JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 557–563 **557**

Enzymatic Synthesis and Identification of Two Trisaccharides Produced from Lactulose by Transgalactosylation

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The enzymatic transgalactosylation during lactulose hydrolysis was studied using the β-galactosidase from *Kluyveromyces lactis* and an initial lactulose concentration of 250 g/L. During hydrolysis of lactulose, the formation of two novel trisaccharides was followed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC−PAD). A maximum trisaccharide yield of 14.05% was observed at 91.9% of lactulose hydrolysis. The two novel trisaccharides obtained by transglycosylation of lactulose were isolated and fully characterized by an extensive nuclear magnetic resonance (NMR) study. Complete structure elucidation and full proton and carbon assignment were carried out using 1D (^{1}H, ^{13}C, and 1D TOCSY) and 2D (gCOSY, TOCSY, ROESY, gHSQC, and gHMBC) NMR experiments. The trisaccharides were shown to be lactulose-based structures; the main one has a Gal unit linked to C-6 of the galactose moiety, and the other one has a Gal unit linked to C-1 of the fructose moiety. Transglycosylation of lactulose allows for the obtention of galacto-oligosaccharides with new glycosidic structures and would open new routes to the synthesis of prebiotics.

KEYWORDS: **β-Galactosidase; trisaccharides; lactulose; transglycosylation**

INTRODUCTION

The importance of the colonic microflora in health and nutrition is well-known. The human large intestine plays an important role as a nutritional organ mainly because of the metabolic activities of the resident microbiota, which is made up of a very complex different bacterial species. A large number of diseases are strongly related to the composition of colonic microbiota (*1*) and *Bifidobacterium* spp., and lactobacilli, present in the colon, can have beneficial effects on constipation, diarrhea, the immune system, cancer, and absorption of minerals (*2, 3*).

The effectiveness of food enrichment with prebiotics to enhance the growth of bifidobacteria and lactobacilli in the intestine has been clearly recognized, and these products are drawing increased interest from consumers. Some carbohydrates that have established their status as prebiotic food ingredients and are currently used in the European market are fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) (*4*). GOS are usually produced by transglycosylation during enzymatic hydrolysis of lactose (*5*), and FOS are obtained by partial hydrolysis of inulin or from sucrose by action of fructosyltransferases (*6, 7*).

The formation of GOS during enzymatic hydrolysis of lactose is well-known, and transfer reactions of β-galactosidases from several sources as well as the effects that different factors, such as temperature and pH (*8–10*), use of organic solvents (*11, 12*), immobilization on different matrices (*13*), etc., have on enzymatic activity and the amount and nature of oligosaccharides formed have been extensively studied.

During the studies of prebiotic properties of carbohydrates, it has been observed that chemical structures of oligosaccharides (the number or type of hexose moieties and the position and conformation of links between the hexoses) may affect the fermentation properties of probiotic microorganisms (*14–17*); therefore, there is a high current interest in the obtention of new prebiotic carbohydrates with improved prebiotic potential.

Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) is a disaccharide currently manufactured by lactose isomerization in basic media. It has several pharmaceutical applications and is also used as a prebiotic ingredient in nutrition (*18–20*). Lactulose may be hydrolyzed by the action of microbial β-galactosidases (*21, 22*), but the study of its use as a substrate for oligosaccharide synthesis has not been undertaken. Because lactulose is resistant to the action of human digestive enzymes,

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10.1021/jf0721343 CCC: $40.75
Published on Web 12/21/2007

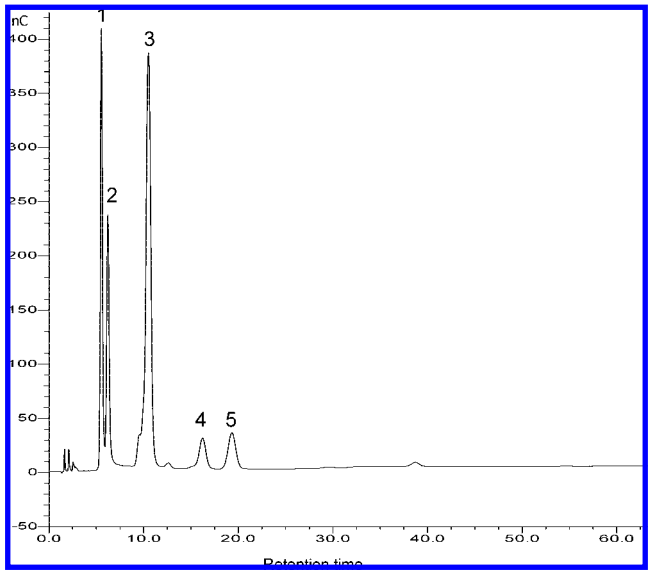

Figure 1. HPAEC−PAD carbohydrate profile obtained from lactulose hydrolysis produced by Lactozym 3000 L HP G at pH 6.5, 50 °C, 250 mg/mL of lactulose, and 3 units/mL of enzyme. The identified compounds are indicated: (1) galactose, (2) fructose, (3) lactulose, (4) trisaccharide **1**, and (5) trisaccharide **2**.

the production of lactulose-derived oligosaccharides by enzyme-mediated transgalactosylation may open new opportunities for the preparation of new complex oligosaccharides. The aim of the present work was to isolate and chromatografically purify new trisaccharides formed during their enzymatic transglycosylation of lactulose and to characterize the structure by an extensive nuclear magnetic resonance (NMR) study using 1D (^{1}H, ^{13}C, and 1D TOCSY) and 2D (gCOSY, TOCSY, ROESY, gHSQC, and gHMBC) NMR experiments.

