11302 *J. Agric. Food Chem.* **2009,** *57,* 11302–11307
DOI:10.1021/jf901834k

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Characterization of Galactosyl Derivatives Obtained by Transgalactosylation of Lactose and Different Polyols Using Immobilized *β*-Galactosidase from *Aspergillus oryzae*

Gabriela Irazoqui,[†] Cecilia Giacomini,[†] Francisco Batista-Viera,[†] Beatriz M. Brena,[†] Alejandra Cardelle-Cobas,[‡] Nieves Corzo,*[‡] and María Luisa Jimeno[§]

[†]Cátedra de Bioquímica, Departamento de Biociencias, Facultad de Química, Universidad de la República, Gral. Flores 2124, CC 1157, Montevideo, Uruguay, [‡]Instituto de Fermentaciones Industriales and [§]Centro Química Orgánica Lora Tamayo (C.S.I.C.), Juan de la Cierva, 3, Madrid 28006, Spain

The synthesis of novel galactosides is interesting because of their important role in several biological processes. Their properties greatly depend upon the configuration and type of galactoside. Therefore, to study biological activity, it is essential to elucidate the structure of the products. Glycosidases are capable of catalyzing glycosidic linkages with absolute stereoselectivity of the anomeric center. We report the enzymatic synthesis of galactosyl-ethylene glycol, galactosyl-glycerol, and galactosyl-erythritol by immobilized *β*-galactosidase from *Aspegillus oryzae*. The obtained galactosides were isolated and fully characterized by an extensive nuclear magnetic resonance (NMR) study. Complete structure elucidation and full proton and carbon assignments were carried out using 1D (^{1}H and ^{13}C) and 2D (gCOSY, TOCSY, multiplicity-edited gHSQC, and gHMBC) NMR experiments. The *β*-galactosidase from *A. oryzae* showed a strong preference for primary alcohols. For galactosyl-glycerol and galactosyl-erythritol, this preference generated one and two chiral centers, respectively, and a mixture of stereoisomers was obtained as a consequence.

KEYWORDS: Galactosyl derivatives; transgalactosylation; lactose; polyols; enzymatic synthesis; immobilized *β*-galactosidase

INTRODUCTION

The use of glycosidases for synthetic purposes is an interesting alternative to the complex chemical synthesis, allowing for the formation of glycosidic bonds in one step, with complete control of the anomeric center configuration (*1*). Glycosidases in nature act as hydrolases, but interestingly, they can catalyze transglycosylation processes, under some specific conditions. In particular, *β*-galactosidase catalyzes the transfer of a galactose moiety from a donor to an acceptor molecule, a principle that has already been used for the synthesis of several galactosides, using both carbohydrates and alcohols as acceptors (*2*−*6*). Glycosidases are stereospecific and synthesize only α or *β* bonds, but they are not regiospecific. A mixture of regioisomers is thus probably obtained when the acceptor presents more than one glycosylation site. The regioisomers produced depend upon the source of the enzyme, the acceptor, and the reaction conditions (*7*−*11*). *Aspergillus oryzae* *β*-galactosidase is a monomeric extracellular enzyme produced by a generally recognized as safe (GRAS) microorganism; therefore, it can be used in food technology. In addition, it is stable in a wide temperature range and in the presence of several organic co-solvents, it has excellent transglycosylation properties, and its performance immobilized onto solid supports has been thoroughly studied (*4*, *12*). All of these properties make it a good choice for galactooligosaccharide synthesis.

The interest in galactosides lies in their important role in biological processes. Galactooligosaccharides (GOS), comprised of 2−20 molecules of galactose and 1 glucose (*13*), are recognized as prebiotics because they can stimulate the proliferation of lactic acid bacteria and bifidobacteria in the human intestine (*14*). Other health benefits, such as a reduction of the level of cholesterol in serum, colon cancer prevention, and enhancement of calcium absorption, have also been described (*14*−*16*). Moreover, several low-molecular-weight glycosides showing interesting biological properties have been reported, such as glycosylglycerol with antitumoral activity (*17*, *18*), lactulose amines with potential anticancer activity (*19*), and galactosyl-aldoximes capable of galectin inhibition (*20*). Because the biologic activity of glycosides is strictly related to its chemical structure, structural characterization of novel compounds is essential (*18*−*23*).

The disaccharide lactose [*β*-D-galactopyranosyl(1→4)D-glucopyranose] is an interesting galactosyl donor, because it makes possible the generation of high added value products from a waste material, such as milk whey (*24*). Alcohols are reported to be better acceptors than carbohydrates (*5*, *6*), favoring the transglycosylation reaction over the hydrolysis. Therefore, we have decided to study the transglycosylation products using lactose as the galactosyl donor and different polyols as acceptors to generate potential bioactive compounds.

*To whom correspondence should be addressed. Telephone: +34-91-258-74-85. Fax: +34-91-564-48-53. E-mail: ncorzo@ifi.csic.es.

 Published on Web 11/06/2009 © 2009 American Chemical Society

Immobilized enzymes offer several benefits, such as better stability than the soluble form, reuse of the biocatalyst, and continuous system application. Moreover, covalently immobilized monomeric enzymes, such as *A. oryzae* β-galactosidase, do not leak from the support; thus, the final product obtained is enzyme-free, and the enzymatic reaction is stopped by a simple filtration step, avoiding the heating stage, which could affect the products (*12*, *25*).

The aim of this work is to perform an extensive nuclear magnetic resonance (NMR) study for characterizing the potentially bioactive galactosyl-polyols obtained by transgalactosylation of lactose (galactosyl-donor) with ethylene glycol, glycerol, and erythritol as acceptors, using immobilized β-galactosidase from *A. oryzae*.

