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Facile chemoenzymatic synthesis of Lewis a (Le^a) antigen in gram-scale and sialyl Lewis a (sLe^a) antigens containing diverse sialic acid forms

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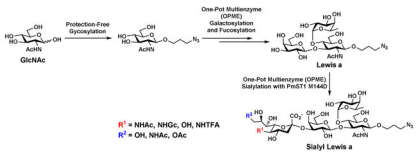
Abstract

An efficient streamlined chemoenzymatic approach has been developed for gram-scale synthesis of Lewis a angtigen (Le^{a}βProN$_3$) and a library of sialyl Lewis a antigens (sLe^{a}βProN$_3$) containing different sialic acid forms. Intially, commercially available inexpensive *N*-acetylglucosamine (GlcNAc) was converted to its *N*′-glycosyl *p*-toluenesulfonohydrazide in one step. Followed by chemical glycosylation, GlcNAcβProN$_3$ was synthesized using this protecting group-free method in high yield (82%). Sequential one-pot multienzyme (OPME) β1–3-galactosylation of GlcNAcβProN$_3$ followed by OPME α1–4-fucosylation reactions produced target Le^{a}βProN$_3$ in gram-scale. Structurally diverse sialic acid forms was successfully introduced using a OPME sialylation reaction containing a CMP-sialic acid synthetase and *Pasteurella multocida* α2–3-sialyltransferase 1 (PmST1) mutant PmST1 M144D with or without a sialic acid aldolase to form sLe^{a}βProN$_3$ containing naturally occurring or non-natural sialic acid forms in preparative scales.

Graphical abstract



Keywords

chemoenzymatic synthesis; glycosyltransferase; Lewis a; sialyl Lewis a; sialic acid; protecting group-free glycosylation

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Supporting Information. ^{1}H and ^{13}C NMR spectra of synthesized glycans.

1. Introduction

Lewis a (Le^a) antigen is a blood group antigen found in glycolipids and glycoproteins on the surface of erythrocytes as well as normal glandular and epithelial cells [1, 2]. Its sialylated form, sialyl Lewis a (sLe^a), is expressed on the cells of some normal tissues such as normal fibroblasts, the luminal side of ductal epithelial cells, and some parenchymatous cells [3]. Nevertheless, overexpression of sLe^a has been found on various types of cancer cells with increased levels of fucosylation [4], including colon cancer, pancreatic cancer, gallbladder/bile duct cancer, breast cancer, cholangiocarcinoma, and small cell lung cancer (SCLC) [3, 5, 6]. In fact, sLe^a was named as carbohydrate antigen 19–9 (CA19–9) [7] and was the first reported tumor-associated carbohydrate antigen (TACA) [8]. It was also discovered in the serum of patients with colon or pancreatic cancers [9] and has been used as a serum biomarker of pancreatic cancer [10] and other gastrointestinal cancers in clinic for prognosis determination, postoperative surveillance, and monitoring clinical responses to therapy [4, 7, 11–14]. Nevertheless, false negative and false positive results are possible and precautions are needed in interpreting the data [7, 10, 11].

sLe^a oligosaccharides and analogs have been synthesized chemically [15–20] or chemoenzymatically [21–24]. Chemoenzymatic synthesis of sLe^a antigens by sialidase (e.g. *Salmonella typhimurium LT2* sialidase)-catalyzed transglycosylation reaction using *p*-nitrophenyl α-*N*-acetylneuraminide (Neu5Acα*p*NP) as the glycosyl donor and Le^a trisaccharide as the acceptor was reported but with poor glycosylation yields [25]. All reported glycosyltransferase-catalyzed chemoenzymatic synthesis of sLe^a antigens were carried out using recombinant mammalian enzymes or human milk enzymes including human α1–4-fucosyltransferase (FucT III) [26, 27] or human milk [28, 29] α1–3/4-fucosyltransferase, rat liver [22, 23, 26] or porcine submaxillary [28] α2–3-sialyltransferase, and/or human β1–3galactosyltransferase [26]. In general, the reactions were carried out by sialyltransferase-catalyzed sialylation of β1–3-linked galactosides followed by fucosylation in the last step [23]. sLe^a derivatives with variations on the aglycon and the *N*-acyl group on the D-GlcNAc or the LFuc residue have been synthesized. However, modified sialic acid forms have not been introduced to sLe^a although variations at C-5 and C-9 positions of sialic acid have been introduced to sialyl Lewis x (sLe^x) structures [22, 23].

