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# Large scale enzymatic synthesis of oligosaccharides and a novel purification process

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ABSTRACT

Herein we report the practical chemo enzymatic synthesis of trisaccharide and derivatives of iGb3 and Gb3, and a novel purification process using immobilized yeast to remove the monosaccharide from the reaction mixture. High purity oligosaccharide compounds were achieved in large scale. This study represents a facile enzymatic synthesis of and novel purification process of oligosaccharide.



Isoglobotrihexosylceramide (iGb3) was regarded as primary endogenous agonist ligand for invariant natural killer T(*i*NKT) cells, because iGb3 stimulates both human VR24 NKT cell and mouse VR14 NKT cells.[1] *i*NKT cells regulate a variety of microbial, allergic, autoimmune, and tumor conditions, through the rapid secretion of interleukin-4 (IL-4), interferon-γ (INF-γ), and other cytokines and chemokines.[2] Such cells specifically recognize microbial and endogenous glycolipid antigens in a CD1d-dependent way.[3,4] In contrast, globotrihexosylceramide (Gb3) with a terminal Gal-α-(1 → 4)Gal instead of Gal-α-(1 → 3)Gal in iGb3 has no stimulatory ability toward *i*NKT cells.

It is also noticed that the hexasaccharide of Globo-H (a member of the globo series of antigenic carbohydrates) has the motif of Gal-α-(1 → 4)Gal-β(1 → 4)Glc. 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.[5–8] Therefore, there has been high demand for iGb3, Gb3, and Globo-H for immunology research and preclinical investigation. Figure 1 shows the structures of iGb3, Gb3, and Globo-H.

Current research on this field is hampered by the rather limited access to these compounds. Purification from natural sources not only requires unique biochemical and analytical apparatus, but also gives milligram quantities of the product.[9] Chemical syntheses of iGb3 and Gb3,[10] Globo-H[11] had been reported. Although the chemical synthesis of the lipid part has been well documented in recent years,[12,13] the chemical preparation of the oligosaccharide donors were tedious, because of selective protections, deprotections, and glycosylations in totally 17–19 steps. Generally, enzymatic glycosylation has several advantages over its chemical counterpart, being one of the most practical methods for oligosaccharide synthesis because of its high stereo- and regioselectivity under mild aqueous conditions.[14–16] Therefore, taking advantage of the high efficiency of enzymatic synthesis of oligosaccharides, we explored the enzymatic synthesis of these trisaccharides in large scale.

According to Leloir pathway,[17] application of glycosyltransferase to preparative synthesis, two factors must be considered: (1) availability of the enzymes; (2) availability of nucleoside phosphate sugars, which serve as activated sugar donors, normally are expensive from commercial source.

The most promising approach of enzymatic synthesis of trisaccharide of isoglobotrihexose and globotrihexose is directly using UDP-galactose (UDP-Gal) and lactose catalyzed by galactotransferase. However, commercially available UDP-Gal is too expensive to be used in large scale oligosaccharide synthesis. Multiple-enzyme sugar nucleotide regeneration systems had been developed to avoid using costly stoichiometric amounts of sugar nucleotides.[18,19] Dr. Wang had reported the enzymatic synthesis of globotrihexose and isoglobotrihexose using superbead or superbug techniques.[20,21] Nowadays, UTP can be used in stoichiometric amounts to generate UDP-Gal because of its commercially low price. Considering the Gal-1-phosphate is much more expensive than Glc-1-phosphate, a two enzymes UDP-Gal generation strategy was used to produce