MATERIALS AND METHODS

Materials. Lactose monohydrate was supplied by Scharlau (Spain). *o*-Nitrophenyl-β-D-galacto pyranoside (ONPG), β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) from *A. oryzae*, galactose, ethylene glycol, glycerol, and erythritol were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade.

Synthesis of Glutaraldehyde−Agarose. Glutaraldehyde−agarose containing 90 μmol of glutaraldehyde/g of suction-dried gel was prepared as it has been previously described by Guisán et al. (*26*).

Immobilization of β-Galactosidase onto Glutaraldehyde−Agarose. The immobilization of the enzyme on to glutaraldehyde−agarose was performed as it was described by Irazoqui et al. (*12*). The enzyme derivative was named Glut90 derivative.

Protein Assay. The protein content of the soluble and immobilized enzyme was estimated by the bicinchoninic acid (BCA) assay (*27*). Immobilized protein was expressed as milligrams of protein per gram of suction-dried gel.

Enzyme Activity. The activity of β-galactosidase was assayed at room temperature using the chromogen ONPG as the substrate. Aliquots of 100 μL of a suitably diluted *A. oryzae* β-galactosidase solution was added to 2.0 mL of 25 mM ONPG in 50 mM sodium acetate buffer at pH 5.5 (*A. oryzae* activity buffer). The rate of formation of free *o*-nitrophenol (ONP) was recorded spectrophotometrically at 405 nm using a 1 cm path length cuvette provided with magnetic stirring. One unit of enzyme activity (EU) was defined as the amount of enzyme hydrolyzing 1 micromol of substrate min^{-1} in the conditions defined above. The extinction coefficient of 7.5×10^2 M^{-1} cm^{-1} for ONP was used for pH 5.5. For immobilized enzyme, activity was measured under identical conditions by incubating 100 μL of an appropriate aliquot of the gel suspensions with 2.0 mL of 25 mM ONPG in 50 mM sodium acetate buffer at pH 5.5. Enzymatic activity was expressed as EU per milliliter for the soluble enzyme and as EU per gram of suction-dried gel for the gel-bound activity of the derivative.

Enzymatic Synthesis of Galactosyl-polyols. Aliquots of 10 mL of 0.75 M lactose solution in 50 mM sodium acetate buffer at pH 5.5 containing 2.0 M of the corresponding acceptor (ethylene glycol, glycerol, and erythritol) were supplemented with Glut90 derivative to a final enzymatic activity of 6 EU/mL. The suspensions were gently agitated at room temperature for 72 h. Aliquots were taken at regular intervals; the reaction was stopped by filtration; and the samples were analyzed for carbohydrates, as described below.

Analysis of Galactosyl-polyols by High-Performance Anion-Exchange Chromatography−Pulsed Amperometric Detection (HPAEC−PAD). The synthesis of the galactosyl-polyols was followed by HPAEC−PAD on an ICS2500 Dionex system consisting of a GP50 gradient pump and an ED50 electrochemical detector with a gold working electrode and Ag/AgCl reference electrode. Acquisition and processing of data were achieved using Chromeleon version 6.7 software (Dionex Corp., Sunnyvale, CA). For eluents preparation, Milli-Q water, 50% (w/v) NaOH, and NaOAc (Fluka, Germany) were used. All eluents were degassed by flushing helium for 25 min. Separations were performed under isocratic conditions using 100 mM NaOH. Elution was at room temperature on a CarboPac PA-1 column (4 × 250 mm) (Dionex) connected to a CarboPac PA-1 (4 × 50 mm) (Dionex) guard column. After each run, the column was washed for 10 min with 100% 100 mM NaOH and 1 M NaOAc and re-equilibrated for 15 min with 100 mM NaOH. Samples and standard solutions were filtered through a nylon Millipore FH (0.22 μm) (Bedford, MA) membrane before injection.

