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Chemical and Enzymatic Synthesis of Glycoconjugates 3: Synthesis of Lactosamine by Thermophilic Galactosidase Catalyzed Galactosylation on a Multigram Scale

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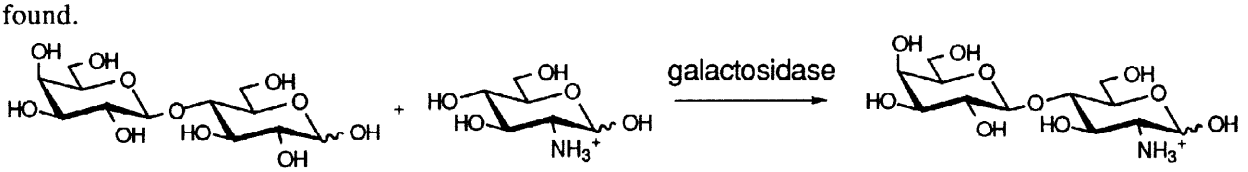
Abstract: A library of thermophilic glycosidases was screened for lactosamine preparation from a transgalactosylation reaction between lactose and glucosamine hydrochloride. Two enzymes showed excellent regioselectivity toward glucosamine hydrochloride. The product could be synthesized on a multigram scale and was easily separated and purified by subsequently eluting through cation and anion exchange resin columns. © 1998 Elsevier Science Ltd. All rights reserved.

Lactosamine sequence, normally in the form of N-acetyllactosamine, exists as the core structure in many glycoproteins and glycolipids.[1,2] Both chemical[3] and enzymatic[4-10] methods have been developed to synthesize this disaccharide sequence. Comparing these two methods, enzymatic synthesis has the advantage of no protection and deprotection over the chemical strategy. Two kinds of enzymes can be used in this synthesis: galactosyltransferases and β-galactosidases. Both enzymes have some drawbacks. In spite of high selectivity and yield in galactosyltransferase-catalyzed reaction, the limited availability of the enzyme, its high cost and instability, and the requirement of expensive uridine 5'-diphosphogalactose (UDP-Gal) as the donor component present major obstacles for large scale synthesis. In comparison, the advantages of low cost, high stability, and simple reaction condition make galactosidase-catalyzed reaction more attractive. However, the difficulty of separating the product from the reaction mixture which contains similar compounds hampers its application. Although approaches have been developed to deal with this problem, which included modifications of the acceptor[9] and the tandem use of galactose oxidase and galactosidase[10], a more efficient and practical route is still necessary. Here we report a new galactosidase-catalyzed reaction which is based on the use of glucosamine hydrochloride salt as the acceptor for the synthesis of lactosamine derivatives (**Scheme 1**). Since the aminosugar is positive charged at neutral condition, a separation procedure using cation exchange resin column can be implemented to separate the positive charged species from the uncharged ones. In addition, the NH_2-containing monosaccharides and disaccharides can also be separated from each other with cation exchange resin if an appropriate eluent can be found.



Scheme 1. Synthesis of lactosamine with a galactosidase catalyzed transgalactosylation reaction.

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Table 1. Screening of a thermophilic enzyme library (Gly001) for the synthesis of lactosamine[a]

Entry	Enzyme	Temperature (°C)	Time (hours)	Total yield[b] (1-4 + 1-6,%)	Regioselectivity[c] (1-4) / (1-6)
1	Gly001-01	85	24	NP[d]	--
2	Gly001-02	85	4.5	8	35/65
3	Gly003-03	85	12	10	45/55
4	Gly003-04	85	24	7	65/35
5	Gly001-05	85	24	NP	--
6	Gly001-06	90	72	19	100/0
7	Gly001-07	85	72	12[e]	--
8	Gly001-08	85	3	13	20/80
9	Gly001-09	85	25	23	100/0
10	Gly001-10	85	24	NP	--
11	Galactosidase[f]	25	2	16	65/35

a. All reactions were conducted as follows: To 20 mmol lactose and 2 mmol glucosamine hydrochloride, the following volume of enzymes was added separately [#1: 2 mL (1.1 mg/mL); #2: 1 mL (1.9 mg/mL); #3: 2 mL (0.87 mg/mL); #4: 2mL (1.0 mg/mL); #5: 3 mL (0.98 mg/mL); #6: 2 mL (1.1 mg/mL); #7: 2 mL (1.7 mg/mL); #8: 2 mL (1.9 mg/mL); #9: 2 mL (1.8 mg/mL); #10: 2 mL (0.8 mg/mL); 10 mg galactosidase from *Bacillus circulans*.]. The volume of the solution was adjusted to 10 mL by phosphate buffer (50 mM, pH 6.0). After reaction, the product(s) was purified by subsequently eluting through cation and anion exchange resin columns.

b. All the yields refer to chromatographically purified compounds, the process of separation and purification was described in the text.

