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Enzymatic Synthesis of Tumor-Associated Carbohydrate Antigen Globo-H Hexasaccharide

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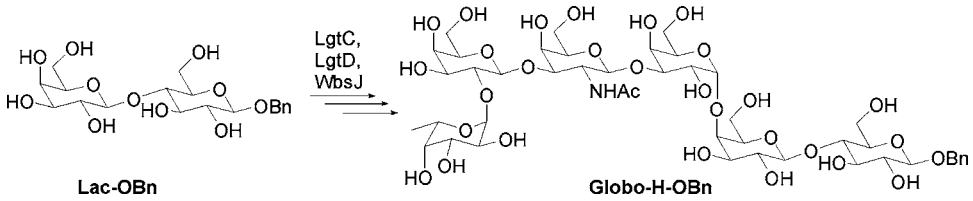
ABSTRACT



We report the enzymatic synthesis of an important tumor-associated carbohydrate antigen, Globo-H hexasaccharide. Starting with Lac-OBn as the initial acceptor, this approach employs three glycosyltransferases: LgtC, an α1,4-galactosyltransferase; LgtD, a bifunctional *β*1,3-galactosyl/*β*1,3-*N*-acetylgalactosaminyltransferase; and WbsJ, an α1,2-fucosyltransferase. In addition, two epimerases, GalE and WbgU, were also employed for the generation of more expensive sugar nucleotides, UDP-Gal and UDP-GalNAc, from their corresponding inexpensive C4 epimers. This study represents a facile enzymatic synthesis of the Globo-H antigen.

Hexasaccharide of Globo-H (Figure 1), a cell surface glycosphingolipid, is a member of the globo series of

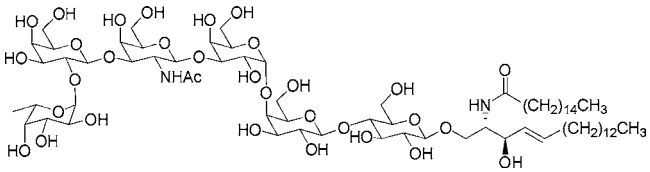


Figure 1. Structure of cell surface glycosphingolipid Globo-H.

antigenic carbohydrates. This carbohydrate antigen is found to be highly expressed on many types of human cancer cell lines, including breast, colon, lung, ovarian and prostate cancers.[1] Globo-H hexasaccharide was initially identified as a breast cancer antigen in 1983 from human MCF-7 breast cancer cells using the monoclonal antibody MBr1.[2] High levels of antibodies against the Globo-H epitope were found in the serum of breast cancer patients. As a distinct tumor-associated antigen marker, this epitope has been emerging as an important sequence for the development of anticancer vaccines.[3] Globo-H has been conjugated to an immunogenic protein carrier, keyhole limpet hemocyanin (KLH).[4] This Globo-H−KLH construct has shown promising results in clinical trials as anti-breast and anti-prostate cancer vaccines.[5]

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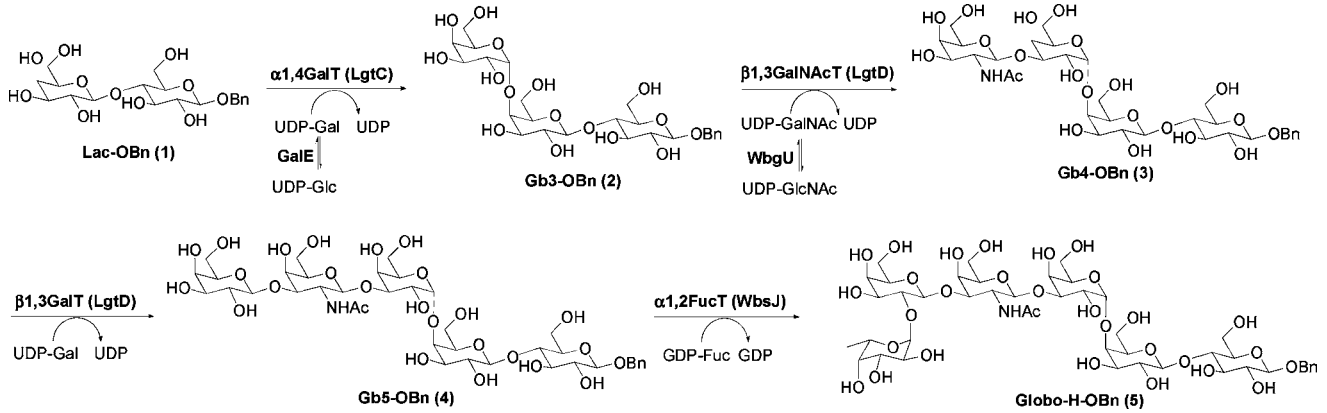
Scheme 1. Enzymatic Synthesis of Globo-H-OBn (**5**) from Lac-OBn (**1**) via Four Steps