MATERIALS AND METHODS

Materials. Lactulose (>98% purity) was supplied by Fluka (Steinheim, Germany), and the standard raffinose was purchased by Sigma-Aldrich (Steinheim, Germany). Soluble commercial preparation of β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G) was kindly provided by Novozymes A/S (Bagsvaerd, Denmark).

Transgalactosylation Reaction. A lactulose solution of 250 g/L was prepared in 50 mM potassium phosphate buffer containing 1 mM $MgCl_2$ at pH 6.5, and β-galactosidase enzyme from *K. lactis* was added at a final concentration of 3 units/mL as described in Martínez-Villaluenga et al. (*23*). The transgalactosylation reaction was performed at a final volume of 1 mL and 50 °C in eppendorfs incubated in an orbital shaker at 300 rpm. Samples (100 μL) were withdrawn at specific time intervals (0, 120, 240, 360, and 480 min) and immediately immersed in boiling water for 5 min to inactivate the enzyme. The samples were stored at −18 °C for subsequent high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC−PAD) analysis. Control samples were prepared in the same manner, except that no enzyme was added and no changes in lactulose were observed.

Chromatographic Determination of Saccharides. The synthesis of the trisaccharides during hydrolysis of lactulose 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. Data acquisition and processing were performed with Chromeleon version 6.7 software (Dionex Corp., Sunnyvale, CA). For eluents preparation, MilliQ water, 50% (w/v) NaOH, and NaOAc (Fluka, Germany) were used. All eluents were degassed by flushing with helium for 25 min.

Separations were performed following the method described by Spletchna et al. (*24*). Elution was at room temperature on a CarboPac PA-1 column (250 × 4 mm) connected to a CarboPac PA-1 (50 × 4 mm) guard column. Eluent A (100 mM NaOH), eluent B (100 mM NaOH and 50 mM NaOAc), and eluent C (100 mM NaOH and 1 M NaOAc) were mixed to form the following gradient: 100% A from 0 to 20 min and 0−100% C from 20 to 70 min. After each run, the column was washed for 10 min with 100% B and re-equilibrated for 15 min with the starting conditions of the employed gradient. Separations were performed at a flow rate of 1 mL/min.

Samples and standard solutions were filtered through a nylon Millipore FH (0.22 μm) (Bedford, MA) membrane before injection. A total of 20 μL was injected using an autosampler, and separations were performed at a rate of 1 mL/min. Quantification of lactulose and trisaccharides was performed by external calibration using a standard solution of lactulose and raffinose, respectively. The regression coefficients of the curves for each standard were always greater than 0.99.

Purification. A total of 2 mL of the reaction mixture, containing 0.5 g of carbohydrates, was diluted to 100 mL of water and stirred for 30 min with 3 g of activated charcoal and then vacuum-filtered through

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts (ppm) and Coupling Constants (Hz) for the Main Isomers of **1** and **2** in D_2O

	1A		**2A**		lactulose (*27*)	
position	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1a	3.42, d, 11.7	64.05	3.73, d, 11.5	71.62	3.65	64.08
1b	3.58, d, 11.7		3.82, d, 11.5		3.49	
2		98.21		97.77		98.26
3	3.79, d, 9.8	66.36	3.82, d, 9.8	66.45	3.84	66.23
4	3.99, dd, 9.8, 3.2	77.99	4.01, dd, 10.0, 3.4	77.25	4.06	77.59
5	4.06, dt, 3.3, 1.5	66.97	4.07, dt, 3.5, 1.5	66.85	4.13	66.97
6a	3.89, dd, 1.4, 13.0	63.19	3.90, dd, 1.4, 12.9	63.21	3.94	63.18
6b	3.61, dd		3.61, overlapped		3.68	
1′	4.43, d, 7.8	101.04	4.42, dd, 7.8	100.98	4.48	101.02
2′	3.47, dd, 7.8, 9.9	70.83	3.47, dd, 7.8, 10.0	70.86	3.53	70.95
3′	3.55, dd, 9.8, 3.4	72.66	3.54, dd, 10.0, 3.5	72.64	3.61	72.79
4′	3.82, dd, 3.3, nm[a]	68.84	3.78, dd, 3.4, 0.9	68.85	3.85	68.88
5′	3.78, ddd, 1.0, 3.8, 8.0	74.39	3.57, ddd, 0.9, 4.6, 8.3	75.52	3.64	75.55
6′a	3.80, dd, 3.8, 12.0	69.16	3.64–3.60, second order	61.31	3.73	61.34
6′b	3.92, dd, 7.9, 12.0				3.67	
1″	4.35, d, 7.9	103.44	4.30, d, 7.8	103.53		
2″	3.38, dd, 7.9, 9.8	70.99	3.43, dd, 7.8, 10.0	70.91		
3″	3.52, dd, 9.8, 3.2	72.77	3.51, dd, 9.9, 3.4	72.76		
4″	3.79, dd, 3.2, nm[a]	68.77	3.78, dd, 3.4, 0.9	68.77		
5″	3.56, ddd, 0.8, 4.4, 8.0	75.28	3.58, ddd, 0.9, 4.5, 8.9	75.41		
6″a/b	3.64–3.60, second order	61.15	3.64–3.60, second order	61.15		

[a] nm = not measurable.

Whatman No. 1 filter paper, and activated charcoal was washed with 50 mL of water. The charcoal sample was treated 3 times with 100 mL of water for complete removal of monosaccharides and almost the total amount of disaccharides. The oligosaccharides adsorbed onto the activated charcoal were 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 oligosaccharide extraction, and the water/ethanol solutions were pooled and concentrated in a rotatory evaporator (Büchi, Switzerland). The partially fractionated reaction products were further purified by high-performance liquid chromatography (HPLC) on amino Kromasil 4.6 × 250 mm i.d., 5 μm size column (Alltech Associates, Inc., Spain). Fractions were eluted in acetonitrile/water (75:25, v/v) as the mobile phase at a flow rate of 1 mL/min, and 1 mL fractions corresponding to GOS were collected. Fractions from 10 runs were pooled and freeze-dried for mass spectrometry and NMR analysis.