c. Calculated from the integration of the ^{1}HNMR spectra. Both products are confirmed by ^{1}H-NMR and ^{13}C-NMR. Selective data for lactosamine hydrochloride: ^{1}H-NMR (400 MHz, D_2O) δ = 5.31 (d, J = 3.6Hz, 1-H α), 4.85 (d, J = 8.4Hz, 1-H β), 4.34 (d, J = 7.6Hz, 1'-H, β); ^{13}C-NMR (100.6 MHz, D_2O) δ = 103.30 (1'-C, β), 92.80 (1-C β), 89.11 (1-C α). For 1-6 isomer: ^{1}H-NMR (400 MHz, D_2O) δ = 5.30 (d, J = 2.8Hz, 1-H α), 4.85 (d, J = 8.0Hz, 1-H β), 4.36 (d, J = 7.2Hz, 1'-H, β); ^{13}C-NMR (100.6 MHz, D_2O) δ = 103.64 (1'-C, β), 93.11 (1-C, β), 89.46 (1-C, α).

d. NP means that no significant amount of product can be separated.

e. The product is a mixture of more than two regioisomers.

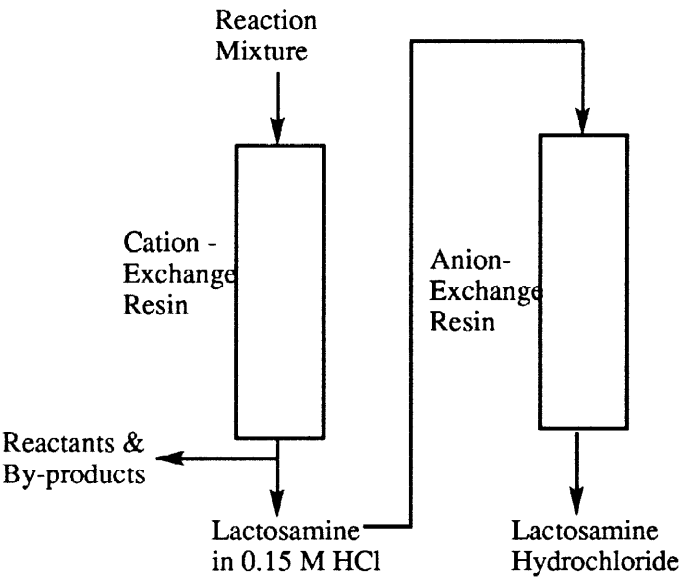
f. From *Bacillus circulans*.

The other significance of this work was the use of thermophilic enzymes. Thermophilic and hyperthermophilic organisms, known as "Thermophiles", can thrive at temperatures above 75 °C.[11] Thermophilic enzymes presenting in these organisms have already evolved to enable them to thrive in harsh environments. Although extremophiles can grow in laboratory cultures, the number of characterized thermophilic enzymes isolated from the organism secretes is limited because of a mismatch between organism and culture. In order to bypass the culture hurdle, Diversa Corporation (San Diego, CA) has succeeded in developing thermophilic enzymes through cloning and automated high-through-put screening systems.[12-13] These thermophilic enzymes have been sorted into libraries according to their character. The library (Gly001) of glycosidases currently contains 10 unique thermostable enzymes; each enzyme displays a variety of activities ranging from

galactosidase, glucosidase to fucosidase.[14] However, little effort has been made to explore the potential synthetic applications of these novel enzymes.[15] In this synthetic work, such a thermophilic glycosidase library was used together with a conventional galactosidase from *Bacillus circulans* to evaluate their synthetic application.

The transgalactosylation between lactose and glucosamine hydrochloride was carried out at 85 °C using one of the ten enzymes in this library (Gly001) which all have β-galactosidase activity. Results (**Table 1**, entry 1-10) demonstrated that Gly001-06 and Gly001-09 gave the only one regioisomer (lactosamine) in 19% and 23% yield (Entry 6 and 9). Gly001-06 had lower activity than Gly001-09. So it required higher temperature and longer reaction time. Three enzymes (Gly001-01, -05 and -10) had no significant activity. All of these active enzymes only had activity toward 4- and/or 6- position of glucosamine except Gly001-07 (Entry 7), in which products were composed of more than two regioisomers. In addition, only Gly001-08 gave the 1-6 isomer as the major product (Entry 8). ß-D-galactosidase from *Bacillus circulans* was also applied to the same reaction at room temperature (Entry 12). According to the literature,[5] this enzyme has good regioselectivity toward N-acetylglucosamine. However, the transgalactosylation studied here gave products containing a mixture of 1-4 and 1-6 regioisomers in a ratio of 2: 1. The amino-containing disaccharide product(s) could be separated by using cation exchange resin column with 0.15 M HCl as eluent. 1-4 and 1-6 isomers also could be separated from each other with low concentration HCl (0.05M) as eluent. The collected solution containing product(s) was neutralized by anion exchange resin. We used several different anion exchange resins and found the weakly basic anion exchange resin of Dowex (MARATHON, WBA) gave a close to neutral solution. This indicated that it could neutralize the HCl but keep the product(s) as hydrochloride salt. The ratios of 1-4 and 1-6 isomers in **Table 1** were calculated from the integration of the ^{1}HNMR spectra of the mixture, and were confirmed by the weight ratio of the separated two regioisomers.