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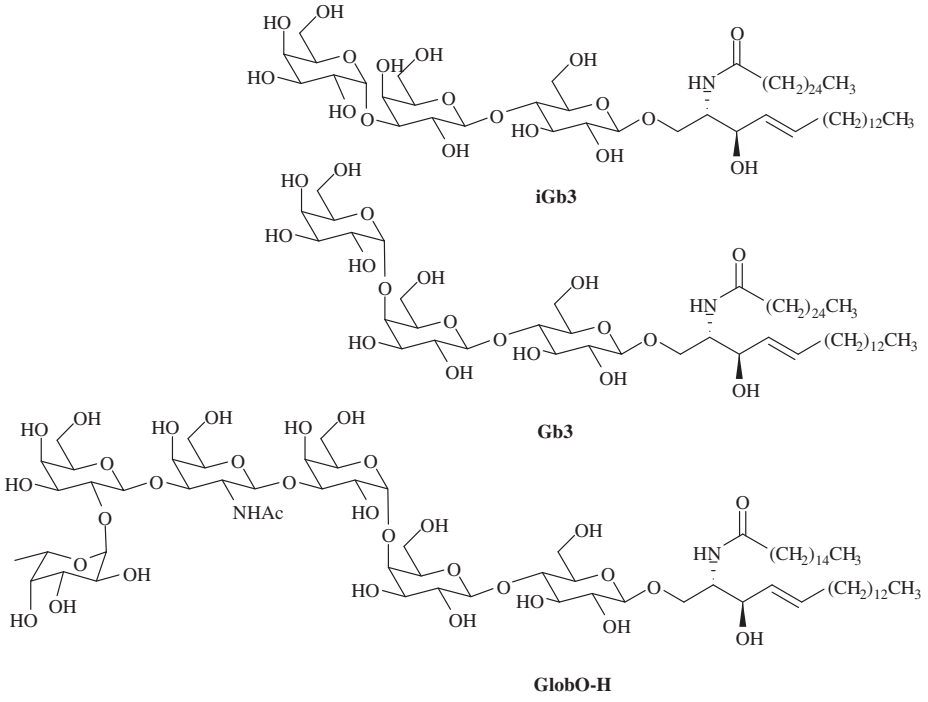
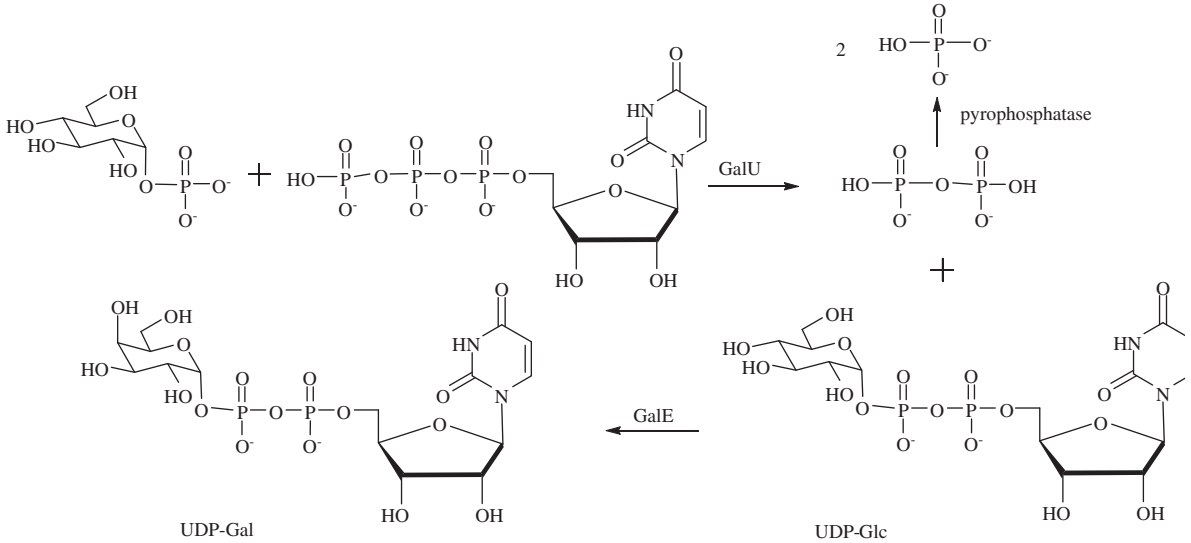



**Figure 1.** Structures of iGb3,Gb3, and Globo-H.

UDP-Gal in large scale.[22] Scheme 1 shows the strategy to prepare UDP-Gal in large scale.