Mass Spectrometry Analysis. The molecular mass of the purified compounds was determined by 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.

NMR Analysis. 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 4.70 (D_2O) relative to tetramethylsilane (TMS). One-dimensional NMR experiments (^{1}H, ^{13}C, and 1D TOCSY) were performed using standard Varian pulse sequences. Two-dimensional [^{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. Two-dimensional [^{1}H, ^{1}H] ROESY NMR experiment used the same conditions with 64 transients for an increment and a mixing time of 200 ms. Two-dimensional [^{1}H−^{13}C] NMR experiments (gHSQC and gHMBC) used the same ^{1}H spectral window, a ^{13}C spectral windows of 15 000 Hz, 1 s of relaxation delay, 1024 data points, and 256 time increments, with a linear prediction to 512. The data were zero-filled to 4096 × 4096 real points. Typical numbers of transients per increment were 4 and 16, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the HPAEC−PAD profile obtained from lactulose hydrolysis produced by Lactozym 3000 L HP G at pH 6.5, 50 °C, 250 mg/mL of lactulose, and 3 units/mL of enzyme. During hydrolysis of lactulose (peak 3) in D-galactose (peak 1) and D-fructose (peak 2), compounds **1** and **2** (peaks 4 and 5, respectively) were formed. Mass spectrometry analysis of the pure compounds **1** and **2** gave a peak at *m/z* 501.1 (M + H) or 527.1 (M + Na). Therefore, trisaccharides were the main GOS formed as a result of transgalactosylation catalyzed by Lactozym 3000 L HP G. Trisaccharides were also dominant among GOS synthesized using lactose as a substrate of the reaction by β-galactosidase from *K. lactis* (*23, 25, 26*).

Figure 2 shows trisaccharide yields (a) and the remaining lactulose content (b) during the time course of the reaction. The maximum yield of trisaccharide **1** referred to the initial lactulose concentration increased up to 9.4% after 360 min at 91.5% of lactulose hydrolysis. Longer times of the reaction brought about the hydrolysis of this compound. With regard to trisaccharide **2**, the highest yield (7.6%) was observed after 120 min at 52.8% lactulose hydrolysis, and after this short time of the reaction, trisaccharide **2** was progressively hydrolyzed. A maximum yield of 14.05% for total trisaccharides was observed after 360 min at 91.5% of lactulose hydrolysis.

Because the prebiotic properties of an oligosaccharide depends, among other factors, upon the number, type, and sequence of its monosaccharide moieties, as well as the

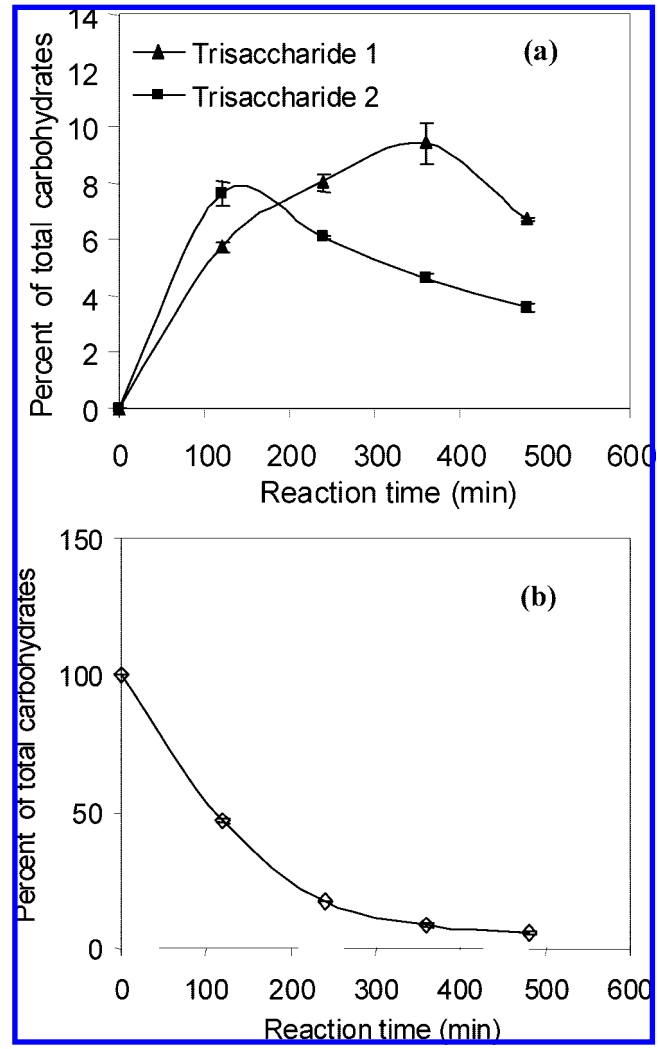

Figure 2. Trisaccharide synthesis (**a**) and the remaining lactulose content (**b**) during the time course of the reaction performed at 50 °C at an initial lactulose concentration of 250 mg/mL, 3 units/mL of enzyme, and pH 6.5. Bars indicate standard deviations.

glycosidic linkage between the monosaccharides, the detailed elucidation of the structural features of oligosaccharides may be useful to understand more precisely their fermentation mechanisms. To establish the overall structure and position of the glycosidic linkages of trisaccharides **1** and **2** (**Figure 1**), an extensive NMR study was performed. Complete structure elucidation and full proton and carbon assignment were carried out using 1D (^{1}H, ^{13}C, and 1D TOCSY) and 2D (gCOSY, TOCSY, ROESY, gHSQC, and gHMBC) NMR (**Table 1**).