Due to the difficulty of isolating such oligosaccharides from natural sources, synthetic approaches represent the only feasible method to obtain homogeneous Globo-H antigen. Danishefsky et al. reported the first total chemical synthesis of the Globo-H hexasaccharide in 1996 using the glycal strategy[6] with later refinements.[7] Many other chemical methodologies have been explored, such as the trichloroacetimidate method,[8] two-directional glycosylation,[9] one-pot strategies,[10] linear synthesis[11] and automated solid-phase assembly.[12] However, chemical synthesis often requires multiple protection/deprotection steps and suffers from poor regio-/stereoselectivity and relatively lower yields. Efficient synthesis of the Globo-H hexasaccharide in large quantities is still in demand for evaluation of these antitumor vaccines. Here, we report an alternative synthesis of Globo-H hexasaccharide using enzymes. It has been shown in literature that enzymatic coupling has several advantages over its chemical counterpart.[13] Enzymatic glycosylation offers high stereo- and regioselectivity under neutral aqueous conditions without functional group protection and higher yields. Furthermore, many enzymatic syntheses of important oligosaccharide antigens have been reported.[14]

In this approach (Scheme 1), we used three glycosyltransferases (LgtC, LgtD, and WbsJ) to synthesize the glycosidic linkages. In addition, two other enzymes (GalE and WbgU) were employed in the glycosylation reactions to convert inexpensive donor substrates, uridine 5′-diphosphoglucose (UDP-Glc) and uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), to expensive donors, uridine 5′-diphosphogalactose (UDP-Gal) and uridine 5′-diphospho-*N*-acetylgalactosamine (UDP-GalNAc), respectively.

Starting from readily available acceptor 1-benzyl-lactose (Lac-OBn) **1**, globotriose (Gb3-OBn) **2** was synthesized with an α1,4-galactosyltransferase (LgtC from *Neisseria meningitidis*) (Scheme 1). This enzyme has been well characterized. LgtC transfers the galactose residue from UDP-Gal onto a variety of acceptors.[15] With UDP-Gal as a sugar nucleotide

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donor, the reaction yield was 95%. In another reaction, we coupled the UDP-Glc C4 epimerase (GalE from *Escherichia coli* K12) with LgtC and used UDP-Glc as the sugar nucleotide donor. GalE has been widely employed in oligosaccharide synthesis for in situ cofactor regeneration to avoid both the stoichiometric use of expensive UDP-Gal and product inhibition.[16] The yield of this coupled reaction is 90% (Table 1), which proves GalE effective in production of Gb3-OBn.

Table 1. Yields of Each Enzyme-Catalyzed Glycosylation Step

step	enzyme(s)	product	yield (%)
1	GalE, LgtC	Gb3-OBn	90
2	LgtD-WbgU	Gb4-OBn	78
3	LgtD	Gb5-OBn	85
4	WbsJ	Globo-H-OBn	95

The subsequent step from Gb3-OBn to globotetraose (Gb4-OBn) **3** was catalyzed by a β1,3-*N*-acetylgalactosaminyltransferase (LgtD from *Haemophilus influenza*). We have previously reported the overexpression and biochemical characterization of LgtD.[17] Furthermore, we have also reported the characterization of UDP-*N*-acetylglucosamine C4 epimerase (WbgU from *Plesiomonas shigelloides*).[18] The LgtD−WbgU fusion protein was constructed and used in coupled enzymatic reactions to synthesize a variety of globotetraose and isoglobotetraose derivatives from corresponding lactoside acceptors.[19] Here, we used this fusion protein to synthesize Gb4-OBn by epimerization of UDP-GlcNAc to UDP-GalNAc by WbgU and subsequent transfer of the GalNAc residue from UDP-GalNAc onto Gb3-OBn by LgtD with 78% yield.

