for catalysis [8,9]. This group is missing in compound **1**, while its relative position in compound **4** is occupied by a hydroxyl group (HO-2) as can be deduced from conformational studies of derivatives of lactose and disaccharides **1** and **4** [10,11]. Compound **7** is also a good substrate for intestinal lactase.

To investigate whether the synthetic galactosyl-xylose disaccharides **1**, **4**, **7** could be efficiently used to evaluate intestinal lactase activity in vivo, D-xylose elimination in the urine after oral administration of the disaccharide mixture to a group of suckling rats of the same litter was measured. The resulting levels of lactase activity in intestinal homogenates were related to the weaning age. As shown in Fig. 2, D-xylose elimination reached a maximum at the 15th day and then it gradually decreased to a plateau at about the 24th day. This pattern paralleled that of the intestinal lactase activity, thus reflecting the physiological decline of the enzyme activity in the postweaning period [12]. We take this experiment as strong evidence that D-xylose was eliminated in the urine as a result of the hydrolysis in vivo of the disaccharides, and therefore it might be of use for the non-invasive diagnosis of lactase deficiency in humans. Additionally, the fact that all

[1] F.J. Cañada and M. Martín-Lomas, unpublished results.

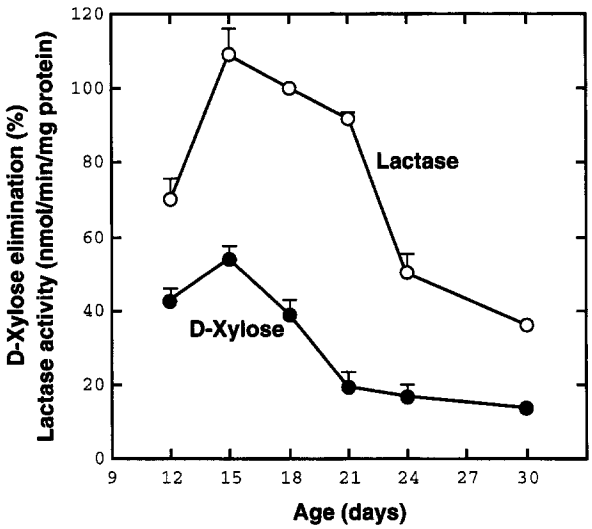

Fig. 2. Elimination of D-xylose in the urine of suckling rats after oral administration of disaccharide mixture **1**, **4**, **7**, and intestinal lactase activity as a function of age. A group of 17 suckling rats of the same litter and age (12 days) were fasted for 6 h and orally administered 16.2 mg of disaccharide mixture **1**, **4**, **7** (8.6:1.4:1.0) in 0.3 mL of water. Urine was collected by intermittent transabdominal bladder pressure during 5 h. D-Xylose eliminated during this time was determined in the urine colorimetrically and intestinal lactase activity was determined postmortem in three rats. The remaining animals were returned to maternal nursing and the experiment was repeated at the age indicated until all the rats were sacrificed. Values corresponding to D-xylose elimination are indicated as percentages of the amount administered. Each point shows the mean ±S.E. of three to seventeen animals. Points without significance values indicate that only one animal was used.

three regioisomers are substrates of lactase, as described above, in conjunction with the relatively high proportion of D-xylose excreted in the urine because of its poor metabolization, will allow the reduction of the dose size of disaccharide administered as compared to other non-invasive methods [13,14].

3. Experimental

Materials and methods.—β-D-Galactosidase from *E. coli* was purchased from Sigma and used without further purification. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were determined with a Perkin–Elmer 141 polarimeter. TLC was performed on Silica Gel GF_{254} (Merck) with detection by charring with H_2SO_4. The ^1H NMR spectra were recorded with a Varian XL-300 (300 MHz) or a Bruker AM-200 (200 MHz) spectrometer. D-Xylose was determined colorimetrically with phloroglucinol [15].

GLC was performed on a fused SE-54 column (15 m) with 0.3-mm i.d. and 0.15-μm film, using a Hewlett–Packard capillary gas chromatograph. A flow rate of 1 mL/min of N_2 and a flame detector were utilized (temperature program: initial temp. 145 °C;

initial time 2 min; rate 5 °C/min; final temp. 250 °C). HPLC was performed on a semipreparative normal phase column μPorasil (Waters) using a HPLC Waters System with refraction index detector.

2-, 3-, and 4-O-β-D-Galactopyranosyl-D-xylose (**7**, **4**, *and* **1**).—*(a) Using* **10**. To a solution of **10** (4 g, 50 mM) and D-xylose (20 g, 500 mM) in buffer (0.05 M KH_2PO_4, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol; 265 mL, pH 7.0) was added β-D-galactosidase from *E. coli* (1.5 mg, 560 U), and the mixture was incubated at 25 °C. When all the substrate was consumed (5 h 45 min) the reaction was stopped by heating for 10 min at 100 °C. The solution was concentrated and fractionated by column chromatography on activated carbon (water → 85:15 water–EtOH) to give, first, D-xylose and D-galactose, then an 8.8:1.4:1.0 mixture of **1**, **4**, and **7** (2.0 g, 50%).

