Biocatalysis and Biotransformation

ISSN: 1024-2422 (Print) 1029-2446 (Online) Journal homepage: http://www.tandfonline.com/loi/ibab20

# Enzymatic synthesis of 3-aminopropyl-1-O-β-D-galactopyranoside catalyzed by Aspergillus oryzae β-galactosidase

**Cecilia Porciúncula González, Ernesto Rodríguez, Silvia Soule, Laura Franco Fraguas, Beatriz M. Brena, Cecilia Giacomini & Gabriela Irazoqui**

**To cite this article:** Cecilia Porciúncula González, Ernesto Rodríguez, Silvia Soule, Laura Franco Fraguas, Beatriz M. Brena, Cecilia Giacomini & Gabriela Irazoqui (2015) Enzymatic synthesis of 3-aminopropyl-1-O-β-D-galactopyranoside catalyzed by Aspergillus oryzae β-galactosidase, Biocatalysis and Biotransformation, 33:4, 197-207, DOI: 10.3109/10242422.2015.1095678

**To link to this article:** http://dx.doi.org/10.3109/10242422.2015.1095678

Published online: 18 Nov 2015.

*Biocatalysis and Biotransformation*, 2015; 33(4):197–207

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

# Enzymatic synthesis of 3-aminopropyl-1-*O*-β-D-galactopyranoside catalyzed by *Aspergillus oryzae* β-galactosidase

CECILIA PORCIÚNCULA GONZÁLEZ[1], ERNESTO RODRÍGUEZ[1], SILVIA SOULE[2], LAURA FRANCO FRAGUAS[1], BEATRIZ M. BRENA[1], CECILIA GIACOMINI[1] & GABRIELA IRAZOQUI[1]

[1]*Cátedra de Bioquímica, Dpto. Biociencias, Facultad de Química, UdelaR, Gral Flores 2124, Montevideo, Uruguay and* [2]*Laboratorio de Carbohidratos, DQO, Facultad de Ciencias/Facultad de Química, UdelaR, Alfredo Navarro 3051, Montevideo, Uruguay*



**Abstract**

Glycosidases represent excellent green chemistry alternatives as catalysts for the synthesis of glycosides, and in particular their stereoselectivity allows the production of anomerically pure glycosides, in only one reaction step using mild reaction conditions. Here, we report the enzymatic synthesis and structural characterization of 3-aminopropyl-1-*O*-β-D-galactopyranoside. Optimal reaction conditions for the transgalactosylation reaction were 100 mM lactose, 500 mM 3-amino-1-propanol and 24 h of incubation at 50 °C with 6 U/mL of β-galactosidase from *Aspergillus oryzae*. The fact that the synthesis of 1-propyl-2-*O*-β-D-galactopyranoside using 1-amino-2-propanol as acceptor was not achieved, and that *N*-glycoside formation was not observed, confirms the selectivity of β-galactosidase for the synthesis of *O*-glycosides, and particularly for primary alcohols. The synthesized galactosides were evaluated for their ability to interact with bovine spleen galectin-1 (Gal-1) by using the hemagglutination inhibition assay; results demonstrated that 3-aminopropyl-1-*O*-β-D-galactopyranoside may be considered as a functionalized galactose moiety more than an efficient Gal-1 inhibitor. The proposed approach constitutes a promising tool for the generation of glycopolymers and glyconanoparticles with potential applications in the development of biosensors as well as construction blocks in chemical synthesis.

**Keywords:** *β-galactosidase*, *galactoside*, *galectins*, *glycosidases*, *transglycosylation*

## Introduction

The study of carbohydrate–lectin interactions is one of the most promising research areas for unveiling the mechanism of biological processes mediated by glycans, for which the availability of glycosides and oligosaccharide of defined structure is essential (Ito et al. 2012; Ogiso et al. 2013; Sunasee and Narain 2013).

Lectins are proteins that possess a carbohydrate recognition domain (CRD) with the ability to interact specifically and reversibly with glycoconjugates. Those with specificity towards β-galactosyl containing glycoconjugates are known as galectins (Varki et al. 1999, Rabinovich 2005; Camby et al. 2006; Boscher et al. 2011). Particularly galectin-1 (Gal-1) is abundantly secreted by almost all malignant tumor cells, and its participation in several processes related to cancer development, including immune suppression, angiogenesis, hypoxia and metastasis has been reported (Hasan et al. 2007; Ito et al. 2012). So, the blocking of the CRD of Gal-1 with glycosides that compete with its natural ligands may convert Gal-1 into a potential target for cancer therapy (Hasan et al. 2007; Giguère et al. 2008; Ito et al. 2012). In this relation, several glycosides capable of inhibiting binding between galectins and their natural ligands have been reported (Rabinovich 2005; Ingrassia et al. 2006; Giguère et al. 2006, 2008; Hasan et al. 2007; Ito et al. 2012).

Glycosidases are excellent alternatives as catalysts for synthetic purposes as their stereoselectivity allows the production of anomerically pure glycosides,

Correspondence: Gabriela Irazoqui, Cátedra de Bioquímica, Dpto. Biociencias, Facultad de Química, UdelaR, Gral. Flores 2124, CC 1157, Montevideo, Uruguay. Tel: +598 29241806. E-mail: mgidrv@fq.edu.uy

*(Received 4 March 2015; accepted 14 September 2015)*

ISSN 1024-2422 print/ISSN 1029-2446 online 
DOI: 10.3109/10242422.2015.1095678

in only one reaction step using mild reaction conditions (Ichikawa et al. 1992; Van Rantwijk et al. 1999; Bojarová and Kren 2008). These enzymes catalyze the transfer of a glycosyl-moiety from a donor compound (glycoside or oligosaccharide) to a nucleophile that works as an acceptor molecule.

The potential of transglycosylation reactions catalyzed by enzymes in synthetic chemistry is enormous, and has not been fully exploited so far. However, these systems are complex and critically dependant on the enzyme specificity, the acceptor molecule and reaction conditions, making the synthesis of new products a challenging objective. Transgalactosylation reactions catalyzed by *Aspergillus oryzae* β-galactosidase have already been applied to the synthesis of different products, mainly galactooligosaccharides (GOS) (Albayrak & Yang 2002; Urrutia et al. 2013). In this work, we studied the transgalactosylation activity of this enzyme using two aliphatic alcoholamines (3-amino-1-propanol and 1-amino-2-propanol) as acceptors and lactose as the galactosyl donor. The biological activity as galectin inhibitor of the synthesized galactosides was evaluated using red blood cells hemagglutination assay. A thorough structural analysis of the obtained products was performed by spectrometric and spectroscopy methods.

## Experimental section

### *Materials*

*o*-Nitrophenyl-β-D-galactopyranoside (ONPG), lactose, galactose, 3-amino-1-propanol, orcinol, β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) from *A. oryzae*, fetuin from calf fetal serum, 1-cyano-4- dimethylamino pyridinium tetrafluoroborate (CDAP-$BF_4$), triethylamine and DEAE-cellulose were purchased from Sigma-Aldrich (St. Louis, MO). Chloroform, 2-amino-1-propanol and ninhydrin were purchased from Merck (Hohenbrunn, Germany). TLC plates were purchased from Machery Nagel (Duren, Germany). Sepharose 4B and PD-10 (Sephadex G25) columns were purchased from GE Health Care (Buckinghamshire, UK). BCA protein assay reagents were purchased from Pierce (Rockford, IL). Heparinized rabbit blood was supplied by the Unidad de Reactivos y Biomodelos Experimentales, Facultad de Medicina, Universidad de la República. Bovine Spleen was donated by Frigorífico Carrasco, Uruguay. All other chemicals used were of analytical grade.