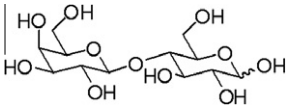
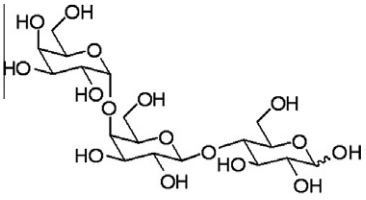
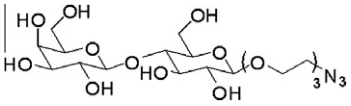
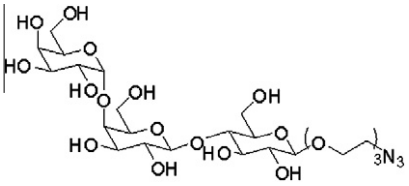
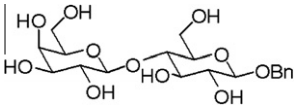
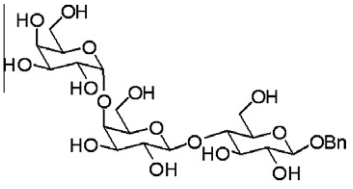
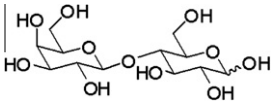
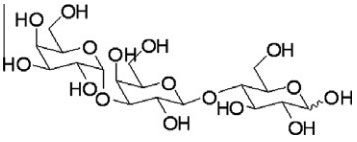
Briefly, glucosyl-1-phosphate reacted with UTP catalyzed by GalU (E.C.2.7.7.9) to yield UDP-Glc, pyrophosphatase here was used to decomposed the PPi into Pi to push the first step of this reaction forward. Then UDP-Glc was converted to UDP-Gal by epimerase, GalE (E.C.5.1.3.2).[23] Two kinds of galactotransferases expressed in *Escherichia coli* BL21(DE3) were employed in the following reactions. The activities of the enzymes were assayed according to previously published protocols.[24,25] One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the conversion of 1 μm of substrate per minute at 37 °C.[26] For the synthesis of isoglobotrihexose, α(1 → 3) galactotransferase was used; and for globotrihexose synthesis, α-(1 → 4) galactotransferase (LgtC) was used. LgtC could transfer the galactose residue from UDP-Gal onto a variety of acceptors. Two lactose derivatives (synthesized in our lab) with linkers at the reducing end were used as acceptor. Synthesized compound Gal-α-(1 → 4)Gal-β(1 → 4)Glc-1-OBn was used as precursor for Globo-H synthesis, compound Gal-α-(1 → 4)Gal-β(1 → 4)Glc-1-$(OC_2H_4)_3$-$N_3$ was used as array of glyconanoparcels study.

In order to utilize UDP-Glc sufficiently, two times acceptor was added into the reaction mixture. For example, the enzymatic synthesis reaction of compound 2 was set up with 100 mM lactose, 50 mM UDP-Glc, 10 mM $MnCl_2$, 10 U GalE, 10 U GalT in Tris–HCl buffer (100 mM, pH 7.0, 100 mL) in 200 mL flask. The reaction mixture was kept at 37 °C under argon atmosphere for 2 days. After the UDP-Glc was used up as monitored by TLC (thin layer chromatography) (isopropanol/$H_2O$/$NH_4OH$ = 7/3/2 (v/v/v)), the mixture was




**Scheme 1.** Strategy to prepare UDP-Gal.

**Table 1**
The oligosaccharides synthesized

| Entry | Acceptor | Enzyme/reaction volume | Product | Qty. | Yield (%) |
|---|---|---|---|---|---|
| 1 | Glc-1-P, UTP | 50 U GalU/500 mL × 5 | UDP-GLC | 25 g | 70[a] |
| 2 |  | 10 U GalE<br>10 U LgtC/100 mL |  | 2.2 g | 90[b] |
| 3 |  | 1 U GalE<br>1 U LgtC/7.5 mL |  | 0.2 g | 81[b] |
| 4 |  | 45 U GalE<br>45 U LgtC/450 mL |  | 10.2 g | 76[b] |
| 5 |  | 30 U GalE<br>30 Uα(1 → 3) galactotransferase/300 mL<br>LgtC/100 mL |  | 5.0 g | 66[b] |

