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Optimizing the enzymatic synthesis of β-D-galactopyranosyl-D-xyloses for their use in the evaluation of lactase activity in vivo

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Abstract—Disaccharides 2-*O*-, 3-*O*-, and 4-*O*-β-D-galactopyranosyl-D-xyloses (**2**, **3**, and **1**, respectively) were obtained by β-galactosidase-catalyzed reactions for their use in the evaluation of intestinal lactase activity in vivo. Their administration to suckling rats followed by determination of the derived D-xylose in the urine and measurement of lactase activity in intestinal homogenates showed **1** to be the most suitable disaccharide for a potential test of the deficiency of intestinal lactase. The synthesis of **1** was further studied by evaluating the effect of different variables on the yield and regioselectivity of the enzymatic galactosylation, and the purification process was optimized.

1. Introduction

Lactose intolerance is caused by deficiency of the enzyme lactase (hypolactasia), which is normally produced by the cells that line the small intestine. Common symptoms of lactose intolerance include bloating, abdominal pain or cramps, flatulence, and diarrhea.[1] The most commonly used test for lactose intolerance in clinic is the hydrogen breath test.[2] This technique requires high doses of administered lactose, which can produce considerable disturbances in the patients, and frequently produce both false-negatives and false-positives, due to individual variations in endogenous gas production capacity.[3]

Over the last years, our group has been working at developing a new diagnostic method to evaluate lactase activity in vivo based on the use of 4-*O*-β-D-galactopyranosyl-D-xylose (**1**, Scheme 1), a structural analog of lactose.[4] This compound was shown to be a substrate of the enzyme in vivo,[4] as its administration to suckling rats led to urinary elimination of D-xylose and to the accumulation of this pentose in plasma, which increased in both fluids in a dose-dependent manner.[5] In addition to **1**, we reported that the regioisomers 2- and 3-*O*-β-D-galactopyranosyl-D-xyloses (**2** and **3**, respectively) were also substrates of the enzyme in vitro.[6] In fact, the kinetic parameters for compounds **2** and **3** showed that the intestinal lactase from lamb hydrolyzes more efficiently these disaccharides than **1**. Therefore, it was necessary to have a procedure to obtain pure preparations of the three disaccharides in amounts enough to perform in vivo experiments, so that their individual characteristics could be later examined under these conditions.

We previously reported[7] the synthesis of different enriched mixtures of disaccharides **1**, **2**, and **3** by galactosylation of D-xylose (**7**) using a β-galactosidase enzyme and *o*-nitrophenyl β-D-galactopyranoside (**6**) (Scheme 1). The ratio of **1**, **2**, and **3** was dependent on the source of the β-galactosidase used. We describe here several modifications of the described enzymatic procedure in order to obtain pure disaccharides in a gram scale. The results of evaluating the efficiency of each of

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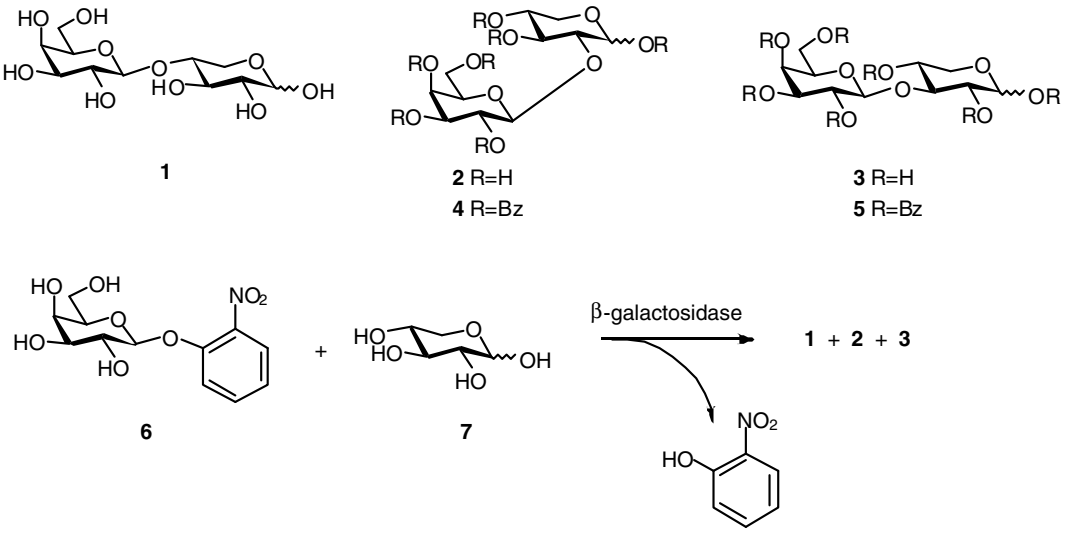