The ^{13}C NMR spectrum of trisaccharide **1** in D_2O displayed three sets of signals, indicating an equilibrium mixture of isomers **1A**, **1B**, and **1C** (**Figure 3**), because of the lactulose mutarotational equilibrium between pyranose and furanose forms (*27*). Assuming that the differences in relaxation of anomeric carbons for the three isomers can be expected to be negligible, we determined the population of the three isomers (75:20:5) approximately. The major set of resonances, corresponding to the most populated isomer (**1A**), contained two anomeric carbons at δ 103.44 and 101.04 and a quaternary carbon at δ 98.21. Accordingly, the ^{1}H NMR spectrum showed signals for two anomeric protons at δ 4.43 and 4.35. From the analysis of the [^{1}H, ^{1}H] TOCSY spectrum, two seven-spin systems were identified. Furthermore, two additional spin systems attributable to the fructose moiety were found. Assignment of the individual

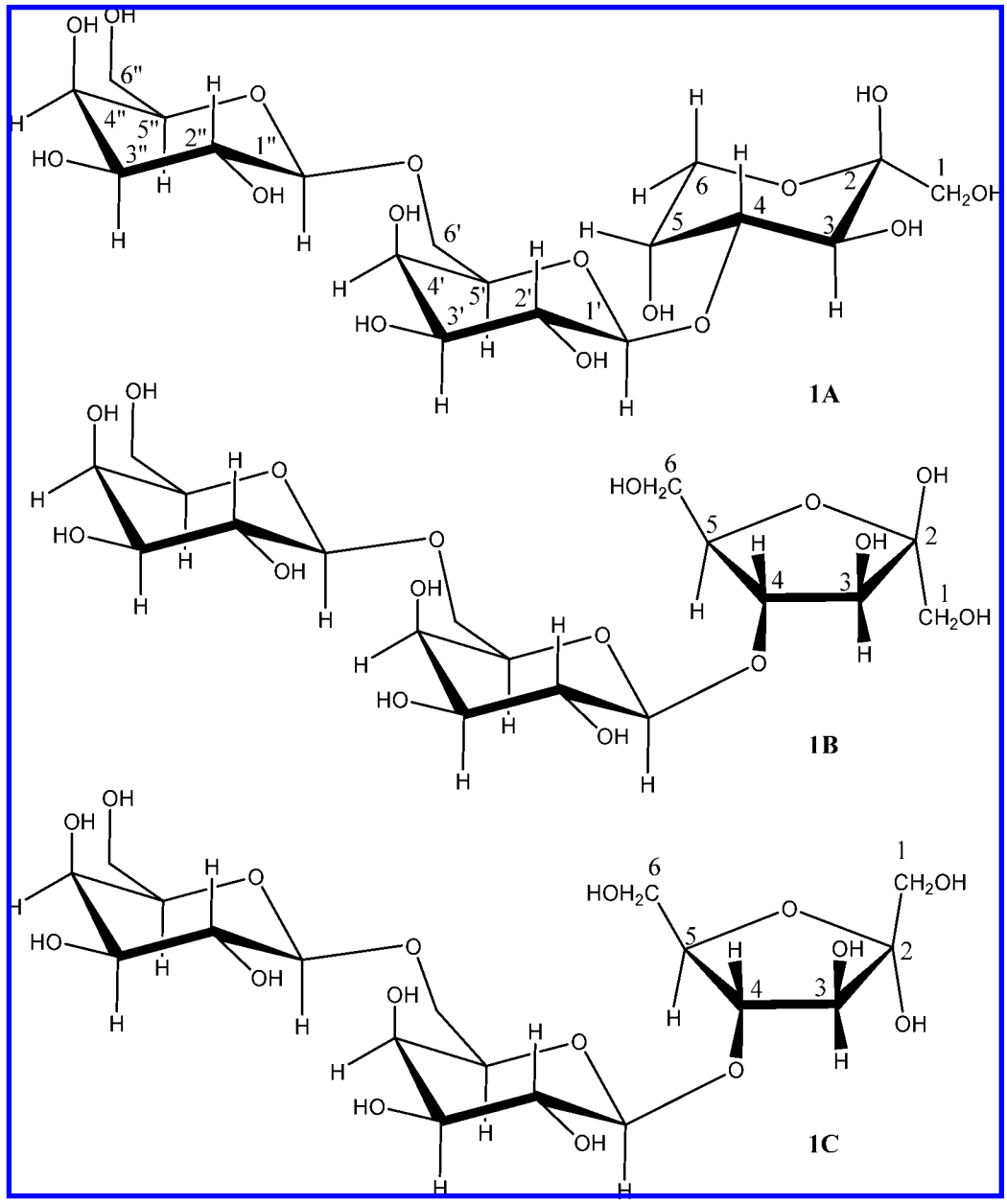

Figure 3. Structures of the mayor isomers of trisaccharide **1**. (**A**) β-D-Galactopyranosyl-(1 → 6)-β-D-galactopyranosyl-(1 → 4)-β-D-fructopyranose, (**B**) β-D-galactopyranosyl-(1 → 6)-β-D-galactopyranosyl-(1 → 4)-β-D-fructofuranose, and (**C**) β-D-galactopyranosyl-(1 → 6)-β-D-galactopyranosyl-(1 → 4)-α-D-fructofuranose.

