Enzymatic Synthesis of Galactosylated 1D-*Chiro*-Inositol and 1D-Pinitol Derivatives Using the β-Galactosidase from *Bacillus circulans*

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Dedicated to Professor András Lipták on his 65th birthday

Abstract: The β-D-galactosidase from *Bacillus circulans* regioselectively galactosylates 1D-*chiro*-inositol and 1D-pinitol (1D-3-*O*-methyl-*chiro*-inositol) to give mono- and digalactosylated inositols in yields of up to 45%. The enzyme does not galactosylate 1L-*chiro*-inositol or 1L-quebrachitol (1L-2-*O*-methyl-*chiro*-inositol). This is the first report of enzymatic glycosylation of *chiro*-inositol and its derivatives.

Key words: carbohydrates, enzymatic glycosylations, *Bacillus circulans*, β-D-galactosidase, inositol oligosaccharides

Oligosaccharides containing inositol sugars such as 1D-*chiro*-inositol (DCI) (**1**) and 1D-pinitol (**2**) are the focus of current interest for their putative role as secondary messengers of insulin and their associated implications in the etiology of Type 2 diabetes.[1-5] Although the detailed structures of these secondary messengers remain elusive, they are believed to contain an amino sugar such as galactosamine linked to a phosphorylated inositol sugar such as **1**, **2** or *myo*-inositol.[6] In addition, DCI and pinitol-based oligosaccharides have been isolated in high levels from the seeds of leguminous plants and are thought to provide protection for the organelles, enzymes, proteins etc. during dessiccation.[7,8]

Glycoside hydrolases (glycosidases) are responsible for the cleavage of oligosaccharides into constituent monosaccharide units in vivo. In vitro, it is possible to drive the reaction in the reverse direction, i.e. to form interglycosidic linkages from monomeric sugar units. By employing glycosidases in this manner it is possible to circumvent classical organic synthesis methods and traditional labour-intensive extraction procedures in the production of oligosaccharides.

The β-D-galactosidase from *Bacillus circulans* (EC 3.2.1.23) has been reported as being an excellent, high yielding catalyst for the formation of di- and trisaccharides with Galβ1→4 linkages.[9-13] This enzyme catalyzes the transfer of Gal from *p*-nitrophenyl β-D-galactopyranoside (GalβO*p*NP) or lactose to Glc, GlcNAc, Gal and GalNAc, predominantly to the OH−4 position.[13]

Herein we describe the enzymatic synthesis and characterisation of the products from the enzyme catalyzed reactions of GalβO*p*NP with **1** and **2** employing the β-D-galactosidase from *B. circulans* (Figures 1 and 2).[14] To our knowledge, this is the first report describing an enzymatic synthesis to prepare inositol containing oligosaccharides.

