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Effective Sugar Nucleotide Regeneration for the Large-Scale Enzymatic Synthesis of Globo H and SSEA4

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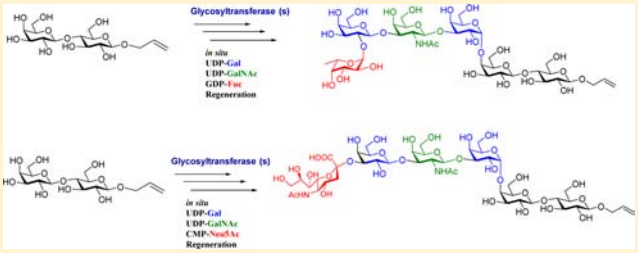
Supporting Information

ABSTRACT: We report here the development of chemoenzymatic methods for the large-scale synthesis of cancer-associated antigens globopentaose (Gb5), fucosyl-Gb5 (Globo H), and sialyl-Gb5 (SSEA4) by using overexpressed glycosyltransferases coupled with effective regeneration of sugar nucleotides, including UDP-Gal, UDP-GalNAc, GDP-Fuc, and CMP-Neu5Ac. The enzymes used in the synthesis were first identified from different species through comparative studies and then overexpressed in *E. coli* and isolated for synthesis. These methods provide multigram quantities of products in high yield with only two or three purification steps and are suitable for the evaluation and development of cancer vaccines and therapeutics.



INTRODUCTION

Glycosphingolipids play important roles in many physiological conditions, including development and differentiation, tumor progression, cell adhesion, and signal transduction. Among the glycosphingolipids, the globo series of gangliosides is strongly associated with malignant diseases such as breast, lung, ovary, stomach, and small-cell lung cancers but is not detectable in normal cells.[1−4] For example, Globo H was first discovered in 1983 from a cultured human teratocarcinoma cell line[5,6] and then from a variety of epithelial tumors such as colon, ovarian, gastric, pancreatic, endometrial, lung, prostate, and breast cancers by staining with the monoclonal antibodies MBr1 and VK9.[7−10] However, the biological function of this molecule is not clear, though its expression is associated with tumor aggressiveness in breast carcinoma, small-cell lung carcinoma, and several malignant cancers.[11,12] These studies have led to the development of a therapeutic cancer vaccine based on synthetic Globo H conjugated to keyhole limpet hemocyanin (KLH) and adjuvant QS-21 for the treatment of breast cancer and prostate cancer, and positive results were observed in phase I clinical trials.[13−15] This vaccine was further advanced to phase III clinical trials for breast cancer with improved synthesis of Globo H by the programmable one-pot method.[16] Our previous studies by flow cytometry also revealed that Globo H was overexpressed in ∼61% of breast cancer patients and Gb5 was overexpressed in ∼77.5% of patients[17] and glycosphingolipids Gb5, Globo H, and SSEA4 were found on breast cancer cells and the cancer stem cells but were not detectable on normal cells.[18] In addition, glycan array analysis demonstrated that higher levels of anti-Globo H antibody exist in the plasma of breast cancer patients.[19,20] Compared to the breast cancer target HER2 that was present in 20% of breast cancer patients,[21] the glycosphingolipid antigens (i.e., Gb5 and its derivatives, Globo H and SSEA4) were much more predominant and are thus better candidates for the development of cancer vaccines or therapeutic antibodies. To improve the vaccine efficacy with regard to its immune response, Globo H has been conjugated to different carrier proteins through different linkers and used in combination with different adjuvants in animal studies.[18] It was found that when Globo H was conjugated to CRM197 (diphtheria toxin) and used in combination with the glycolipid adjuvant C34, the vaccine elicited a significant IgG response to recognize not only Globo H but also Gb5 and SSEA4, as compared to the Globo H-KLH-QS21 vaccine that elicited a significant IgM antibody response with less selectivity toward tumor-associated glycans.[18]

Currently, the synthesis of complex carbohydrates for clinical study is available by chemical methods; however, it has been a challenge in clinical development because of the lack of methods available for large-scale synthesis.[22] The traditional chemical synthesis is tedious and labor-intensive because the complex protection, deprotection, and extensive purification steps are necessary to achieve high purity and structural integrity. The problem has stimulated the development of an enzymatic method.[23] Nevertheless, several methods have been