spin systems was performed by 1D TOCSY and 2D COSY NMR experiments. Selective excitation of anomeric protons at δ 4.43 and 4.35 showed resonances at δ 3.47 (H-2′), 3.55 (H-3′), 3.82 (H-4′), and 3.78 (H-5′) for the former seven-spin system and at δ 3.38 (H-2″), 3.52 (H-3″), and 3.79 (H-4″) for the latter one. From the vicinal coupling constant values (**Table 1**), the existence of two β-galactose units was confirmed. The position of glycosidic linkages was established from gHMBC and ROESY spectra. Therefore, HMBC correlations between both H-1′ at δ 4.43 and C-4 at δ 77.99 (fructose) and between both C-1′ at δ 101.04 and H-4 (fructose) at δ 3.99 unambiguously established the 1 → 4 linkage between a unit of galactose and the fructose unit in the lactulose moiety. In addition, HMBC correlations between both H-1″ at δ 4.35 and C-6′ at δ 69.16 and between both C-1″ at δ 103.44 and H-6′a and H-6′b at δ 3.80 and 3.92, respectively, established the 1 → 6 linkage between both galactose units. Correlations obtained from the ROESY spectrum (**Figure 4a**) and the low-field shift observed for C-6′ confirmed the proposed structure.

Finally, ring size and anomeric configuration of the fructose unit were established. From the comparison between the chemical shifts of the fructose unit and those proposed for isomers of lactulose (*27*), we determined that the most populated isomer for trisaccharide **1** was β-D-galactopyranosyl-(1 → 6)-β-D-galactopyranosyl-(1 → 4)-β-D-fructopyranose (**1A**). It was also possible to measure C-2 [(**1B**, δ 102.42) and (**1C**, δ 105.11)] and C-4 [(**1B**, δ 84.31) and (**1C**, δ 85.54)] resonances for minor isomers (results not shown in **Table 1**). Values found for δ C-4 of **1B** and **1C** were typical of furanosides (*28*). From the comparison of those values, we established the second populated isomer as β-D-galactopyranosyl-(1 → 6)-β-D-galactopyranosyl-(1 → 4)-β-D-fructofuranose (**1B**) and the third one as β-D-galactopyranosyl-(1 → 6)-β-D-galactopyranosyl-(1 → 4)-α-D-fructofuranose (**1C**).

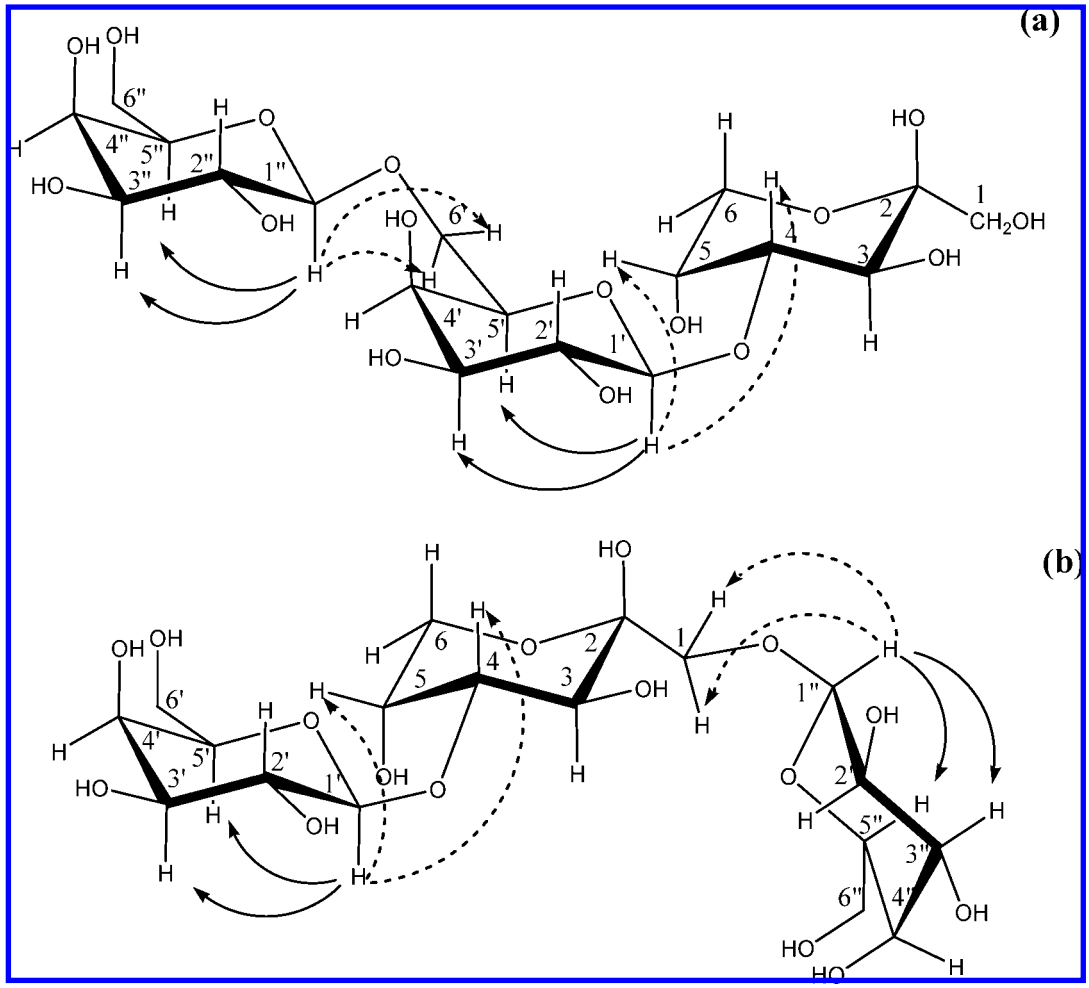

Figure 4. Important intraresidual (solid arrows) and inter-residual (dashed arrows) ROESY correlations for major isomers of (**a**) trisaccharide **1** and (**b**) trisaccharide **2** in D_2O.