We have been interested in developing an efficient approach for systematically synthesizing sLe^a antigens containing different sialic acid forms which can be used as important probes to understand the structure-function relationship of sLe^a and the important roles of sialic acid modification. Unlike the common glycosylation sequence of sialylation followed by fucosylation for the formation of sLe^a in nature by glycosyltransferase-catalyzed reactions, the most efficient strategy to obtain sLe^a containing different sialic acid forms is to alter the glycosylation sequence by obtaining Le^a followed by α2–3-sialylation using a suitable α2–3-sialyltransferase-catalyzed reaction. This strategy will streamline the synthetic scheme and simplify the purification process as demonstrated previously for the synthesis of a library of sialyl Lewis x (sLe^x) antigens containing different sialic acid forms [30]. We show here that Le^a trisaccharide Galβ1–3(Fucα1–4)GlcNAcβProN_3 can be readily assembled in gram-scale by a bacterial disaccharide phosphorylase-containing one-pot multienzyme (OPME)

galactosylation reaction followed by a bacterial α1–3/4-fucosyltransferase-containing OPME fucosylation reaction. A previously constructed bacterial α2–3-sialyltransferse mutant is shown to be efficient in tolerating Le^a as the acceptor substrate to introduce different sialic acid forms with modifications at C-5, C-7, and/or C-9 positions for the synthesis of desired sLe^a antigens in good yields.

2. Results and discussion

2.1 Gram-scale chemoenzymatic synthesis of Le^a antigen Galβ1–3(Fucα1–4)GlcNAcβProN_3

To synthesize Le^a trisaccharide which could be potentially used as an acceptor substrate for sialyltransferase-catalyzed sialylation reaction for the production of desired sLe^a tetrasaccharides containing different sialic acid forms, monosaccharide glycoside GlcNAcβProN_3 was chemically synthesized. Comparing different strategies for obtaining the desired compound, a protecting group-free glycosylation strategy using *N*′-glycosyl *p*-toluenesulfonohydrazides (GSHs) as glycosyl donors reported by Gudmundsdottir and Nitz [31] was identified as an excellent choice. This method was shown to be especially useful for 2-acetamido-sugars such as free *N*-acetylglucosamine (GlcNAc) and unprotected disaccharides containing a free GlcNAc at the reducing end. The reactions resulted in good yields and excellent β-selectivity [32]. The GSH donors can be easily prepared from unprotected sugars and *p*-toluenesulfonyl hydrazide under mild acidic conditions in high yields. They can then be readily activated by *N*-bromosuccinimide (NBS) to couple with various alcohols to form glycosides [31]. We applied this glycosylation method for efficient synthesis of GlcNAcβProN_3 from commercially available and inexpensive GlcNAc. The azido functional group introduced can be used as a versatile chemical handle for bio-conjugation with alkyne-containing molecules [33]. It can also be readily reduced to an amine for immobilization or conjugation [34, 35].