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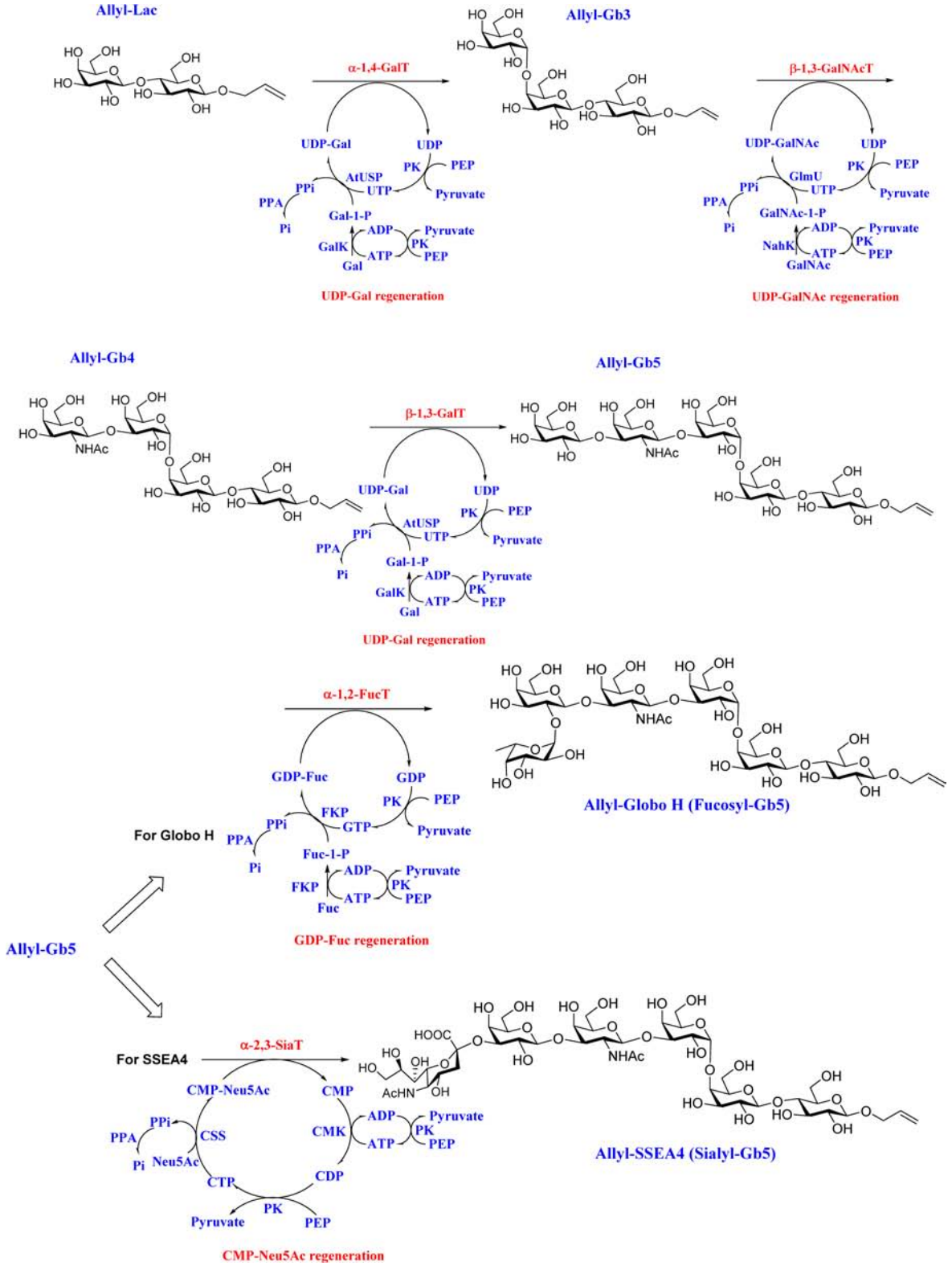

Figure 1. Enzymatic synthesis of (a) Globo H and (b) SSEA4 with sugar nucleotide regeneration.

developed to synthesize Globo H chemically, including the glycal[22,24] and Schmidt trichloroacetimidate strategies,[25] two-directional glycosylation methodology,[13] linear solution-phase synthesis,[26] automated solid-phase synthesis,[27] multicomponent one-pot synthesis,[28] total synthesis,[29] and programmable one-pot synthesis.[16,19] Of these methods, the programmable one-pot method was used in the phase II/III clinical development of the Globo H vaccine. However, only two methods have been reported for SSEA4 synthesis, including the methods of one-pot chemical synthesis (~30%)[30] and chemoenzymatic synthesis (~34%).[31]

The enzymatic synthesis of Globo H based on the Leloir pathway glycosyltransferases requires the use of only sugar nucleotides as donors, but the cofactors are too expensive to be

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Table 1. Enzymatic Reactions Used to Prepare Allyl-Gb3, Allyl-Gb4, Allyl-Gb5, Allyl-Globo H, and Allyl-SSEA4

reactor characteristics	allyl-Lac	allyl-Gb3	allyl-Gb4	allyl-Gb5 for Globo H	allyl-Gb5 for SSEA4
solution volume (mL)	200	220[a]	250[a]	150[a]	150[a]
quantity (mmol)	10	10	10	4.5	4.5
pH, temp (°C)	7.0, 25	7.0, 25	7.0, 25	7.0, 25	7.0, 25
reaction time (days)	2	4	10	4	3
Synthetic Enzyme					
	GalK	NahK	GalK	FKP	CMK
$TTN_{sugar\ kinase}$	5.6×10^{5b}	5.5×10^{5b}	5.6×10^{5b}	1.5×10^{5b}	2.2×10^{5b}
	AtUSP	GlmU	AtUSP	FKP	PmCSS
$TTN_{nucleotide\ pyrophosphorylase}$	8.9×10^{5b}	$8.5 \times 10^{3b,e}$	8.9×10^{5b}	1.5×10^{5b}	9.1×10^{4b}
	LgtC	LgtD	LgtD	FutC	JT-FAJ-16
$TTN_{glycosyltransferase}$	7.2×10^{5b}	2.1×10^{4b}	2.1×10^{4b}	3×10^{4b}	2.9×10^{5b}
Substrate (mmol)					
Gal	10		10		
GalNAc		10			
Fuc				5	
Neu5Ac					5
Cofactors (mmol)					
ATP, TTN_{ATP}	0.05, 200	0.05, 200[c]	0.05, 200	0.05, 200[c]	0.05, 200[c]
UTP, TTN_{UTP}	0.125, 80	0.125, 80[c]	0.125, 80		
GTP, TTN_{GTP}				0.5, 20[c]	
CTP, TTN_{CTP}					0.5, 20[c]
Isolated Products (mmol)					
	allyl-Gb3	allyl-Gb4	allyl-Gb4	allyl-Globo H	allyl-SSEA4
yield from allyl-lac (%)	99%	95%	94%	94%[d]	54%[d]

[a]These volumes are the initial solutions. Volumes at the end of the reactions were slightly increased because of the addition of reagents and enzymes with pH control. [b]TTN (total turnover number) is the moles of product generated per mole of enzyme (cofactor) originally present in the reaction solution. The turnover numbers are not corrected for residual enzyme (cofactor) activity remaining at the conclusion of the reaction. [c]These nucleoside triphosphates remain in the solution from a previous reaction. [d]Yield calculated from the fractional part of allyl-Gb4. [e]GlmU is less stable under low salt conditions in synthetic solution.

used as stoichiometric reagents for large-scale synthesis. Moreover, the byproduct nucleoside diphosphate or monophosphate released from the reaction inhibits the glycosyltransferase; therefore, regeneration of sugar nucleotide in situ is necessary to reduce the cost and eliminate the problem of product inhibition.[23,32,33] In 2008, an enzyme-based strategy was developed for the synthesis of Globo H.[34] However, the epimerase activity was relatively low and became the rate-limiting step in the process, making it unfeasible for the large-scale production of Globo H. Here, we report a new enzymatic strategy for the large-scale synthesis of Gb5, Globo H, and SSEA4 using overexpressed glycosyltransferases coupled with effective sugar nucleotide regeneration in situ (Figure 1).