### *Enzymatic activity assay*

Aliquots of 100 μL of suitably diluted *A. oryzae* β-galactosidase solution were added to 2.0 mL of 25 mM ONPG in 50 mM sodium acetate buffer at pH 5.5 (activity buffer), at room temperature. The rate of formation of free *o*-nitrophenol (ONP) was recorded spectrophotometrically at 405 nm using a 1 cm path length. One enzyme unit (U) was defined as the amount of enzyme hydrolyzing 1 μmol of substrate per minute in the above defined conditions. The extinction coefficient used for ONP at pH 5.5 was $7.5 \times 10^2$ $M^{-1}$ $cm^{-1}$. Enzyme activity was expressed as U/mL.

All the enzymatic assays were performed in triplicate.

### *Protein determination assay*

Protein was determined by the bicinchoninic acid (BCA) assay (Smith et al. 1985; Giacomini et al. 1998). All the protein assays were preformed in triplicate.

### *Enzymatic synthesis of galactosides*

To volumes of 10 mL of reaction mixtures containing lactose (100–347 mM) and 3-amino-1-propanol hydrochloride or 1-amino-2-propanol hydrochloride (100–500 mM) in 0.5 M sodium acetate buffer at pH 5.5, soluble *A. oryzae* β-galactosidase was added to a final concentration of 6 U/mL. The reaction mixtures were incubated at 50 °C and gently stirred. Aliquots of the mixture were taken at regular intervals and the reaction was stopped by heating at 100 °C for 5 min. The samples were analyzed by thin layer chromatography (TLC).

All the experiments were performed in triplicate.

### *TLC analysis*

Analytical TLC was performed on 20 cm × 10 cm × 0.25 mm precoated Alugram Silica Gel 60/$UV_{254}$ indicator TLC plates. Aliquots of 5 μL of samples (diluted 1:4) and standards (glucose, galactose and lactose) were spotted onto the TLC plates and developed to 10 cm in an 22.0 cm × 11.0 cm × 6.2 cm chamber (saturation time 30 min), using MeOH:$CHCl_3$:$C_3H_6O$:$NH_4OH$ (42:17:25:17) as mobile phase. The TLC plates were air-dried, sprayed with orcinol 0.2% (w/v) in EtOH:$H_2SO_4$ (90:10), then heated for 2 min at 110 °C or sprayed with ninhydrin spray reagent (0.2% (w/v) in 1-butanol:10% acetic acid (95:5) for free amino group detection (Garófalo et al. 2011). All the experiments were performed in triplicate.

*Purification of the compounds*

Synthesized galactosides were purified in two steps. The first one consisted of size exclusion chromatography in a Sephadex G10 column (1.6 cm id × 83 cm height) and isocratically eluted with deionized water at a flow rate of 0.06 mL/min (1.8 cm $h^{-1}$ linear flow rate). Fractions were collected, lyophilized and analyzed by TLC as described previously. The second purification step consisted in a preparative TLC performed in 20 cm × 10 cm × 0.25 mm precoated with Alugram Silica Gel 60/$UV_{254}$ indicator TLC plates in the same conditions as the analytical TLC. In order to identify the area of the plate containing the compound of interest, the edges of the plate were stained with orcinol. The silica from the zone containing the compound of interest was removed and the compound was extracted by sonication during 10 min in methanol:water (20:80 v/v) solution. Then the suspension was centrifuged for 10 min at 8700*g*. The extraction procedure was repeated three times. The supernatants were pooled, the methanol was rotary evaporated and the sample was further lyophilized and the purity was checked by analytical TLC as described previously.

*MS analysis*

ESI-MS analyses were performed using a Thermo LTQ Velos spectrometer equipped with a linear detector. The electrospray-ionization ion source was operated in the positive-ion mode. The capillary conditions were: voltage 5 kV and temperature 275 °C. The sample was diluted in a 30% acetonitrile (v/v), 0.1% formic acid (v/v) aqueous solution and introduced in direct injection mode at 4 μL/min.

*NMR experiments*

The chemical structures of synthesized galactosides were identified by an extensive $^{1}H$ and $^{13}C$ NMR study. NMR spectra were recorded at 30 °C at 400 and 100 MHz, respectively, using deuterated methanol ($CD_3OD$) as solvent on a Bruker Avance DPX 400 NMR. $^{1}H$ and $^{13}C$ chemical shifts were expressed in ppm using TMS ($\delta = 0.00$) and $Me_2CO$ ($\delta = 31.00$) as internal reference. 2D experiments [correlation spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC)] were carried out according to the standard pulse sequences of the instrument software. Proton signals were assigned based on COSY spectra. $^{13}C$ signals were assigned with HSQC spectra, based on the assignment of proton signals. Finally, the linkage position in each structure was determined by detecting interring cross-peaks in HMBC spectrum.

*Determination of kinetic parameters*

Kinetic parameters were determined using ONPG as galactose donor (1.0–15.0 mM) in the presence of 3-amino-1-propanol (100–1000 mM) in activity buffer at 25 °C. The rate of ONP formation was determined as previously described. Results were analyzed using Hanes linearization (Cornish-Bowden 1995).

All the activity assays were performed in triplicate.

*Purification of Gal-1*

Gal-1 was purified from bovine spleen as previously reported by Ahmed et al. (1996) with the following modifications. The bovine spleen membrane was removed and the organ was cut into small pieces and suspended in 75 mM sodium phosphate buffer pH 7.2 containing 75 mM NaCl, 2 mM EDTA, 4 mM mercaptoethanol, 0.3M lactose (buffer B) in a ratio of 2 mL/g of spleen. The mixture was homogenized at 4 °C and then was centrifuged at 26 000*g* for 60 min at 4 °C. Aliquots of 30 mL of clarified extract twice diluted in buffer were incubated with 30 g of DEAE-cellulose previously swollen in 75 mM sodium phosphate buffer pH 7.2 containing 75 mM NaCl, 2 mM EDTA, 4 mM mercaptoethanol (buffer A) under mild stirring for 4 h at room temperature. The non-bound material of the ionic exchange was gel filtered in PD-10 columns pre-equilibrated in buffer A. The void volume fraction containing lectin activity was applied to the asialofetuin (ASF)–sepharose affinity column, previously equilibrated in buffer A, at a flow rate of 0.25 mL/min. The column was washed with buffer A until the $A_{280}$ of the eluate was lower than 0.05, then Gal-1 was eluted with buffer B. The ASF–sepharose was synthesized in our laboratory as previously described by Plá et al. (2003).

