Regiospecific synthesis of lactose analog Gal-(β 1,4)-Xyl by transgalactosylation

Wolf-Dieter Fessner and Juan Manuel Juárez Ruiz

Abstract: A short enzymatic synthesis of disaccharide 4-*O*-β-D-galactopyranosyl-D-xylose (**1**) has been developed, which is of interest as a lactose analog for a non-invasive medicinal determination of lactose intolerance. The starting material, benzyl α-D-xyloside, was obtained by a Fischer-type glycosidation of D-xylose with benzyl alcohol, followed by anomeric differentiation of mixed glycosides using a glycosidase from *Aspergillus oryzae*. From several commercial β-galactosidases, which were screened for their transgalactosylation capacity, the enzyme from *Escherichia coli* was found to catalyze a virtually regio- and stereospecific galactosyl transfer from donor compounds *o*-nitrophenyl β-D-galactoside or lactose to the α-D-xyloside. Subsequent hydrogenolytic deprotection furnished desired disaccharide **1**.

Key words: oligosaccharide synthesis, β-galactosidase, lactose intolerance.

Résumé : On a mis au point une courte synthèse du disaccharide 4-*O*-β-D-galactopyranosyl-D-xylose qui présente de l'intérêt comme analogue de lactose pour procéder à une évaluation médicinale non invasive de l'intolérance au lactose. On a obtenu le produit de départ, l'α-D-xyloside de benzyle, par une glycosylation de type Fischer du D-xylose par l'alcool benzylique suivie d'une différentiation anomérique des glycosides mixtes à l'aide d'une glycosidase provenant d'*Aspergillus oryzae*. Diverses β-galactosidases commerciales ont été évaluées pour leur capacité à provoquer une transgalactosylation; on a trouvé que l'enzyme provenant d'*Escherichia coli* catalyse le transfert du galactosyle d'une façon virtuellement régio- et stéréospécique à partir de β-D-galactoside d'*o*-nitrophényle ou de lactose agissant comme composés donneurs vers l'α-D-xyloside. Une déprotection hydrogénolytique subséquente conduit au disaccharide **1** recherché.

Mots clés : oligosaccharide, synthèse, β-galactosidase, intolérance au lactose.

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Introduction

Lactose is a milk disaccharide produced by nearly all mammalian species. For digestion, it must be hydrolyzed to D-galactose and D-glucose by lactase, an enzyme which is present on the brush border of small intestinal enterocytes. Evaluation of an enzyme deficiency (lactase insufficiency, lactose intolerance) (1, 2) is important in pediatrics and gastroenterology due to the high frequency of a genetic disposition. Because many of the standard diagnostic procedures are invasive, quite drastic, and unacceptable or dangerous for the testing of infants and young children, a non-invasive method for lactase determination has been proposed on the basis of 4-*O*-β-D-galactopyranosyl-D-xylose (**1**) (3), which can be administered as a substrate analog to lactose (4, 5). The xylose released from small quantities of this disaccharide by lactase activity is eliminated in the urine where it can be quantified colorimetrically. The β-Gal-(1,4)-Xyl disaccharide (**1**) is also well known as a fragment of the highly conserved linkage region between the core protein and the polysaccharide chain (glycosaminoglycan) in proteoglycans of connective tissue (6).

Chemical syntheses of **1** require a judicious installation of protective groups and suffer from long, tedious reaction sequences with little overall productivity (7–9). For a choice of alternative enzymatic strategies, the biosynthetic route involving specific UDP-Gal dependant galactosyltransferase enzymes (e.g., β4GalT7) (10) is burdened by technical difficulties, high costs, and the ambident reactivity of xylose (11, 12). On the other hand, glycosidases have been shown by numerous examples to allow the facile formation of glycosides by transglycosylation at low cost (13, 14). Disadvantages of this approach, however, include yield limitations due to competing hydrolysis reactions, and separation problems because of the formation of regioisomers and other by-products with similar physical properties, which is due to the low acceptor selectivity of glycosidases. For example, attempts to form Gal-(β 1,4)-linkages by transglycosylation to D-xylose (15, 16) or to different aryl- or alkyl-β-D-xylosides (17–20) as acceptors were generally met by concomitant

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Dedicated to J. Bryan Jones on the occasion of his 65th birthday in recognition of his pioneering contributions to preparative biocatalysis.

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formation of the corresponding (β 1,3)-disaccharides in significant proportions.

Here we report a new enzymatic synthesis of **1** based on a successful regiospecific transgalactosylation to α-configurated benzyl D-xyloside.