The ^{13}C NMR spectrum of trisaccharide **2** (**Figure 5**) in D_2O displayed three sets of signals, with the population of the three isomers (**2A**, **2B**, and **2C**) being 80:15:5 approximately. The major set of resonances, corresponding to **2A**, contained two anomeric carbons at δ 103.53 and 100.98 and a quaternary carbon at δ 97.77. Accordingly, the ^1H NMR spectrum showed signals for two anomeric protons at δ 4.42 and 4.30 (**Table 1**). Using the same procedure, we confirmed the existence of a fructose and two β-galactose units. HMBC correlations between both H-1′ at δ 4.42 and C-4 (fructose) at δ 77.25 and between both C-1′ at δ 100.98 and H-4 (fructose) at δ 4.01 confirmed the 1 → 4 linkage between a unit of galactose and the fructose unit in the lactulose moiety. In addition, HMBC correlations between both H-1″ at δ 4.30 and C-1 at δ 71.62 and between both C-1″ at δ 103.53 and H-1a and H-1b at δ 3.73 and 3.82, respectively, established the 1 → 1 linkage between the fructose and the second galactose unit. This result was further supported by the correlations obtained from the ROESY spectrum (**Figure 4b**) and the low-field shift observed for C-1. In the same way as shown above for trisaccharide **1**, we determined that the most populated isomer for trisaccharide **2** was β-D-galactopyranosyl-(1 → 4)-β-D-fructopyranosyl-(1 → 1)-β-D-galactopyranose (**2A**). We also measured C-2 [(**2B**, δ 102.97) and (**2C**, δ 104.98)] and C-4 [(**2B**, δ 84.08) and (**2C**, δ 86.20)] resonances for minor isomers (results not shown in **Table 1**). From the comparison of those values, we established the second populated isomer as β-D-galactopyranosyl-(1 → 4)-β-D-fructofuranosyl-(1 → 1)-β-D-galactopyranose (**2B**) and the third one as β-D-galactopyranosyl-(1 → 4)-α-D-fructofuranosyl-(1 → 1)-β-D-galactopyranose (**2C**).

The resulting β-(1 → 6) glycosidic linkages observed in the trisaccharide 6′ galactosyl-lactulose were also previously found in GOS produced from lactose using β-galactosidase from *K. lactis* (*23, 26*). However, to our knowledge, β-(1 → 1) glycosidic linkage presented in the trisaccharide β-D-galactopyranosyl-(1 → 4)-β-D-fructose-(1 → 1)-β-D-galactopyranose was not previously described in the literature. These differences may be due to the specificity of glycosidases and the dependence of the products formed on the acceptor structure (*29*).

Several reports have demonstrated that glycosidic linkages and molecular weights of carbohydrates contribute toward the selectivity of fermentation by beneficial gut bacteria. GOS produced by transgalactosylation with linkages β-(1 → 6) have been shown to be selective for bifidobacteria (*30*), while no information was reported in relation to β-(1 → 1) glycosidic linkages. Moreover, in general, carbohydrates with the degree of polymerization (DP) of 3 showed the highest selectivity toward bifidobacteria (*31, 32*). On the basis of this information, 6′-galactosyl-lactulose and β-D-Galp-(1 → 4)-β-D-Fru-(1 → 1)-β-D-Galp could be good candidates to consider as potential prebiotics. Conceivably, higher molecular-weight oligosaccharides may be more slowly fermented (*33*); therefore, these trisaccharides derived from lactulose could exhibit higher colonic persistence than lactulose, reaching the most distal regions where most of the chronic intestinal disorders originate.