*Hemagglutination assay (HAG)*

Gal-1 activity was determined by the HAG assay using rabbit red cells and estimated by the twofold serial dilution assay (Franco Fraguas et al. 2003). Erythrocytes were prepared from fresh blood collected in heparin and washed four times with sodium phosphate buffer 50 mM pH 7.4, containing 0.15 M NaCl (PBS buffer) by centrifugation during 3 min at 1500*g*. Glutaraldehyde-fixed and trypsin-treated red cells were prepared as described by Nowak et al. (1976) and diluted to give a 4% suspension in PBS. In the experiment, 25 μl NaCl 0.15 M, 25 μl 1% BSA in 0.15 M NaCl, 25 μl Gal-1 and 25 μl 4% suspension of the glutaraldehyde-fixed and trypsin-treated

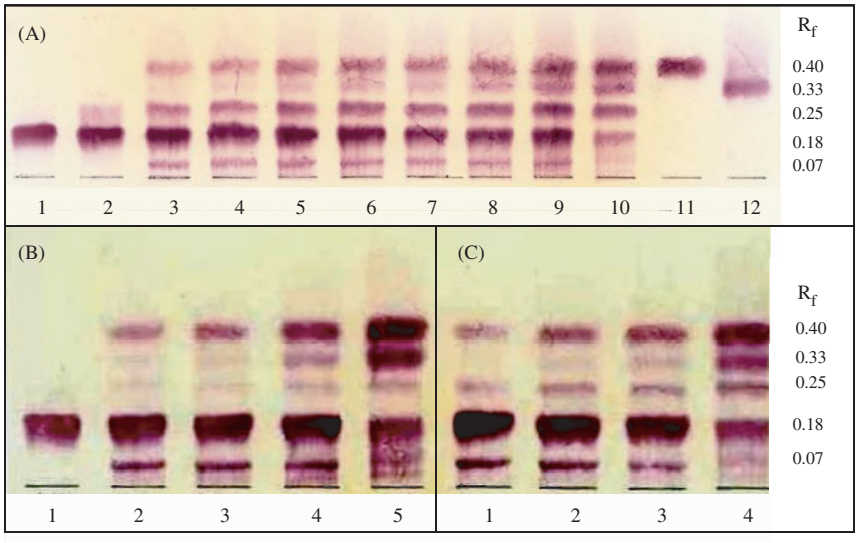



Figure 1. Time course reaction using lactose 200 mM, 6 U/mL at 50° C and pH 5.5, and different 3-amino-1-propanol concentrations. (A) 3-Amino-1-propanol 500 mM; Lanes: 1 – lactose standard (50 mM), 2 – 0 h, 3 – 0.25 h, 4 – 0.5 h, 5 – 1 h, 6 – 2 h, 7 – 3 h, 8 – 4 h, 9 – 5 h, 10 – 24 h, 11 – glucose standard (50 mM), 12 – galactose standard (50 mM). (B) 3-Amino-1-propanol 100 mM; Lanes: 1 – 0 h; 2 – 0.5 h; 3 – 1 h; 4 – 3 h; 5 – 24 h. (C) 3-Amino-1-propanol 250 mM; Lanes: 1 – 0.5 h; 2 – 1 h; 3 – 3 h; 4 – 24 h.



rabbit red cells were homogenized and incubated in a U-shaped microtiter plate at room temperature during 30 min and examined for agglutination. Titer per mL was defined as the reciprocal of the highest dilution giving visible agglutination.

All the experiments were performed in triplicate.

### *HAG inhibition assay*

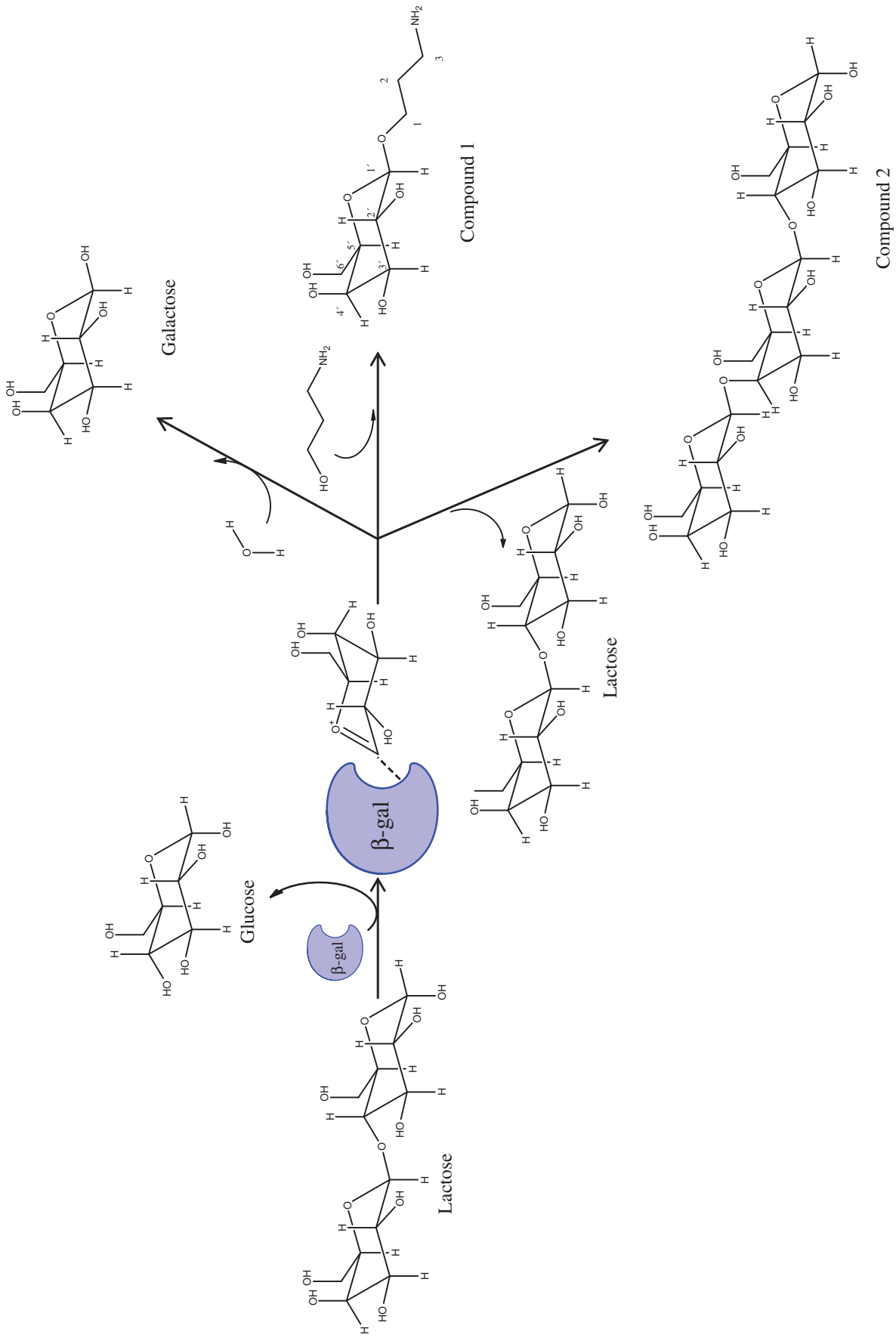
The ability of the synthesized galactosides to interact with Gal-1 was determined by the HAG inhibition assay. The lectin dilution used for the end point was the highest dilution causing 50% HAG (dilution before the previous causing visible HAG) defined as $HAG_{50\%}$ (Franco Fraguas et al. 2003). The minimum inhibitory concentration (MIC) of the galactoside that inhibits the $HAG_{50\%}$ was determined by using a serial twofold dilution, starting from 100 mM galactoside, in the HAG inhibition assay. Relative inhibitory power (RIP) was defined as the ratio between the galactose MIC and the galactoside MIC.

All the experiments were performed in triplicate.