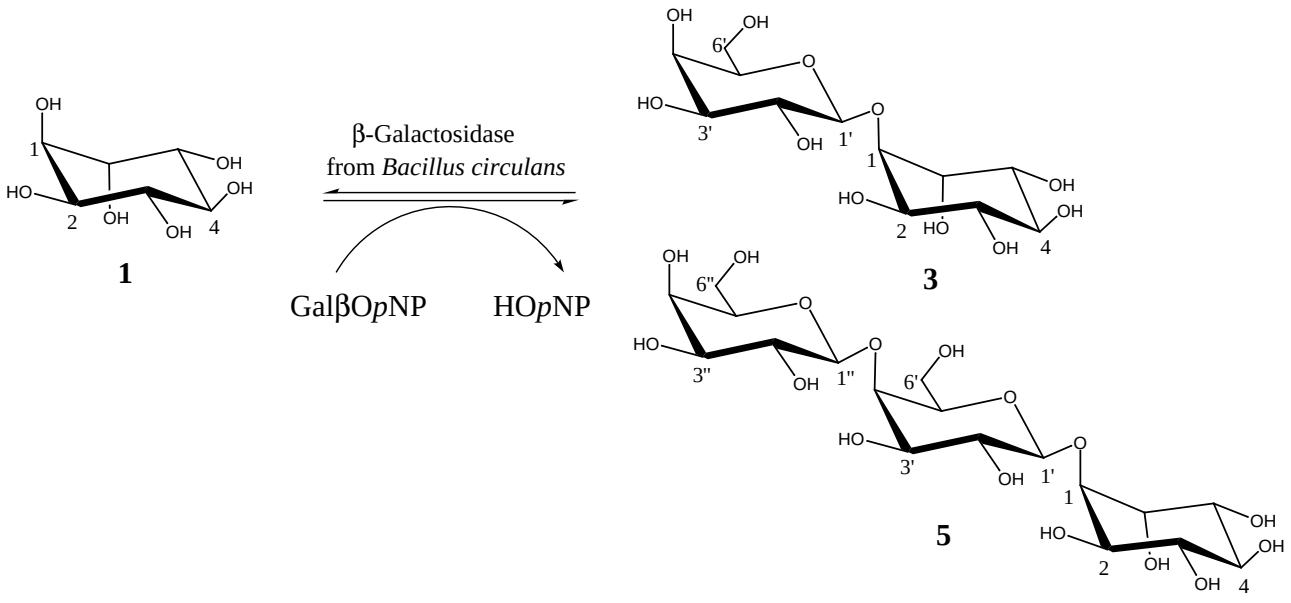

Figure 1 Galactosylation Reaction with DCI as Acceptor

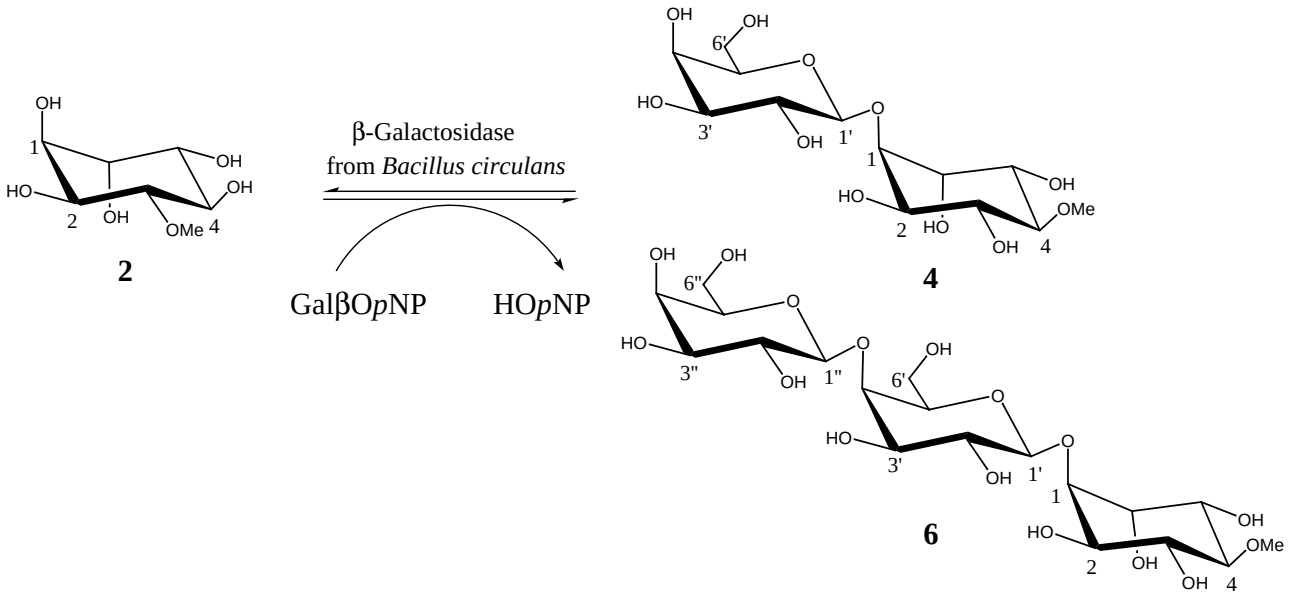

Figure 2 Galactosylation Reaction with Pinitol as Acceptor

Galactosylations using GalβO*p*NP[14] as the donor were performed in sodium acetate buffer (pH 5.0) at 50 °C with 4.4 eq. of the acceptor **1**.[15] Galactosylations using 4.4 eq. of **2** were performed at 37 °C in sodium phosphate buffer (pH 7.0) with acetonitrile as a co-solvent.[15] These are optimized reaction conditions. Previous results obtained using acetonitrile as a co-solvent in glycosylation reactions with glycoside acceptors (eg. OAll and SPh) improved the acceptor solubility and the overall reaction yields.[13] The reactions were worked up after 2 to 3 days and the products isolated and purified by column chromatography (Biogel P2). The monogalactosylated products, 1D-1-*O*-(β-D-galactopyranosyl)-*chiro*-inositol (**3**) and 1D-1-*O*-(β-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol (**4**),[16] were obtained in 43% and 45% respective yields and the digalactosylated products, 1D-1-*O*-(β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl)-*chiro*-inositol (**5**) and 