Scheme 1. Synthesis of disaccharides **1**, **2**, and **3** by galactosidation of D-xylose (**7**) using a β-galactosidase.

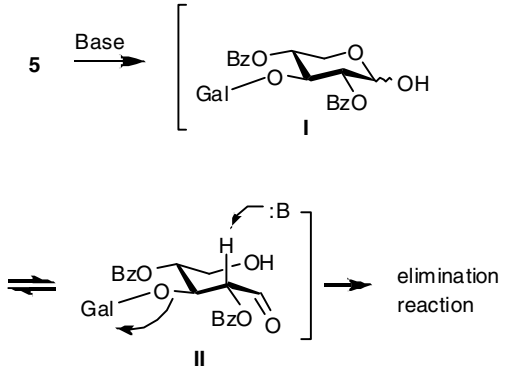
the three disaccharides in detecting the changes of rat lactase activity in vivo are discussed.

2. Results and discussion

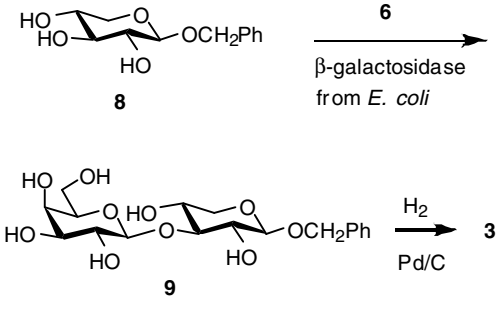
Following our previous results,[7] the galactosylation of D-xylose using β-galactosidase from *Aspergillus oryzae* gave a mixture of **2** and **3** as main products, accompanied by some minor amount of **1** (5:49:46 ratio of **1**, **2**, and **3**, respectively, determined by gas chromatography). To separate the disaccharide products, the mixture was benzoylated and fractionated on silica gel column chromatography, to give benzoyl derivatives **4** and **5** (see Scheme 1) separately. Debenzoylation of **4** afforded **2**. However, attempts to obtain **3** from **5** under a variety of conditions (NaOMe/MeOH; guanidine/NaOMe/MeOH[8]; KCN/MeOH[9]) led to a partial cleavage of the glycosidic bond with formation of galactose. The easy cleavage of the glycosidic bond can be explained by considering that debenzoylation at the anomeric position takes place more rapidly due to electronic factor. Hemiacetal **I** (Scheme 2) equilibrates with the acyclic carbonyl species **II**, which leads to elimination by abstraction of the acidic proton at C-2. In view of these difficulties, we changed the route to obtain disaccharide **3**, and the enzymatic galactosylation was carried out on a xylose derivative with a protecting group at the anomeric position. In previous studies on the regioselectivity of disaccharide synthesis using β-galactosidase from *Escherichia coli*,[10] we showed that the presence of an aromatic ring at the anomeric position of β-D-xylopyranoside acceptors drives the reaction toward the formation of 3-*O*-β-D-galactopyranosyl derivatives. Thus, when the galactosylation was performed on benzyl β-D-xylopyranoside as substrate (**8**, Scheme 3) the disaccharide **9** was obtained as main product, which after hydrogenolysis afforded **3**.