## Results and discussion

### *Synthesis of 3-aminopropyl-1-O-β-D-galactopyranoside*

The time course of the enzymatic reaction using lactose (200 mM), 3-amino-1-propanol (500 mM) and 6 U/mL of *A. oryzae* β-galactosidase in 0.5 M sodium acetate buffer pH 5.5 at 50 °C is shown in Figure 1(A). As expected, the release of glucose ($R_f = 0.40$) by hydrolysis is proportional to the decrease of lactose ($R_f = 0.18$). However, during the first five hours of reaction, the concentration of galactose released ($R_f = 0.33$) was notoriously less pronounced than that of glucose. This is an indication of the coexistence of transgalactosylation and hydrolysis reaction, as glucose is released in the first reaction step of both processes, while galactose is released during the second reaction step, only when water is the galactosyl acceptor (Scheme 1). On the other hand, two new spots with $R_f$ of 0.25 (compound 1) and 0.07 (compound 2) were observed and they could not be assigned neither to the substrate nor to lactose hydrolysis products, inferring that they may correspond to transgalactosylation products. It should be highlighted that upon 24 h of reaction the intensity of the spot corresponding to compound 2 notoriously decreases, while that of compound 1 remains unchanged. This could point to enzymatic hydrolysis of compound 2; on the other hand compound 1 either could not be hydrolyzed by the enzyme, or steady state between its synthesis rate and its hydrolysis rate was achieved. In order to detect the presence of free amino groups in the new compounds, the TLC was developed with ninhydrin (specific developer for primary amine groups). Only compound 1 ($R_f = 0.25$) and 3-amino-1-propanol ($R_f = 0.67$) were revealed by ninhydrin (data not shown). This was the first evidence that compound 1 could be preliminarily identified as the galactoside: 3-aminopropyl-1-*O*-β-D-galactopyranoside.




Scheme 1. Transglycosylation mechanism for β-galactosidase using lactose as galactosyl donor and 3-amino-1-propanol as acceptor.

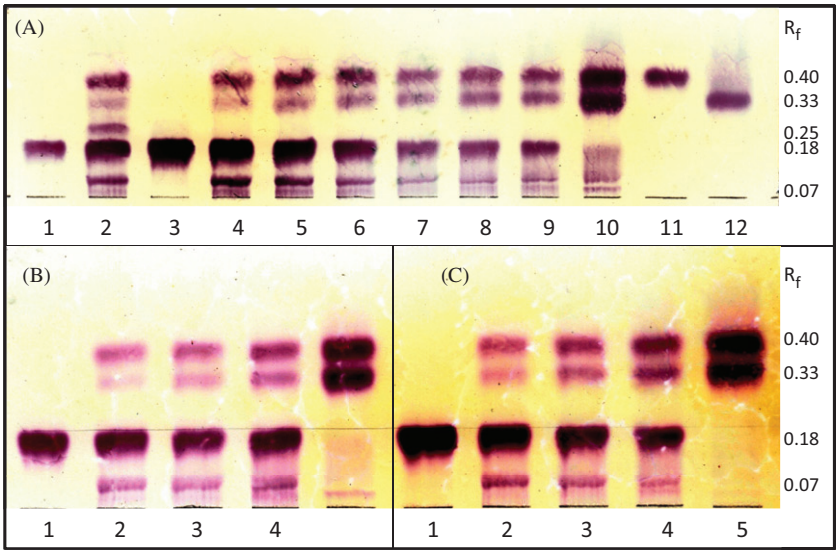



Figure 2. Time course reactions using different lactose concentrations, 6 U/mL at 50° C, pH 5.5, in the absence of 3-amino-1-propanol. (A) Lactose 347 mM, Lanes: 1 – lactose standard (50 mM), 2–2 h of reaction synthesis with 500 mM 3-amino-1-propanol (as control), 3 – 0 h, 4 – 0.25 h, 5 – 0.5 h, 6 – 1 h, 7 – 2 h, 8 – 3 h, 9 – 4 h, 10 – 24 h, 11 – glucose standard (50 mM), 12 – galactose standard (50 mM). (B) Lactose 200 mM: Lanes: 1 – 0 h, 2 – 0.25 h, 3 – 0.5 h, 4 – 1 h, 5 – 24h. (C) Lactose 100 mM: Lanes: 1 – 0 h, 2 – 0.25 h, 3 – 0.5 h, 4 – 1 h, 5 – 24 h.



*Synthesis reaction in the absence of the alcoholamine*

The transgalactosylation reaction was evaluated in the absence of 3-amino-1-propanol and for this purpose the enzyme (6 U/mL) was incubated with different lactose concentrations (100, 200 and 347 mM) in 50 mM sodium acetate buffer pH 5.5 at 50 °C for 24 h. Spots corresponding to hydrolysis products, glucose and galactose, as well as to compound 2 could be observed for all the lactose concentrations studied (Figure 2).

The fact that compound 2 ($R_f = 0.07$) could be synthesized in the absence of 3-amino-1-propanol denotes that it could be the result of a transglycosylation reaction where lactose fulfilled the double role of galactosyl donor and acceptor, resulting in the formation of a trisaccharide (Scheme 1). Interestingly, this transglycosylation reaction took place in aqueous medium even at low lactose concentrations (Figure 2C). On the other hand, the fact that compound 1 was not generated in the absence of 3-amino-1-propanol, reinforced the hypothesis that it could be the 3-aminopropyl-1-*O*-β-D-galactopyranoside. A point to be considered is that total lactose conversion was achieved after 24 h of reaction, when initial lactose concentrations were 100 and 200 mM (Figure 2C and 2B), and almost completely achieved when lactose concentration was 347 mM (Figure 2A).

*Study of the optimal reaction conditions for the synthesis of 3-aminopropyl-1-O-β-D-galactopyranoside*

The transgalactosylation reaction for the synthesis of 3-aminopropyl-1-*O*-β-D-galactopyranoside was evaluated under different conditions. The synthesis of both, compounds 1 ($R_f = 0.25$) and 2 ($R_f = 0.07$), was achieved at all lactose concentrations studied (100–347 mM) at a constant 3-amino-1-propanol concentration (500 mM) (data not shown). These results showed that the lactose concentration was not a critical parameter for the galactoside synthesis, in the concentration range studied. Nevertheless, an increase in 3-amino-1-propanol concentration from 100 to 500 mM at a fixed lactose concentration (200 mM) resulted in an increase in the amount of 3-aminopropyl-1-*O*-β-D-galactopyranoside, shown by the higher intensity of the TLC spots with $R_f = 0.25$ (Figure 1). This performance shows that high concentrations of 3-amino-1-propanol favor transglycosylation over hydrolysis.

The comparison between the reaction synthesis at 24 h in the absence of acceptor (Figure 2), and in the presence of all the 3-amino-1-propanol concentrations studied (Figure 1), at the same lactose concentration (200 mM), clearly showed that lactose conversion was lower in the latter cases. In order to evaluate if 3-amino-1-propanol was an enzyme inhibitor, kinetic parameters in the presence

of different concentrations of the aminoalcohol (100–1000 mM) were determined. As the enzyme kinetic parameters ($K_M = 2.6 \pm 0.2$ mM, $K_{cat} = (5.7 \pm 0.5) \times 10^3\ min^{-1}$) did not change significantly in the presence of 3-amino-1-propanol (data not shown) enzyme inhibition was discarded as the cause of lactose conversion slowing-down.