RESULTS AND DISCUSSION

The selection of enzymes for glycosylation and cofactor regeneration is based on several criteria, including stability, specificity, specific activity, optimal pH, temperature, metal ion requirements, and their compatibility. In our initial study, we exploited a new route to producing UDP-Gal through Gal-1-phosphate as a means to regenerate UDP-Gal for the enzymatic incorporation of the galactose residue, a major component of glycosphingolipids, into an acceptor. The regeneration of UDP-Gal from UDP was first developed in 1982 via UDP-Glc epimerase to interconvert UDP-Glc and UDP-Gal.[23] Another method was based on the Glc-1-phosphate uridylyltransferase-catalyzed interchange of Gal-1-phosphate and UDP-Glc to Glc-1-phosphate and UDP-Gal.[35] In 2004, Kotake et al. discovered an enzyme from *Pea Sprouts*, called UDP-sugar pyrophosphorylase with broad substrate specificity toward monosaccharide-1-phosphate to form UDP-sugar.[36] Two years later, Kotake's and Somers' groups independently reported another enzyme, AtUSP, from *Arabidopsis* with similar function,[37,38] and the homologous enzymes were also found in parasites and bacteria such as *Leishmania, Trypanosoma*, and *Bifidobacterium*.[39,40] More recently, Chen et al. utilized the promiscuous UDP-sugar pyrophosphorylase (BLUSP) and galactokinase (GalK) to synthesize UDP-sugar analogs in a one-pot reaction with multienzymes.[39,41] After the initial analysis, we chose AtUSP to further evaluate its catalytic property to convert Gal-1-phosphate with UTP directly to UDP-Gal.

Large-Scale Synthesis of Allyl-Gb3. To develop a process for the large-scale synthesis of allyl-Globo H (multiple-gram scales), the allyl glycoside of the disaccharide lactose was chosen as the starting material. In a representative reaction, to a 180 mL solution containing allyl-lactose (3.82 g, 10 mmol) ATP (28 mg, 0.05 mmol), UTP (69 mg, 0.125 mmol), galactose (1.8 g, 10 mmol), phosphoenolpyruvate (4.55 g, 22 mmol, monopotassium salt), and $MgCl_2$ (final 10 mM) were added the enzymes galactokinase (GalK, 50 U), UDP-sugar pyrophosphorylase (AtUSP, 150 U), α1,4-galactosyltransferase[42] (LgtC, 100 U), pyruvate kinase (PK, 200 U), and inorganic pyrophosphatase (PPA, 200 U). The final volume of the mixture was adjusted to 200 mL, and the reaction was carried out at room temperature over the course of 2 days with the pH controlled at 7.0. The reaction was monitored by thin-layer chromatography (5:3:2 butanol/acetate/water). The turnover numbers of each enzyme and cofactor were measured

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Table 2. Kinetics Parameter of Enzymes in Allyl-Globo H and Allyl-SSEA4 Synthesis

type	species	acceptor	K_m (mM)	K_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	donor	K_m (mM)	K_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	product
A. Allyl-Gb3 Synthesis Enzymes										
GalK	*E. coli*	Gal	1.125	49.62	44.11	ATP	0.0454	14.85	327.45	Gal-1-P
AtUSP	*A. thaliana*	Gal-1-P	0.0067	0.5666	84.61	UTP	0.5302	0.006	0.011	UDP-Gal
LgtC	*N.meningitidis*	allyl-Lac	2.535	1.781	0.70	UDP-Gal	0.01715	0.1996	11.64	allyl-Gb3
B. Allyl-Gb4 Synthesis Enzymes										
NahK	*Bifidobacterium longum subsp. infantis*	GalNAc	0.3572	11.52	32.25	ATP	0.0047	0.4255	89.94	GalNAc-1-P
GlmU	*E. coli*	GalNAc-1-P	3.532	0.2260	0.064	UTP	2.061	0.2528	0.12	UDP-GalNAc
LgtD	*H.influenzae Rd*	allyl-Gb3	1.489	5.701	3.83	UDP-GalNAc	0.1031	1.868	18.12	allyl-Gb4
C. Allyl-Gb5 Synthesis Enzymes										
GalK	*E. coli*	Gal	1.125	49.62	44.11	ATP	0.0454	14.85	327.45	Gal-1-P
AtUSP	*A. thaliana*	Gal-1-P	0.0067	0.5666	84.61	UTP	0.5302	0.006	0.011	UDP-Gal
LgtD	*H.influenzae Rd*	allyl-Gb4	0.052	0.065	1.25	UDP-Gal	1.620	0.025	0.016	allyl-Gb5
D. Allyl-Globo H Synthesis Enzymes										
FKP	*B. fragilis*	Fuc	0.0485	7.809	160.91	ATP	3.330	12.03	3.61	Fuc-1-P
FKP	*B. fragilis*	Fuc-1-P	0.04244	0.8998	21.20	GTP	1.079	1.595	1.48	GDP-Fuc
FutC	*H. pylori*	allyl-Gb5	0.1211	1.069	8.83	GDP-Fuc	0.005467	0.9085	166.18	allyl-Globo H
E. Allyl-SSEA4 Synthesis Enzymes										
CMK	*E. coli*	CMP	0.061	248.5	4,074.44	ATP	0.0122	158.9	13,045.98	CDP
PmCSS	*P. multocida*	Neu5Ac	0.0042	0.1801	43.07	CTP	0.01291	0.2372	18.37	CMP-Neu5Ac
JT-FAJ-16	*V.sp.JT-FAJ-16*	allyl-Gb5	0.2286	44.04	192.65	CMP-Neu5Ac	0.05590	35.42	633.63	allyl-SSEA4

