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SYNTHESIS OF LACTOSAMINE DERIVATIVES USING β-D-GALACTOSIDASE FROM *BACILLUS CIRCULANS*

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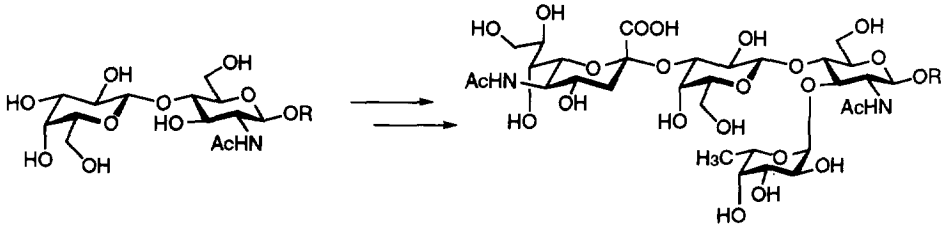
Abstract: Various lactosamine derivatives that are versatile building blocks for oligosaccharide synthesis were obtained with excellent regioselectivity and moderate to high yields using β-D-galactosidase from *Bacillus circulans* as the biocatalyst. Copyright © 1996 Elsevier Science Ltd

N-Acetyl-D-lactosamine (Galβ(1,4)GlcNAc or LacNAc) is an important structural component in glycoproteins and glycolipids.[1] LacNAc and their derivatives are useful for the study of biological recognition processes[2] and have been the objective of much synthetic effort by means of standard chemical[3] and enzymatic methods.[4] Enzymatic approaches to the synthesis of these compounds have proven to be useful, especially that based on β(1,4)-galactosyltransferase coupled with in situ regeneration of sugar nucleotides.[5] Recently, β-D-galactosidase from *Bacillus circulans* has been used in forming Galβ(1,4) linkages to GlcNAc and its derivatives.[6] The advantages of the galactosidase-catalyzed reactions are the enzyme's low cost ($350 per kg, 5 x 10^6 units, Daiwa Kasei, Osaka, Japan), high stability ($t_{1/2}$ = 262 h at pH 7 and 22°C), low cost of the donors (no need for expensive sugar nucleotides), and simple reaction conditions. Galactosidases also have a broad acceptor specificity and allow the synthesis of compounds that are not accessible with galactosyltransferases. There are also some disadvantages in using galactosidase catalyzed reactions, however. Because of the hydrolytic nature of the enzyme, the yields hitherto reported were low and the desired compound was difficult to isolate from the reaction mixture containing very similar products.[6, 7]

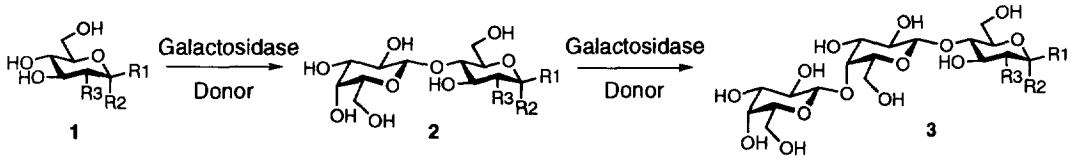


N-Acetyl-D-lactosamine Sialyl Lewis^x

We became interested in using β-D-galactosidase from *Bacillus circulans* for the synthesis of lactosamine derivatives that are building blocks for the synthesis of sialyl Lewis^x (SLe^x) conjugates with higher affinity and selectivity compared to free SLe^x. SLe^x is a terminal tetrasaccharide fragment of membrane glycoproteins and

glycolipids that mediates cell-cell binding in biological processes, such as inflammation and cancer development.[8] Though SLe^x is crucial in these interactions, it is only the minimum recognition element in these processes[9] and some SLe^x bearing ligands with additional reducing end substituents have higher affinities and selectivities.[10]

Scheme 1[11]



Condition A (phosphate buffer : CH_3CN = 1:1 v/v, pH 7) Donor: lactose (3.4 equiv)