1D-1-*O*-(β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol (**6**),[16] were obtained in 9% and 7% respective yields (Table). The products (**3**-**6**) were fully characterized by 1D and 2D NMR spectroscopy (HH-COSY, HMQC, HMBC, TOCSY, HMQC-TOCSY and ROESY experiments) and ES-MS.[17-20] Key correlations in the NMR spectra were observed across the glycosidic bonds, clearly defining the inositol-Gal and Gal-Gal linkages. For example, in com-

Table Summary of Enzymatic Galactosylation Results with the β-Galactosidase from *B. circulans*

donor	acceptor	product	isolated yield (%)
p-Nitrophenyl β-D-Gal*p*	DCI **1**	β-D-Gal*p*-(1→1)-DCI **3**	43
		β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→1)-DCI **5**	9
p-Nitrophenyl β-D-Gal*p*	D-Pinitol **2**	β-D-Gal*p*-(1→1)-4-OMe-DCI **4**	45
		β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→1)-4-OMe-DCI **6**	7
p-Nitrophenyl β-D-Gal*p*	LCI **7**	nr	-
p-Nitrophenyl β-D-Gal*p*	1L-Quebrachitol **8**	nr	-

nr = no reaction

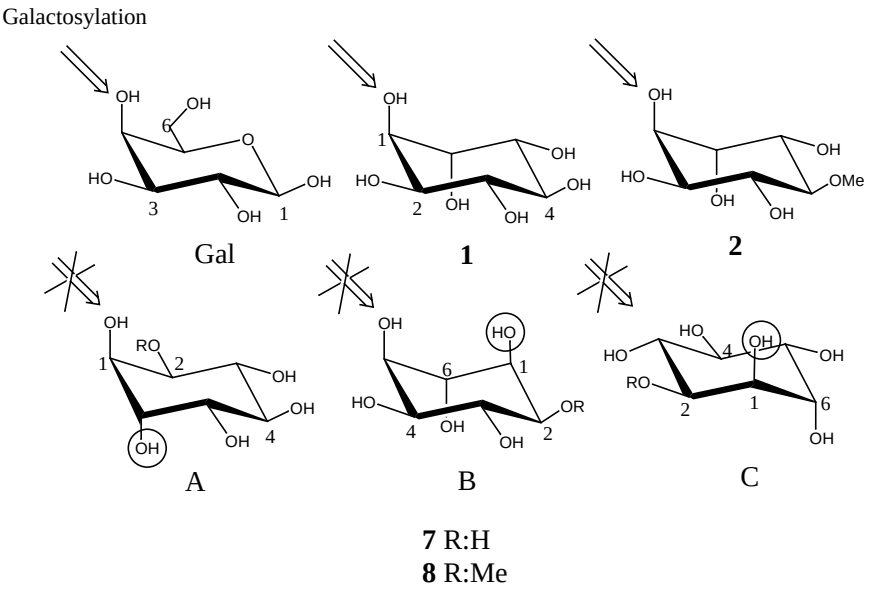

Figure 3 Structure Comparison of Selected Inositol Sugars with Galactose

pound **3**, HMBC correlations were observed from the C−1' of Gal to H−1 of DCI and from C−1 of DCI to the β-linked H−1' proton of Gal.

