

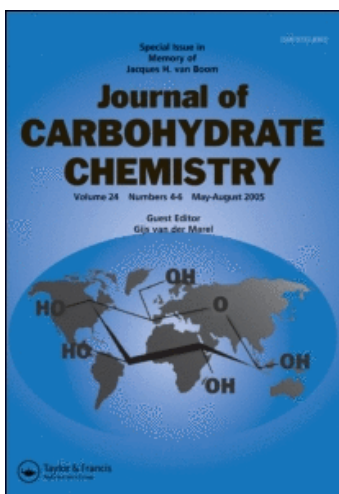
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### On the Effect of the Aglycon Structure of Three Aryl $\beta$ -D-Galactosides on the Yield and the Regioselectivity of the Transglycolytic Synthesis of *N*-Acetyllactosamine

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**ON THE EFFECT OF THE AGLYCON STRUCTURE OF THREE ARYL  $\beta$ -D-GALACTOSIDES ON THE YIELD AND THE REGIOSELECTIVITY OF THE TRANSGLYCOLYTIC SYNTHESIS OF *N*-ACETYLLACTOSAMINE<sup>1</sup>**

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**ABSTRACT**

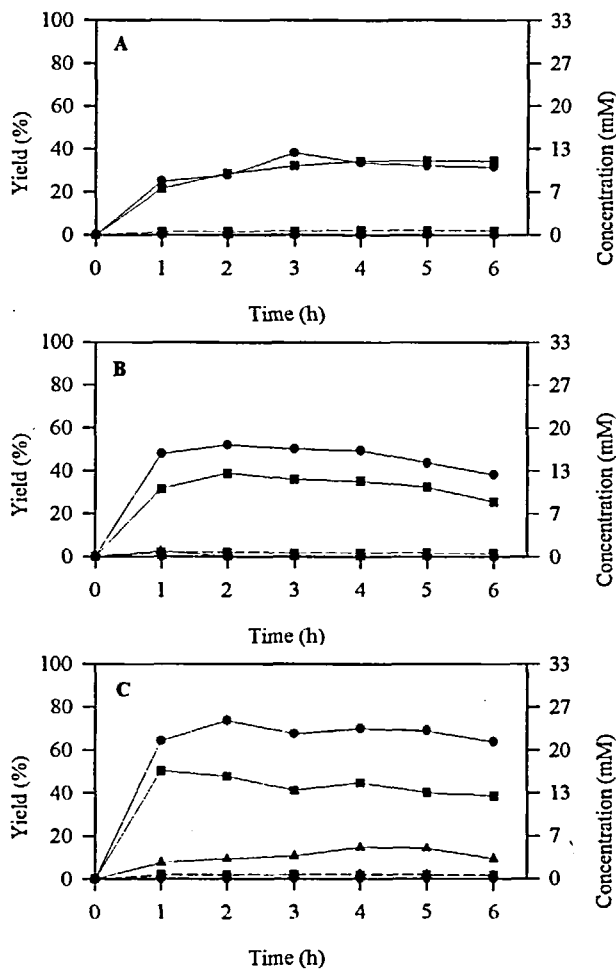
We examined the effect as donors of three aryl  $\beta$ -D-galactosides (*i.e.* *p*-nitrophenyl  $\beta$ -D-galactopyranoside, *o*-nitrophenyl  $\beta$ -D-galactopyranoside and phenyl  $\beta$ -D-galactopyranoside) on the regioselectivity and the yield of the synthesis of *N*-acetyllactosamine obtained from the transglycosylation reaction catalyzed by a crude preparation of  $\beta$ -D-galactosidase from *Bacillus circulans* at 25 °C, 37 °C and 55 °C, respectively. Using *p*-nitrophenyl  $\beta$ -D-galactopyranoside the reaction results were fully regiospecific at all the temperatures considered: the maximum molar yield (74%) was obtained at an incubation temperature of 55 °C. Using *o*-nitrophenyl  $\beta$ -D-galactopyranoside as the donor the reaction was still highly regioselective and the maximum molar yield (50%) was achieved at an incubation temperature also of 55 °C. Using phenyl  $\beta$ -D-galactopyranoside transglycolytic products appear only at an incubation temperature of 55 °C but at very low molar yield (about 14%) and lower regioselectivity.