1a R_1= $O(CH_2)_5CO_2Me$	R_2= H	R_3= NHAc	**2a** 12% (75%)*	**3a** 2.4% (15%)*
1b R_1= OBn	R_2= H	R_3= NHAc	**2b** 14% (74%)*	**3b** 4% (24%)*
1c R_1= H	R_2= OBn	R_3= NHAc	**2c** 14% (60%)*	**3c** 4% (18%)*
1d R_1= $O(CH_2)_7CH_3$	R_2= H	R_3= NHAc	**2d** 10% (42%)*	**3d** not isolated
1e R_1= SPh	R_2= H	R_3= NHAc	**2e** 14% (62%)*	**3e** 1.7% (10%)*
1f R_1= SPh	R_2= H	R_3= N_3	**2f** 4% (29%) *	**3f** not isolated

Condition B (phosphate buffer, pH 7) Donor: GalβOPNP (0.1 equiv)

1a R_1= $O(CH_2)_5CO_2Me$, R_2= H, R_3= NHAc **2a** 46%**

Condition C (phosphate buffer : CH_3CN = 1:1, pH 7) Donor: GalβOPNP (0.1 equiv)

1a R_1= $O(CH_2)_5CO_2Me$, R_2= H, R_3= NHAc **2a** 57%**

* Yield based on acceptor used, the yield in () is based on unrecovered acceptor
** Yield based on donor used

Scheme 1 shows the D-glucosamine derivatives used as acceptors along with the obtained di- and tri-saccharide products. At the beginning, we used a 20 mM pH 7 phosphate buffer/CH_3CN (1/1) as the solvent system with excess of lactose as the donor[6a] (Condition A). The trans-galactosylation reaction proceded with excellent regioselectivity and good yields (based on unconsumed acceptor) with all *N*-acetyl-D-glucosamine derivatives regardless of the anomeric configuration[12] to give Galβ(1,4)GlcNAc disaccharides and Galβ(1,4)Galβ(1,4)GlcNAc trisaccharides which are produced by two sequential transgalactosylations.[6a] Acceptor **1f** which has a 2-azido group instead of a 2-acetamido group, however, gave a lower yield and regioselectivity. Due to the hydrophobic nature of the aglycons, the disaccharide and trisaccharide products as well as the unconsumed acceptors were easily isolated from the reaction mixture by silica gel chromatography.

Even though we were able to obtain various lactosamine derivatives from glucosamine derivatives in one step by using Condition A, the efficiency of transgalactosylation to the desired acceptor was low and significant amounts of side products were produced. Since the donor, lactose, is also an efficient acceptor and exists in excess, the side reaction in which transgalactosylation occured to lactose actually seemed to be the major reaction under Condition A. To improve the efficiency and reduce the side reactions, we decided to try using the acceptor, instead of the donor, in excess (10 eq). The transgalactosylation reaction proceeded nicely with

excellent regioselectivity and good yield using *p*-nitrophenyl β-D-galactopyranoside (GalβOPNP) as the donor either in pure buffer (Condition B) or buffer/acetonitrile (1/1) mixture (Condition C). The reaction was much faster, even with much less enzyme under Conditions B (13 mg enzyme/mmol donor, 3 h compared to 230 mg enzyme/mmol donor, 108 h). However, the yield was better under Condition C (57% compared to 46%).

In summary, we have shown an improved strategy for the high yielding, highly regioselective synthesis of lactosamine building blocks. The galactosidase catalyzed reactions were very regioselective, forming the β-galactosyl linkage at the 4-position of *N*-acetyl-D-glucosamine and also at the 4-position of D-galactose regardless of the anomeric configuration of the acceptor. The various aglycons not only serve as handles for synthesizing various glycoconjugates but also improve the (1,4) regioselectivity compared to free *N*-acetyl-D-glucosamine.[6, 13] The hydrophobicity of the aglycons also facilitated the isolation of products and recovery of unconsumed acceptor. Compounds **2e** and especially **2f** could be useful as lactosamine donors in chemical glycosylations.[14] We also note that neither the disaccharide **2c** nor any of the trisaccharide products **3a-f** can be synthesized by galactosylation utilizing β(1,4)-galactosyltransferase because the enzyme does not work well when D-galactose or α-D-glucosaminides are used as the acceptors.[15] Work is now in progress for the synthesis of various SLe^x conjugates using these D-lactosamine derivatives.