In contrast to the reactions observed for the D-enantiomers, no reaction was observed when either 1L-*chiro*-inositol (LCI) (**7**) or 1L-quebrachitol (1L-2-*O*-methyl-*chiro*-inositol) (**8**) were employed as acceptors (Table). From these results it appears that DCI (**1**) and 1D-pinitol (**2**) are able to mimic the hydroxyl configuration of the natural acceptor, Gal, resulting in galactosylation at the axial OH−1 or OH−6 groups of **1** and the axial OH−6 group of **2**[16] which is remote from that of the methyl group (Figure 3). This is consistent with the predominant OH−4 galactosylation pattern observed for the β-galactosidase from *B. circulans*.[13] 1L-*chiro*-inositol (**7**), and its 2-*O*-methyl ether quebrachitol (**8**), are not substrates for the enzyme, despite containing arrangements of OH groups (A, B, C Figure 3) which mimic Glc and Gal. The presence of axial OH groups in **7** and **8** (denoted as circled OH's in Figure 3), appears to prevent their binding as acceptors in the enzyme catalyzed reaction.

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(14) β-Galactosidase from *Bacillus circulans* (EC 3.2.1.23) was purchased from "Biolacta" Daiwa Kasei Co., Ltd., Osaka, Japan, and *p*-nitrophenyl β-D-galactopyranoside from Sigma, Germany.
(15) Experimental procedure: Compounds **3** and **5**: GalβO*p*NP (20 mg, 0.066 mmol) and 1D-*chiro*-inositol (53 mg, 0.29 mmol, 4.4 eq.) were incubated in 1 mL sodium acetate buffer (pH 5.0, 50 mM) with 0.5 U β-galactosidase from *Bacillus circulans* at 50 °C. After 5 h the donor was not completely hydrolyzed so the reaction was further incubated at 37 °C. After 2 days the enzyme was denaturated by heating at 90 °C for 10 min. The solution was extracted with ethyl acetate to remove *p*-nitro-

phenol and lyophilized. The residue was applied to a Biogel P2 column and eluted with water to give compound **3** (9 mg, 43%) and compound **5** (2 mg, 9%);
Compounds **4** and **6**: *G*alβO*p*NP (17 mg, 0.056 mmol) and 1D-pinitol (52 mg, 0.27 mmol, 4.8 eq.) were incubated in a 1:1 mixture of sodium phosphate buffer (0.4 mL, pH 7.0, 0.1 M) and acetonitrile with 0.5 U β-galactosidase from *Bacillus circulans* at 37 °C. After 3 days the enzyme was denaturated by heating at 90 °C for 10 min. The solution was extracted with ethyl acetate to remove *p*-nitrophenol and lyophilized. The residue was applied to a Biogel P2 column and eluted with water to give compound **4** (9 mg, 45%) and compound **6** (2 mg, 7%).

(16) Note on numbering: Pinitol is formally described as 1D-3-*O*-methyl-*chiro*-inositol under the IUPAC rules applicable to the naming of inositols. Galactosylation occurs at the 6−position of pinitol, giving a molecule for which the correct numbering places the galactosyl substituent on the *O*-1-position of the *chiro*-inositol molecule, renumbering the methyl at the 4−position i.e. the correct numbering is 1-*O*-galactosyl-4-*O*-methyl- and the methyl group has not changed position.

