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# *In situ* generated O-Glycan Core 1 Structure as Substrate for Gal(β1-3)GalNAc β-1,6-GlcNAc Transferase

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**Abstract:** β-Galactosidase from bovine testes was used in a one pot reaction together with a recombinant β-1,6-GlcNAc transferase for the synthesis of GlcNAc(β1-6)GalNAc(α1-OBn) (core 6-Bn). The galactosidase, which reversibly links galactose via a (β1-3) linkage to *N*-acetylgalactosamine, provides the substrate for the GlcNAc transferase *in situ*. The synthesis was carried out with a yield >90%.

As glycoconjugates play an important role in cell recognition processes,[1,2] there is an increasing need for the production of these structures *in vitro*. A β-1,6-GlcNAc transferase (GlcNAcT, EC.2.4.1.102), which is involved in the biosynthesis of O-glycans,[3] was cloned by Bierhuizen and Fukuda[4] and has recently been expressed in pilot scale.[5] However, the use of this enzyme has been limited by the availability of the core 1 acceptor substrate [Gal(β1-3)GalNAc(α1-OR)]. On the one hand, the appropriate β-1,3-galactosyltransferase for its synthesis is only available from natural sources[6-8] since its cDNA has not been cloned yet. On the other hand, the formation of the (β1-3) linkage by a β-galactosidase suffers from poor regioselectivity and insufficient yields, which rarely exceed 30%.[9,10] A recently cloned β-galactosidase obtained from *B. circulans* was employed in a transgalactosylation reaction yielding 68% with regard to the donor substrate.[11] In order to overcome product hydrolysis, the combined use of β-galactosidases and sialyltransferases in multi enzyme systems has been reported,[12,13] but the acceptor was generally used in large excess and yields could not be raised over 50%. These methods therefore proved inappropriate for processing high-priced acceptor substrates such as glycopeptides.

The goal of our work was the development of an enzymatic reaction system for the production of O-glycan core structures in high yields, which can be used as building blocks for Sialyl Lewis$^x$ structures. We chose GalNAc(α1-OBn) (**1**) as a model compound for serine/threonine linked glycans. Here, we present a one pot synthesis of GlcNAc(β1–6)GalNAc(α1-OBn) (core 6-Bn, **4**) by the combined use of β-galactosidase from bovine testes (EC.3.2.1.23) and recombinant Gal(β1-3)GalNAc β-1,6-GlcNAc transferase. Figure 1 shows the simplified reaction scheme.

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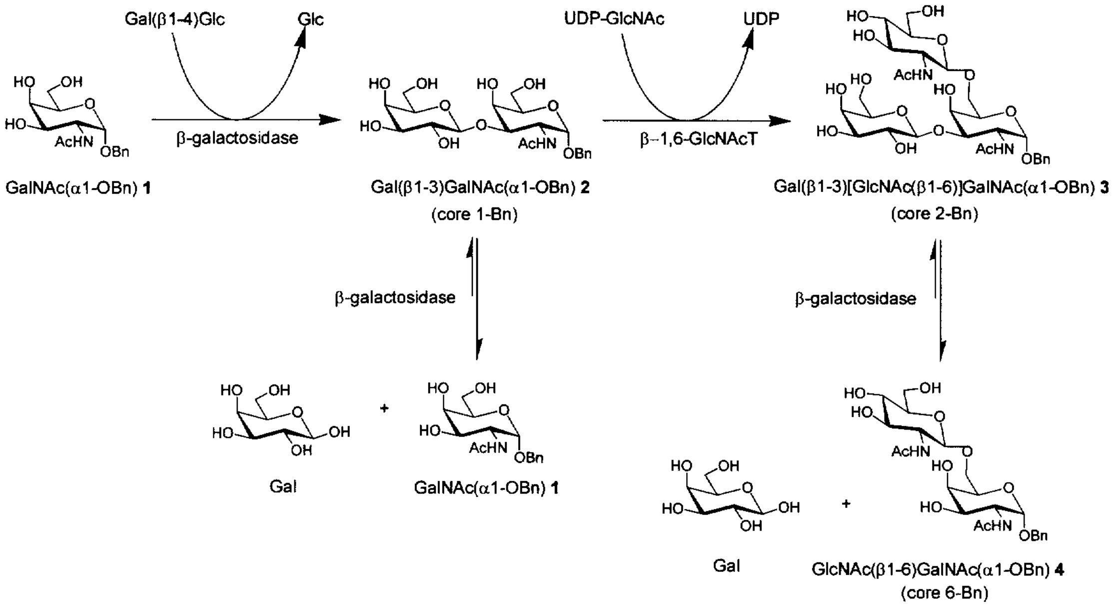