Besides lactose, *o*-nitrophenyl-ß-D-galactopyranoside (ONP-Gal) was also used as a donor but resulted in no significant formation of lactosamine. The reason might be that the hydrolysis of ONP-Gal was faster than the transgalactosylation reaction. In contrast, the hydrolysis of lactose had a comparable rate with the transgalactosylation. Thus lactosamine could be produced when lactose was used as a donor.



Scheme 2. Procedure of separation and purification of lactosamine from an enzymatic reaction mixture.

We also succeeded in scaling this reaction up to 40 mmol glucosamine as acceptor. The typical procedure for the multigram scale synthesis is as follows (**Scheme 2**): To a solution of 8.6 g (40 mmol) glucosamine hydrochloride and 72 g (200 mmol) lactose in 100 mL phosphate buffer (50 mM, pH 6.0), 15 mL (1.8 mg/mL) Gly001-09 was added. After incubation at 85°C for 74 hours, the reaction mixture was diluted with 300 mL distilled water and then loaded to a cation exchange resin column (Dowex 50, H^+ form, 200-400 mesh, 400 mL). Unreacted lactose and other products (glucose and

galactose) were removed by washing the column with distilled water. Then the column was washed with 0.15 M HCl. The elution was monitored by TLC. The fractions containing lactosamine were collected and then flowed through an anion exchange resin column (Dowex, Marathon WBA, OH^- form, 20-50 mesh, 300 mL) in order to neutralize the acid. Then the anion exchange resin column was washed with distilled water. The collected solution was concentrated under reduced pressure at 40 °C to a volume of 50 mL, and lyophilized to give the pure product as hydrochloride salt (3.2 g, 20% yield). The reactant glucosamine hydrochloride (5.9 g, 69%) could be recovered by eluting the cation exchange resin column with 0.6 M HCl and the resin (H^+ form) was regenerated at the same time. If based on the glucosamine consumed, the yield of lactosamine was 65%.

In summary, we succeeded in multigram scale synthesis of lactosamine by using glucosamine as an acceptor. With unprotected amino group in the acceptor, we could separate the product by using cation exchange resin column. Another advantage of this method is that the amino group of lactosamine can be protected with a variety of protecting groups[16] for further synthetic application. Further work is in progress to use the synthetic lactosamine for oligosaccharide synthesis.

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References and Notes

1. Varki, A. *Glycobiology* **1993**, 3, 97.
2. Feizi, T.; Solomon, J. C.; Yuen, C. -T.; Jeng, K. C. G.; Frigeri, L. G.; Hsu, D. K.; Liu, F.-T. *Biochemistry* **1994**, 33, 6342.
3. (a) Toshima, K.; Tatsuta, K. *Chem. Rev.* **1993**, 93, 1503. (b) Schmidt, R. R. *Angew. Chem. Int. Ed.* **1986**, 25, 212.
4. (a) Wong, C.-H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem. Int. Ed. Engl.* **1995**, 34, 412 and 521. (b) Toone, E. J.; Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *Tetrahedron* **1989**, 45, 5365. (c) Takayama, S.; Wong, C.-H. *Bioorg. Med. Chem. Lett.* **1996**, 1123.
5. Wong, C.-H.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1982**, 5416.
6. Thiem, J.; Weimann, T. *Synthesis.* **1992**, 141.
7. Zervosen, A.; Elling, L. *J. Am. Chem. Soc.* **1996**, 118, 1836-1840.
8. (a) Usui, T.; Kubota, S.; Ohi, H. *Carbohydr. Res.* **1993**, 244, 315. (b) Herrmann, G. F.; Kragl, U.; Wandrey, C. ; Gaeta, F. C. A.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, 114, 9283.
9. Nilsson, K. G.; Eliasson, A.; Lorek, U. *Biotechnol. Lett.* **1995**, 17, 717.
10. Kimura, T.; Takayama, S.; Wong, C.-H. *Angew. Chem. Int. Engl.* **1996**, 35, 2348.
11. Adams, M. W. W.; Kelly, R. M. *Chem. Eng. News* **1995**, December 18, 32.
12. Brennan, M. B. *Chem. Eng. News* **1996**, October 14, 31.
13. Robertson, D. E.; Mathur, E. J.; Swanson, R. Y.; Marrs, B. L.; Short, J. M. *SIMS News*, **1996**, 46, 3.
14. For more information, see homepage of Diversa Corp.: http://www.biocat.com.
15. Li, J.; Wang, P. G. *Tetrahedron Lett.* **1997**, in press.
16. Alais, J.; Veyrieres, A. *Tetrahedron Lett.* **1983**, 5223.