(17) 1D-1-*O*-(β-D-galactopyranosyl)-*chiro*-inositol (**3**): $[\alpha]^{21}_D$ +39 ° (*c* 0.9, H_2O); ^1H NMR (500 MHz, D_2O): δ = 4.48 (d, 1 H, $J_{1',2'}$ = 7.8 Hz, H−1'), 4.27 (dd, 1 H, $J_{6,1}$ and $J_{6,5}$ = 3.3 Hz, H−6), 4.02 (dd, 1 H, $J_{1,2}$ = 3.3 Hz, H−1), 3.88 (d, 1 H, $J_{4',3'}$ = 3.4 Hz, H−4'), 3.79 (dd, 1 H, $J_{5,4}$ = 10.0 Hz, H−5), 3.77 (dd, 1 H, $J_{2,3}$ = 10.0 Hz, H−2), 3.72 (m, 2 H, H6'/H6'), 3.65 (dd, 1 H, $J_{5'6'/6'}$ = 4.2, 7.8 Hz, H−5'), 3.62 (dd, 1 H, $J_{3'2'}$ = 9.5 Hz, H−3'), 3.60 (dd, 1 H, $J_{3,4}$ = 10.0 Hz, H−3), 3.53 (dd, 1 H, H−4), 3.52 (dd, 1 H, H−2'); ^{13}C NMR (125 MHz, D_2O): δ = 105.3 (C−1'), 81.9 (C−1), 75.6 (C−5'), 73.3 (C−3), 73.2 (C−4), 73.0 (C−3'), 71.6 (C−2'), 71.1 (C−6), 70.9 (C−5), 70.8 (C−2), 68.9 (C−4'), 61.4 (C−6'); HR ES-MS: Calcd. for $C_{12}H_{23}O_{11}$ $[M+H]^+$ 343.1240, found 343.1235.

(18) 1D-1-*O*-(β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl)-*chiro*-inositol (**5**): ^1H NMR (500 MHz, D_2O): δ = 4.57 (d, 1 H, $J_{1'',2''}$ = 7.8 Hz, H−1''), 4.53 (d, 1 H, $J_{1',2'}$ = 8.0 Hz, H−1'), 4.23 (dd, 1 H, $J_{6,5}$ and $J_{6,1}$ = 3.5 Hz, H−6), 4.15 (d, 1 H, $J_{4',3'}$ = 3.4 Hz, H−4'), 4.04 (dd, 1 H, $J_{1,2}$ = 3.5 Hz, H−1), 3.88 (d, 1 H, $J_{4'',3''}$ = 3.4 Hz, H−4''), 3.80 (obsc., 1 H, H−2), 3.79 (m, 2 H, H−6''/6''), 3.77 (obsc., 1 H, H−5'), 3.76 (obsc., 1 H, H−5), 3.74 (obsc., 1 H, H−3'), 3.72 (m, 2 H, H−6'/6'), 3.64 (obsc., 1 H, H−3''), 3.64 (obsc., 1 H, H−5''), 3.61 (obsc., 1 H, H−2'), 3.60 (dd, 1 H, $J_{3,2}$ and $J_{3,4}$ = 9.7 Hz, H−3), 3.57 (obsc., 1 H, H−2''), 3.54 (dd, 1 H, $J_{4,5}$ = 9.7 Hz, H−4); ^{13}C NMR (125 MHz, D_2O): δ = 105.4 (C−1'), 104.7 (C−1''), 82.2 (C−1), 77.6 (C−4'), 75.7 (C−5'), 74.9 (C−5''), 73.4 (C−3'), 73.3 (C−3), 73.2 (C−3''), 73.2 (C−4), 72.0 (C−2'), 71.9 (C−2''), 71.1 (C−6), 70.8 (C−5), 70.8 (C−2), 69.1 (C−4''), 61.5 (C−6''), 61.0 (C−6'); HR ES-MS: Calcd. for $C_{18}H_{32}O_{16}ONa$ $[M+Na]^+$ 527.1583, found 527.1578.

