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CHEMICAL AND ENZYMATIC SYNTHESIS OF GLYCOCONJUGATES 5: ONE-POT REGIOSELECTIVE SYNTHESIS OF BIOACTIVE GALACTOBIOSIDES USING A CLONEZYME™ THERMOPHILIC GLYCOSIDASE LIBRARY

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Abstract: Enzymatic synthesis of galactobiosides using a versatile CLONEZYME™ thermostable glycosidase library was studied. One-pot transglycosylation reactions were demonstrated to synthesize β(1→4), β(1→6), and α(1→6) disaccharide sequences with high regioselectivity and moderate to high yields.

Over the past few years, glycosidases have been used widely for the preparation of a broad spectrum of glycoconjugates and derivatives employing either transglycosylation or reverse hydrolysis reactions.[1] In order to find biocatalysts with unprecedented properties from vast untapped resources of extremophiles, recombinant technologies and robotic screening approaches have been developed by Diversa Inc for the discovery of a novel glycosidase library, namely CLONEZYME™ library, which contains 10 unique thermostable glycosidases.[2] Recently, we have demonstrated useful transformations for the synthesis of *N*-acetyllactosamine, lactosamine, and β-D-fucopyranosyl-β-D-xylopyranosides with the CLONEZYME™ library.[3] It is particularly useful to explore the synthetic repertoire of these robust enzymes catalyzing one-pot oligosaccharide synthesis via either self-transglycosylation or tandem transglycosylation (Figure 1). Using the above strategy, we herein report efficient synthesis of biologically important galactobiosides with this versatile glycosidase library.

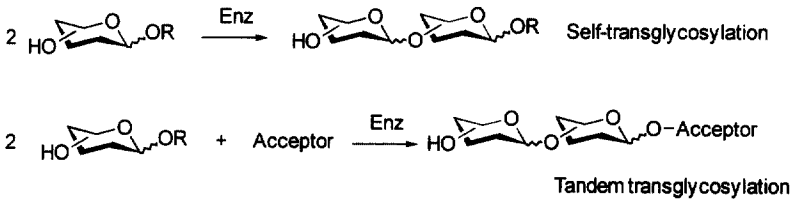

Figure 1. One-Pot Oligosaccharide Synthesis Catalyzed by Transglycosylation Reactions.

So far, self-transglycosylation reactions using nitrophenyl glycosides as donors with relatively large scale have been limited to the synthesis of Galα1→3 Gal and Manα1→2 Man as major sequences.[4] Tandem transglycosylation reactions using lactose and raffinose as donors were reported to mainly afford Galβ1→3 Gal and Galα1→3 Gal sequences, respectively.[5] In the course of our screening the commercially available CLONEZYME™ glycosidase library, we found efficient synthesis of three bioactive galactobioside disaccharide sequences with β(1→4), β(1→6), and α(1→6) linkages. Galβ(1→4)Gal, as a major component of

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galactooligosaccharide, can promote the growth of Bifidobacteria in human large intestine. Bifidobacteria is a friendly bacteria in human intestine, which has the following therapeutic effects ranging from antitumorigenic activity, reduction of serum cholesterol levels, synthesis of B-complex vitamins, to enhanced absorption of calcium in the prevention of bone loss.[6] Galβ(1→6)Gal was found to be responsible for the binding of myeloma immunoglobulins.[7] Galα(1→6) Gal is an important disaccharide sequence in glycosylphosphatidylinositol (GPI) anchor of *Trypanosoma brucei* (Figure 2).[8] Comparing to chemical synthesis of β(1→4),[9] β(1→6),[10] and α(1→6)[11] linked galactobiosides elaborated with multistep protecting, glycosylation, and deprotecting sequences, one-pot enzymatic reactions via self-and tandem-transglycosylation are extremely attractive in assembling these bioactive oligosaccharides.