On other hand, since β-galactosidase from *A. oryzae* showed to be relatively stable in the most drastic conditions assayed (50 °C, 500 mM 3-amino-1-propanol) keeping near to 50% of residual enzyme activity upon 24 h, this could be the cause of the decreased rate of the lactose conversion. Moreover, given that the enzyme was completely stable at 50 °C during 24 h in the absence of the acceptor, this decrease may be partially attributed to the presence of the 3-amino-propanol in the bulk reaction.

Despite this enzyme inactivation, 500 mM of 3-amino-1-propanol was selected as the optimal condition as it was the one that provided the highest production of galactoside. Taking into account that the galactoside purification process was easier at low residual lactose concentration, and considering that initial lactose concentration was a parameter that had low significance over the amount of the synthesized galactoside, the optimal conditions selected for the synthesis were 100 mM lactose, 500 mM 3-amino-1-propanol, 6 U/mL, 50 °C, 24 h.

### *Galactoside purification and structural characterization*

The purification of the galactoside was performed in two chromatographic steps. The first one by size exclusion chromatography using a Sephadex G10 column; here compound 2 was isolated while compound 1 was co-eluted with lactose. In the second step, compound 1 was purified by preparative TLC. The purity of both compounds was analyzed by TLC (Figure 3).

Structural elucidation of the purified compounds 1 and 2 was performed by combining the use of mono ($^1$H NMR, $^{13}$C NMR) and bidimensional (COSY, HSQC, HMBC) techniques of nuclear magnetic resonance as well as mass spectrometry.

### *Structural characterization of 3-aminopropyl-1-O-β-D-galactopyranoside*

#### *NMR analysis*

The $^1$H NMR spectrum of compound 1 in water-*d2* at 30 °C showed only one anomeric proton at $\delta_H = 4.32$ ppm ($J = 8$ Hz), confirming the presence of a monosaccharide moiety. Proton decoupled HSQC experiments allowed the assignment of the

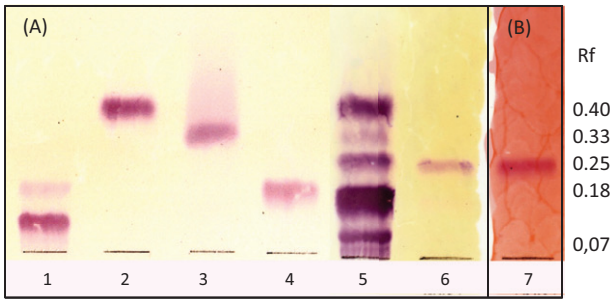



Figure 3. Thin layer chromatography of purified compounds 1 and 2. (A) Lanes: 1 – purified compound 2; 2 – glucose standard (50 mM); 3 – galactose standard (50 mM); 4 – lactose standard (50 mM); 5 – 2 h of reaction synthesis with 347 mM lactose, 500 mM 3-amino-1-propanol, 50°C, 6 U/mL; 6 – purified compound 1. All lines developed with orcinol. (B) Lane: 7 – purified compound 1 developed with ninhydrin.

corresponding carbon signal (Table 1). This information, together with the values of $^3J_{H1,H2}$ for the anomeric proton and published chemical shift data for sugar residues allowed the assignment of the spin system for a specific sugar residue and the determination of the anomeric configuration. The data showed that the galactose residue was a β-pyranoside (Jansson et al. 1989; Agrawal 1992).

The $^1$H NMR spectrum of compound 1 showed, in addition to the signals of the monosaccharide residue, signals corresponding to the 3-amino-1-propanol $C_2$ protons bonded to the amino terminal through a methylene carbon at $\delta_H = 1.87$ ppm (*m*), and two important signals at $\delta_H = 3.63$ ppm (*t*) and $\delta_H = 3.07$ ppm (*t*) corresponding to two methylene carbons.

Moreover the $^{13}$C NMR spectrum showed signals for nine carbons, three of which arose from the aglycon moiety. Signals observed in the HSQC spectrum indicated the presence of four methylene and five methine carbons, which confirmed the presence of the aminopropyl aglycon and a sugar residue. The proton spin of the pyranoside ring was assigned by H–H correlation COSY. These data together with the proton–carbon correlation from the HSQC experiment allowed the assignment of the carbon signals. The absence of a correlation of the proton signal at $\delta_H = 2.5$ with a carbon signal confirmed that it corresponds to the amino protons. The glycosidic linkage between both molecules was evidenced by the strong correlation observed among the $H_1$ proton $\delta_H = 3.63$ and the anomeric carbon ($\delta_C = 102.8$) by the HBMC experiment.

#### *MS analysis*

Compound 1 ESI-MS spectrum revealed the presence of intense ions $[M + H]^+$ and $[M + Na]^+$ at *m/z* 238.12 and 260.13, respectively, which are in accordance to the molecular weight of the proposed

Table 1. $^{1}H$ and $^{13}C$ NMR Chemical Shift Values of Compound 1, β-D-galactose and methyl-1-O-β-D-galactopyranoside.

| Compound 1 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Galactose | | | | | | | 3-amino-1-Propanol | | |
| 1′ | 2′ | 3′ | 4′ | 5′ | 6′ | 6″ | 1 | 2 | 3 |
| 4.32 d (8.0)[a] | 3.42 dd (8.0, 8.0)[a] | 3.60 dd (3.2, 3.6)[a] | 3.83 d (3.2)[a] | 3.58 d | 3.72 | 3.95 m | 3.63 t | 1.87 m | 3.07 t |
| 102.80 | 70.71 | 72.67 | 68.59 | 75.17 | 67.81 | | 60.98 | 26.64 | 37.64 |
| **β-D-galactose**[b] | | | | | | | | | |
| *1′* | 2′ | 3′ | 4′ | 5′ | 6′ | 6″ | | | |
| 4.53 | 3.45 | 3.59 | 3.89 | 3.65 | 3.64 | 3.72 | | | |
| 97.4 | 72.9 | 73.8 | 69.7 | 75.9 | 61.8 | | | | |
| **Methyl-1-O-β-D-galactopyranoside**[b] | | | | | | | | | |
| *1′* | 2′ | 3′ | 4′ | 5′ | 6′ | 6″ | | | |
| 4.20 | 3.39 | 3.53 | 3.81 | 3.57 | 3.69 | 3.64 | | | |
| 104.5 | 71.7 | 73.8 | 69.7 | 76.0 | 62.0 | | | | |

Multiplicity: d-doublet; dd- double doublet; t-triplet; m-multiplet.
[a]*J*(Hz) in brackets.
[b]Chemical shifts reported by Agrawal, 1992.