Synthesis of globopentaose (Gb5-OBn) **4** was also achieved by using LgtD. Randriantsoa et al. reported a novel β1,3-galactosyltransferase activity of LgtD.[20] They demonstrated that in the presence of globotriose, LgtD prefers UDP-GalNAc as the donor, and acts as a GalNAc transferase. On the other hand, donor specificity was changed to prefer UDP-Gal in the presence of globotetraose, and LgtD acts as a Gal transferase, resulting in the production of globopentaose. With purified LgtD and UDP-Gal, we also demonstrated the formation of Gb5-OBn with 85% yield.[21] Another possible candidate that has gathered much interest for the synthesis of globopentaose is the β1,3-galactosyltransferase CgtB from *Campylobacter jejuni*. Bernatchez et al.[22] reported the substrate specificities of CgtB from three *C. jejuni* strains. The activity of CgtB from strain HS:10 with a GA2 ganglioside mimic was significantly higher when compared to sialylated or monosaccharide acceptors. GA2 and globotetraose both have terminal GalNAc residues. However, the GalNAcβ1,4Gal linkage of GA2 differs from the GalNAcβ1,3Gal linkage in globotetraose.

The final step of enzymatic transformation is the addition of a fucose residue from guanosine 5-diphosphofucose (GDP-Fuc) via an α1,2- linkage to the galactose residue of Gb5-OBn by an α1,2-fucosyltransferase (WbsJ from *Escherichia coli* O128:B12). WbsJ is involved in the synthesis of the *E. coli* O128 O-antigen.[23] This enzyme was cloned and biochemically characterized in our group.[24] WbsJ has relaxed substrate specificity toward Galβ1,4Fru (lactulose), Galβ1,4Glc (lactose), Galβ1,3GalNAc (T antigen) and Galβ1,4Man. The broad acceptor specificity of WbsJ reveals its potential application in the synthesis of important fucosylated glycoconjugates. WbsJ was cloned and transformed into *E. coli* BL21(DE3) and expressed as a GST-fusion protein. Using purified WbsJ protein, we completed the transfer of a fucose residue to Gb5-OBn with 95% yield. The structure of Globo-H-OBn **5** was analyzed by ^{1}H NMR, ^{13}C NMR, H−H COSY, HMQC and HRMS (see Supporting Information).[25] It was also confirmed by comparing with the reported hexasaccharides.

In this study, we report the enzymatic synthetic route to obtain Globo-H hexasaccharide with Lac-OBn as the starting material. The whole synthetic route contains three glycosyltransferases and two epimerases with an overall yield of 57%. In views of high stereo- and regioselectivity under mild