[a]All reaction were assayed at room temperature.

to evaluate the efficiency of the catalytic reaction (Table 1). In a nonoptimized process in allyl-Gb3 synthesis, the total turnover numbers (TTN = moles of product produced per mole of cofactor or enzyme present in the reaction solution) were UTP = 80, ATP = 200, GalK = 5.6×10^5, AtUSP = 8.9×10^5, and LgtC = 7.2×10^5 (Table 1). On the basis of the characteristics of individual enzymes in this reaction, all enzymes require magnesium ions; however, manganese ions would activate galactokinase and α-1,4-galactosyltransferase but inactivate UDP-sugar pyrophosphorylase. We also observed that when excess Gal was used, more than one galactose residue would be incorporated into the lactose derivative, resulting in a reduced yield of the product and the necessity of an additional purification process. However, when equal equivalents of Gal and allyl-Lac were utilized, this complication could be avoided. In the fermentation method reported by Samain et al., a similar problem was observed because the amount of galactose used in the culture medium was difficult to control. However, the Gb3 synthesis with in situ UDP-Gal regeneration illustrated here gave the product in a very high yield (~99%) and the purification of allyl-Gb3 for further glycosylation became unnecessary (Table 1).

Large-Scale Synthesis of Allyl-Gb4. For the synthesis of globotetraose (Gb4) as allyl glycoside, a glycosyltransferase catalyzing the transfer of GalNAc from UDP-GalNAc coupled with UDP-GalNAc regeneration is necessary. The biosynthesis of UDP-GalNAc was exploited from different pathways, including a combination of C4 epimerase and UDP-GlcNAc synthesis enzymes,[43,44] or a direct conversion from GalNAc to its 1-phosphate coupled with a pyrophosphorylase reaction.[45] However, an effective kinase for GalNAc-1-phosphate synthesis has not been found in bacteria since the discovery of NahK in the *Bifidobacterium* species,[46] which could phosphorylate GlcNAc and GalNAc directly to form the corresponding 1-phosphate, with a slightly better catalytic activity (low K_m and high K_{cat}) than that of GalK2 from a mammalian source (Table 2).[47] Another potentially useful enzyme in the UDP-GalNAc biosynthesis pathway is GlmU (a bifunctional enzyme from *E. coli*), which has been shown to accept both GlcNAc-1-phosphate and GalNAc-1-phosphate.[48] In this study, NahK was exploited to phosphorylate GalNAc, and GlmU was used to catalyze the subsequent reaction to generate UDP-GalNAc. Thus, the UDP-GalNAc synthesis could be achieved with only two enzymes expressed in *E. coli*. In the glycosyltransferase reaction step, we tried to use the glycosyltransferase LgtD from *Haemophilus influenza*[49,50] to generate Gb4 from Gb3. Thus, to a solution (220 mL, pH 7.0) containing *N*-acetylgalactosamine (2.21 g, 10 mmol) and phosphoenolpyruvate (4.55 g, 22 mmol, monopotassium salt) was added *N*-acetylhexosamine kinase (NahK, 50 U), *N*-acetyl glucosamine-1-phosphate uridyltransferase (GlmU, 200 U), and β1,3- *N*-acetylgalactosaminyltransferase (LgtD, 100 U) (Table 1). The ATP regeneration enzymes, including pyruvate kinase (PK) and inorganic pyrophosphatase (PPA), and cofactors ATP and UTP were all conserved and reused from the previous reaction. In this reaction, we found that, unlike the UDP-GalNAc synthesis enzymes, NahK and GlmU prefer mildly acidic conditions (pH 6.5) and LgtD prefers mildly basic conditions (pH 9.0) (Figure S2a). From kinetic analysis of the glycosylation reaction, GlmU is the rate-limiting enzyme, about 500-fold less active than kinase NahK (Table 2). We also found that this glycosylation step requires magnesium ion but manganese or others would abolish the phosphotransfer activity entirely (Figure S2a–c). Though sugar kinase NahK and glycosyltransferase LgtD have high turnover numbers, pyrophosphorylase GlmU is less stable under low salt conditions and requires additional amounts to accomplish the reaction. The TTN for ATP and UTP are the same as for the previous glycosylation reaction, and the TTNs

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for enzymes are NahK = 5.5×10^5, GlmU = 8.5×10^3, and LgtD = 2.1×10^4. Compared to other pyrophosphorylases, GlmU has a lower TTN, probably caused by the low salt effect.

After the completion of the reaction, the proteins in the reaction mixture were removed by holding the temperature at 90 °C for 30 min, followed by centrifugation (5000 rpm, 20 min) and filtration with a 0.22 μM filter. The filtrate was then purified by C-18 gel chromatography and eluted by a gradient from 100% H_2O to 10% methanol in H_2O. Fractions were collected and monitored by TLC, and the fractions containing allyl-Gb4 were pooled and lyophilized. The product with more than 99% purity could be obtained by HPLC using a Cosmosil 5SL-II column in (19:81 H_2O/acetonitrile) in an isocratic mode. This purification process is essential for the large-scale synthesis of allyl-Globo H and allyl-SSEA4 because it would eliminate galactosyltransferases LgtC and LgtD, which would mediate unwanted side products from repeating galactosylation, and most importantly, this two-step reaction can significantly increase the yield (7.02 g, ~95%) compared to that in the previous study using commercial UDP-Glc/UDP-GlcNAc and epimerase.[24]