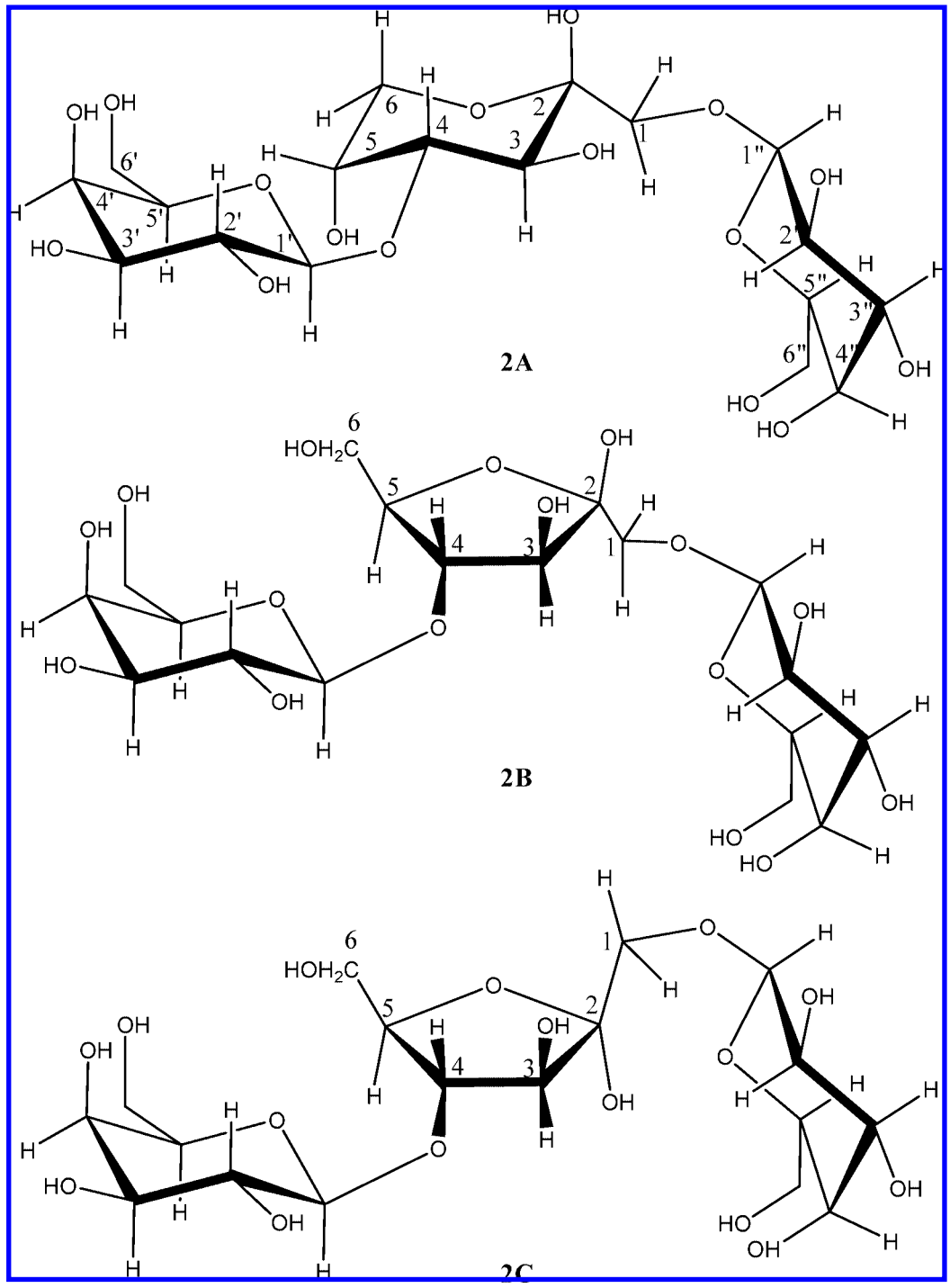

Figure 5. Structures of the mayor isomers of trisaccharide **2**. (**A**) β-D-Galactopyranosyl-(1 → 4)-β-D-fructopyranosyl-(1 → 1)-β-D-galactopyranose, (**B**) β-D-galactopyranosyl-(1 → 4)-β-D-fructofuranosyl-(1 → 1)-β-D-galactopyranose, and (**C**) β-D-galactopyranosyl-(1 → 4)-α-D-fructofuranosyl-(1 → 1)-β-D-galactopyranose.

Thus, further work is in progress to study their physiological properties, as well as the obtention of other oligosaccharides from lactulose.

Lactulose could be used as a substrate for the enzymatic synthesis of novel *trans*-galactooligosaccharides by β-galactosidase from *K. lactis*. This β-galactosidase was able to produce mainly trisaccharides with lactulose-based structures, which exhibited a galactose unit linked to C-6 of the galactose moiety of lactulose, while the other one presented a galactose unit linked to C-1 of the fructose moiety of lactulose. Because, dependent upon the origin of the β-galactosidase, different linkages between monosaccharides may be formed, the use of different enzymes would open new routes for synthesizing lactulose-derived oligosaccharides.

ACKNOWLEDGMENT

This work has been financed under a research and development program of the Spanish Ministry of Education and Science, project AGL-2004-07227-C02 and project ALIBIRD S-0505/AGR/000153, from the Comunidad de Madrid. A. Cardelle-Cobas thanks MEC for the FPU Grant, and C. Martínez-Villaluenga is a recipient of a I3P-CSIC contract. The authors thank Ramiro Martínez for the Lactozym supply.

LITERATURE CITED

(1) Steer, T.; Carpenter, H.; Tuohy, K.; Gibson, G. R. Perspectives on the role of the human gut microbiota and its modulation by pro- and prebiotics. *Nutr. Res. Rev.* **2000**, *13*, 229–254.

(2) Mountzouris, K. C.; McCartney, A. L.; Gibson, G. R. Intestinal microflora of human infants and current trends for its nutritional modulation. *Br. J. Nutr.* **2002**, *87*, 405–420.

(3) Madsen, K. L. The use of probiotics in gastrointestinal disease. *Can. J. Gastroenterol.* **2001**, *15*, 817–822.

(4) Drakoularakou, A.; McCartney, A.; Rastall, R.; Gibson, G. R. Established aid emerging prebiotics and their effects on the gut microflora. *Agro-Food-Ind. Hi-Tech* **2004**, *15*, 18–20.

(5) Panesar, P. S.; Panesar, R.; Singh, R. S.; Kennedy, J. F.; Kumar, H. Microbial production, immobilization and applications of β-D-galactosidase. *J. Chem. Toxicol. Biotechnol.* **2006**, *81*, 530–543.

(6) Yoshikawa, J.; Amachi, S.; Shinoyama, H.; Fujii, T. Multiple β-fructofuranosidases by *Aureobasidium pullulans* DSM2404 and their roles in fructooligosaccharide production. *FEMS Microbiol. Lett.* **2006**, *265*, 159–163.

(7) Katapodis, P.; Kalogeris, E.; Kekos, D.; Macris, B. J.; Christakopoulos, P. Production of β-fructofuranosidase from *Sporotrichum thermophile* and its application in the synthesis of fructooligosaccharides. *Food Biotechnol.* **2003**, *17*, 1–14.

(8) Mahoney, R. R. Galactosyl-oligosaccharide formation during lactose hydrolysis: A review. *Food Chem.* **1998**, *63* (2), 147–154.