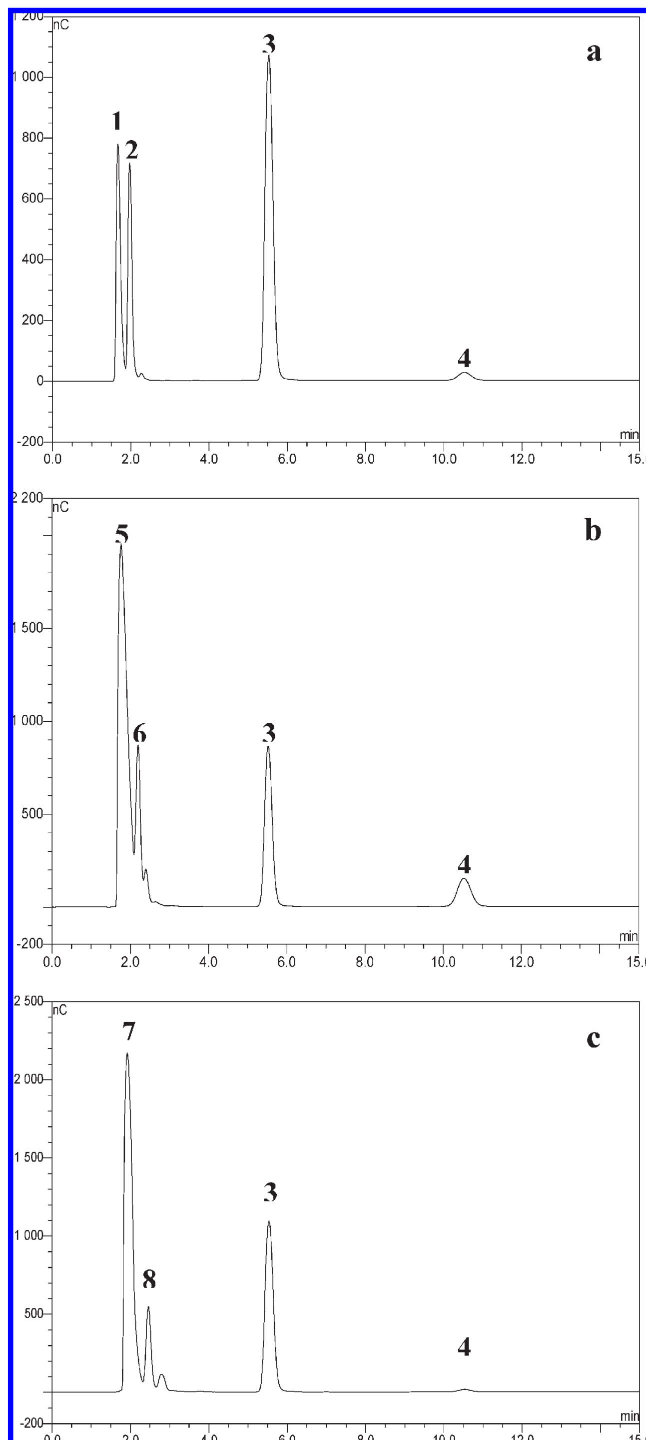

Figure 1. HPAEC−PAD chromatograms of galactosyl-polyol synthesis starting from lactose (0.75 M) and (a) 2 M ethylene glycol, (b) 2 M glycerol, and (c) 2 M erythritol, using immobilized β-galactosidase from *A. oryzae*, after 48 h of reaction at room temperature. The identified compounds are indicated: (1) ethylene glycol, (2) galactosyl-ethylene glycol, (3) galactose and glucose, (4) lactose, (5) glycerol, (6) galactosyl-glycerol, (7) erythritol, and (8) galactosyl-erythritol.

A total of 20 μL was injected using an autosampler, and separations were performed at a rate of 1 mL/min. The detection time and voltage parameters were set according to waveform A: $E_1 = 0.1$ V ($t_1 = 400$ ms), $E_2 = -2$ V ($t_2 = 10$ ms), $E_3 = 0.6$ V, and $E_4 = -0.1$ ($t_3 = 60$ ms) (*28*).

Quantification was performed by external calibration using a standard solution of lactose and galactose. The regression coefficients of the curves for each standard were always higher than 0.99.

Galactosyl-polyols Purification. *Charcoal Treatment*. To reduce the concentration of monosaccharides, a reaction mixture of the galactosyl-glycerol was treated with activated charcoal (*29*). A total of 2 mL of the reaction mixture, containing 0.5 g of carbohydrates, was diluted to 100 mL with water, stirred for 30 min with 3 g of activated charcoal, and then vacuum-filtered through Whatman No. 1 filter paper. Afterward, the activated charcoal was washed with 50 mL of water. The galactosyl-glycerol adsorbed onto the activated charcoal was extracted by stirring for 30 min with 100 mL of a 50:50 water/ethanol solution and then filtered. The procedure was repeated up to the total galactosyl-glycerol extraction, and the water/ethanol solutions were pooled and concentrated in a rotatory evaporator (Büchi, Switzerland) at 30 °C.

Semi-preparative High-Performance Liquid Chromatography−Refractive Index (HPLC−RI). Galactosyl derivatives were further purified by semi-preparative HPLC−RI using an amino Kromasil 100 NH_2, 5 μm, 10 × 250 mm column (Tecknocroma, Spain). Compounds were separated under isocratic conditions with acetonitrile/water (75:25, v/v) at a flow rate of 4 mL/min. Acquisition and processing of data were achieved with Chromeleon version 6.7 software (Dionex Corp., Sunnyvale, CA). The signal corresponding to the RI detector was registered as μA. Fractions corresponding to each galactosyl derivative were collected, analyzed by HPAEC−PAD as previously described, and freeze-dried for mass spectrometry (MS) and NMR analyses.

Characterization of Galactosyl-polyols. The molecular mass of the purified compounds was determined by MS. Spectra were recorded using a quadrupole HP 1100 mass detector in the electrospray positive mode (API−ES). The mass spectrometer was operating with a 4000 V needle potential, 330 °C gas temperature, drying gas flow of 10 L/min, and 40 psi nebulizer pressure. Scan m/z was from 100 to 1500.

The chemical structures of synthesized galactosyl-polyols were identified by an extensive ^{13}C and ^{1}H NMR study. NMR spectra were recorded at 293 K, using D_2O as the solvent, on a Varian System 500 NMR spectrometer equipped with a 5 mm HCN cold probe. ^{1}H chemical shifts were referenced to the residual solvent signal at δ_H 4.72 (D_2O) relative to DSS. ^{13}C chemical shifts were referenced using dioxane as an internal reference (δ_C 66.6 relative to external TMS in water). ^{1}H spectra were obtained using presaturation solvent suppression. 2D [^{1}H−^{1}H] NMR experiments (gCOSY and TOCSY) were carried out with the following parameters: a delay time of 1 s, a spectral width of 3000 Hz in both dimensions, 4096 complex points in t2 and 4 transients for each of 256 time increments, and linear prediction to 512. The data were zero-filled to 4096 × 4096 real points. 2D [^{1}H−^{13}C] NMR experiments (multiplicity-edited gHSQC and gHMBC) used the same ^{1}H spectral window, a ^{13}C spectral window of 15000 Hz, 1s of relaxation delay, 1024 data points, and 256 time increments, with linear prediction to 512. The data was zero-filled to 2048 × 1024 real points. Typical numbers of transients per increment were 4 and 16, respectively. Spectra were optimized for coupling constants of 145 Hz (gHSQC) and 8 Hz (gHMBC), respectively.