Large-Scale Synthesis of Allyl-Gb5. The galactosyltransferase for Gb5 synthesis was first identified unexpectedly by Samain et al.[51] According to their report, LgtD is a bifunctional glycosyltransferase that exhibits β-1,3-*N*-acetylgalactosaminyltransferase (β-1,3-GalNAcT) activity if Gb3 is used as the acceptor and UDP-GalNAc is used as the donor, whereas it shows β-1,3-galactosyltransferase (β-1,3-GalT) activity if Gb4 is used as the acceptor and UDP-Gal is used as the donor. However, this assay was based on an *E. coli* crude extract, which could not sufficiently reflect the actual in vitro synthesis condition. Therefore, to obtain the kinetics for further study, we purified and characterized the enzyme again and found that it contains a typical DXD motif, Asn92 and Asn94, for divalent metal ion binding. We found that either the magnesium or manganese ion was absolutely required for the β-1,3-GalNAcT activity if Gb3 was used as an acceptor; however, the divalent metal ion could increase the β-1,3-GalT activity only slightly when Gb4 was used as the acceptor (Figures S2b and S3b). Also, this bifunctional glycosyltransferase LgtD is 3-fold more active ($K_{cat,UDP\text{-}Gal}/k_{m,UDP\text{-}Gal}$) with allyl-Gb3 than with allyl-Gb4 as the acceptor (Table 2).

The synthesis of allyl-Gb5 was similar to allyl-Gb3 synthesis except for a replacement of LgtC with LgtD. In a representative reaction, allyl-Gb4 (7.47 g, 10 mmol), ATP (28 mg, 0.05 mmol), UTP (69 mg, 0.125 mmol), galactose (1.8 g, 10 mmol), phosphoenolpyruvate (4.55 g, 22 mmol, monopotassium salt), and $MgCl_2$ (final 10 mM) were mixed with enzymes galactokinase (GalK), UDP-sugar pyrophosphorylase (AtUSP), β-1,3-galactosyltransferase (LgtD, 200 U), pyruvate kinase (PK), and inorganic pyrophosphatase (PPA) in a 250 mL solution, and the reaction was initiated at room temperature over the course of 10 days with the pH controlled at 7.0. More enzymes were added if necessary, and the reaction was monitored by TLC until no more product could be observed. In the galactosylation step, the TTN numbers of cofactors (ATP and UTP) and enzymes (GalK and AtUSP) for allyl-Gb5 are similar to those in allyl-Gb3 synthesis. The TTN for glycosyltransferase LgtD was 2.1×10^4. During the synthesis of allyl-Gb5, it was observed that the reaction mixture of allyl-Gb4 prepared from allyl-Gb3 could be used directly for the synthesis of allyl-Gb5, but the product yield was only moderate (~40%, based on TLC observation), perhaps as a result of the presence of certain inhibitors in the mixture. However, using purified allyl-Gb4 as an acceptor can eliminate side-product formation and significantly increase the product yield. The purification step for allyl-Gb5 is the same as that for allyl-Gb4, and 94% purity could be achieved.

Large-Scale Synthesis of Allyl-Globo H. Our strategy for GDP-Fuc regeneration was based on a bifunctional enzyme (FKP) found in *Bacteroides* species with L-fucose-1-phosphate guanyltransferase and L-fucose kinases activities.[52] Although *Arabidopsis* has a similar bifunctional enzyme, the maximal activities of the kinase and pyrophosphorylase were significantly lower than that from *Bacteroides*.[53] This approach would require only two nucleotides (ATP and GTP), one monosaccharide (L-Fucose), and one enzyme (FKP) for GDP-Fuc regeneration, bypassing the longer and less efficient synthesis from GDP-Man by the de no pathway.[54] Our study also showed that the kinase activity was 4-fold more enhanced by the manganese ion than by the magnesium ion but had a significantly smaller pyrophosphorylase activity (Figure S4b). It was also reported that the α-1,2-fucosyltransferase (FutC) from *Helicobacter pylori* has a broad substrate specificity, including accepting Gb5 as a substrate.[55] Unlike the other glycosyltransferases used in this study ,which prefer mild basic conditions (pH 8.0), the fucosyltransferase is more active under the moderately acidic condition (pH 5.5) (Figure S4a). In this large-scale fucosylation reaction, the bifunctional fucokinase/GDP-L-fucose pyrophosphorylase (FKP, 200 U) used for GDP-Fuc synthesis was mixed with α-1,2-fucosyltransferase (FutC, 200 U), pyruvate kinase (PK), pyrophosphatase (PPA), allyl-Gb5(4.09 g, 4.5 mmol), L-Fuc (0.82 g, 5 mmol), ATP (2.7 mg, 0.05 mmol), GTP (27 mg, 0.5 mmol), and phosphoenolpyruvate (2.07 g, 10 mmol, monopotassium salt) in a mixture of 150 mL of Tris buffer at pH 7.0. The glycosylation reaction was monitored by TLC until completion. The turnover numbers were GTP = 20, FKP = 1.5×10^5, and FutC = 3×10^4.

Purification of Allyl-Globo H. Because the preparation of pure allyl-Globo H on large scales is important for further utility, an efficient and simple purification method is necessary to replace the tedious and multiple steps of the HPLC procedure. In the beginning, the proteins in the reaction solution were removed by heating and centrifugation, followed by lyophilization. Next, the residual product was redissolved in a 20 mM Tris buffer at pH 9.0. All unwanted charged species in the starting materials and intermediates, which contained phosphates (nucleotide di- and triphosphate and sugar-1-phosphate), were further removed with anion exchange column Q (pre-equilibrated under the same buffer condition). Further purification was performed by silica gel column chromatography (1:5 to 1:3 H_2O/acetonitrile) in a gradient mode. Fractions were collected and monitored by TLC, and the fractions containing allyl-Globo H were pooled and lyophilized. The oligosaccharide was further characterized by HRMS and NMR (Supporting Information, S11−S2, and S24−S28). This process gave allyl-Globo H in 94% yield (4.46 g) and 98% purity, which are comparable to those from HPLC.