(9) Rustom, I. Y. S.; Foda, M. I.; López-Leiva, M. H. Formation of oligosaccharides from whey UF-permeate by enzymatic hydrolysis—Analysis of factors. *Food Chem.* **1998**, *62*, 141–147.

(10) Jurado, E.; Camacho, F.; Luzón, G.; Vicaria, J. M. Kinetic models of activity for β-galactosidases: Influence of pH, ionic concentration and temperature. *Enzyme Microbiol. Technol.* **2004**, *34*, 33–40.

(11) Finch, P.; Yoon, J. H. The effects of organic solvents on the synthesis of galactose disaccharides using β-galactosidases. *Carbohydr. Res.* **1997**, *303*, 339–345.

(12) Yoon, J. H.; Mckenzie, D. A. comparison of the activities of three β-galactosidases in aqueous-organic solvent mixtures. *Enzyme Microbiol. Technol.* **2005**, *36*, 439–446.

(13) Gaur, R.; Pant, H.; Jain, R.; Khare, S. K. Galacto-oligosaccharide synthesis by immobilized *Aspergillus oryzae* β-galactosidase. *Food Chem.* **2006**, *97*, 426–430.

(14) Djouzi, Z.; Andrieux, C. Compared effects of three oligosaccharides on metabolism of intestinal microflora in rats inoculated with a human faecal flora. *Br. J. Nutr.* **1997**, *78*, 313–324.

(15) Palframan, R.; Gibson, G. R.; Rastall, R. A. Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. *Lett. Appl. Microbiol.* **2003**, *37*, 281–284.

(16) Delzenne, N. M. Oligosaccharides: State of the art. *Proc. Nutr. Soc.* **2003**, *62*, 177–182.

(17) Tzortzis, G.; Goulas, A. K.; Gibson, G. R. Synthesis of prebiotic galactooligosaccharides using whole cells of a novel strain, *Bifidobacterium bifidum* NCIMB 41171. *Appl. Microbiol. Biotechnol.* **2005**, *68*, 412–416.

(18) Méndez, A.; Olano, A. Lactulose. A review of some chemical properties and application in infant nutrition and medicine. *Dairy Sci. Abstr.* **1979**, *41*, 531–535.

(19) Kozempel, M. F.; Kurantz, M. J.; Craig, J. C., Jr.; Hiks, K. B. Development of a continuous lactulose process—Separation and purification. *Biotechnol. Prog.* **1995**, *11*, 592–595.

(20) Aider, M.; de Halleux, D. Isomerization of lactose and lactulose production: Review. *Trends Food Sci. Technol.* **2007**, *18*, 356–364.

(21) Harju, M. Lactulose as a substrate for β-galactosidases. II. Results and discussion. *Milchwissenschaft* **1986**, *41*, 349–352.

(22) Berger, J. L.; Lee, B. H.; Lacroix, C. Purification, properties and characterization of a high-molecular-mass β-galactosidase isoenzyme from *Thermus aquaticus* YT-I. *Biotechnol. Appl. Biochem.* **1997**, *25*, 29–41.

(23) Martínez-Villaluenga, C.; Cardelle-Cobas, A.; Corzo, N.; Olano, A.; Villamiel, M. Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G). *Food Chem.* **2007**, *107*, 258–264.

(24) Splechtna, B.; Nguyen, T. H.; Steinböck, M.; Kulbe, K. D.; Lorenz, W.; Haltrich, D. Production of prebiotic galactooligosaccharides from lactose using β-galactosidase from *Lactobacillus reuteri*. *J. Agric. Food Chem.* **2006**, *54*, 4999–5006.

(25) Boon, M. A.; Janssen, A. E. M.; van't Riet, K. Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides. *Enzyme Microbiol. Technol.* **2000**, *26*, 271–281.

(26) Chockchaisawasdee, S.; Athanasopoulos, V. I.; Niranjan, K.; Rastall, R. A. Synthesis of galacto-oligosaccharide from lactose using β-galactosidase from *Kluyveromyces lactis*: Studies on batch and continuous UF membrane-fitted bioreactors. *Biotechnol. Bioeng.* **2005**, *89*, 434–443.

(27) Mayer, J.; Conrad, J.; Klaiber, I.; Lutz-Wahl, S.; Beifuss, U.; Fischer, L. Enzymatic production and complete nuclear magnetic resonance assignment of the sugar lactulose. *J. Agric. Food Chem.* **2004**, *52*, 6983–6990.

(28) Kalinowski, H. O.; Berger, S.; Braun S. *Carbon-13 NMR Spectroscopy*; John Wiley and Sons: New York1988; Chapter 4.

(29) Watt, G. M.; Lowden, P. A. S.; Flitsch, S. L. Enzyme catalyzed formation of glycosidic linkages. *Curr. Opin. Struct. Biol.* **1997**, *7*, 652–660.

(30) Rowland, I. R.; Tanaka, R. The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with human faecal microflora. *J. Appl. Bacteriol.* **1993**, *74*, 667–674.

(31) Kaneko, T.; Kohmoto, T.; Kikuchi, H.; Shiota, M.; Iino, H.; Mitsuoka, T. Effects of isomaltooligosaccharides with different degrees of polymerization on human faecal *Bifidobacteria*. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 2288–2290.

(32) Kaplan, H.; Hutkins, R. W. Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. *Appl. Environ. Microbiol.* **2000**, *66*, 2682–2684.

(33) Sanz, M. L.; Cote, G. L.; Gibson, G. R.; Rastall, R. A. Influence of glycosidic linkages and molecular weight on the fermentation of maltose-bases oligosaccharides by human gut bacteria. *J. Agric. Food Chem.* **2006**, *54*, 9779–9784.

Received for review July 17, 2007. Revised manuscript received October 4, 2007. Accepted October 22, 2007.

JF0721343