As shown in Scheme 1, starting from GlcNAc (**1**) and *p*-toluenesulfonyl hydrazide (1.2 equiv.) in the presence of a catalytic amount of acetic acid in dimethylformamide (DMF), the GSH donor (**2**) was synthesized on a multi-gram (26.35 g) scale in a quantitative yield. The product was easily isolated by precipitation using diethyl ether. Glycosylation of 3-chloro-1propanol using the GSH donor (**2**) in the presence of NBS formed glycoside GlcNAcβProCl which was subsequently converted to the desired GlcNAcβProN_3 (**3**) by treating with NaN_3 in DMF. Compared to a previously reported method for synthesizing GlcNAcβProN_3 (**3**) which involved converting GlcNAc to peracetylated GlcNAc trichloroacetimidate followed by glycosylation and deprotection (5 steps, 28% yield) [36, 37], the current route (3 steps, 82% yield) is a much more efficient method which allows easy scaling up.

The obtained GlcNAcβProN_3 (**3**) was used for gram-scale enzymatic synthesis of disaccharide Galβ1–3GlcNAcβProN_3 (**4**) in the presence of galactose (Gal) and adenosine 5′triphosphate (ATP) using a one-pot two-enzyme galactosylation system containing *Streptococcus pneumoniae* (SpGalK) [38] and *Bifidobacterium infantis* D-galactosyl-β1–3-*N*-acetyl-D-hexosamine phosphorylase (BiGalHexNAcP) [39]. An excellent yield (90%) was achieved after product purification by silica gel column followed by BioGel P-2 gel

filtration column chromatography. Compared to β1–3-galactosyltransferase-catalyzed reactions, the strategy using BiGalHexNAcP avoided the need to produce uridine 5'-diphosphate-galactose (UDP-Gal) from uridine 5'-triphosphsate (UTP). Instead, galactose-1-phosphate formed by galactokinase-catalyzed reaction was directly used for the production of the disaccharide product. In addition, BiGalHexNAcP was able to be expressed at a level of 55 mg/L in *Escherichia coli* culture [39], providing an ample amount of enzyme for large-scale synthesis.

For the synthesis of Le^a trisaccharide from the resulting disaccharide Galβ1–3GlcNAcβProN_3 (**4**), a highly efficient fucosyltransferase was needed. We reported recently the cloning and application of a recombinant *Helicobacter pylori* α1–3/4-fucosyltransferase (Hp3/4FT) in a highly efficient one-pot three-enzyme (OP3E) fucosylation system for the preparative-scale (up to 100 mg) synthesis of diverse α1–3- and α1–4-linked fucosides [40]. The Hp3/4FT-containing OP3E fucosylation system was applied for gram scale synthesis of Le^a trisaccharide Galβ1–3(Fucα1–4)GlcNAcβProN_3 (Le^aβProN_3, **5**) which was achieve in high-yield (91%) in the presence of L-fucose, adenosine 5'-triphosphate (ATP), and guanosine 5'triphosphate (GTP). In this system, fucosyltransferase donor substrate guanosine 5'-diphosphateL-fucose (GDP-Fuc) was generated *in situ* from L-fucose, ATP, and GTP using a bifunctional *Bacteroides fragilis* enzyme (BfFKP) with both L-fucokinase and GDP-Fuc pyrophosphorylase activities [41]. To degrade the by-product pyrophosphate formed in the GDP-fucose formation process to inorganic phosphate, *Pasteurella multocida* inorganic pyrophosphatase (PmPpA) [37] was used. Hp3/4FT-catalyzed fucosylation of disaccharide Galβ1–3GlcNAcβProN_3 (**4**) using *in situ* generated GDP-fucose as the donor substrate produced the desired fucoside Le^aβProN_3 (**5**). The OPME fucosylation approach generated high cost GDP-Fuc *in situ* from inexpensive materials without the purification of intermediates, making the gram-scale synthesis of Le^a an economic and highly efficient process.