[a] Yield was based on Glc-1-P.
[b] Yield based on UDP-Glc.

worked up. The protein was removed by centrifugation after the mixture was heated at 100 °C for 10 min, then the solution was passed through Dowex Cl anion-exchange resin, only acceptor and trisaccharide were remaining inside the filtration. The following trisaccharides in Table1 were prepared according to the same method.

Purification of oligosaccharides mixtures was conventionally proceeded by chromatography using activated charcoal and ion-exchange resins or Bio-Gel P-Gels column, actually these procedures were not only complicated, but also could hardly separate the remaining lactose from the filtration, especially in large scale. It was worthy noted that an efficient way of producing high-purity oligosaccharide was carried out by immobilized yeast cells (*Saccharomyces cereviside* L1) under fermentation condition. This yeast cells consumed only monosaccharide, such as glucose and galactose.[27] Inspired by this new technology, a novel purification strategy was developed: using β-galactosidase to digest lactose into glucose and galactose, then using this immobilized yeast to consume the monosaccharide, so there will be only trisaccharide remaining in the solution. The *S. cereviside* L1 was cultured in medium containing 10 g malt extract in 1000 mL water (pH 6.4) at 30 °C. Cells were harvested at log phase growth by centrifugation at 10,000 rpm for 20 min and were immobilized in calcium alginate. Five grams crude cells were mixed with 10 mL sodium alginate solution (4%) and then extruded into a 3.0% $CaCl_2$ solution to form beads at 4 °C. The beads were washed with distilled water until no $Ca^{2+}$ was detected in the wash. The syrup of the oligosaccharide mixture was fermented with 2 g immobilized cells. Batch reactions were performed in an incubator shaker at 100 rpm at 30 °C. The reaction was monitored by TLC. TLC indicated that only trisaccharide and protected monosaccharide were inside the filtration after all natural monosaccharide was consumed. Since the yeast cells can only digest natural monosaccharide, the monosaccharide protected at the reduced end of the entries 3 and 4 had to be removed by Bio-Gel P-2 gel (Bio-Rad) filtration to achieve purified trisaccharide with water as the mobile phase. The desired fractions were pooled and lyophilized to provide the target compound. Table 1 shows the oligosaccharides synthesized and purified through this novel process.[28]

In this study, we report the practical enzymatic synthetic route to obtain trisaccharide and its conjugates in large-scale, and a novel efficient method using β-galactosidase and immobilized yeast to remove lactose and monosaccharides. Further studies will be carried out by using this process to synthesize and purify more oligoasaccharides for research.

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## References and notes


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28. Compound 3: white solid; $^{1}$H NMR (500 MHz, $D_2O$) $\delta$ 3.29–3.32 (m, 1H), 3.46–3.48 (m, 2H), 3.52–3.56 (m, 2H), 3.60–3.63 (2H), 3.66–3.76 (m, 12H), 3.77–3.81 (m, 4H), 3.86–3.91 (m, 2H), 3.94–4.03 (m, 4H), 4.31 (t, $J$ = 6.25 Hz, 1H), 4.49 (t, $J$ = 7.5 Hz, 2H), 4.91 (t, $J$ = 3.3, 1H). $^{13}$C NMR (125 MHz, $D_2O$) $\delta$ 50.2, 60.1, 60.4, 60.6, 68.6, 68.8, 69.0, 69.2, 69.22, 69.5, 69.6, 69.7, 70.9, 71.0, 72.2, 72.9, 74.4, 74.8, 75.4, 77.4, 78.7, 100.4, 102.1, 103.3 ppm. HRMS: calcd for $C_{24}H_{43}N_3O_{18}Na^+$ (M+Na$^+$) 684.2439, found 684.2439.