**Figure 1.** Simplified reaction scheme of the synthesis of core 6-Bn (**4**).

The intermediate Gal(β1-3)GalNAc(α1-OBn) (core 1-Bn, **2**) is formed by transgalactosylation from lactose as a donor to GalNAc(α1-OBn) (**1**). Gal(β1-3)[GlcNAc(β1-6)]GalNAc(α1-OBn) (core 2-Bn, **3**), the product of the consecutive reaction, is readily hydrolysed again by the galactosidase thus forming the product GlcNAc(β1-6)GalNAc(α1-OBn) (core 6-Bn, **4**). Because of the significant difference of pH optima of the employed enzymes – pH 4.3 for the galactosidase[14] and a broad maximum at pH 7 for the GlcNAcT – optimisation regarding activity and stability of the enzymes was carried out. Measurements at pH 5 and 6 showed that a two fold increase in galactosidase stability at pH 6 exceeds the loss of activity (40%). At pH 6, the use of MES buffer, which is applied for GlcNAcT assays, increases the galactosidase activity 1.4 fold in comparison to the use of citrate buffer, which is the standard buffer for this enzyme. Under the chosen conditions, GlcNAcT does not show reduced stability or activity. Calf intestine alkaline phosphatase (EC.3.1.3.1) was added as a third enzyme in order to overcome GlcNAcT inhibition by UDP.[15]

After optimisation of the reaction system and a kinetic characterisation of the enzymes, which will be published elsewhere, reactions with different ratios of galactosidase to GlcNAcT were carried out. In order to obtain an economic process, care was taken to keep enzyme consumption low. Figure 2 shows the best result with a total yield (**3** + **4**) of >90% after 6 days.[16] A ten fold dilution and incubation with additional galactosidase led to complete hydrolysis of the trisaccharide **3** to the desired product **4**.

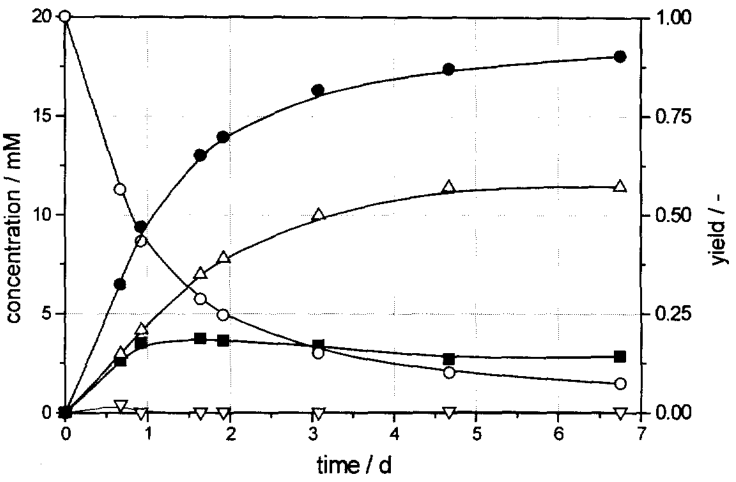

**Figure 2.** Course of reaction of the one pot synthesis of core 6-Bn (**4**).
●yield (**3** + **4**), △core 6-Bn (**4**), ■core 2-Bn (**3**), ▽core1-Bn (**2**), ○GalNAc-OBn (**1**).

For structural analysis, a synthesis with a reaction volume of 2 mL and a doubled specific activity of GlcNAcT was carried out and stopped after 60 hours in order to yield a maximum amount of the by-product **3**. 3.4 mg of core 2-Bn (**3**) and 4.8 mg of core 6-Bn (**4**) were purified on preparative HPLC[17] and characterised by NMR spectroscopy and mass spectrometry.[18,19]

In summary, we have developed a method that enables us to use a β-1,6-GlcNAcT for the synthesis of O-glycosidic core structures. As far as we know, this is the first example employing a glycosidase trans-glycosylation reaction that leads to an almost quantitative yield. Thus, we have shown that the severe limitations of glycosidases as synthetic enzymes concerning regioselectivity and conversion can be overcome by combined use with a glycosyltransferase and accurate optimisation of the reaction system. In ongoing studies, we will use the reaction system for the enzymatic synthesis of O-glycosylated peptides.