## INTRODUCTION

The important role of carbohydrates, in general, and of oligosaccharides in particular, in many biological processes is well established.<sup>2-4</sup> The possibility to synthesize the oligosaccharides involved in biological functions can also allow for a better understanding of the biological phenomena that they mediate. Regioselective glycosylation of carbohydrates can be achieved both by chemical and enzymatic methods. In the last decade transglycosylation reactions catalyzed by glycosidases demonstrated a useful biotechnological tool for the synthesis of a number of biologically important oligosaccharides and glycosides.<sup>5-11</sup> The main problem of the transglycosylation is control of the regioselectivity. Glycosidases show a good tendency to transfer a carbohydrate molecule to primary hydroxyl groups whereas examples of glycosidases with a regioselectivity shifted towards secondary hydroxyl groups are few. Among the latter, the  $\beta$ -D-galactosidase from *Bacillus circulans* is one of the most interesting enzymes. It catalyzes the transfer of galactose predominantly to the OH-4 position of GlcNAc to afford *N*-acetylactosamine (Gal $\beta$ 1-4GlcNAc), but *N*-acetylallolactosamine (Gal $\beta$ 1-6GlcNAc) is also formed to a lesser but not negligible extent.<sup>12-18</sup> There are various means to control the regioselectivity of the transglycosylation reactions. The correct choice of the enzyme is of primary importance and the nature of the aglycon part of the acceptor appears to be an important parameter.<sup>19-22</sup> Recently it has also been shown that temperature and pH can be useful modulating factors.<sup>11, 14</sup> On the other hand no data are available about the effect of the structure of the aglycon portion of the donors used in the transglycosylation reactions on regioselectivity and the yield. Here we report the comparative results regarding the effect of the structure of three aryl  $\beta$ -D-galactosides, *i.e.* *p*-nitrophenyl  $\beta$ -D-galactopyranoside (Gal $\beta$ Op-NO<sub>2</sub>Ph), *o*-nitrophenyl  $\beta$ -D-galactopyranoside (Gal $\beta$ Oo-NO<sub>2</sub>Ph), phenyl  $\beta$ -D-galactopyranoside (Gal $\beta$ O-Ph), on the regioselectivity and yield of the synthesis of *N*-acetylactosamine obtained by use of the transglycosylation reaction catalyzed by the *Bacillus circulans*  $\beta$ -D-galactosidase.

## RESULTS AND DISCUSSION

Kinetic analyses of the transglycosylation reactions using different donors were carried out at different temperatures, in the range from 25 °C to 55 °C. In all cases the value of pH was 5.0, already determined in a previous study<sup>14</sup> to be optimal for the synthesis of *N*-acetylactosamine using *Bacillus circulans*  $\beta$ -D-galactosidase. The results are reported in Figure 1. The optimal values of the results obtained with the different aryl



**Figure 1** Kinetic analysis of the synthesis of *N*-acetyllactosamine (—) and *N*-acetylalloctosamine (----) at 25 °C (A), 37 °C (B) and 55 °C (C) using Gal $\beta$ O $p$ -NO $_2$ Ph (●), Gal $\beta$ O $o$ -NO $_2$ Ph (■) and Gal $\beta$ O-Ph (▲) as the donor.

$\beta$ -D-galactosides are summarised in Table 1, together with those obtained with lactose as the donor,<sup>14</sup> for comparison purposes.

Among the three aryl donors, only Gal $\beta$ O $p$ -NO $_2$ Ph is able to give a wholly regiospecific reaction: only *N*-acetyllactosamine was detected at all the temperatures considered. The molar yield, based on the donor, is always rather high, ranging from 30%, for an incubation at 25 °C, to 74%, for that conducted at 55 °C. The use of the Gal $\beta$ O $o$ -NO $_2$ Ph, namely a position-isomer in the structure of the aglycon portion, yields a

**Table 1.** Regioselectivity and yield of the Gal $\beta$ 1-4GlcNAc and Gal $\beta$ 1-6GlcNAc obtained in the transglycosylation reactions conducted at 55 °C and pH =5.0.