Results and discussion

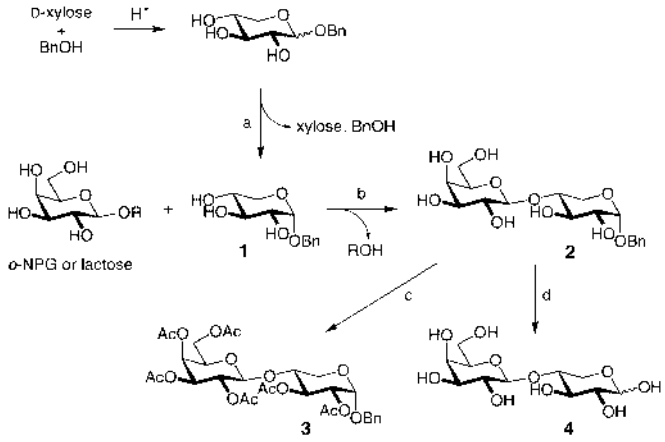
The synthetic strategy for constructing free-reducing disaccharide **1** (for possible use in clinical diagnosis) called for an anomeric protection group that ideally should confer several important properties to the synthetic intermediates: (*i*) it needs to be easily introduced in the starting material at low cost; (*ii*) it needs to be efficiently removed in the last step with avoidance of toxic reagents; (*iii*) it should facilitate monitoring of conversion and characterization at intermediate stages; (*iv*) it should simplify purification of the intermediates by a sufficiently high tendency to crystallize (particularly in view of a larger scale synthesis); and (*v*) it should exert pronounced stereoelectronic effects for substrate recognition during the transglycosylation process for an enhanced regioselectivity. Thus, from a choice of allyl, 4-pentenyl, and benzyl protection, the latter was given preference because it was expected to best meet these criteria.

Recently, we have developed a simple and efficient procedure for the anomeric differentiation of mixed α,β-glycosides using the hydrolytic function of glycosidases (21). Such anomeric mixtures are readily obtainable by acid-catalyzed Fischer-type glycosidation. Because many glycosidases with suitable substrate specificity and complementary anomeric preference are readily available from various sources (14), pure unprotected glycosides are efficiently accessible. Specifically, the α-configurated anomeric form of benzyl xylopyranoside **1** (Scheme 1) can be isolated in high yield at low cost using crude commercial enzyme preparations from *Aspergillus oryzae* (21).

Since prior attempts for D-xylopyranoside transgalactosylation were mainly performed on the β-configurated benzyl glycoside with unsatisfactory regioselectivity (17–20), α-anomer **1** was chosen for our study. Several commercial β-galactosidases (EC 3.2.1.23, e.g., from *A. oryzae*, *E. coli*, and CloneZyme® library enzymes of undisclosed origin) were screened for transgalactosylation activity in phosphate buffer at pH 7.0 using **1** as the acceptor component and *o*-nitrophenyl β-D-galactoside (*o*-NPG) as the donor component. Because individual regioisomers were not revealed upon TLC analyses using various solvents, crude product mixtures were analyzed by NMR spectroscopy for composition. Practically all enzymes tested were found to catalyze the desired reaction to a varying extent, producing disaccharide mixtures composed of **2** and considerable fractions of the undesired (β 1,3)-regioisomer. No formation of the (β 1,2)-disaccharide was observed, which is probably due to the steric inaccessibility to the biocatalysts. The enzyme from *E. coli* was unique, however, because it yielded the desired 1,4-regioisomer **2** as the only disaccharide product detectable by high-resolution NMR spectroscopy (de ≥ 95%).

Although the transgalactosylation proceeded with excellent regioselectivity, the presence of unreacted acceptor glycoside and free galactose from competing hydrolysis of the donor complicated product purification by silica gel column chromatography. Residual acceptor **1** could, however, be easily removed by selective extraction with ethyl acetate, while the more hydrophilic disaccharide derivative **2** remained in the aqueous phase. Further purification was performed by column filtration through a plug of reversed-phase silica gel. Free galactose was readily removed upon washing with water, while ethanol elution furnished pure benzyl-protected disaccharide **2** in 23% yield as a crystalline solid. Unambiguous structural proof was obtained by full characterization of corresponding peracetate **3**.

Scheme 1. Chemoenzymatic synthesis of disaccharide **4**. Reagents and conditions: (*a*) crude β-galactosidase from *A. oryzae*, H_2O, >60%; (*b*) β-galactosidase from *E. coli*, 23%; (*c*) Ac_2O, pyridine, 95%; (*d*) H_2–Pd/C, aq. EtOH, 80%.