Large-Scale Synthesis of Allyl-SSEA4. SSEA4 is the sialylated form of Gb5 with the α-2,3 linkage. The biosynthesis of CMP-Neu5Ac in prokaryotic and eukaryotic systems are subtly different.[56] In eukaryotic cells, CMP-Neu5Ac is produced from UDP-GlcNAc as a precursor via several steps, including dehydration, epimerization, phosphorylation, condensation with phosphoenolpyruvate, dephosphorylation, and

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finally condensation with CTP. However, in prokaryotic cells, CMP-Neu5Ac is generated directly from Neu5Ac and CTP is catalyzed by CMP-Neu5Ac synthetase (CSS). Regarding the sialylation reaction,[57−59] the marine α-2,3-sialyltransferase, JT-FAJ-16, which does not exhibit the intrinsic neuraminidase activity and prefers mildly basic conditions at pH 8.0 (Figure S4a), is a good candidate for the reaction. We thus utilize this α-2,3-sialyltransferase,[60] coupled with the CMP-Neu5Ac regeneration system comprising cytidine monophosphate kinase (CMK) and the CMP-Neu5Ac synthetase from *Pasteurella multocida*,[60] for the synthesis of allyl-SSEA4.

Allyl-SSEA4 was synthesized by adding allyl-Gb5 (4.09 g, 4.5 mmol), Neu5Ac (1.4 g, 4.5 mmol), ATP (2.7 mg, 0.05 mmol), CTP (0.27 g, 0.5 mmol), phosphoenolpyruvate (2.07 g, 10 mmol, monopotassium salt), cytidine monophosphate kinase (CMK, 50 U), CMP-sialic acid synthetases (CSS, 120 U), and α-2,3-sialyltransferase (SiaT, 150 U) to a 150 mL, pH 7.0 solution of Tris buffer. The glycosylation reaction was monitored by TLC until completion, and the turnover numbers were CTP = 40 (ATP was conserved from Gb5 synthesis in this following reaction), CMK = 2.2×10^5, CSS = 9.1×10^4, and SiaT = 2.9×10^5. Compared to the synthesis of allyl-Globo H, the sialylation of allyl-Gb5 gave a higher yield due to the higher TTN and specific activity (K_{cat}/K_m) of the sialyltransferase (Tables 1 and 2). After removing the proteins by heating and centrifugation, followed by chromatography, further purification was performed by silica gel chromatography (19:81 H_2O/acetonitrile), and the fractions containing allyl-SSEA4 were collected, lyophilized, and characterized by HRMS and NMR (Supporting Information, Figures S22 and S23, S28 and S29). The synthesis of allyl-SSEA4 after purification was achieved in 54% yield (~2.9 g).

■ CONCLUSIONS

We have devised an effective enzymatic approach that combines sugar nucleotide regeneration and glycosyltransferases for the syntheses of Gb5, Globo H, and SSEA4. Except for CMP-Neu5Ac, the more efficient salvage pathway enzymes were used to regenerate UDP-Gal, UDP-GalNAc, and GDP-Fuc from their corresponding monosaccharides through the reactions with kinase and sugar nucleotide pyrophosphorylase. With this new method available, we can obtain desired products Gb5 and Globo H in gram quantities in just two steps and three steps, respectively. The transferases as well as the other enzymes used in the regeneration systems were identified from different species through comparative studies and then overexpressed in *E. coli* and isolated for the synthesis study. These enzymatic methods are more effective than the previous systems and should be useful for the large-scale preparation of oligosaccharides for discovery research and clinical development.

■ EXPERIMENTAL SECTION

Materials and Methods. *Materials.* All nucleotides, sugars, sugar nucleotides, and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Restriction enzymes and T4 DNA ligase were from NEB (Beverly, MA), primers were from Proligo Singapore Pte Ltd. (Singapore), Ni-NTA Agarose was from Qiagen (Santa Clarita, CA), and Bio-Gel P2 gel was from Bio-Rad (Hercules, CA). Plasmids pET28a and pET47b and precoated glass plates covered with silica gel 60, F254 with 0.25 mm layer thickness were from EMD Chemicals Inc. (Carlsbad, CA). ArcticExpress/RIL competent cells were obtained from Agilent Genomics (La Jolla, CA).

Cloning of Genes for Sugar Nucleotide Synthesis, Glycosyltransferases, and ATP Regeneration. All genes were obtained via PCR from genomic DNA or the cDNA library using a primer (Table s1), and PCR products were ligated into the modified pET47b or pET28a with N- or C-terminal His-tag for further affinity purification.[61] To increase the gene expression level, the four glycosyltransferases were synthesized by codon optimization in *E. coli*. The plasmid with a correct sequence was transformed into ArcticExpress/RIL competent cells by a chemical transformation method. Single colonies were picked and inoculated in a TB medium with kanamycin antibiotics overnight, the cell culture was refreshed with TB medium, and the target protein expression was induced by IPTG (0.1 mM) until OD 600 reached 0.5. After that, the culture was grown at 16 °C for 24 h. The *E. coli* cells were harvested and disrupted in a buffer containing 50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, and 10 mM imidazole by a microfluidizer. The cells were centrifuged at 10 000 rpm for 30 min at 4 °C. Then, the supernatant was mixed with Ni-NTA agarose. The bound protein was eluted in the same buffer containing a higher concentration of imidazole (250 mM). The protein concentration was determined by Qubit Protein Quantitation kit (Invitrogen, CA, USA), and the purity was confirmed by SDS-PAGE.

Enzyme Assay. To maintain the assay condition constant, all activity measurements were made at 25 °C with 10 mM $MgCl_2$ and 100 mM Tris, pH 7.5.