<i>Donor</i>	<i>Acceptor</i>	<i>Regioselectivity,</i> [(1-4)/(1-4 + 1-6)]•100	<i>Yield,</i> %
Gal $\beta$ Op-NO <sub>2</sub> Ph <sup>a</sup>	GlcNAc	100	74
Gal $\beta$ Oo-NO <sub>2</sub> Ph <sup>a</sup>	GlcNAc	95	46
Gal $\beta$ O-Ph <sup>a</sup>	GlcNAc	88	14
Lactose <sup>b</sup>	GlcNAc	98	42

a. This work

b. Reference 14.

regioselective reaction, since a low but significant (about 2%) by-product of the 1-6 isomer is present. The yield of *N*-acetylglucosamine remains high, in the range of 40%. The best result is obtained at an incubation temperature of 55 °C. Decrease of the polarity of the aglycon by the use of the Gal $\beta$ O-Ph, produces a dramatic effect. *N*-Acetylglucosamine was produced only at an incubation temperature of 55 °C. The maximum molar yield, *i.e.* 14.5%, was obtained after 5 hours of incubation. The regioselectivity is lower: the presence of the (1-6) isomer amounts to about 2% (on mole basis), thereby giving a (1-4):(1-6) ratio of 7:1.

The kinetic analysis data provided the best set of the physical parameters to be used to produce additional amounts of the desired products for the molecular characterization. In all cases examined the temperature was 55 °C, whereas the incubation time, *t*, was *t*=2 hours for Gal $\beta$ Op-NO<sub>2</sub>Ph, *t*=2 hours for Gal $\beta$ Oo-NO<sub>2</sub>Ph and *t*=5 hours for Gal $\beta$ O-Ph, respectively. The products were purified as described in the experimental section and then characterized by NMR spectroscopy. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of the transglycosylation products were found to be completely superimposable over the respective standards.

In conclusion, the present results confirm that the structure of the aglycon plays an important role in achieving both a high yield and a complete regiospecificity for the transglycosylation reaction catalyzed by *Bacillus circulans*  $\beta$ -D-galactosidase. Although previous studies addressed this point as far as the acceptor was concerned,<sup>19-22</sup> our results

demonstrate (for the first time) that a similar important role is played also by the donor. Within the aryl *donors*, the most hydrophobic one, *i.e.*, Gal $\beta$ O-Ph, is the least effective both as to regioselectivity and yield. This finding is at interesting variance with those of Nilsson,<sup>19</sup> which indicated a considerable hydrophobic interaction at the *acceptor* site. As in the quoted study,<sup>19</sup> however, the *ortho* isomer seems to impair the reaction performance, in comparison to the *para* isomer, probably because of an effect of steric hindrance.

Lactose gives regioselectivity and yield values very similar to those of Gal $\beta$ Oo-NO<sub>2</sub>Ph, *i.e.*, lower than those of Gal $\beta$ Op-NO<sub>2</sub>Ph. Whether the *p*-nitrophenol aglycon is favoured by specific effects related to its aromatic nature, or just since its hindrance is anyway lower than that of glucose is not a matter which can be safely defined on the basis of the present results only. Theoretical calculations have already been undertaken to try to get a deeper insight into the problem.

## EXPERIMENTAL

**Materials.** *N*-acetyllactosamine, Gal $\beta$ Op-NO<sub>2</sub>Ph, Gal $\beta$ Oo-NO<sub>2</sub>Ph, Gal $\beta$ O-Ph, 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) were obtained from Sigma (St. Louis, MO, USA).  $\beta$ -D-galactosidase from *Bacillus circulans* was from Daiwa Kasei Co. (Osaka, Japan). Sep-Pak C<sub>18</sub> (light and Vac 35 cc) cartridges were from Waters (Milford, MA, USA). LiChrosorb-NH<sub>2</sub>, analytical HPLC column was from Merck (Darmstadt, Germany). Spherisorb S5 amino, semi-prep HPLC column was from Waters (Milford, MA, USA). Acetonitrile HyperSolv for HPLC was from BDH (Poole, UK).