For the synthesis of **1**, the galactosylation of D-xylose was carried out in the presence of β-galactosidase from *E. coli* at 25 °C, pH 7.0, to give a mixture of **1**, **2**, and **3** in a ratio of 8.0:1.5:0.5, respectively. After purification using a carbon–Celite column compound **1** was obtained.

Galactosylation at the anomeric position of xylose was not observed in any of the enzymatic reactions investigated. The lack of formation of 1-*O*-β-D-galactopyranosyl-α,β-D-xylopyranosides could be due to the low nucleophilicity of the anomeric hydroxyl group.



Scheme 2. Proposed mechanism for the cleavage of the glycosidic bond during the debenzoylation of **5**.



Scheme 3. Synthetic pathway for disaccharide **3**.

The above-described procedures allowed us to obtain gram quantities of pure disaccharides utilizable for in vivo experiments. As seen in Table 1, administration of disaccharides **1**–**3** separately to suckling rats followed by determination of the derived D-xylose in the urine and measurement of lactase activity in intestinal homogenates elicited a gradual decrease in the elimination of urinary pentose as a function of age, that was markedly different with the three compounds. Thus, although maximal elimination of D-xylose was similar for each disaccharide at the 15th day, its decrease with compound **1** was practically similar to the physiologic decline of lactase activity along the weaning age, whereas the decrease of D-xylose elimination was lower with compound **2**, and much lower with compound **3**, as compared with the enzyme decline. Consequently, correlation between D-xylose elimination and lactase activity was the highest with **1**, lower with **2**, and very low with **3**. These results agree with our previous data describing the individual behavior of these disaccharides in greater details.[5] Steady-state kinetics of rat intestinal lactase in vitro showed the highest V_{max}/K_m ratio for compound **3** (even greater than that for lactose), intermediate for **2**, and very low for **1**.[5] Therefore, the high catalytic efficiency with compound **3** accounts for its extensive hydrolysis in vivo even with low levels of lactase activity, whereas the low activity for compound **1** accommodates the changes of D-xylose elimination to those of enzyme activity. Thus, disaccharide **1** resulted the most effective compound to evaluate the changes in lactase activity in vivo, despite being the worst substrate. These observations are in agreement with previous reports on the function of the hydroxyl groups of the lactose molecule in its interaction with lactase.[11,12] Thus, the HO-6 was found important for hydrolysis of the glycosidic bond,[11,12] and so its absence in compound **1** makes this disaccharide a relatively poor substrate. This position is occupied by HO-2 in compound **3**.[13] which improves the enzyme catalytic efficiency, making it a substrate even better than lactose. In compound **2**, HO-3 is equivalent to the HO-6 group, but the anomeric nature of HO-1 appears to hinder its interaction with the enzyme. Compound **1** was, therefore, chosen as the most suitable tool for a potential test of the deficiency of intestinal lactase in noninvasive way.

Regarding the synthesis of **1**, a regiospecific synthesis of this disaccharide by galactosylation of benzyl α-D-xylopyranoside (the anomer of **8**) using β-galactosidase from *E. coli* has been reported.[14] However, this synthesis requires the preparation of the xylopyranoside acceptor and subsequent removal of the benzyl group after glycosylation. We pursued a straightforward synthesis of **1** by optimizing the enzymatic galactosylation of unprotected xylose. Thus, using the enzyme from *E. coli*, we evaluated the effect of addition of cosolvents (DMF, DMSO, THF, diethyleneglycoldiethylether, and acetonitrile), pH, and temperature, on the yield and regioselectivity of disaccharides formed (Table 2). Glycosylation was observed only when DMF and DMSO were used as cosolvents (20% v/v) (entries 1 and 2). However, reactions took longer and the yield of disaccharides decreased. The pH changes from 7.0 to 5.0 or 8.5 (entries 3–5) had also a negative effect on the yield, although an increase of selectivity toward the formation of **1** was observed at pH 5.0. In the case of temperature, an appreciable change of regioselectivity in