Activity of Galactokinase (GalK), N-Acetylhexosamine Kinase (NahK), Fucokinase (FKP), and Cytidine Monophosphate Kinase (CMK). The fluorometric assay was based on ADP production (ATP consumption) using the pyruvate kinase/lactate dehydrogenase coupled enzymatic assay for NADH consumption.[62,63] (NADH has an excitation wavelength of 340 nm and an emission wavelength of 450 nm). The assay was performed in a 100 μL reaction mixture containing the coupling enzyme (5 units of pyruvate kinase and 7 units of lactate dehydrogenase from rabbit muscle), substrates, and cofactors (0.2 mM NADH, 0.8 mM PEP, 10 mM $MgCl_2$) in 100 mM Tris (pH 7.5), and the reaction was initiated by the addition of kinase and sugar. The kinetic parameters, K_{cat} and K_m, were calculated by curve fitting the experimental data with a theoretical equation, using Grafit version 7 (Erithacus Software, Middlesex, UK). One unit of sugar kinase activity was defined as 1 μmol of sugar-1-P formed per minute at 25 °C. Sugar kinases GalK, NahK, FKP, and CMK have specific activities of 25.63, 1.37, 16.18, and 229.59 units/mg, respectively. All reactions were performed in triplicate for statistical evaluation.

Activity of UDP-Sugar Pyrophosphorylase (AtUSP), N-Acetyl Glucosamine-1-phosphate Uridyltransferase (GlmU), GDP-L-Fucose Pyrophosphorylase (FKP), and CMP-Sialic Acid Synthetases (CSS). The production of pyrophosphate was measured using an EnzCheck pyrophosphate assay kit (Invitrogen, CA, USA). Assay components include 200 μM 2-amino-6-mercapto-7-methylpurine ribonucleoside, 1 unit of nucleoside phosphorylase, 0.03 unit of inorganic pyrophosphatase, and 10 mM $MgCl_2$, 50 mM Tris, pH 7.5 with sufficient amounts of UTP, GTP, or CTP combined with their respective sugar-1-phosphoate in 100 μL of UV-Star microplates (Greiner Bio One, Germany). All components except enzymes were mixed in the microplates and allowed to equilibrate until a flat baseline was achieved. Reactions were initiated by the addition of enzyme. One unit of enzyme activity was defined as the production of 1 μmol of nucleotide sugar from the respective sugar-1-P per minute at 25 °C, except CMP-sialic acid synthetase, which was defined as 1 μmol of pyrophosphate formed per minute at 25 °C. Sugar nucleotide pyrophosphorylase AtSUP, GlmU, FKP, and CSS have specific activities of 63.77, 9.99, 78.69, and 57.63 units/mg, respectively. All reactions were performed in triplicate for statistical evaluation.

Activity Measurement of Glycosyltransferase: α-1,4-Galactosyltransferase (LgtC), β-1,3-N-Acetylgalactosaminyltransferase (β1,3GalNAcT, LgtD), β-1,3-Galactosyltransferase (β1,3GalT, LgtD), α-1,2-Fucosyltransferase (FutC), and α-2,3-Sialyltransferase (JT-FAJ-16). The fluorometric assay method was used to monitor UDP, GDP, or CDP production by using the pyruvate kinase/lactate dehydrogenase coupled assay for NADH consumption. The assay components except sugar nucleotide were simultaneously incubated in the multiple plate fluorometer (SpectraMax M2 Readers, Molecular Devices) at 25 °C. Reactions were initiated by the addition of

corresponding nucleotide sugar. Kinetic parameters K_{cat} and K_m were calculated by curve fitting the experimental data with a theoretical equation, using Grafit version 7 (Erithacus Software, Middlesex, U.K.). One unit of activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of sugar from the respective sugar nucleotide to acceptor per minute at 25 °C. Glycosyltransferase LgtC for allyl-Gb3 synthesis, LgtD for allyl-Gb4 synthesis, LgtD for allyl-Gb5 synthesis, FutC for allyl-Globo H synthesis, and JT-FAJ-16 for allyl-SSEA4 synthesis have their specific activities of 2.82, 11.40, 1.19, 5.82, and 11.89 units/mg, respectively. All reactions were performed in triplicate for statistical evaluation.

Activity Measurement of Pyruvate Kinase (PK). The pyruvate kinase assay was slightly modified from the sugar kinase measurement as previous mentioned on the basis of NADH consumption. A 100 μL reaction mixture contained 0.8 mM ADP, 0.8 mM PEP, 0.2 mM NADH, 10 mM $MgCl_2$, and 5 units of lactate dehydrogenase from rabbit muscle in 100 mM Tris (pH 7.5). Reaction was initiated by adding a suitable amount of recombinant *E. coli* pyruvate kinase. One unit of pyruvate kinase was defined as the conversion of 1.0 μmole of phosphoenolpyruvate to pyruvate per minute at 25 °C. Pyruvate kinase has a specific activity of 326 units/mg. The reactions were performed in triplicate for statistical evaluation.

Activity Measurement of Inorganic Pyrophosphatase (PPA). The pyrophosphatase assay is slightly modified from the pyrophorylase protocol in the EnzCheck pyrophosphate assay kit (Invitrogen, CA, USA). Assay components include 1 mM pyrophosphate, 200 μM 2-amino-6-mercapto-7-methylpurine ribonucleoside, 1 unit of nucleoside phosphorylase, and 10 mM $MgCl_2$, 50 mM Tris, pH 7.5 on a 100 μL scale in UV-Star microplates (Greiner Bio One, Germany) with a suitable amount of recombinant *E. coli* pyrophosphatase. One unit of pyrophosphatase activity was defined as the liberation of 1.0 μmole of inorganic pyrophosphate per minute at 25 °C. Inorganic pyrophosphatase has a specific activity of 842 units/mg. The reactions were performed in triplicate for statistical evaluation.