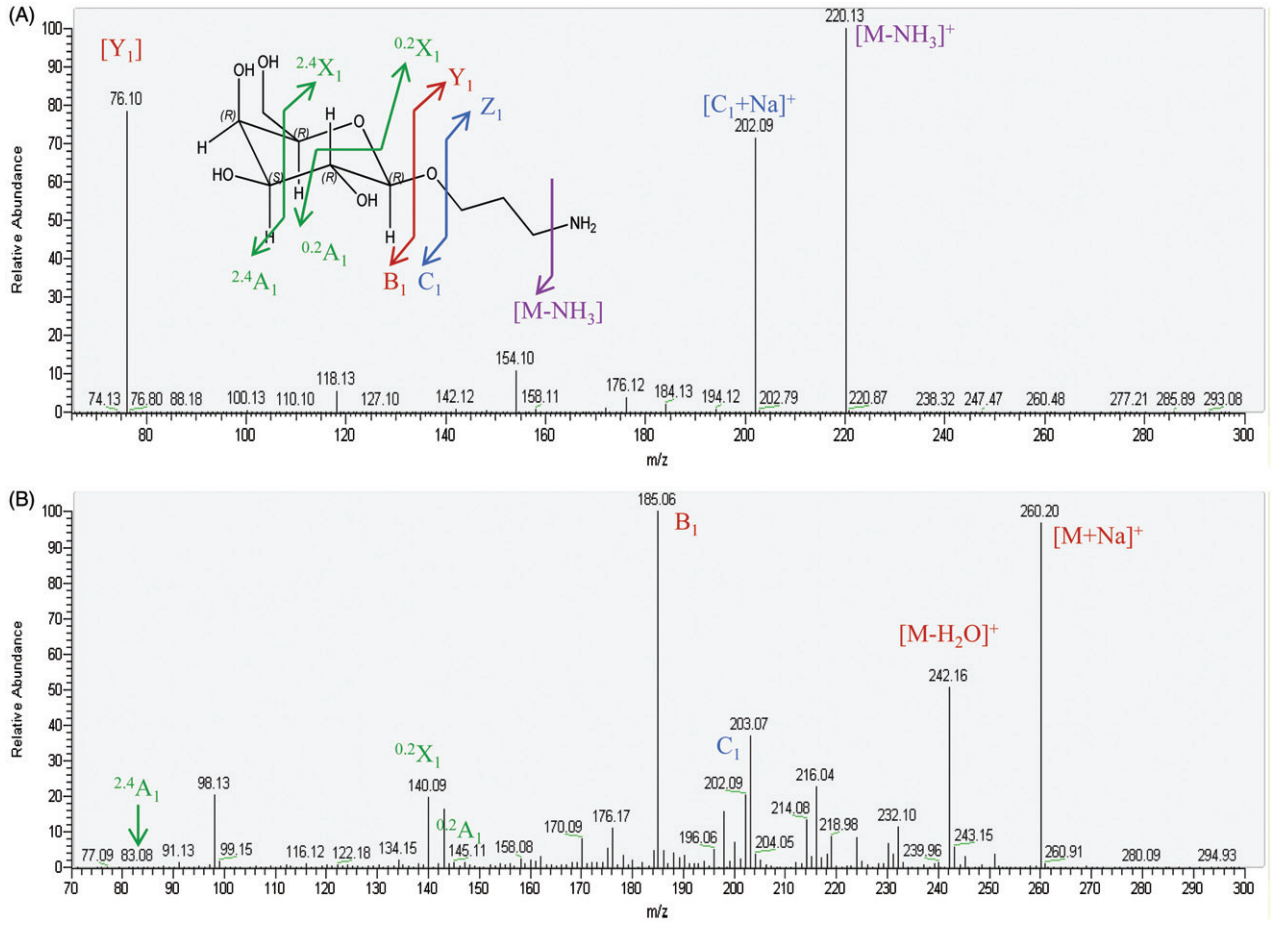



Figure 4. 3-Aminopropyl-1-*O*-β-D-galactopyranoside MS-analysis, (A) ion $m/z = 238$ MS/MS spectrum; (B) ion $m/z = 260$ MS/MS spectrum.

galactoside. According to the Domon and Costello (1988) nomenclature, we analyzed the MS–MS spectrum of the $[M+H]^+$ shown in Figure 4(A). It can be observed that the ion $m/z = 76.10$ ($Y_1$) generated due to the release of the galactose moiety lacking the oxygen atom of the glycosidic bond

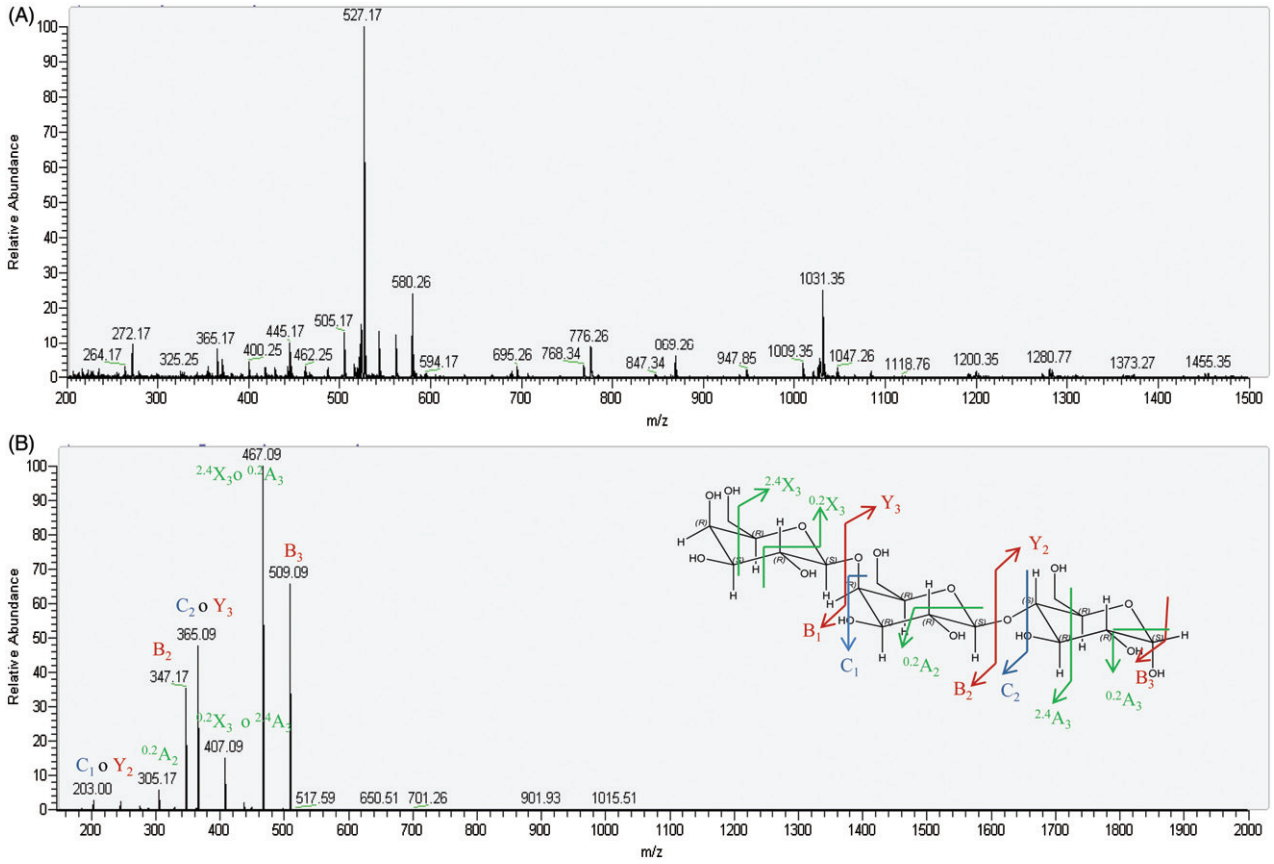



Figure 5. β-D-galactopyranosyl-(1-4)-β-D-galactopyranosyl-(1-4)-α-D-glucose MS-analysis. (A) Compound 2 MS; (B) compound 2 ion $m/z = 527$ MS/MS.

corresponds to the molecular weight of the 3-amino-1-propanol. The fragments $m/z = 202.09$ (C1) and $m/z = 220.13$ were also identified. The latter was assigned to the $[M - NH_3]^+$ ion which evidenced the loss of the terminal amino as an ammonium molecule according to a polyfunctional compound (McLafferty 1980). The $[M + Na]^+$/MS–MS spectrum (Figure 4B) showed that the ions $m/z = 242.16$ arose as a consequence of the loss of a water molecule $[M + Na - H_2O]$. The fragments assigned to the split of the pyranoside ring $^{2.4}A_1$ ($m/z = 83.08$), $^{0.2}A_1$ ($m/z = 143.0$) and $^{0.2}X_1$ ($m/z = 140.09$) confirmed the presence of a galactose moiety (McLafferty 1980; Hofmeister et al. 1991). Finally, the glycosidic bond excision was supported by the ions $m/z = 185.06$ ($B_1$) and $m/z = 202.09$ ($C_1$).