Table 2. Yield and regioselectivity (as the ratio of **1**:[**2** + **3**]) of the disaccharides **1**, **2**, and **3** from the β-galactosidase-catalyzed reaction of **6** and **7**

Entry	Cosolvent	*T* (°C)	pH	Yield (%)	Ratio of **1**:(**2** + **3**)
1	DMSO	37	7.0	35	70:30
2	DMF	37	7.0	38	72:28
3	—	37	7.0	50	71:29
4	—	37	5.0	22	81:19
5	—	37	8.5	36	68:32
6	—	45	7.0	48	68:32
7	—	25	7.0	45	79:21
8	—	5	7.0	48	80:22
9	—	−5	7.0	46	83:17

Table 1. Elimination of D-xylose in the urine of suckling rats after oral administration of the disaccharides **1**, **2**, and **3**, and intestinal lactase activity as a function of age[a]

Age (days)	D-Xylose elimination in urine (%)[b]			Lactase activity (U/mg protein)
	From **1**	From **2**	From **3**	
15	23.66 ± 0.60	23.10 ± 0.28	23.30 ± 0.94	0.172 ± 0.0026
18	6.99 ± 0.55	20.71 ± 0.64	20.63 ± 0.23	0.070 ± 0.0023
30	3.05 ± 0.21	9.43 ± 1.05	19.39 ± 0.28	0.015 ± 0.0005
	$r = 0.98$[c]	$r = 0.84$[c]	$r = 0.58$[c]	
	$r^2 = 0.96$[c]	$r^2 = 0.71$[c]	$r^2 = 0.33$[c]	
	$P < 0.0001$[c]	$P < 0.001$[c]	$P < 0.05$[c]	

[a] Distinct groups of four suckling rats from the same litter and of the indicated age were used for each disaccharide. Rats were fasted for 6 h in metabolic cages at 30 °C and orally administered 4 mg of the corresponding disaccharide **1**–**3** in 0.3 mL of water. Urine was collected by intermittent transabdominal bladder pressure during 6 h after disaccharide administration. D-Xylose elimination during this time was determined in the urine colorimetrically and intestinal lactase activity was determined post mortem in all animals. Data are means ± SE.

[b] D-Xylose eliminated in the urine is indicated as percentage of the amount of the administered disaccharide.

[c] Correlation parameters were calculated by linear regression analysis of urinary D-xylose after administration of each disaccharide and intestinal lactase activity. Statistical significance was set up at $P < 0.05$.

favor of **1** was also observed as the reaction temperature was lowered (entries 7–9). However, at 5 and −5 °C a greater amount of enzyme had to be added in order to proceed the reactions until completion (6 and 12 times more enzyme, respectively). The best results in terms of enzyme efficiency and yield were obtained at 37 °C in buffer pH 7.0 (entry 3), under which the disaccharide **1** was formed with satisfactory regioselectivity. Therefore, these were the conditions of choice for the synthesis of **1**.

We next focused on the isolation of **1** from the reaction mixture. Fractionation on a carbon–Celite column required large volumes of isopropanol–water that may be troublesome for industrial application. We quantified the adsorption preference on carbon of the different sugars present in the reaction mixture, in order to minimize the amount of carbon added and, therefore, the volume of solvent during the elution step. To a typical crude reaction mixture (66 mL volume), carbon was added in 0.5–1 g portions. Aliquots from the solution were analyzed by gas chromatography. As can be seen in Figure 1, the relative selectivity of the adsorption on carbon is *o*-nitrophenyl β-D-galactopyranoside (**6**) > disaccharides (**1**, **2**, **3**) > xylose (**7**), galactose. About 90% of disaccharides were adsorbed after addition of 10 g of carbon to the reaction mixture, while around 80% of xylose and galactose remained in solution. In view of these results, we modified the procedure in the following manner. After the reaction was finished, carbon (0.12 g/mL of solution) was added, then the solution, containing the monosaccharides and buffer salts, was filtered, and the carbon washed with 5% isopropanol in water. Washing fractions containing the disaccharide **1** were concentrated and crystallized from acetone–water. This modified procedure has several advantages. The volume of fractions containing the disaccharide is reduced in more than tenfold. In addition, heating the mixture to stop the reaction by deactivation of the enzyme is not required. Application of this procedure allows to obtain multi-gram quantities of **1** in only two working days.