RESULTS AND DISCUSSION

The synthesis of the galactosyl derivatives by transgalactosylation of lactose with immobilized β-galactosidase from *A. oryzae* using different polyols was followed by HPAEC−PAD. **Figure 1** shows a chromatographic profile of each reaction mixture of lactose and (**a**) ethylene glycol, (**b**) glycerol, and (**c**) erythritol after 48 h of reaction at room temperature. The first peak found in all chromatograms corresponded to the alcohol acceptor, ethylene glycol (peak 1), glycerol (peak 5), and erythritol (peak 7). From a comparison of the retention time with standards, peak 3 was assigned to glucose and galactose, which co-eluted, and peak 4 was assigned to lactose. Moreover, unknown compounds (peaks 2, 6, and 8) eluting in the region between the polyols and monosaccharides were also detected in the three reaction mixtures analyzed; they could be tentatively assigned as galactosyl-ethylene glycol (peak 2; compound **1**), galactosyl-glycerol (peak 6; compound **2**), and galactosyl-erythritol (peak 8; compound **3**). To identify these compounds, fractionation of each reaction mixture was carried out by semi-preparative HPLC−RI.

Figure 2 shows a preparative HPLC−RI profile obtained from analysis of the reaction mixture of lactose (0.75 M) and ethylene glycol (2.0 M) incubated at room temperature during 48 h using immobilized β-galactosidase. The peaks observed corresponded to glucose and galactose (peak 3) and lactose (peak 4). Peak 2 was tentatively assigned as galactosyl-ethylene glycol and was collected and analyzed by MS and NMR. This purification process was also carried out for each enzymatic reaction mixture of lactose/glycerol (previously treated with activated charcoal) and lactose/erythritol.

The three HPAEC−PAD profiles showed that the major compound synthesized in each case was the galactosyl-polyol of

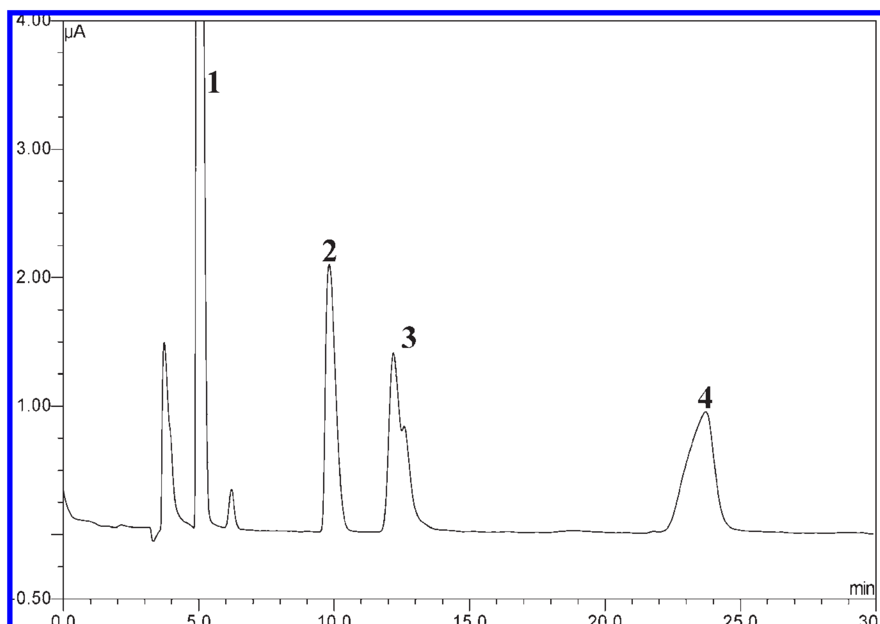

Figure 2. Preparative HPLC−RI chromatogram of galactosyl-ethylene glycol synthesis, at room temperature, starting from lactose (0.75 M) and ethylene glycol (2.0 M), using immobilized β-galactosidase from *A. oryzae*, after 48 h of reaction. The identified compounds are indicated as (1) ethylene glycol, (2) galactosyl-ethylene glycol, (3) glucose and galactose, and (4) lactose.

interest. This might indicate that ethylene glycol, glycerol, and erythritol were excellent galactosyl acceptors. In a previous work, we have already reported that ethylene glycol was a more suitable acceptor than water, allowing for 100% of transgalactosylation in similar synthesis conditions, starting from *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the galactosyl donor (*5*).