Consequently the structure 3-aminopropyl-1-*O*-β-D-galactopyranoside was deduced for compound 1.

*Structural characterization of β-D-galactopyranosyl-(1-4)-β-D-galactopyranosyl-(1-4)-α-D-glucose*

*MS analysis*

Compound 2 ESI-MS spectrum revealed the presence of an intense ion $[M + Na]^+$ at $m/z$ 527.17, which is in accordance with the molecular weight of a trisaccharide (Figure 5A). This was reinforced by the MS–MS fragmentation pattern of this ion (Figure 5B). The generation of the ions corresponding to the cleavage of the glycosidic bonds ($m/z = 203.00$ ($C_1$ o $Y_2$), $m/z = 365.09$ ($C_2$ o $Y_3$), $m/z = 347.17$ ($B_2$), $m/z = 509.09$ ($B_3$)), as well as those assigned to the pyranoside rings internal cleavage ($m/z = 305.17$ ($^{0.2}A_2$), $m/z = 407.09$ ($^{0.2}X_3$ o $^{2.4}A_3$) and $m/z = 467.09$ ($^{0.2}A_3$ o $^{2.4}X_3$)), evidenced that the trisaccharide was formed by a galactose bonded to lactose moiety trough (1 → 4) bond. The presence of ion $^{0.2}A_n$ in the absence of the ions $^{0.3}A_n$ y $^{0.4}A_n$ is characteristic of (1 → 4) bonds between monosaccharide moieties (McLafferty 1980; Hofmeister et al. 1991; Matamoros Fernández et al. 2004s).

*NMR analysis*

In order to confirm the structure proposed for compound 2 according to MS–MS experiments we included a brief analysis by NMR.

The $^{1}$H NMR spectrum of compound 2 showed three signals corresponding to three anomeric protons at $\delta_H = 4.35$ ppm ($J = 7.6$ Hz), $\delta_H = 4.53$ ppm ($J = 8.0$ Hz) and $\delta_H = 5.10$ ppm ($J = 3.6$ Hz), this information is consistent with a trisaccharide structure assigned to the three anomeric protons. The coupling constant evidenced the presence of two β anomers ($J = 7.6$, $J = 8.0$) and one α anomer ($J = 3.6$). The HSQC experiment showed the corresponding anomeric carbons at $\delta_C = 103.32$, $\delta_C = 95.64$ and $\delta_C = 91.81$ ppm, respectively. This information, together with the values of $^{3}J_{H1,H2}$ for each anomerics protons and published chemical shift data for the sugars residues allowed the determination of each anomeric configuration. The data showed that galactose residues were β-pyranosides forms; instead the glucoside residue was α-pyranoside (Jansson et al. 1989; Agrawal 1992).

Based on the experimental data, the enzyme mechanism (Scheme 1) as well as the fact that galactosidases preserve the anomeric configuration, we propose that the trisaccharide structure is β-D-galactopyranosyl-(1 → 4)-β-D-galactopyranosyl-(1 → 4)-α-D-glucopyranoside. The ability of *A. oryzae* β-galactosidase to generate galactooligosaccharides in aqueous medium with lactose as unique substrate is well known. Urrutia et al. (2013) reported a detailed characterization of the GOS synthesized at 40 °C, using 400 g/L lactose and 15 U/mL of enzyme. In this case, the main trisaccharides formed were: Gal-β(1 → 6)-Gal-β(1 → 4)-Glc, Gal-β(1 → 3)-Gal-β(1 → 4)-Glc and Gal-β(1 → 4)-Gal-β(1 → 4)-Glc. The comparison with our results suggested that the presence of high concentrations of 3-amino-1-propanol was a determinant parameter since it led to the formation of a single trisaccharide identified as Gal-β(1 → 4)-Gal-β(1 → 4)-αGlc.

### *Study of 1-amino-2-propanol as acceptor*

The use of 1-amino-2-propanol as acceptor in the transgalactosylation system was explored using identical reaction conditions as those previously described for 3-amino-1-propanol. In this case, only one new spot with rf 0.07 was observed when the reaction performance was followed by TLC. This compound was purified by molecular exclusion chromatography, analyzed by mass spectrometry and NMR, confirming it was a trisaccharide with the same chemical structure as compound 2 previously reported. No additional spots which pointed to the synthesis of the 1-amino-2-propanol galactoside was observed, reinforcing the hypothesis that β-galactosidase from *A. oryzae* exhibited preference for primary hydroxyl as previously reported (Woudenberg-van Oosterom et al. 1998; Irazoqui et al. 2009).

Table 2. Galectin-1 inhibitory properties of compounds 1 and 2.

| Compound | MIC[a] (mM) | RIP[b] |
|---|---|---|
| Galactose | 50.0 | 1 |
| Lactose | 3.1 | 16 |
| 1 | 25.0 | 2 |
| 2 | 6.3 | 8 |

[a]MIC: Minimal inhibition concentration, defined as the minimal inhibitor concentration that cause hemagglutination inhibition.
[b]RIP: Relative inhibitor, defined as the quotient between Galactose MIC and compound MIC.

### *Interaction between galactosides and bovine spleen Gal-1*

The interaction between compounds 1 and 2 and Gal-1 was evaluated by using the HAG inhibition assay. This assay is reported as a useful tool for the study of carbohydrate–lectin interactions. The HAG inhibition assay was performed as described in the experimental section, in the presence of the potential inhibitors; those with affinity for Gal-1 will bind to its CRD, thus inhibiting the HAG. Minimal inhibition concentration (MIC) and relative inhibitor power (RIP) were determined (Table 2). The MIC for 3-aminopropyl-1-*O*-β-D-galactopyranoside (compound 1) and β-D-galactopyranosyl-(1 → 4)-β-D-galactopyranosyl–(1 → 4)-α-D-glucopyranoside (compound 2) was higher than the MIC for galactose, being lower than for the case of lactose, a natural ligand for Gal-1.

## Conclusions

Transgalactosylation catalyzed by the *A. oryzae* β-galactosidase demonstrated to be useful for the enzymatic synthesis of 3-aminopropyl-1-*O*-β-D-galactopyranoside. Optimal reaction conditions for this synthesis reaction were achieved with 100 mM lactose, 500 mM 3-amino-1-propanol and 6 U/mL incubated during 24 h at 50 °C.

The synthesis of 1-aminopropyl-2-*O*-β-D-galactopyranoside was not achieved when using 1-amino-2-propanol as acceptor and *N*-glycoside formation was not observed. These results evidenced the selectivity of β-galactosidase for the synthesis of *O*-glycosides as well as the selectivity of the enzyme from *A. oryzae* towards primary alcohols (Woudenberg-van Oosterom et al. 1998; Irazoqui et al. 2009; Porciúncula González et al. 2013).