2.2 Enzymatic synthesis of sLe^a tetrasacchrides containing different sialic acid forms

The most efficient route for systematic synthesis of sLe^a antigens containing different sialic acid forms would be sialylating Le^a using a one-pot multienzyme (OPME) α2–3sialylation system [42]. To do this, the sialyltransferase used in the system has to tolerate fucosylated galactosides (e.g. Le^aβProN_3, **5**) as acceptor substrates. Previously, we reported that a single mutant of *Pasteurella multocida* multifunctional α2–3sialyltransferase 1 [43], PmST1 M144D, was able to tolerate fucosylated galactoside Le^xβProN_3 as the acceptor substrates for the synthesis of sLe^x structures containing different sialic acid forms [30]. To our delight, Le^aβProN_3 (**5**) that differs from Le^xβProN_3 on both galactosidic and fucosidic linkages was also a suitable acceptor substrates for PmST1 M144D. Therefore, the OPME sialylation system was readily used for the construction of the desired library of sLe^a antigens.

An shown in Scheme 2, the OPME sialylation system containing *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) and PmST1 M144D with (A) or without (B) *Pasteurella multocida* sialic acid aldolase (PmNanA), allowed the synthesis of α2–3-linked sialosides containing diverse sialic acid forms from Le^aβProN_3 (**5**) and sialic acid precursors with in situ generation of a diverse array of CMP-sialic acids. sLe^aβProN_3 tetrasaccharides

(**6–12**) containing *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), 3-deoxy-2-ketononulosonic acid (Kdn), *N*-trifluoroacetylneuraminic acid (Neu5NTFA), 9-*N*-acetyl-9-deoxyNeu5Ac (Neu5Ac9NAc), 9-*O*-acety-Neu5Ac (Neu5,9Ac_2), or 9-*O*-acety-Neu5Gc (Neu5Gc9Ac) were successfully synthesized from the corresponding six-carbon (Man/ManNAc derivatives) or 9-carbon (Neu5Ac derivatives) precursors, respectively.

The synthesis of sLe^a tetrasaccharides **6–10** was carried out in the one-pot three-enzyme system at pH 8.5. As shown in Table 1, sLe^{a}βProN_3 containing naturally occurring sialic acid forms Neu5Acα2–3Le^{a}βProN_3 (**6**), Neu5Gcα2–3Le^{a}βProN_3 (**7**), and Kdnα2–3Le^{a}βProN_3 (**8**) were obtained in 85%, 82%, and 86% yields, respectively, from *N*-acetylmannosamine (ManNAc), *N*-glycolylmannosamine (ManNGc) [44], and mannose (Man) as sialic acid precursors. sLe^{a}βProN_3 containing non-natural sialic acid forms Neu5NTFAα2–3Le^{a}βProN_3 (**9**) and Neu5Ac9NAcα2–3Le^{a}βProN_3 (**10**) were obtained in good yields (80% and 76%, respectively) from C2- or C6-modified ManNAc derivatives *N*-trifluoroacetylmannosamine (ManNTFA) [45] or 6-acetamido-6-deoxy-*N*-acetylmannosamine (ManNAc6NAc) [46, 47], respectively. sLe^{a}βProN_3 containing naturally occurring 9-*O*-acetylated sialic acid forms Neu5,9Ac_2α2–3Le^{a}βProN_3 (**11**) and Neu5Gc9Acα2–3Le^{a}βProN_3 (**12**) were obtained in 62% and 51% yields from Neu5,9Ac_2 and Neu5Gc9Ac [48, 49], respectively, in a one-pot two-enzyme reaction containing NmCSS and PmST1 M144D at pH 7.5. The lower yields for the synthesis of **11** and **12** were due to partial de-*O*-acetylation during the reaction and purification process.

3. Conclusions

In conclusion, an efficient chemoenzymatic method for systematic synthesis of synthetically challenging sialyl Lewis a antigens (sLe^{a}βProN_3, **6–12**) containing different sialic acid forms was developed successfully. The protecting group-free chemial glycosylation reaction combed with BiGalHexNAcP-catalyzed OPME β1–3-galactosylation and Hp3/4FT-catalyzed OPME fucosylation reactions succeffully produced Lewis a angtigen (Le^{a}βProN_3, **5**) in a gram scale. PmST1 M144D was proven to be an efficient catalyst for sialylating Le^a with different sialic acid forms. The study presented here provides a facile chemoenzymatic route for synthesizing Le^a antigen in gram-scale in high yield and for systematic synthesis of sLe^a antigens containing different sialic acid forms.