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(21) Spectroscopic data for Gb5-OBn: ^{1}H NMR (500 MHz, D_2O): δ 7.47−7.38 (m, 5H, Ph), 4.91 (d, J = 11.7 Hz, 1H, $PhCH_2$), 4.88 (d, J = 3.8 Hz, H-1‴), 4.74 (d, J = 11.8 Hz, 1H, $PhCH_2$), 4.66 (d, J = 8.6 Hz, 1H, H-1), 4.52 (d, J = 8.1 Hz, 1H, H-1), 4.48 (d, J = 7.8 Hz, 1H, H-1), 4.42 (d, J = 7.7 Hz, 1H, H-1), 4.35 (t, J = 6.3 Hz, 1H), 4.22 (d, J = 1.7 Hz, 1H), 4.15 (d, J = 2.7 Hz, 1H), 4.03 (m, 1H), 4.01 (m, 1H), 3.97 (dd, J = 12.5, 1.6 Hz, 1H), 3.94 (dd, J = 10.1, 2.9 Hz, 1H), 3.91−3.85 (m, 4H), 3.82 (d, J = 4.4 Hz, 1H), 3.79 (d, J = 4.4 Hz, 1H), 3.78−3.69 (m, 6H), 3.68−3.65 (m, 3H), 3.64−3.61 (m, 2H), 3.60−3.53 (m, 4H), 3.50 (dd, J = 9.9, 7.9 Hz, 1H), 3.32 (t, J = 8.6 Hz, 1H), 1.99 (s, 3H, CH_3CONH); ^{13}C NMR (125 MHz, D_2O): δ 175.2, 136.6, 128.8, 128.76, 128.5, 104.8, 103.3, 103.0, 101.0, 100.4, 79.6, 78.8, 78.7, 77.3, 75.5, 75.0, 74.9, 74.6, 73.0, 72.5, 72.2, 71.5, 70.9, 70.6, 70.3, 69.0, 68.6, 68.0, 67.6, 61.4, 61.0, 60.98, 60.4, 60.3, 60.1, 59.4, 51.5, 22.3; HRMS calcd for $C_{39}H_{61}NO_{26}Na$ ($[M + Na]^+$) 982.3380, found 982.3405.

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(25) Spectroscopic data for Globo-H-OBn: ^{1}H NMR (500 MHz, D_2O): δ 7.46−7.38 (m, 5H, Ph), 5.20 (d, J = 4.1 Hz, 1H, H-1⁗), 4.91 (d, J = 11.7 Hz, 1H, $PhCH_2$), 4.86 (d, J = 3.9 Hz, 1H, H-1‴), 4.74 (d, J = 11.6 Hz, 1H, $PhCH_2$), 4.59 (d, J = 7.7 Hz, 1H, H-1), 4.53 (d, J = 7.4 Hz, 1H, H-1), 4.51 (d, J = 6.7 Hz, 1H, H-1), 4.48 (d, J = 7.7 Hz, 1H, H-1), 4.35 (t, J = 6.4 Hz, 1H), 4.22−4.18 (m, 2H), 4.07 (d, J = 2.1 Hz, 1H), 4.00 (d, J = 3.0 Hz, 1H), 3.98−3.94 (m, 3H), 3.91 (dd, J = 10.6, 2.8 Hz, 1H), 3.88−3.84 (m, 3H), 3.83−3.79 (m, 4H), 3.77−3.72 (m, 7H), 3.70−3.65 (m, 5H), 3.64−3.60 (m, 5H), 3.58−3.53 (m, 3H), 3.33 (t, J = 8.6 Hz, 1H), 2.01 (s, 3H, CH_3CONH), 1.19 (d, J = 6.6 Hz, 3H, CH_3 of fucose); ^{13}C NMR-DEPT (125 MHz, D_2O): δ 128.8, 128.77, 128.5, 104.0, 103.3, 102.1, 101.0, 100.5, 99.3, 78.9, 78.4, 77.2, 76.4, 76.2, 75.5, 75.1, 74.9, 74.7, 74.6, 73.6, 73.0, 72.7, 71.9, 71.5, 70.9, 70.7, 70.2, 69.6, 69.2, 69.17, 68.5, 68.1, 67.9, 66.8, 61.0, 61.00, 60.4, 60.37, 60.1, 51.7, 22.3, 15.4; HRMS calcd for $C_{45}H_{71}NO_{30}Na$ ($[M + Na]^+$) 1128.3959, found 1128.3981.

conditions without protecting group manipulation, our study paves a way for large-scale enzymatic synthesis of the Globo-H antigen. Further studies will be geared toward conjugation of the hexasaccharide to carrier proteins, lipids, polymers, and nanoparticles for development of anti-cancer vaccines.

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Supporting Information Available: Full experimental details as well as spectra for Gb5-OBn and Globo-H-OBn. This material is available free of charge via the Internet at http://pubs.acs.org.

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