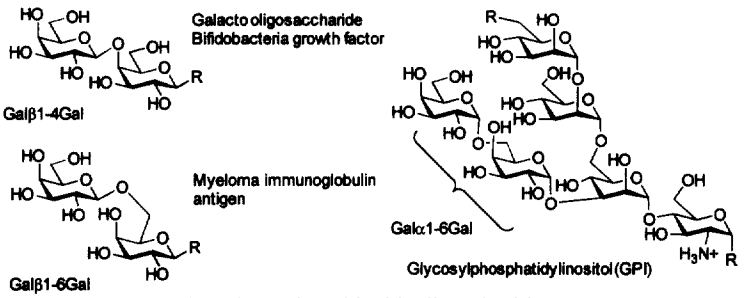
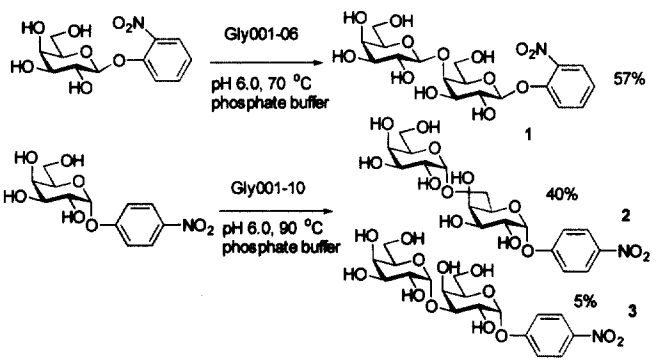


Figure 2. Bioactive galactobioside disaccharide sequences.

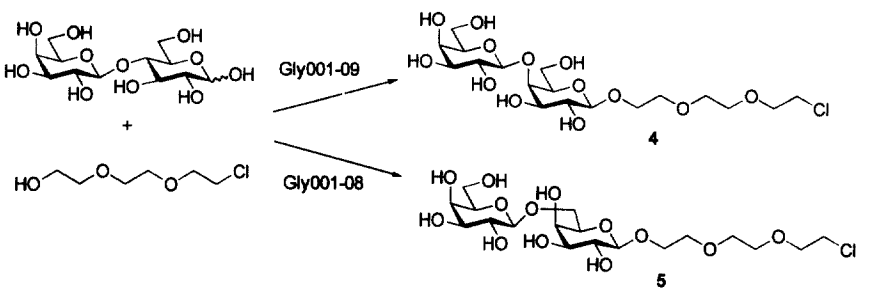
The one-pot reactions via self-transglycosylation were demonstrated using glycosidase Gly-001-06 and Gly-001-10 (Scheme 1). The latter is the only enzyme in the library showing α-galactosidase activity. It is noteworthy that Gly-001-06 is especially efficient for the regioselective synthesis of β1→4 linked galactobioside, while Gly-001-10 is specific for the synthesis of α1→6 linked galactobioside. Both reactions were in good yields up to 57%. Nevertheless, minor product of α1→3 linked galactobioside was observed in the use of Gly-001-10. A general procedure is described as follows: 0.36 mmol nitrophenyl D-galactopyranoside was dissolved in 6 mL sodium phosphate buffer (50 mM, pH 6.0). Glycosidase (0.2 mg/mL) from CLONEZYME™ library was added. The reaction was incubated at 70 or 90 °C for 3 h and terminated by freeze-drying. The residue was loaded on silica gel column chromatography and eluted with ^{i}PrOH/EtOAc/H_2O (2/6/1) to afford products **1**, **2**, and **3**. The glycosidic linkages of the products were identified by marked down-field shift of the corresponding C-3, C-4, and C-6 resonances.[12]



Scheme 1. Regioselective synthesis of galactobiosides via self-transglycosylation.

The β1→4 linked galactobioside was found to obtain in high yield in our studies comparing to the results obtained previously from a similar transglycosylation using conventional β-galactosidases.[13] To the best of our knowledge, Gly001-10 is the only enzyme reported so far to catalyze the high yielding formation of α1-6 linked galactobioside using self-transglycosylation scheme. The *p*-nitrophenyl group can be selectively removed by a sequence of hydrogenation, *N*-acetylation, and CAN (cerium ammonium nitrate) oxidation reactions.[14] Thus, *p*-nitrophenyl-α-D-galactopyranosyl-(1→6)-α-D-galactopyranoside, **2**, can be used as a disaccharide building block for the total synthesis of GPI.