4. Experimental

4.1. Materials and general methods

Chemicals were purchased and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance-800 NMR spectrometer. High resolution electrospray ionization (ESI) mass spectra were obtained using Thermo Electron LTQOrbitrap Hybrid MS at the Mass Spectrometry Facility in the University of California, Davis. Silica gel 60 Å (230–400 mesh, Sorbent Technologies) was used for flash column chromatography. Thin-layer chromatography (Sorbent Technologies) was performed on silica gel plates using *p*-anisaldehyde sugar staining with 5% sulfuric acid in ethanol stain for detection. Gel filtration chromatography was performed with a column (100 cm × 2.5

cm) packed with Bio-Gel P-2 Fine resins (Bio-Rad). Recombinant enzymes *Streptococcus pneumoniae* (SpGalK) [38], *Bifidobacterium infantis* D-galactosyl-β1–3-*N*-acetyl-Dhexosamine phosphorylase (BiGalHexNAcP) [39], *Bacteroides fragilis* strain NCTC9343 bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (BfFKP) [41], *Pasteurella multocida* inorganic pyrophosphatase [37], *Helicobacter pylori* α1–3/4-fucosyltransferase (Hp3/4FT) [40], *Pasteurella multocida* sialic acid aldolase (PmNanA) [50], *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) [44], and *Pasteurella multocida* α2–3sialyltransferase 1 M144D mutant (PmST1 M144D) [30] were expressed and purified as described previously.

4.2. Gram-scale synthesis of trisaccharide Le^{a}βProN_3 (5)

4.2.1 N'-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-p-toluenesulfono-hydrazide (2)—GlcNAc (15.0 g, 67.8 mmol) and *p*-toluenesulfonylhydrazide (13.9 g, 74.6 mmol) were dissolved in DMF (30 mL) and H_2O (15 mL), 3 mL of acetic acid was added. The mixture was incubated at 37 °C without stirring for 2 days. After all solids were dissolved, the resulting solution was poured into diethyl ether (1 L) and the mixture was stirred vigorously for overnight. The precipitate was collected to produce compound **2** (26.35 g, 99.8%) as a light yellow solid. ^{1}H NMR (800 MHz, D_2O) δ 7.71 (d, *J* = 8.0 Hz, 2H), 7.46 (d, *J* = 8.0 Hz, 2H), 3.91 (d, *J* = 8.8 Hz, 1H, H-1), 3.86 (dd, *J* = 1.6 and 12.0 Hz, 1H), 3.68 (dd, *J* = 5.6 and 12.0 Hz, 1H), 3.49 (t, *J* = 9.6 Hz, 1H), 3.45 (t, *J* = 9.6 Hz, 1H), 3.34 (t, *J* = 9.6 Hz, 1H), 3.30–3.27 (m, 1H), 2.44 (s, 3H), 2.02 (s, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.10, 145.48, 132.93, 129.85, 129.74, 127.80, 127.68, 89.64 (C-1), 76.50, 74.06, 69.68, 60.63, 53.09, 22.07, 20.63. HRMS (ESI) *m/z* calcd for $C_{15}H_{24}N_3O_7S$ (M+H) 390.1335, found 390.1366.