(19) 1D-1-*O*-(β-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol (**4**): $[\alpha]^{21}_D$+38 ° (*c* 0.9, H_2O), ^1H NMR (500 MHz, D_2O): δ = 4.47 (d, 1 H, $J_{1',2'}$ = 7.8 Hz, H−1'), 4.27 (dd, 1 H, $J_{6,1}$ and $J_{6,5}$ = 3.7 Hz, H−6), 4.01 (dd, 1 H, $J_{1,2}$ = 3.7 Hz, H−1), 3.88 (d, 1 H, $J_{4',3'}$ = 3.4 Hz, H−4'), 3.84 (dd, 1 H, $J_{5,4}$ = 9.5 Hz, H−5), 3.77 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H−2), 3.74 (dd, 1 H, $J_{6',5'}$ = 4.4 Hz, $J_{6',6'}$ = 11.7 Hz, H−6') 3.70 (dd, 1 H, $J_{6',5'}$ = 7.8 Hz, H−6'), 3.68 (dd, 1 H, $J_{3,4}$ = 9.5 Hz, H−3), 3.65 (dd, 1 H, H−5'), 3.62 (dd, 1 H, $J_{3',2'}$ = 10.0 Hz, H−3'), 3.57 (s, 3 H, OCH_3), 3.51 (dd, 1 H, H−2'), 3.31 (dd, 1 H, H−4); ^{13}C NMR (125 MHz, D_2O): δ = 105.3 (C−1'), 83.1 (C−4), 81.6 (C−1), 75.6 (C−5'), 72.9 (C−3'), 72.7 (C−3), 71.6 (C−2'), 71.1 (C−6), 70.8 (C−2), 70.2 (C−5), 68.9 (C−4'), 61.4 (C−6'), 60.1 (OCH_3); HR ES-MS: Calcd. for $C_{13}H_{24}O_{11}Na$ $[M+Na]^+$ 379.1216, found 379.1211.

(20) 1D-1-*O*-(β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol (**6**): ^1H NMR (500 MHz, D_2O): δ = 4.57 (d, 1 H, $J_{1'',2''}$ = 7.7 Hz, H−1''), 4.52 (d, 1 H, $J_{1',2'}$ = 7.8 Hz, H−1'), 4.22 (dd, 1 H, $J_{6,1}$ and $J_{6,5}$ = 3.7 Hz, H−6), 4.14 (d, $J_{4',3'}$ = 3.3 Hz, H−4'), 4.02 (dd, $J_{1,2}$ = 3.7 Hz, H−1), 3.88 (d, $J_{4'',3''}$ = 2.7 Hz, H−4''), 3.84 (obsc., 1 H, H−5), 3.82 (dd, 1 H, $J_{2,3}$ = 9.6 Hz, H−2), 3.82 (obsc., 1 H, H−6'), 3.76 (obsc., 2 H, H−6''/6''), 3.75 (obsc., 1 H, H−6'), 3.74 (obsc., 1 H, H−3'), 3.72 (obsc. 1 H, H−5'), 3.68 (dd, 1 H, $J_{3,4}$ = 9.6 Hz, H−3), 3.66 (obsc., 1 H, H−5''), 3.66 (s, 3 H, OCH_3), 3.64 (obsc., 1 H, H−3''), 3.60 (obsc., 1 H, H−2'), 3.57 (obsc., 1 H, H−2''), 3.31(dd, 1 H, $J_{4,5}$ = 9.6 Hz, H−4); ^{13}C NMR (125 MHz, D_2O): δ = 105.4 (C−1'), 104.7 (C−1''), 83.1 (C−4), 81.9 (C−1), 77.5 (C−4'), 75.6 (C−5''), 74.9 (C−5'), 73.4 (C−3'), 73.2 (C−3''), 72.6 (C−3), 72.0 (C−2'), 71.9 (C−2''), 71.1 (C−6), 70.9 (C−2), 70.2 (C−5), 69.1 (C−4''), 61.5 (C−6''), 61.0 (C−6'), 60.1 (OCH_3); HR ES-MS: Calcd. for $C_{19}H_{34}O_{16}Na$ $[M+Na]^+$ 541.1739, found 541.1750.

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