The one-pot reactions via tandem transglycosylation were demonstrated using lactose as a cheap donor and CLONEZYME™ glycosidases as catalysts for double galactosylation of acceptor 2-[2-(2-chloroethoxy)-ethoxy]ethanol which can be served as a biocompatible spacer with a good leaving chloride group at one end of the chain. Generally, the yield of double transglycosylation reaction is low. Moreover, the reaction is also plagued by the poor regioselectivity observed at the second transglycosylation step. Here we found two unique enzymes Gly-001-08 and Gly-001-09 catalyzed the double galactosylation reactions with regioselectivity exclusively for β1→6 and β1→4 linkages, respectively (Scheme 2). Although the overall yields are relatively low without optimization, the reactions can be readily scaled up in view of the efficiency of this regioselective approach and the use of cheap donor and acceptor. A general approach for this transglycosylation is shown as follows: To a solution of donor lactose (1 g, 2.9 mmol) and acceptor (212 μL, 1.4 mmol) in 2.9 mL of sodium phosphate buffer (50 mM, pH 6.0) was added CLONEZYME™ glycosidase (100 μL, 1.8-1.9 mg/mL). The reaction was incubated at 75 °C for 4 h to overnight when the maximum formation of disaccharides was reached. The reaction mixture was concentrated and the residue was fractionated and separated by Biogel P-2 gel. Only β1→4 linked galactobioside **4** (50 mg, 7% yield) and β1→6 linked galactobioside **5** (31 mg, 4% yield) were found for Gly-001-09 and Gly-001-08, respectively.[12] No other types of linkage were observed in the reaction mixtures.



Scheme 2. One-pot regioselective synthesis of galactobiosides via tandem transglycosylation.

In summary, we demonstrated here a versatile CLONEZYME™ thermophilic glycosidase library could be applied for the synthesis of biologically important galactobiosides via highly efficient one-pot regioselective tranglycosylation routes. The yields of the reactions were achieved from relatively low to high with remarkable regioselectivities. All the one-pot reactions shown here provide us with a simple and useful method for the synthesis of complex galactobiosides.

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12. ^{13}C NMR for **1** (100 MHz, D_2O/5% CD_3OD): δ 171.4, 150.4, 136.0, 126.4, 124.0, 118.5, 105.3 (C-1'), 102.0 (C-1), 77.8 (C-4), 76.1, 75.7, 74.0, 73.9, 72.5, 71.7, 69.6, 61.9 (C-6'), 61.2 (C-6). **2** (100 MHz, D_2O) δ 161.8, 142.7, 126.3, 117.5, 98.1 (C-1'), 96.8 (C-1), 71.2, 71.1, 69.83, 69.80 (2C), 69.5, 68.5, 68.2, 67.0, 61.4 (C-6'). **3** (100 MHz, D_2O) δ 161.2, 142.1, 125.8, 116.6, 96.6 (C-1'), 95.0 (C-1), 73.9 (C-3), 71.9, 70.9, 69.2, 69.1, 68.1, 66.2, 65.2, 61.1 (C-1'), 60.8 (C-1). **4** (100 MHz, D_2O/20% CD_3OCCD_3) δ 104.7 (C-1'), 103.1 (C-1), 77.5 (C-4), 75.4, 74.5, 73.5, 73.2, 71.8, 71.6, 71.1, 70.1, 69.84, 69.80, 69.0, 68.9, 61.3 (C-6'), 60.7 (C-6), 43.5 ($\underline{C}H_2Cl$). **5** (100 MHz, D_2O/20% CD_3OCCD_3) δ 103.7 (C-1'), 103.2 (C-1), 75.5, 74.1, 73.2, 73.0, 71.1, 71.0, 70.1, 69.9, 69.8, 69.1, 69.0 (2C), 61.3 (C-6'), 43.5 ($\underline{C}H_2Cl$).
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