**Purification of the transglycosylation products.** The products of transglycosylation were purified on a Sep-Pak C<sub>18</sub> (Vac 35 cc) cartridge previously conditioned with three volumes of methanol followed by three volumes of water. Unreacted GlcNAc, free Gal and the transglycosylation product(s) were eluted by washing with water. Less polar compounds like aryl  $\beta$ -D-galactosides and the free aglycons were eluted with methanol. The water-eluted fraction was dried under reduced pressure, redissolved in water and then purified using a Jasco HPLC system consisting of a BIP-I pump equipped with an UVDEC-100-V UV-visible detector monitoring at 210 nm and a Spherisorb S5 amino semi-preparative HPLC column (5  $\mu$ m, 250 x 10 mm I.D.). The column was eluted under isocratic conditions at a flow rate of 3 mL/min, using a mixture of CH<sub>3</sub>CN: H<sub>2</sub>O (80: 20) as mobile phase. The peaks corresponding to the product were pooled and dried under reduced pressure. The residue was dissolved in 2 mL of water and freeze-dried.

**Kinetic analysis of the synthesis of *N*-acetylactosamine using the three different aryl  $\beta$ -D-galactosides as donors.** The following general protocol was used: 100  $\mu$ moles of aryl  $\beta$ -D-galactosides and 1.3 mmoles of GlcNAc were dissolved in 1.5 mL of 50 mM sodium acetate buffer pH 5.0. The solution was divided in three aliquots of 0.5 mL each. To each aliquot, 490  $\mu$ L of 50 mM sodium acetate buffer pH 5.0 and 10  $\mu$ L (0.06 U) of a 10 mg/mL solution of *Bacillus circulans*  $\beta$ -D-galactosidase were added, respectively. The three aliquots were incubated at 25 °C, 37 °C and 55 °C for 6 hours, respectively. During incubation, every hour, 50  $\mu$ L of each sample were collected, added to 450  $\mu$ L of distilled water and heated in a boiling water bath for 10 min and then immediately cooled in ice. After centrifugation at 11,000 rpm for 5 min the clear supernatants of each sample were separately passed through a Sep-Pak C<sub>18</sub> (light) cartridge prepared as described before. The water-eluted fractions were freeze-dried. The residues were resuspended in 500  $\mu$ L of distilled water and analyzed by the same HPLC system as described above but equipped with a LiChrosorb-NH<sub>2</sub>, analytical HPLC column (5  $\mu$ m, 250 x 4.00 mm I.D.). The column was eluted under isocratic conditions at a flow rate of 1 mL/min, using a mixture of CH<sub>3</sub>CN: H<sub>2</sub>O (80: 20) as mobile phase.

**Preparation, purification and characterization of *N*-acetylactosamine using the three different aryl  $\beta$ -D-galactosides as donors.** The following general protocol was used: 133  $\mu$ moles of aryl  $\beta$ -D-galactosides and 1.8 mmoles of GlcNAc were dissolved in 4.0 mL of 50 mM sodium acetate buffer pH 5.0 to which 40  $\mu$ L (0.24 U) of a 10 mg/mL solution of *Bacillus circulans*  $\beta$ -D-galactosidase were added. The mixture was incubated at 55 °C for 2 hours (for the Gal $\beta$ Op-NO<sub>2</sub>Ph and Gal $\beta$ Oo-NO<sub>2</sub>Ph) and for 5 hours (for the Gal $\beta$ O-Ph). After that the mixture was heated in a boiling water bath for 10 min, to inactivate enzyme and then immediately cooled in ice. After centrifugation at 11,000 rpm for 5 min the clear supernatant was purified as described above.

**Structural identification methods.** <sup>1</sup>H and <sup>13</sup>C NMR experiments were performed on a Bruker AC 200 spectrometer equipped with a 5 mm Multinuclear probe. 500  $\mu$ L of deuterated water were used for all the measurements with a typical sample concentration of 5 mg/mL for <sup>1</sup>H measurements and 30 mg/mL for <sup>13</sup>C measurements. The spectra were obtained at 297K.

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