4.2.2 Synthesis of GlcNAcβProN_3 (3)—To a solution of compound **2** (2.0 g) in anhydrous DMF (10 mL), 3-chloropropanol (8.6 mL) was added. NBS (2.2 g) was then added at room temperature. After stirring the mixture for 10 minutes, Dowex (OH-) resins were added to quench the reaction. The mixture was stirred until the yellow color disappeared. After filtration, the filtrate was concentrated and purified by flash chromatography (EtOAc: MeOH = 4:1, by volume) to produce 3-chloropropyl GlcNAc as a syrup residue. The obtained product was dissolved in DMF (10 mL) and NaN_3 (1.6 g) was then added. The reaction mixture was stirred at 65 °C for overnight before it was concentrated. The residue was purified by flash chromatography (EtOAc:MeOH = 4:1, by volume) to provide pure GlcNAcβProN_3 (**3**, 1.28 g, 82%) as a white solid. ^{1}H NMR (800 MHz, D_2O) δ 4.50 (d, *J* = 8.8Hz, 1H, H-1), 3.98–3.95 (m, 1H, H-5), 3.92 (dd, *J* = 2.4 and 12.8 Hz, 1H),), 3.74 (dd, *J* = 5.6 and 12.8 Hz, 1H), 3.69–3.65 (m, 2H, O$\underline{CH_2}CH_2CH_2N_3$), 3.53 (t, *J* = 8.0 Hz, 1H), 3.46–3.41 (m, 2H), 3.40–3.34 (m, 2H, $OCH_2CH_2\underline{CH_2}N_3$), 2.04 (s, 3H, NHCO$\underline{CH_3}$), 1.86–1.80 (m, 2H, $OCH_2\underline{CH_2}CH_2N_3$). ^{13}C NMR (200 MHz, D_2O) δ 174.42, 101.06 (C-1), 75.73, 73.63, 69.80, 66.96, 60.61, 55.46, 47.67, 27.99, 22.03. HRMS (ESI) *m/z* calcd for $C_{11}H_{21}N_4O_6$ (M+H) 305.1461, found 305.1456.

4.2.3 One-pot two-enzyme chemoenzymatic synthesis of Galβ1–3GlcNAcβProN_3 (4)—A reaction mixture with 30 mM acceptor GlcNAcβProN_3 (**3**, 1.04 g, 3.42 mmol), containing Tris-HCl buffer (100 mM, pH 6.55), galactose (1.23 g, 6.84

mmol), ATP (3.77 g, 6.84 mmol), $MgCl_2$ (20 mM), SpGalK (4.65 mg), and BiGalHexNAcP (5.74 mg) was incubated in a water bath at 37 °C. The reaction progress was monitored by thin-layer chromatography (TLC) and mass spectrometry analyses. When an optimal yield was achieved (after 2 day), the enzyme reaction was terminated by adding same volume of methanol as the reaction mixture and incubating at 4°C for 30 minutes. The mixture was centrifuged and the precipitates were removed. The supernatant was concentrated and passed through a silica gel column (EtOAc:MeOH:H_2O = 6:2:1, by volume) followed by a Bio-Gel P-2 gel filtration column purification to produce disaccharide Galβ1–3GlcNAcβProN$_3$ (**4**, 1.43 g, 90%). ^{1}H NMR (800 MHz, D_2O) δ 4.53 (d, *J* = 8.0 Hz, 1H, H_{GlcNAc}-1), 4.41 (d, *J* = 8.0 Hz, 1H, H_{Gal}-1), 3.97–3.88 (m, 3H), 3.81 (t, *J* = 10.4 Hz, 1H), 3.78–3.61 (m, 7H), 3.52 (t, *J* = 10.4 Hz, 1H), 3.51–3.45 (m, 2H), 3.38–3.28 (m, 2H, $OCH_2CH_2\underline{CH_2}N_3$), 2.02 (s, 3H, $NHCO\underline{CH_3}$), 1.84–1.81 (m, 2H, $OCH_2\underline{CH_2}CH_2N_3$); ^{13}C NMR (200 MHz, D_2O) δ 174.46, 103.40 (C_{Gal}-1), 100.80 (C_{GlcNAc}-1), 82.25, 75.22, 75.14, 72.34, 70.53, 68.58, 68.39, 67.03, 60.89, 60.58, 54.44, 47.65, 27.97, 22.09. HRMS (ESI) *m/z* calcd for $C_{17}H_{31}N_4O_{11}$ (M+H) 467.1989, found 467.1986.