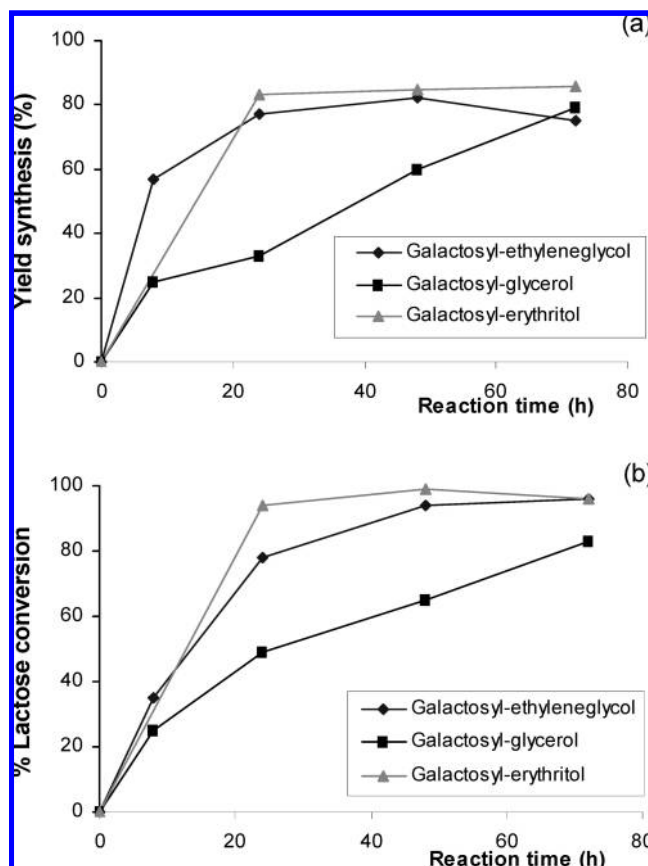

Figure 3. (a) Galactosyl-polyol synthesis yield and (b) percentage of lactose conversion during the time course of the enzymatic reaction performed at room temperature at an initial lactose concentration of 0.75 M, 2 M of each polyalcohol, and 6 EU/mL of immobilized β-galactosidase at pH 5.5. The synthesis yield was defined as the amount (moles) of galactosyl-polyol obtained as a percentage of the total amount (moles) of initial lactose (galactosyl donor). The lactose conversion was defined as the amount (moles) of reacted lactose (difference between the amount of lactose initially applied and the amount of unreacted lactose) as a percentage of the total amount (moles) of lactose initially applied.

The time course of lactose conversion has been studied for the three galactosyl-polyols. The synthesis yield was defined as the amount (moles) of galactosyl-polyol obtained as a percentage of the total amount (moles) of initial lactose (galactosyl donor). The conditions of synthesis used were taken from our previous work (*5*), where galactosyl-ethylene glycol synthesis was carried out using 50 mM ONPG and 8.9 M ethylene glycol, containing 1 EU/mL of β-galactosidase from *A. oryzae* as the biocatalyst and incubating at room temperature for 4 h. Because lactose hydrolysis had slower kinetics than the ONPG hydrolysis, in the present work, galactosyl-polyol synthesis was carried out using a longer period of time and greater amount of enzyme. The lactose conversion was defined as the amount (moles) of reacted lactose (difference between the amount of lactose initially applied and the amount of unreacted lactose) as a percentage of the total amount (moles) of lactose initially applied. **Figure 3** shows that the maximum yields for galactosyl-erythritol (85%) and galactosyl-ethylene glycol (82%) were reached after 48 h of reaction, corresponding to 99 and 94% of lactose conversion, respectively. The kinetic of the galactosyl-glycerol synthesis and lactose conversion was lower than that of the other two galactosyl-polyols, because after 48 h of reaction, the synthesis yield was 60% and the percentage of lactose conversion was 65%. Therefore, in this case, a longer reaction time was convenient, which allowed for 79% of the synthesis yield and 83% of lactose conversion after 72 h.

Structural Characterization of Galactosyl-polyols. *MS Analysis.* MS analysis of pure galactosyl-polyols gave intense ions (M + Na) at *m*/*z* 247.1 for galactosyl-ethylene glycol, *m*/*z* 277.0 for galactoysl-glycerol, and *m*/*z* 307.0 for galactosyl-erythritol. This confirms that these compounds were the main galactosides formed as a result of transgalactosylation catalyzed by β-galactosidase from *A. oryzae*.

NMR Analysis. Complete structural elucidation of pure galactosyl-polyols was carried out by the combined use of 1D and 2D [^{1}H−^{1}H] and [^{1}H−^{13}C] NMR experiments (gCOSY, TOCSY, multiplicity-edited gHSQC, and gHMBC). ^{1}H and ^{13}C NMR assignments for synthesized galactosyl-polyols are given in **Table 1**. A full set of spectra are collected in the Supporting Information.

For compound **1**, the ^{1}H NMR spectrum showed one doublet in the anomeric region. Moreover, the ^{13}C NMR spectrum displayed eight signals. A multiplicity-edited gHSQC spectrum was used to link the carbon signals to the corresponding proton

Table 1. ^{1}H and ^{13}C NMR Chemical Shift Values of Compounds **1**−**3**

	galactose						polyol			
compound	1′	2′	3′	4′	5′	6′	1	2	3	4
1	4.33 (7.9)[a]	3.45	3.56	3.82	3.60	3.69 3.76	3.68	3.91 3.68		
2a	4.30 (7.8)[a]	3.437	3.55	3.81	3.59	3.70 3.89	3.49 3.55	3.82	3.66 3.81	
2b	4.30 (7.9)[a]	3.439	3.55	3.81	3.59	3.71 3.89	3.49	3.85	3.57 3.90	
3a	4.30 (7.8)[a]	3.43	3.53	3.80	3.57	3.66 3.63	3.51	3.70	3.61	3.89 3.74
3b	4.30 (7.8)[a]	3.43	3.53	3.80	3.57	3.66 3.63	3.51	3.70	3.61	3.98 3.64
1	103.15	71.03	72.85	68.82	75.32	61.16	60.89	71.30		
2a	103.20	70.93	72.77	68.77	75.30	61.14	62.47	70.59	70.88	
2b	103.46	70.98	72.77	68.77	75.30	61.14	62.47	70.80	71.09	
3a	103.20	71.01	72.84	68.84	75.35	61.20	62.72	70.58	71.77	71.28
3b	103.58	71.01	72.84	68.84	75.35	61.20	62.72	70.85	71.92	71.07

[a] *J* (H1′, H2′) in parentheses.