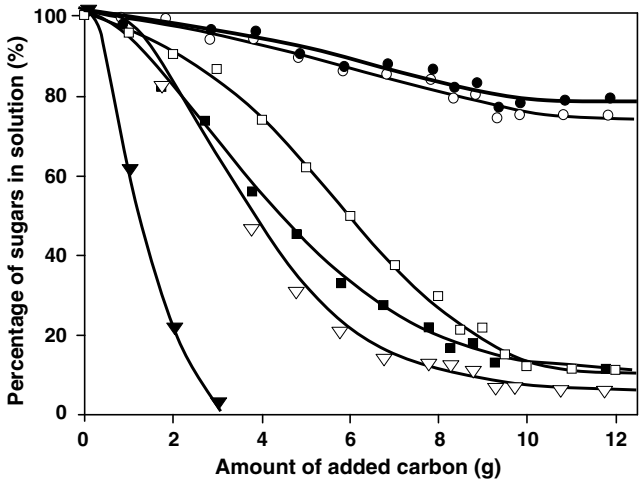


Figure 1. Percentage of remaining sugars in solution after addition of activated carbon to the reaction mixture: ●, D-xylose; ○, D-galactose; ■, **1**; □, **2**; ▽, **3**; ▼, **6**.

3. Conclusions

The first synthesis of disaccharide **1** was carried out using a multi-step approach[15] requiring toxic chemical reagents, which was unpractical regarding a possible industrial application. The synthesis procedure described in this work involves a simple enzymatic process using readily available substrates, and is presently being adapted for production at industrial scale. The synthesis of **1** optimized herein, in conjunction with the recent optimization of the measurement of the derived D-xylose in either urine or blood from animals,[5] as an estimate of the overall lactase activity in vivo, provides a new method for the noninvasive diagnosis of hypolactasia which is now ready for clinical trials.

4. Experimental

4.1. General remarks

β-Galactosidases were purchased from a commercial source and used without further purification. *o*-Nitrophenyl β-D-galactopyranoside, D-xylose, and other chemicals were from commercial sources. Melting points are uncorrected. TLC was performed on silica gel GF_{254} with detection by charring with H_2SO_4. ^1H NMR spectra were measured at 300 or 400 MHz. ^{13}C NMR were obtained at 125 MHz. Gas–liquid chromatographic analysis was carried out on a chromatograph with FID detector using fused SE-54 capillary column (5% Ph Me silicone, 12 m, 0.2 mm id, and 0.33 μm film). A flow rate of 1 mL/min of nitrogen was utilized. Samples were previously silylated. D-Xylose was determined colorimetrically with phloroglucinol.[16]

4.1.1. 2-O-β-D-Galactopyranosyl-D-xylose (2). To a solution of **6** (10 g, 50 mM) and D-xylose (**7**, 50 g, 500 mM) in buffer (0.2 M KH_2PO_4, pH 5.5) was added β-galactosidase from *A. oryzae* (85 mg, 450 U), and the mixture was incubated at 30 °C for 2.5 h. When all the substrate was consumed (2.5 h, TLC isopropanol/NH_3/H_2O, 7.5:0.5:2.5) the reaction was stopped by heating at 100 °C for 10 min, washed with CH_2Cl_2, and concentrated. The residue was fractionated on a carbon–Celite column (H_2O/isopropanol, 100:0 → 95:5) to give a separated fraction containing the mixture of disaccharides **1**, **2**, and **3** (3.6 g). This mixture was dissolved in CH_2Cl_2 (30 mL) and treated with pyridine (7.0 mL), *N*,*N*-dimethyl-4-aminopyridine (cat.), and benzoyl chloride (10.0 mL, 86.0 mmol), stirring at 5 °C for 24 h. After this time, the mixture was diluted with CH_2Cl_2, washed with H_2O, dried (Na_2SO_4), and concentrated. The residue was eluted through a silica gel column chromatography (toluene/AcOEt, 10:1 → 10:1). Fractions containing **4** were crystallized from CH_2Cl_2/Et_2O/hexane (1:20:20). Crystals were dissolved in MeOH (180 mL) and treated with 0.5 M NaOMe in MeOH (12 mL) at room temperature for 2 h. The solution was neutralized with Amber-