Fractions containing mixtures of compounds **1**, **4**, and **7** were acetylated conventionally with Ac_2O–pyridine to give a mixture of **2**, **3**, **5**, **6**, **8**, and **9**. Each peracetylated disaccharide was purified from the mixture by semipreparative HPLC (55:45 hexane–EtOAc). ^1H NMR spectra, see Table 1.

Deacetylation of each anomeric pair **2–3**, **5–6**, and **8–9** gave pure **1**, **4**, and **7**, respectively.

Compound **1**: spectroscopic and physical data were identical to those previously reported [1].

Compound **4**: $[\alpha]_D^{25}$ +12° (*c* 1.0, H_2O), lit. [16] +25 → +10° (*c* 1.0, H_2O); ^1H NMR (300 MHz, D_2O, δ 4.71): δ 5.19, 4.65 (2 d, m, 1 H, $J_{1,2}$ 3.56 and 7.42 Hz, H-1α,1β), 4.62, 4.61 (2 d, 1 H, $J_{1',2'}$ 7.45 and 7.87 Hz, H-1'α,1'β), 3.97 (dd, $J_{5eq,4}$ 5.15, $J_{5eq,5ax}$ 11.52 Hz, H-5eq,β), 3.92 (d, 1 H, $J_{4',3'}$ 3.32 Hz, H-4'α,β), 3.80–3.55 (m, 8 H), and 3.45–3.30 (m, 1 H); ^{13}C NMR (50 MHz, D_2O): δ 103.8 and 103.7 (C-1'α,β), 96.7 (C-1β), 92.4 (C-1α), 84.8 (C-3β), 82.4 (C-3α), 75.7, 73.9, 72.9, 71.7, 71.1, 68.9, 67.4, 67.3, 65.0, 61.4, 61.0, 59.2.

Compound **7**: $[\alpha]_D^{25}$ +17° (*c* 1.0, H_2O), lit. [16] +25° (constant; *c* 1.4, H_2O); ^1H NMR (300 MHz, D_2O, δ 4.71): δ 5.39, 4.70 (2 d, 1 H, $J_{1,2}$ 3.45 and 7.61 Hz, H-1α,1β), 4.66, 4.54 (2 d, 1 H, $J_{1',2'}$ 7.49 and 7.19 Hz, H-1'α,1'β), 3.91 (d, 1 H, $J_{4',3'}$ 3.31 Hz, H-4'α,β), and 3.87–3.30 (m, 10 H); ^{13}C NMR (50 MHz, D_2O): δ 104.8 (C-1'α), 103.7 (C-1'β), 95.8 (C-1β), 92.4 (C-1α), 81.6 (C-2β), 81.0 (C-2α), 76.12, 75.7, 75.4, 72.9, 72.2, 71.7, 71.4, 69.6, 69.5, 69.1, 69.0, 65.5, 61.5, 61.3, 61.1.

(b) Using lactose. To a solution of lactose (9.6 g, 100 mM) and D-xylose (20 g, 500 mM) in buffer (0.05 M KH_2PO_4, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol; 265 mL, pH 7.0) was added β-D-galactosidase from *E. coli* (4 mg), and the mixture was incubated at 25 °C for 48 h. The reaction was stopped and the solution worked up as described above. Fractions containing **1**, **4**, and **7** (5.5:3.1:1.0, estimated by GLC) were lyophilized to afford a mixture of lactose, allolactose, **1**, **4**, and **7** (5.5 g). The amount of **1**, **4**, and **7** in the mixture was estimated by GLC (1.0 g, 25%).

Enzymatic assays. Kinetic parameters of the hydrolysis of **1**, **4**, **7**, and lactose by lamb small-intestinal lactase were obtained as previously reported [8]. Substrate hydrolysis was evaluated as the xylose released, measured by GLC as described above. A variable amount of substrate (1–200 mM) in sodium phosphate (50 mM, pH 5.9) was incubated in the presence of the purified enzyme. After incubation for 10 min at 37 °C, the reaction mixture (25 μL) was frozen and lyophilized. The dry residue was

derivatized for GLC analysis. Preliminary assays were performed with each substrate to determine the conditions for a linear releasing of xylose. For the measurement of rat intestinal lactase, animals were sacrificed by a blow on the neck, the small intestine was rapidly removed, washed with 100 mM sodium maleate (pH 6.5), and cut open, and the mucus was scraped off with a glass slide. Homogenization of the mucus and determination of lactase activity by measuring the liberated galactose were carried out as described previously [2]. Protein was determined by the method of Lowry et al. [17].

Acknowledgements

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