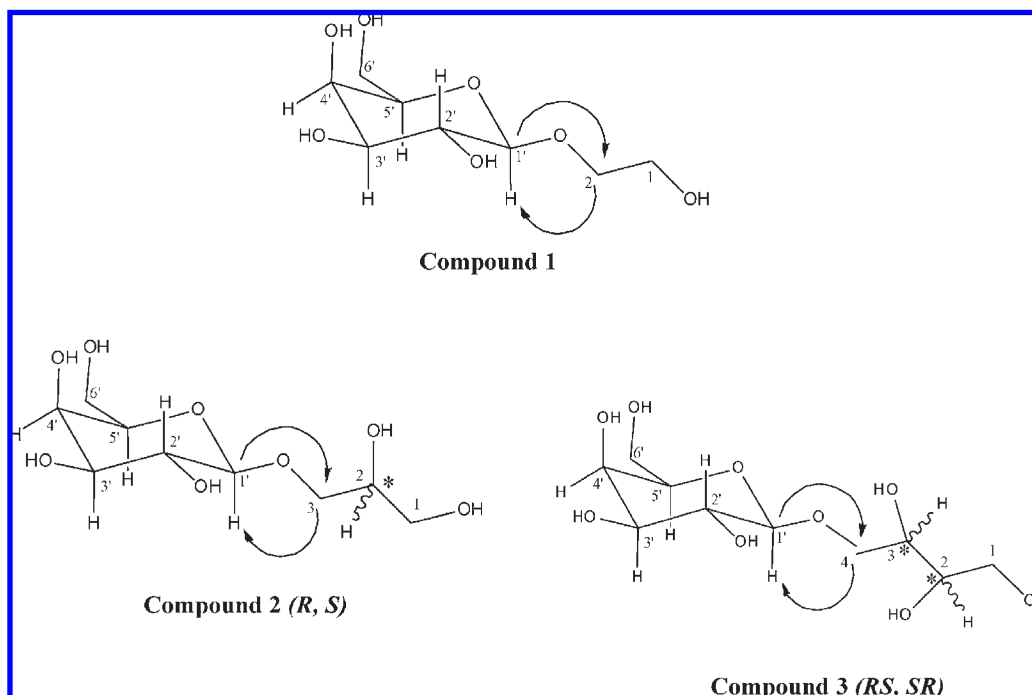

Figure 4. Structure of compound **1** (2-*O*-β-D-galactopyranosyl-ethylene glycol), compound **2** [(2*R*)- and (2*S*)-3-*O*-β-D-galactopyranosyl-glycerol], and compound **3** [(2*R*,3*S*)- and (2*S*,3*R*)-4-*O*-β-D-galactopyranosyl-erythritol]. Relevant interglycosidic gHMBC NMR correlations are indicated by arrows.

resonances, indicating the presence of three methylene and five methine carbons. From these results and taking the anomeric proton as a starting point, gCOSY and TOCSY experiments revealed the presence of a sugar moiety, which was determined to be galactose with a β configuration [J (H1′, H2′) = 7.8 Hz]. In addition, the remaining two methylene carbons were assigned to the existence of an ethylene glycol unit. The linkage between the ethylene glycol unit and the sugar was established from gHMBC correlations between the anomeric proton at δ_H 4.33 and the methylene carbon at δ_C 71.30 and between the methylene proton at δ_H 3.91 and the anomeric carbon at δ_C 103.15 (see **Figure 4**). Consequently, the structure 2-*O*-β-D-galactopyranosyl-ethylene glycol was deduced for compound **1**.

Compound **2** displayed a ^{1}H NMR spectrum similar to that of compound **1**. However, the spectrum was complicated, owing to the formation of a mixture of isomers (1:1), and some signals appeared duplicated {two overlapped doublets [J (H1, H2) = 7.8−7.9 Hz] for the anomeric proton that was observed}. Similarly, the ^{13}C NMR spectrum displayed two sets of peaks. From the multiplicity-edited gHSQC spectrum, three methylene and six methine carbons were identified for each set. gCOSY and TOCSY experiments allowed for the assignments of the ^{1}H chemical shifts for both isomers. These assignments were consistent with the presence of a β-galactosyl and glycerol moiety for each isomer. For both isomers, the linkage between glycerol and galactose moieties was established from gHMBC correlations (see **Figure 4**) between the anomeric proton (δ_H 4.30 and 4.30) and the methylene carbon (δ_C 70.88 and 71.09) and between the methylene proton (δ_H 3.81 and 3.90) and the anomeric carbon (δ_C 103.20 and 103.46). These data indicated that, in both isomers, the β-galactosyl residue was linked to a primary hydroxyl of glycerol. Taking into account that glycosylation would make the C-2′ of the glyceryl residue asymmetric, which would thus lead to the formation of a mixture of two diastereomeric galactosyl- glycerol molecules, and that the integrated areas of the two anomeric carbon signals in the ^{13}C NMR spectrum showed a 1:1 ratio, compound **2** was identified as a mixture of (2*R*)- and (2*S*)-3-*O*-β-D-galactopyranosyl-glycerol.

For compound **3**, ^{13}C and ^{1}H NMR spectra were very similar to those of compound **2**, displaying two sets of peaks, owing to the presence of two isomers. From the multiplicity-edited gHSQC spectrum, three methylene and seven methine carbons were identified for each set. Using the same set of NMR techniques, total assignments of ^{1}H and ^{13}C NMR spectra were established (see **Table 1**). On the basis of these data, the presence of a β-galactosyl and an erythritol moiety was deduced for each isomer. For both isomers, the linkage between erythritol and galactose residues was established from gHMBC correlations (see **Figure 4**) between the anomeric proton (δ_H 4.30 and 4.30) and one of the erythritol methylene carbons (δ_C 71.28 and 71.07) and between one of the erythritol methylene protons (δ_H 3.89 and 3.98) and the anomeric carbon (δ_C 103.20 and 103.58). These data indicated that, in both isomers, the β-galactosyl residue was linked to a primary hydroxyl of erythritol. Therefore, compound **3** was identified as a mixture of (2*R*,3*S*)- and (2*S*,3*R*)-4-*O*-β-D-galacto-pyranosyl-erythritol.