lite IR-120 (H^+) and concentrated to give pure **2** (1.2 g). ^1H and ^{13}C NMR spectra of **2** were identical to those previously reported[6]; $[\alpha]_D$ +10° (*c* 1.0, H_2O), lit.[6] $[\alpha]_D$ +12° (*c* 1.0, H_2O).

4.1.2. 3-O-β-D-Galactopyranosyl-D-xylose (3). To a solution of **6** (5.6 g, 50 mM) and **8**[15] (9.0 g, 100 mM) in buffer (0.2 M K_2HPO_4, pH 7.0) was added β-galactosidase from *E. coli* (3.6 mg, 1152 U), and the mixture was left at 37 °C. After 1 and 3.5 h more **6** was added (2.8 g at the time). When all the substrate was consumed (TLC isopropanol/NH_3/H_2O, 7.5:0.5:2.5) the reaction was stopped by heating at 100 °C for 10 min. The solution was concentrated, and the residue was fractionated by silica gel column chromatography (AcOEt/MeOH, 30:1 → 5:1), to give **9** (1.9 g). Hydrogenation of **9** (1.85 g) with 10% Pd–C (0.5 g) in H_2O/MeOH (1:1) at atmospheric pressure afforded **3** (1.4 g). ^1H and ^{13}C NMR spectra of **3** were identical to those previously reported[6]; $[\alpha]_D$ +22° (*c* 1.0, H_2O), lit.[6] $[\alpha]_D$ +17° (*c* 1.0, H_2O).

4.1.3. 4-O-β-D-Galactopyranosyl-D-xylose (1). To a solution of **6** (25 g, 50 mM) and D-xylose (**7**, 125 g, 500 mM) in buffer (0.2 M K_2HPO_4, pH 7.0) was added β-galactosidase from *E. coli* (1 mg, 312 U), and the mixture was left at 37 °C for 22 h. After this time, activated carbon (200 g) was added and the mixture was stirred for 30 min. Then, the mixture was filtered, and the solid was washed with H_2O/isopropanol (100:0 → 95:5). Fractions containing disaccharide (TLC isopropanol/NH_3/H_2O, 7.5:0.5:2.5) were concentrated, and the residue was crystallized from acetone–H_2O, to give **1** (5.15 g). The ^1H NMR spectrum of **1** was identical to that previously reported[15]; mp 160–168 °C; $[\alpha]_D$ +17° (*c* 1.0, H_2O), lit.[15] $[\alpha]_D$ +15° (*c* 1.0, H_2O); ^{13}C NMR(125 MHz, D_2O): δ 101.8 (C-1′), 96.6 (C-1_β), 92.1 (C-1_α), 76.8, 76.6, 75.4, 74.2, 74.0, 72.7, 71.4, 71.3, 70.8, 68.7, 63.2, 61.26, 58.9.

4.1.4. Assay of lactase activity. To measure intestinal lactase, the mucosa from the medium region of the small intestine from Sprague–Dawley rats was obtained as described[4] and homogenized with 10 volumes of 100 mM sodium maleate, pH 6.0. Lactase activity was determined in the homogenate by measuring the liberated galactose as described.[4] One unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1 μmol of substrate/min at 25 °C.

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