It should be emphasized that the judicious choice of benzyl protection did indeed strongly facilitate product recovery and purification by easily scalable extractive manipulations and thus obviated the need for an exacting high-performance chromatographic separation. Although reaction conditions have not been further optimized, the yield is within the range commonly obtainable by preparative-scale transglycosylations (13, 14). An improvement may be expected from applications of glycosynthases that have been engineered to prevent product hydrolysis (22). Concerning the regioselectivity of the galactosyl transfer, it should be compared with the unfavorable 5:2 ratio of 3-/4-regioisomers obtained for the corresponding benzyl β-xyloside with the same enzyme. The unusually high regioselectivity in the case of α-xyloside may be due to a pronounced bend in the molecular shape of **1** induced by the steric bulk of the α-anomeric benzyl group. Rather than the linearly extended geometry of the β-xyloside, this seems to render the relative setting of the 3- and 4-hydroxyl groups sufficiently different from each other to facilitate discrimination by the galactosidase in favor of the desired compound.

The transgalactosylation was also tested using lactose as an alternative, inexpensive galactosyl donor. As expected, the reactions proceeded more slowly compared with those with the highly reactive *o*-NPG donor. Following the same work-up procedure, pure disaccharide **2** was obtained in 15% yield. The lower yield, as compared with the *o*-NPG donor, is in line with the relatively lower conversion rate because of the increasing likelihood of product hydrolysis at longer reaction times. Attempts were also made for an extended usage of the biocatalyst. Because a continuous operation

of the transglycosylation would be difficult to achieve given the propensity of the product to be hydrolyzed under the reaction conditions, batchwise recycling of the enzyme offered a better solution. Indeed, when the protein was recovered from the reaction mixture by ultrafiltration, it could be reused for at least four identical consecutive runs without noticeable detrimental effects on the reaction velocity or product yield.

Hydrogenolytic cleavage of the benzyl protection group in **2** was performed on 10% Pd/C catalyst. Complete conversion was only achieved by applying quite forcing conditions (60 bar H_2, 50°C), which may be related to the lower reactivity of an α-glycoside. The resultant product was purified by filtration through reversed-phase silica gel column using water as the eluent to furnish the free disaccharide **4** in 80% yield. The spectroscopic data of this compound was identical to values published for the disaccharide from chemical synthesis (7).

Conclusions

A short, versatile enzymatic synthesis has been developed for the lactose analog Gal-(β 1,4)-Xyl **4** by a highly regio- and stereoselective transgalactosylation of benzyl α-D-xyloside **1** as the acceptor. Unlike the situation for free xylose or the corresponding β-D-xyloside, the β-galactosidase from *E. coli* efficiently directs galactosyl transfer to 4-OH of the acceptor, which is likely supported by the steric influence of the bulky α-anomeric substituent that is presumed to restrict a competing access to 3-OH. The benzyl group is of further advantage for simple and effective isolation of the target disaccharide **2** from complex product mixtures by allowing group-specific extraction procedures. Thus, this synthesis is promising for the preparation of larger disaccharide quantities.

Experimental

General

NMR spectra were recorded on Bruker ARX-300 and Bruker AVANCE 500 spectrometers; chemical shifts are referenced to internal TMS ($CDCl_3$) or TSP (D_2O; 0.00 ppm). Mass spectra were recorded on a Bruker Esquire-LC system (ESI), and elemental analyses were performed on a Heraeus CHN-O-Rapid system. Optical rotations were measured with a PerkinElmer 241 apparatus in a 10-cm cuvette at λ = 589 nm. For ultrafiltration, an Amicon 8400 cell equipped with a YM 10 membrane was used. Column chromatography was performed on Merck 60 silica gel (0.063–0.200 mesh), and analytical thin-layer chromatography was performed on Merck silica gel plates 60 GF_{254} using anisaldehyde stain for detection. C_{18}-silica gel 100 (Fluka, 0.040–0.063 mm) was used for reversed-phase (RP) adsorption. β-Galactosidases from *A. oryzae* (Sigma, G-5160 or G-7138) and from *E. coli* (Roche Molecular Biochemicals, 150797 or Sigma, G-6008) were used as commercially supplied. The CloneZyme® glycosidase kit from Recombinant Biocatalysts Inc. (now Diversa) was used for transglycosylation screening.