The results obtained here show the characterization of the following galactosyl-polyols: 2-*O*-β-D-galactopyranosyl-ethylene glycol, (2*R*)- and (2*S*)-3-*O*-β-D-galactopyranosyl-glycerol, and (2*R*,3*S*)- and (2*S*,3*R*)-4-*O*-β-D-galactopyranosyl-erythritol.

The use of β-galactosidase from *A. oryzae* as a biocatalyst for galactosyl-glycerol synthesis, starting from lactose and glycerol, gives only the galactosyl product bounded by the primary alcohol function of glycerol, in agreement with previous reports (*30*). It has been reported that the specificity of each glycosidase is dependent upon its origin and plays a main role in the structure of the products obtained (*8*, *10*, *11*). Thus, Stevenson et al. (*31*) have reported the synthesis of galactosyl-glycerol catalyzed by the β-galactosidase from *Kluyveromyces lactis*, obtaining galactosyl-glycerol bounded by both the primary and secondary alcohol functions.

The structure of the galactosyl-erythritol obtained indicates, once again, a total selectivity of the *A. oryzae* β-galactosidase to bind the galactose moiety to the primary alcohol of the poly-alcohol. Even though synthesis of galactosyl-erythritol using

K. lactis, *Kluyveromyces fragilis*, and *A. oryzae* β-galactosidases has been previously reported (*2*, *32*), this is the first time that a complete identification and a structural characterization of these products have been carried out.

In this work, we achieved the synthesis of a series of galactosyl-polyols by lactose transglycosylation with a series of polyols using β-galactosidase from *A. oryzae*. The obtained galactosides were isolated and fully characterized by an extensive NMR study. The following galactosides were identified: 2-*O*-β-D-galactopyranosyl-ethylene glycol, a (1:1) mixture of (2*R*) and (2*S*)-3-*O*-β-D-galactopyranosyl-glycerol, and a (1:1) mixture of (2*R*,3*S*) and (2*S*,3*R*)-4-*O*-β-D-galactopyranosyl-erythritol. The immobilized β-galactosidase from *A. oryzae* showed a preference for primary alcohols in all of the acceptors studied. Because the biological effects can vary greatly for different isomers, the knowledge of the detailed structure of the galactosyl-polyols reported here is of crucial importance in connection with its possible biological application as prebiotics or antitumor-promoting agents. The enzymatic synthesis process will be further optimized and scaled up to allow for a thorough study of their potential biological properties, which will be the subject of future studies.