Measurement of Optimum pH. The optimum pH for enzyme activity was determined in the standard enzyme assay mentioned above in the pH range 4.0−10.0, including sodium acetate, MES, MOPS, HEPES, Tris-HCl, and CHES buffer. The pH of the buffer was adjusted at the temperature of incubation. All reactions were performed in triplicate for statistical evaluation.

Measurement of Optimum Divalent Metal Ion. The assay for the metal requirement was conducted under standard assay conditions. Enzymes were mixed with metal ions (Mg^{2+}, Mn^{2+}, Mg^{2+} + Mn^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, or Ni^{2+}) in a final concentration of 10 mM in the presence and absence of EDTA. All reactions were performed in triplicate for statistical evaluation.

Measurement of Optimum Temperature. The effect of temperature on the activity of enzymes was determined by incubating an appropriate amount of purified enzyme in MOPS buffer (pH 7.0), 10 mM $MgCl_2$, and the respective substrates. To keep the assay consistent, all components were mixed well and preheated at assay temperature for 5 min, and the reaction was started by adding the enzyme and recorded by multimode plate readers (SpectraMax M5, Molecular Devices) at constant temperature. The temperature ranged from 20 to 60 °C. All reactions were performed in triplicate for statistical evaluation.

Synthesis of Allyl-Gb3. The reaction mixture (200 mL) contained 10 mmol of allyl-lac,[64] 10 mmol of galactose, 22 mmol of phosphoenolpyruvate (PEP), 0.05 mmol of ATP, 0.125 mmol of UTP, and 10 mM $MgCl_2$ in a 100 mM Tris-HCl buffer solution (pH 7.0). The reaction was initiated by the addition of 100 U of α-1,4-galactosyltransferase (LgtC), 50 U of galactokinase (GalK), 150 U of UDP-sugar pyrophosphorylase (AtUSP), 200 U of pyruvate kinase (PK), and 200 U of pyrophosphatase (PPA). The flask was incubated at 25 °C, and the reaction progress was monitored by TLC and *p*-anisaldehyde staining. More enzymes were added if necessary until the reaction was complete, and the products were confirmed by TLC and ESI-MS.

Synthesis of Allyl-Gb4. Following the allyl-Gb3 synthesis, additional components were added to the soution, including 9.9 mmol of *N*-acetylgalactosamine (GalNAc), 22 mmol of PEP, 100 U of β-1,3-*N*-acetylgalactosaminyltransferase (β1,3GalNAcT, LgtD), 50 U of *N*-acetylhexosamine 1-kinase (NahK), 200 U of *N*-acetylglucosamine 1-phosphate uridylyltransferase (GlmU), 100 U of PK, and 100 U of PPA (final volume = 220 mL). The mixture was incubated at 25 °C and monitored by TLC and ESI-MS as before until the reaction was complete. The product was further purified by a C-18 gel column and characterized by NMR.

Synthesis of Allyl-Gb5. To a reaction mixture (250 mL) containing 10 mmol of allyl-Gb4, 10 mmol of galactose, 22 mmol of PEP, 0.05 mmol of ATP, 0.125 mmol of UTP, 10 mM $MgCl_2$, and 100 mM Tris (pH 7.0) we added 200 U of β-1,3-galactosyltransferase (β1,3GalT, LgtD), 50 U of GalK, 150 U of AtUSP, 100 U of PK, and 100 U of PPA, and the mixture was incubated at 25 °C until the reaction was complete. The product was further purified by a C-18 gel column and characterized by NMR.

Synthesis of Allyl-Globo H. To a solution containing 4.5 mmol of allyl-Gb5, 5 mmol of fucose, 0.05 mmol of ATP, 0.5 mmol of GTP, 10 mmol of PEP, and 10 mM $MgCl_2$ in a 100 mM Tris buffer (pH 7.0) we added 200 U of L-fucokinase/GDP-fucose pyrophosphorylase (FKP), 200 U of PK, 200 U of PPA, and 200 U of α-1,2-fucosyltransferase (FutC), and the mixture was incubated at 25 °C until the reaction was complete. The product was purified by C-18 gel chromatography as before and characterized.

Synthesis of Allyl-SSEA4. Another half of the allyl-Gb5 (4.5 mmol) reaction mixture was used for the synthesis of allyl-SSEA4 by adding 5 mmol of *N*-acetylneuraminic acid (Neu5Ac), 0.05 mmol of ATP, 0.5 mmol of CTP, 10 mmol of PEP with 10 mM $MgCl_2$ in 100 mM Tris-HCl buffer (pH 8.0) followed by 50 U of cytidine monophosphate kinase (CMK), 120 U of CMP-sialic acid synthetase (CSS), 100 U of PK, 100 U of PPA, and 150 U of α-2,3-sialyltransferase (JT-FAJ-16). The progress was monitored by TLC, and the product was purified and characterized as described above.

Purification and Characterization of Oligosaccharides. The proteins in the reaction mixture were removed by holding at 90 °C for 30 min, followed by centrifugation (5000 rpm, 20 min). The filtrate was then purified by C-18 gel chromatography and eluted by a gradient from 100% H_2O to 10% methanol in H_2O. Fractions were collected and monitored by TLC (5:3:2 v/v/v butanol/ammonium hydroxide/water), and the fractions with allyl-oligosaccharides were pooled and lyophilized. The product with more than 99% purity of product could be gathered by HPLC using a Cosmosil 5SL-II column in (19:81 H_2O/acetonitrile) in an isocratic mode. The structures of allyl-Lac, allyl-Gb3, allyl-Gb4, allyl-Gb5, allyl-Globo H, and allyl-SSEA4 were analyzed by ^1H NMR, ^{13}C NMR, and mass spectrometry (Avance 600 and APEX-ultra 9.4 T FTICR-MS, Bruker Daltonics) (Supporting Information S10−S29).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

List of primers, detailed parameters of enzymes, and ^1H and ^{13}C NMR and HRMS spectra of synthetic molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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