Benzyl α-D-xylopyranoside (1)

The glycosidation mixture (21) from acidic equilibration of D-xylose with benzyl alcohol (0.1 M) was hydrolyzed in phosphate buffer (0.2 M, pH 4.5) at 37°C using the crude β-galactosidase preparation from *A. oryzae* with TLC monitoring. The unreacted benzyl α-D-xyloside (**1**) was isolated according to the published procedure (21) as a colorless crystalline solid in >60% absolute yield.

Benzyl β-D-galactopyranosyl-(1,4)-α-D-xylopyranoside (2)

o-NPG donor

Compound **1** (100 mg, 0.4 mmol) and *o*-nitrophenyl β-D-galactoside (250 mg, 0.8 mmol) were dissolved in phosphate buffer (8.0 mL, 50 mM; 1.0 mM $MgCl_2$, 5.0 mM mercaptoethanol, pH 7.0), and the mixture was incubated with β-galactosidase from *E. coli* (130 μL, 215 U) on a rotary shaker (50 rpm) at room temperature. Reaction was monitored for product formation by TLC (MeOH–toluene, 2:1, R_f (**2**) = 0.50). After 4 h, an additional equivalent of *o*-NPG was added to the solution, and agitation was continued for 1 h. The enzyme was removed by heating the solution at 80°C for 3 min, followed by centrifugation. Free nitrophenol and residual acceptor were removed by extraction with ethyl acetate (3 × 10 mL), and the aqueous phase was filtered through a plug of RP-silica gel (5.0 g). After washing with water (20 mL), the product was eluted with ethanol to furnish **2** as a colorless solid (37 mg, 23%).

Lactose donor

Compound **1** (23 mg, 0.1 mmol) and D-lactose (65 mg, 0.2 mmol) were dissolved in phosphate buffer (2.0 mL, 50 mM; 1.0 mmol L^{-1} $MgCl_2$, 5.0 mM mercaptoethanol, pH 7.0), and the mixture was incubated with β-galactosidase from *E. coli* (30 μL, 50 U) with shaking. After 3 h, a further equivalent of lactose was added to the solution, and agitation was continued for 2 h. The product was isolated as above to give pure **2** (4 mg, 15%).

Repetitive-batch operation

To a solution containing D-lactose (3.7 g, 10.8 mmol) in phosphate buffer (20 mL, 50 mM; 1.0 mmol L^{-1} $MgCl_2$, 5.0 mM mercaptoethanol, pH 7.0) was added a solution of **1** (1.00 g, 4.2 mmol) in acetonitrile (1.0 mL). The mixture was placed in an ultrafiltration cell and was incubated with β-galactosidase from *E. coli* (1.3 mL, 2150 U) at room temperature for 3.5 h with slow stirring. The product-containing solution was collected by pressure application, and the enzyme that was retained by the membrane was re-used for a subsequent, identical conversion.

The combined reaction volumes from consecutive batches were processed by extractive removal of **1** using ethyl acetate (3 × 30 mL), followed by solid-phase extraction of **2** on RP-silica gel essentially as described above. Isolated yield 334 mg (10%); $[\alpha]_D^{20}$ +63.7 (*c* 1.0, water); R_f = 0.42 (EtOAc–isopropanol–H_2O, 9:4:2). ^1H NMR (500 MHz, D_2O) δ: 7.49–7.41 (m, 5H, H_{ar}), 5.00 (d, 1H, 1′-H), 4.77 (d, 1H, 1-H_a), 4.62 (d, 1H, 1-H_b), 4.46 (d, 1H, 1″-H), 3.92 (d, 1H, 4″-H), 3.87–3.78 (m, 4H, 3′-H, 5′-H_a, 5″-H, 6″-H_a), 3.75 (dd, 1H, 5′-H_b), 3.70 (dd, 1H, 4′-H), 3.66–3.63 (m, 2H, 3″-, 6″-H_b), 3.60 (dd, 1H, 2′-H), 3.52 (dd, 1H, 2″-H) ($J_{1a,1b}$ =

11.7, $J_{1',2'}$ = 3.7, $J_{2',3'}$ = 9.3, $J_{3',4'}$ = 8.1, $J_{4',5b'}$ = 3.9, $J_{5a',5b'}$ = 11.8, $J_{1'',2''}$ = 7.8, $J_{2'',3''}$ = 9.8, $J_{3'',4''}$ = 3.3 Hz). ^{13}C NMR (125.7 MHz, D_2O) δ: 139.8 (C_i), 131.6 (C_o), 131.3 (C_m), 131.16 (C_p), 104.6 (C-1″), 100.3 (C-1′), 79.6 (C-3′), 78.1 (C-4′), 75.5 (C-3″), 74.3 (C-5″), 74.0 (C-2′), 73.5 (C-2″), 72.6 (C-1), 71.5 (C-4″), 63.9 (C-5′), 62.0 (C-6″). MS *m*/*z* (%): 425.3 ([M + Na]$^+$100).

Benzyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1,4)-2,3-di-*O*-acetyl-α-D-xylopyranoside (3)

Disaccharide **2** (40 mg, 0.1 mmol) was taken up in dry pyridine (3.0 mL), acetic anhydride (2.0 mL) was added, and the solution was stirred at room temperature for 24 h. Water was added (20 mL), and the mixture was extracted with EtOAc (3 × 10 mL). The combined organic phases were washed consecutively with water, 1.0 N HCl, sat. $NaHCO_3$, and brine, and then dried over Na_2SO_4. The solvent was removed in vacuo, and the residue recrystallized from Et_2O–petrol ether mixtures to give peracetate **3** as colorless crystals (95 mg, 95%); mp 102°C; R_f = 0.47 (chloroform–MeOH, 40:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.35–7.23 (m, 5H, H_{ar}), 5.42 (t, 1H, 3′-H), 5.33 (dd, 1H, 4″-H), 5.06 (dd, 1H, 2″-H), 4.96 (dd, 1H, 3″-H), 4.93 (d, 1H, 1′-H), 4.73 (dd, 1H, 2′-H), 4.71 (d, 1H, 1-H_a), 4.47 (d, 1H, 1-H_b), 4.46 (d, 1H, 1″-H), 4.08 (m, 2H, 6″-H) 3.86 (dd, 1H, 5″-H), 3.78 (dd, 1H, 4′-H), 3.62 (d, 2H, 5′-H_{ab}) 2.13, 2.02, 2.01, 2.00, 1.99, 1.95 (6 × s, 18H, Ac) ($J_{1a,1b}$ = 12.2, $J_{1',2'}$ = 3.3, $J_{2',3'}$ = 10.2, $J_{3',4'}$ 9.4, $J_{4',5a'}$ = 7.6, $J_{5a',5b'}$ = 8.0, $J_{1'',2''}$ = 7.6, $J_{2'',3''}$ = 10.4, $J_{3'',4''}$ 3.4, = $J_{4'',5''}$ = 0.9, $J_{5'',6a''}$ = 6.6, $J_{5'',6b''}$ = 7.1 Hz) ^{13}C NMR (75.4 MHz, $CDCl_3$) δ: 172.2 (C=O), 138.9 (C_i), 130.4 (C_o), 129.9 (C_m), 129.7 (C_p), 103.1 (C-1″), 96.9 (C-1′), 79.1 (C-4′), 73.1 (C-5″), 72.9 (C-2′), 72.8 (C-3″), 72.1 (C-3′), 71.6 (C-1), 71.1 (C-2″), 68.9 (C-4″), 63.2 (C-6″), 61.4 (C-5′), 22.5, 22.6, 22.8 (6 × CH_3). Anal. calcd. for $C_{30}H_{38}O_{16}$ (654.61): C 55.04, H 5.85; found C 55.12, H 5.88.

β-D-Galactopyranosyl-(1,4)-D-xylose (4)

To a solution of benzyl disaccharide **2** (160 mg, 0.4 mmol) in 50% aqueous ethanol (10 mL) containing acetic acid (0.25 mL) was added Pd/C catalyst (10%; 400 mg), and the suspension was stirred in an autoclave under hydrogen (60 bar) at 50°C. After 24 h (TLC control EtOAc–isopropanol–water, 3:2:2), the reaction mixture was filtered through Celite, and the solution was concentrated in vacuo. The resultant residue was taken up in water (10 mL) and filtered through a plug of RP silica gel (2.0 g). After further elution with water (20 mL), the combined aqueous phases were freeze-dried to provide free disaccharide **4** as a colorless solid, which could be recrystallized from acetone. Yield 99 mg (80%); mp 164°C (mp 160–168°C (7)); $[\alpha]_D^{20}$ +21.5 (*c* 1.0, water; $[\alpha]_D^{20}$ +22 (7)). ^{1}H NMR (300 MHz, D_2O) δ: 4.56 (d, $J_{1'',2''}$ = 7.3 Hz, 1″-H), 3.97–3.56 (m, 12H). ^{13}C NMR (75.4 MHz, D_2O) δ: 105.19 (C-1″), 82.91 (C-1′), 77.95, 75.40, 74.71, 73.76, 72.70, 71.45, 65.48, 63.89, 63.44.

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