Supporting Information Available: Full set of spectra for compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Crout, D. H. G.; Mac Manus, D. A. Enzymatic synthesis of glycosides using the β-galactosidase of *Escherichia coli*: Regio- and stereochemical studies. *J. Chem. Soc., Perkin Trans. 1* **1990**, *1*, 1865–1868.
(2) Klewicki, R. Formation of gal-sorbitol during lactose hydrolysis with β-galactosidase. *Food Chem.* **2007**, *100*, 1196–1201.
(3) Schröder, S.; Schmidt, U.; Thiem, J.; Kowalczyk, J.; Kunz, M.; Vogel, M. Synthesis of oligosaccharides as potential novel food components and up scaled enzymatic reaction employing the β-galactosidase from bovine testes. *Tetrahedron* **2004**, *60*, 2601–2608.
(4) Reuter, S.; Nygaard, A. R.; Zimmermann, W. β-Galactooligosaccharide synthesis with β-galactosidases from *Sulfolobus solfataricus*, *Aspergillus oryzae*, and *Escherichia coli*. *Enzyme Microb. Technol.* **1999**, *25*, 509–516.
(5) Giacomini, C.; Irazoqui, G.; Gonzalez, P.; Batista-Viera, F.; Brena, M. B. Enzymatic synthesis of galactosyl-xylose by *Aspergillus oryzae* β-galactosidase. *J. Mol. Catal. B: Enzym.* **2002**, *19*−*20*, 159–165.
(6) Tricone, A.; Tramice, A.; Giordano, A.; Andreotti, G. Glycoside hydrolases in *Aplysia fasciata*: Analysis and applications. *Biotechnol. Genet. Eng. Rev.* **2008**, *25*, 129–148.
(7) Ichikawa, Y.; Look, G. C.; Wong, C.-H. Enzyme-catalyzed oligosaccharide synthesis. *Anal. Biochem.* **1992**, *202*, 215–238.
(8) Palcic, M. C. Biocatalytic synthesis of oligosaccharides. *Curr. Opin. Biotechnol.* **1999**, *10*, 616–624.
(9) Usui, T.; Morimoto, S.; Hayakawa, Y.; Kawaguchi, M.; Murata, T.; Matahira, Y.; Nishida, Y. Regioselectivity of β-D-galactosyl-disaccharide formation using the β-D-galactosidase from *Bacillus circulans*. *Charbohydr. Res.* **1996**, *285*, 29–39.
(10) Yoon, J. H.; Ajisaka, K. The synthesis of galactopyranosyl derivatives with β-galactosidases of different origins. *Charbohydr. Res.* **1996**, *292*, 153–163.
(11) Watt, G. M.; Lowden, P. A. S.; Flitsch, S. L. Enzyme catalyzed formation of glycosidic linkages. *Curr. Opin. Struct. Biol.* **1997**, *7*, 652–660.
(12) Irazoqui, G.; Giacomini, C.; Batista-Viera, F.; Brena, B. M. Hydrophilization of immobilized model enzymes suggests a widely applicable method for enhancing protein stability in polar organic cosolvents. *J. Mol. Catal. B: Enzym.* **2007**, *46*, 43–51.
(13) Miller, J. N.; Whistler, R. L. Carbohydrates. In *Food Chemistry*; Fennema, O., Ed.; Marcel Dekker: New York, 2000; p 207.
(14) Sako, T.; Matsumoto, K.; Tanaka, R. Recent progress on research and applications of non-digestible galacto-oligosaccharides. *Int. Dairy J.* **1999**, *9*, 69–80.
(15) Perugino, G.; Trincone, A.; Rossi, M.; Moracci, M. Oligosaccharide synthesis by glycosynthases. *Trends Biotechnol.* **2004**, *22*, 31–37.
(16) Tuohy, K. M.; Rouzaud, G. C.; Brück, W. M.; Gibson, G. R. Modulation of the human gut microflora towards improved health using prebiotics—Assessment of efficacy. *Curr. Pharm. Des.* **2005**, *11*, 75–90.
(17) Colombo, D.; Compostella, F.; Ronchetti, F.; Scala, A.; Toma, L.; Kuchide, M.; Tokuda, H.; Nishino, H. Anti-tumor-promoting effects of glycoglycerolipid analogues on two-stage mouse skin carcinogenesis. *Cancer Lett.* **2000**, *161*, 201–205.
(18) Colombo, D.; Scala, A.; Taino, I. M.; Toma, L.; Ronchetti, F.; Tokuda, H.; Nishino, H.; Nagatsu, A.; Sakakibara, J. 1-*O*-, 2-*O*- and 3-*O*-β-glycosyl-*sn*-glycerols: Structure−anti-tumor-promoting activity relationship. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1187–1190.
(19) Rabinovich, G. A.; Cumashi, A.; Bianco, G. A.; Ciavardelli, D.; Lurisci, I.; Dégidio, M.; Piccolo, E.; Tinari, N.; Nifantiev, N.; Iacobelli, S. Synthetic lactulose amines: novel class of anticancer agents that induce tumor-cell apoptosis and inhibit galectin-mediated homotypic cell aggregation and endothelial cell morphogenesis. *Glycobiology* **2006**, *16*, 210–220.
(20) Tejler, J.; Leffler, H.; Nilsson, U. J. Synthesis of *O*-galactosyl-aldoximes as potent LacNac-mimetic galectin 3 inhibitors. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2343–2345.
(21) Sanz, M. L.; Gibson, G. R.; Rastall, R. A. Influence of disaccharide structure on prebiotic selectivity in vitro. *J. Agric. Food Chem.* **2005**, *53*, 5192–5199.
(22) Giguère, D.; Sachiko, S.; St. Peirre, C.; Sirois, S.; Roy, R. Aryl *O*- and *S*-galactosides and lactosides as specific inhibitors of human galectins-1 and -3: Role of electrostatic potential at O-3. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1668–1672.
(23) Salameh, B. A.; Leffler, H.; Nilsson, U. J. 3-(1,2,3-Triazol-1-yl)-1-thio-galactosides as small, efficient, and hydrolytically stable inhibitors of galectin-3. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3344–3346.
(24) Rastall, R. A.; Maitin, V. Prebiotics and synbiotics: Towards the next generation. *Curr. Opin. Biotechnol.* **2002**, *13*, 490–496.
(25) Giacomini, C.; Irazoqui, G.; Batista-Viera, F.; Brena, B. M. Influence of the immobilization chemistry on the properties of immobilized β-galactosidases. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 597–606.
(26) Guisán, J. M.; Penzol, G.; Armisén, P.; Bastida, A.; Blanco, R. M.; Fernández-Lafuente, R.; García-Junceda, E. Immobilization of enzyme acting on macromolecular substrates. In *Immobilization of Enzymes and Cells*; Bickerstaff, G. F., Ed.; Human Press, Inc.: Totowa, NJ, 1997; pp 261−275.
(27) Giacomini, C.; Villarino, A.; Franco-Fraguas, L.; Batista-Viera, F. Immobilization of β-galactosidase from *Kluyveromyces lactis* on silica and agarose: Comparison of different methods. *J. Mol. Catal. B: Enzym.* **1998**, *4*, 313–327.
(28) Dionex. Technical Note 21. Optimal settings for pulsed amperometric detection of carbohydrates using the Dionex ED40 electrochemical detector.
(29) Morales, V.; Sanz, M. L.; Olano, A. Rapid separation on activated charcoal of high oligosaccharides in honey. *Chromatographia* **2006**, *64*, 233–238.
(30) Woudenberg-van Oosterom, M.; van Belle, H. J. A.; van Rantwijk, F.; Sheldon, R. A. Immobilised β-galactosidases and their use in galactoside synthesis. *J. Mol. Catal. A: Chem.* **1998**, *134*, 267–274.
(31) Stevenson, D. E.; Stanley, R. A.; Furneaux, R. H. Optimization of alkyl β-D-galactopyranoside síntesis from lactose using commercially available β-galactosidases. *Biotechnol. Bioeng.* **1993**, *42*, 657–666.
(32) Klewicki, R. Transglycosylation of a β-galactosyl radical, in the course of enzymic hydrolysis of lactose, in the presence of selected polyhydroxyalcohols. *Biotechnol. Lett.* **2000**, *22*, 1063–1066.

Received for review June 1, 2009. Revised manuscript received October 16, 2009. Accepted October 16, 2009. This work was supported by the "Programa de Desarrollo de las Ciencias Básicas" (PEDECIBA) of Uruguay and the Cooperation Programme between Consejo Superior de Investigaciones Cientificas (España) and the Universidad de la República (Uruguay) (Project 2007UY0013).