The synthesized galactoside did not show better inhibitor properties for Gal-1 than the natural inhibitors, yet the 3-aminopropyl-1-*O*-β-D-galactopyranoside may be considered as a functionalized galactose moiety. This is very interesting for the generation of glycopolymers and glyconanoparticles for the development of biosensors, as well as for building blocks in chemical synthesis (Ito et al. 2012; Ogiso et al. 2013; Sunasee and Narain 2013).

## Acknowledgements

Authors thank to Dra. Rosario Durán (Institute Pasteur Montevideo, Uruguay) for performing MS analyses.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.



## References

Agrawal PK. 1992. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. Phytochemistry 31:3307–3330.

Ahmed H, Fink NE, Pohl J, Vasta, GR. 1996. Galectin-1 from bovine spleen: biochemical characterization, carbohydrate specificity and tissue specific isoform profiles. J Biochem 120:1007–1019.

Albayrak N, Yang ST. 2002. Production of galactooligosaccharides from lactose by *Aspergillus oryzae* β-galactosidase immobilized on cotton cloth. Biotechnol Bioeng 77:8–19.

Bojarová P, Kren V. 2008. Glycosidases: a key to tailored carbohydrates. Trends Biotechnol 27:199–209.

Boscher C, Dennis JW, Nabi IR. 2011. Glycosylation, galectins and cellular signaling. Curr Opin Cell Biol 23:383–392.

Camby I, Le Mercier M, Lefranc F, Kiss R. 2006. Galectin-1: a small protein with major functions. Glycobiology 16:137R–157R.

Cornish-Bowden A. 1995. Introduction of enzyme kinetics. In: Cornish-Bowden A, Ed. Fundamentals of enzyme kinetics. London: Portland Press; 31–32.

Domon B, Costello CE. 1988. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. Glycoconjugate J 5:397–409.

Franco Fraguas L, Batista-Viera F, Carlsson J. 2003. Isolation of a β-galactoside-binding lectin from cat liver. Braz J Med Biol Res 36:447–457.

Garófalo L, Vázquez D, Ferreira F, Soule S. 2011. Wheat flour non-starch polysaccharides and their effect on dough rheological properties. Ind Crops Prod 34:1327–1331.

Giacomini C, Villarino A, Franco-Fraguas L, Batista-Viera F. 1998. Immobilization of β-galactosidase from *Kluyveromyces lactis* on silica and agarose: comparison of different methods. J Mol Catal B: Enzym 4:313–327.

GiguèreD, SatoS, St-PierreC, SiroisS, RoyR. 2006. 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 16:1668–1672.

Giguère D, Bonin M-A, Cloutier P, Patnam R, St-Pierre C, Sato S, Roy R. 2008. Synthesis of stable and selective inhibitors of human galectins-1 and -3. Bioorg Med Chem 16:7811–7823.

Hasan SS, Ashraf GM, Banu N. 2007. Galectins – potential targets for cancer therapy. Cancer Lett 253:25–33.

Hofmeister GE, Zhou Z, Leary J. 1991. Linkage position determination in lithium-cationized disaccharides: tandem mass spectrometry and semiempirical calculations. J Am Chem Soc 113:5964–5970.

Ichikawa Y, Look GC, Wong CH. 1992. Enzyme-catalyzed oligosaccharide synthesis. Anal Biochem 202:215–238.

Ingrassia L, Camby I, Lefranc F, Mathieu V, Nshimyumukiza P, Darro F, Kiss R. 2006. Anti-galectin compounds as potential anti-cancer drugs. Curr Med Chem 13:3513–3527.

Irazoqui G, Giacomini C, Batista-Viera F, Brena BM, Cardelle-Cobas A, Corzo N, Jimeno ML. 2009. Characterization of galactosyl derivatives obtained by transgalactosylation of lactose and different polyols using immobilized β-galactosidase from *Aspergillus oryzae*. J Agric Food Chem 57:11302–11307.

Ito K, Stannard K, Gabutero E, Clark AM, Neo S-Y, Onturk S, Blanchard H, Ralph SJ. 2012. Galectin-1 as a potent target for cancer therapy: role in the tumor microenvironment. Cancer Metastasis Rev 31:763–778.

Jansson PE, Kenne L, Widmalm G. 1989. Computer-assisted structural analysis of polysaccharides with an extended version of CASPER using 1H and 13C-NMR data. Carbohyd Res 188:169–191.

Matamoros Fernández L, Obel N, Scheller HV, Roepstorff P. 2004. Differentiation of isomeric oligosaccharide structures by ESI tandem MS and GC–MS. Carbohyd Res 339:655–664.

McLafferty FW. 1980. Interpretation of mass spectra. 3rd ed. California: University Science Books.

Nowak TP, Haywood PL, Barondes SH. 1976. Developmentally regulated lectin in embryonic chick muscle and a myogenic cell line. Biochem Biophys Res Commun 68:650–657.

Ogiso M, Kobayashi J, Imai T, Matsuoka K, Itoh M, Imamura T, Okada T, Miura H, Nishiyama T, Hatanaka K, Minoura N. 2013. Carbohydrate immobilized on a dendrimer-coated colloidal gold surface for fabrication of a lectin sensing device based on localized surface plasmon resonance spectroscopy. Biosens Bioelectron 41:465–470.

Plá A, Alonso E, Batista-Viera F, Franco Fraguas L. 2003. Screening for carbohydrate-binding proteins in extracts of Uruguayan plants. Braz J Med Biol Res 36:851–860.

Porciúncula González C, Castilla A, Garófalo L, Soule S, Irazoqui G, Giacomini C. 2013. Enzymatic synthesis of 2-aminoethyl β-D-galactopyranoside catalyzed by *Aspergillus oryzae* β-galactosidase. Carbohyd Res 368:104–110.

Rabinovich GA. 2005. Galectin-1 as a potential cancer target. Br J Cancer 92:1188–1192.

Smith PK, Khron RI, Hermanson GF, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurements of protein using bicinchoninic acid. Anal Biochem 150:76–85.

Sunasee R, Narain R. 2013. Glycopolymers and glyconanoparticles in biomolecular recognition processes and vaccine development. Macromol Biosci 13:9–27.

Urrutia P, Rodriguez-Colinas B, Fernandez-Arrojo L, Ballesteros AO, Wilson L, Illanes A, Plou FJ. 2013. Detailed analysis of galactooligosaccharides synthesis with β-galactosidase from *Aspergillus oryzae*. J Agric Food Chem 61:1081–1087.

Varki A, Cummings R, Esko J, Freeze H, Hart G, Marth J. 1999. S-type lectins (Galectins). In: Varki A, Cummings R, Esko J, Freeze H, Hart G, Marth J, Eds. Essentials of glycobiology. New York: Cold Spring Harbor Laboratory Press; 417–427.

Van Rantwijk F, Woudenberg-Van Oosterom M, Sheldon RA. 1999. Glycosidase-catalysed synthesis of alkyl glycosides. J Mol Catal B: Enzym 6:511–532.

Woudenberg-van Oosterom M, Van Belle HJA, van Rantwijk F, Sheldon RA. 1998. Immobilized β-galactosidases and their use in galactoside synthesis. J Mol Catal A: Chem 134:267–274.