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11. **Condition A:** In a typical reaction, crude β-D-galactosidase from *Bacillus circulans* (Daiwa Kasei) (450 mg) was added to a mixture of α-lactose monohydrate (18 g, 50 mmol) and GlcNAcβO$(CH_2)_5CO_2$Me (**1a**) (4.85 g, 14.5 mmol) in a buffered solution (20 mM pH 7.0 phosphate buffer/acetonitrile, 1/1, 150 ml). The reaction mixture was stirred under an argon atmosphere for 48 h at room temperature. The reaction mixture was then concentrated *in vacuo* and the residue was chromatographed (silica gel; $CHCl_3$/MeOH/30%NH_4OH_{aq}, 3/1/0.3) to give recovered **1a** (4.08 g) and the disaccharide Galβ(1,4)GlcNAcβO$(CH_2)_5CO_2$Me (**2a**) (820 mg, 12% or 75% based on unrecovered **1a**). Further elution (*i*-PrOH/H_2O/30%NH_4OH_{aq}, 7/2/1) afforded Galβ(1,4)Galβ(1,4)GlcNAcβO$(CH_2)_5CO_2$Me (**3a**) (233 mg, 2.4% or 15% based on unrecovered **1a**).
Condition B: Crude β-D-galactosidase from *Bacillus circulans* (Daiwa Kasei) (2 mg) was added to a mixture of GalβOPNP (45 mg, 0.15 mmol) and GlcNAcβO$(CH_2)_5CO_2$Me (**1a**) (500 mg, 1.5 mmol) in a buffered solution (20 mM pH 7.0 phosphate buffer 3 ml). The reaction mixture was stirred for 3 h at room temperature. The reaction mixture was then concentrated in vacuo and the residue was chromatographed (silica gel; EtOAc/MeOH/H_2O, 30/4/1 to 5/2/1) to give recovered **1a** (413 mg) and Galβ(1,4)GlcNAcβO$(CH_2)_5CO_2$Me (**2a**) (38 mg, 46% based on GalβOPNP used).
Condition C: Crude β-D-galactosidase from *Bacillus circulans* (Daiwa Kasei) (50 mg, contains 22.5 mg of lactose) was added to a mixture of GalβOPNP (135 mg, 0.45 mmol) and GlcNAcβO$(CH_2)_5CO_2$Me (**1**) (1.50 g, 4.48 mmol) in a buffered solution (20 mM pH 7.0 phosphate buffer/acetonitrile, 1/1, 12 ml). The reaction mixture was stirred for 108 h at room temperature with periodic addition of additional enzyme (100 mg, contains 45 mg of lactose). Work up as in Condition B gave recovered **1a** (1.15 g) and Galβ(1,4)GlcNAcβO$(CH_2)_5CO_2$Me (**2a**) (186 mg, 57% based on GalβOPNP plus lactose contained in the crude enzyme).
All new compounds gave satisfactory mass spectral and NMR (^1H and ^{13}C) data in accord with proposed structures. The new structures were also confirmed through extensive decoupling experiments on their peracetylated derivatives.
Data for **2a**: ^1H NMR (400 MHz, D_2O) δ 1.32-1.37 (m, 2H), 1.53-1.65 (m, 4H), 2.04 (s, 3H, $C(O)CH_3$), 2.40 (t, 2H, J = 7.2 Hz, -$CH_2C(O)$-), 3.52-3.60 (m, 3H), 3.65-3.93 (m, 13H), 3.99 (d, 1H, J = 8.2 Hz, 6-H), 4.47 (d, 1H, J = 7.8 Hz, 1 or 1'-H), 4.52 (d, 1H, J = 8.4 Hz, 1 or 1'-H). ^{13}C NMR (100 MHz, D_2O) δ 22.6, 24.4, 25.1, 28.7, 34.1, 52.5, 55.6, 60.5, 61.5, 69.0, 70.7, 71.4, 72.9, 73.0, 75.2, 75.8, 78.9, 101.5, 103.3, 174.8, 178.0. HRMS calcd for $C_{21}H_{37}O_{13}NCs$ ($M+Cs^+$) 644.1319, found 644.1297.
Compounds **1a** and **1d** were prepared from GlcNAc by glycosylation and deacetylation. Also see Lemieux, R. U.; Bundle, D. R.; Baker, D. A. *J. Am. Chem. Soc.* **1975**, *97*, 4076. **1b**, **1c**: Paulsen, H.; Kolar, C.; Stenzel, W. *Chem. Ber.* **1978**, *111*, 2370. **1e**, **1f**: Buskas, T.; Garegg, P.; Konradsson, P.; Maloisel, J.-L. *Tetrahedron: Asymmetry* **1994**, *5*, 2187.
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