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## References and notes

1. A. Varki. *Glycobiology* **1993**, *3*, 97-130.

2. M. Fukuda. *Bioorg. Med. Chem.* **1995**, *3*, 207-215.
3. I. Brockhausen. Biosynthesis of O-glycans of the *N*-Acetylgalactosamine-α-Ser/Thr linkage type. In *Glycoproteins;* J. Montreuil, J.F.G. Vliegenthart, H. Schachter Eds.; Elsevier Science: Amsterdam, **1995**; Vol. 29a; pp. 201-260.
4. M.F.A. Bierhuizen, M. Fukuda. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 9326-9330.
5. S. Zeng, A. Dinter, D. Eisenkrätzer, M. Biselli, C. Wandrey, E.G. Berger. *Biochem. Biophys. Res. Commun.* **1997**, *237*, 653-658.
6. J. Mendicino, S. Sivakami, M. Davila, E.V. Chandrasekaran. *J. Biol. Chem.* **1982**, *257*, 3987-3994.
7. K. Furukawa, S. Roth. *Biochem. J.* **1985**, *227*, 573-582.
8. I. Brockhausen, G. Moller, A. Pollex-Krüger, V. Rutz, H. Paulsen, K.L. Matta. *Biochem. Cell. Biol.* **1992**, *70*, 99-108.
9. L. Hedbys, E. Johansson, K. Mosbach, P.-O. Larsson. *Carbohydr. Res.* **1989**, *186*, 217-223.
10. A. Fernández-Mayoralas. *Topics Curr. Chem.* **1997**, *186*, 1-21.
11. K. Suzuki, H. Fujimoto, Y. Ito, T. Sasaki, K. Ajisaka. *Tetrahedron Lett.* **1997**, *38*, 1211-1214.
12. G.F. Herrmann, Y. Ichikawa, C. Wandrey, F.C.A. Gaeta, J.C. Paulson, C.-H. Wong. *Tetrahedron Lett.* **1993**, *34*, 3091-3094.
13. V. Kren, J. Thiem. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 893-895.
14. J.J. Distler, G.W. Jourdian. *J. Biol. Chem.* **1973**, *248*, 6772-6780.
15. C. Unverzagt, H. Kunz, J.C. Paulson. *J. Am. Chem. Soc.* **1990**, *112*, 9308-9309.
16. Reaction conditions: 200 μL MES buffer (50 mM, pH 6), 0.5 M lactose, 20 mM GalNAc(α1-OBn), 25 mM UDP-GlcNAc, 10 mM $MgCl_2$, 0.5 mg/mL BSA, 10 U/mL calf intestine alkaline phosphatase, 250 mU/mL β-galactosidase, 50 mU/mL β-1,6-GlcNAcT, 37°C. HPLC analysis: HPX-87 cation-exchange column (300 mm, Biorad), 6 mM $H_2SO_4$.
17. Reversed phase column (ODS Hypersil 10 μm, Gyncotek), eluent: 12% aqueous acetonitrile.
18. Product **3**: FAB-MS (positive ion mode, glycerol) *m/z* 699.2 (M+Na), 677.2 (M+H), 569.2 (M+H – BnOH), 515.1 (GlcNAc-GalNAc-OBn+H), 474.2 (Gal-GalNAc-OBn+H), 407.1 (515.1 – BnOH), 312.1 (GalNAc-OBn+H), 204.1 (M+H – Gal-GalNAc-OBn). $^1H$ NMR ($D_2O$, 500 MHz, 300K, 2D TOCSY, referenced to internal acetate δ 1.908), A = GalNAc, B = GlcNAc, C = Gal, δ 7.429 (m, 5H, $C_6H_5$), 4.973 (d, $^3J_{1,2}$ 3.5 Hz, A-1), 4.706 and 4.500 (2d, 2H, $^3J$ 11.5 Hz, $CH_2C_6H_5$), 4.543 (d, $^3J_{1,2}$ 8.5 Hz, B-1), 4.430 (d, $^3J_{1,2}$ 7.5 Hz, C-1), 4.313 (A-2), 4.226 (A-4), 4.148 (A-5), 4.070 (A-6b), 4.015 (A-3), 3.930 (B-6b), 3.893 (C-4), 3.746 (B-2), 3.730 (B-6a), 3.704 (A-6a), 3.630 (C-5), 3.601 (C-3), 3.539 (B-3), 3.499 (C-2), 3.474 (B-5), 3.447 (B-4), 1.964 (s, B-NAc), 1.959 (s, A-NAc). C-6a and C-6b not determined.

    Product **4**: FAB-MS (positive ion mode, glycerol) *m/z* 537.1 (M+Na), 515.1 (M+H), 407.1 (M+H – BnOH), 312.1 (GalNAc-OBn+H), 204.1 (M+H – GalNAc-OBn). $^1H$ NMR ($D_2O$, 500 MHz, 300K, 2D TOCSY, referenced to internal acetate δ 1.908), A = GalNAc, B = GlcNAc, δ 7.440 (m, 5H, $C_6H_5$), 4.973 (d, $^3J_{1,2}$ 3.8 Hz, A-1), 4.692 and 4.508 (2d, 2H, $^3J$ 11.5 Hz, $CH_2C_6H_5$), 4.518 (d, $^3J_{1,2}$ 8.5 Hz, B-1), 4.124 (A-2), 4.081 (A-5), 4.045 (A-6b), 3.959 (A-4), 3.934 (B-6b), 3.893 (A-3), 3.741 (B-6a), 3.733 (B-2), 3.703 (A-6a), 3.542 (B-3), 3.478 (B-5), 3.448 (B-4), 1.972 (s, A-NAc), 1.968 (s, B-NAc).
19. A. Pollex-Krüger, B. Meyer, R. Stuike-Prill, V. Sinnwell, K.L. Matta, I. Brockhausen. *Glycoconjugate J.* **1993**, *10*, 365-380.