4.2.4 One-pot three-enzyme chemoenzymatic synthesis of Le^{a}βProN$_3$ (5)—

A reaction mixture containing Tris-HCl buffer (100 mM, pH 7.5), Galβ1–3GlcNAcβProN$_3$ (**4**, 1.18 g, 30 mM, 2.53 mmol), L-fucose (0.62 g, 3.80 mmol), ATP (2.10 g, 3.80 mmol), GTP (2.15 g, 3.80 mmol), $MgCl_2$ (20 mM), BfFKP (4.49 mg), PmPpA (1.35 mg), and Hp3/4FT (10.55 mg) was incubated in a water bath at 37 °C. The reaction was monitored by TLC and mass spectrometry analyses. When an optimal yield was achieved (after 2 days), the same volume (115 mL) of methanol was added to the reaction mixture. This mixture was incubated at 4 °C for 30 min and then centrifuged. The supernatant was concentrated and passed through a silica gel column (EtOAc:MeOH:H_2O = 5:2:1, by volume) followed by a Bio-Gel P-2 gel filtration column purification to produce Le^{a}βProN$_3$ (**5**, 1.41g, 91%) as a white powder. ^{1}H NMR (800 MHz, D_2O) δ 5.01 (d, *J* = 4.0 Hz, 1H, H_{Fuc}-1), 4.88–4.85 (m, 1H, H_{Fuc}-5), 4.52 (d, *J* = 8.8 Hz, 1H, H_{GlcNAc}-1), 4.48 (d, *J* = 8.0 Hz, 1H, H_{Gal}-1), 4.07–3.34 (m, 19H), 2.04 (s, 3H, $NHCO\underline{CH_3}$), 1.86–1.82 (m, 2H, $OCH_2\underline{CH_2}CH_2N_3$), 1.17 (d, *J* = 6.4 Hz, 3H, H_{Fuc}-6); ^{13}C NMR (200 MHz, D_2O) δ 174.24, 102.77 (C_{Gal}-1), 100.93 (C_{GlcNAc}-1), 97.96 (C_{Fuc}-1), 75.96, 75.29, 74.70, 72.31, 72.18, 71.83, 70.35, 69.01, 68.24, 67.66, 67.08, 66.75, 61.55, 59.57, 55.65, 47.66, 28.01, 22.16, 15.25. HRMS (ESI) m/z calculated for $C_{23}H_{40}N_4NaO_{15}$ (M+Na) 635.2388, found 635.2353.

4.3. General procedure of one-pot multienzyme synthesis of sLe^a containing different sialic acid forms (6–12):

Le^{a}βProN$_3$ (**5**, 10–15 mM, 1 equiv.), a sialic acid precursor (mannose, ManNAc, or their derivatives, 1.5 equiv.), sodium pyruvate (5–7.5 equiv. when six-carbon sugars used as substrates), CTP (1.3–2.0 equiv.) were dissolved in water in a 50 mL centrifugal tube. The pH of this mixture was adjusted to neutral. $MgCl_2$ (20 mM) and Tris-HCl buffer (100 mM, pH 8.5 for reactions using ManNAc, Man, or derivatives as the sialyltransferase donor precursor; or pH 7.5 for reactions using Neu5,9Ac_2 or Neu5Gc9Ac as the sialyltransferase donor precursor) were then added. After adding NmCSS (1–3 mg) and PmST1 M144D (1–3.5 mg) with (1–3 mg, for reactions using ManNAc, Man, or derivatives as the sialyltransferase donor precursor) or without PmNanA, water was added to bring the

concentration of the acceptor to the range of 10–15 mM. The reactions were carried out for 12–36 hours before they were stopped by adding the same volume of ice-cold methanol followed by incubation at 4 °C for 30 min. After centrifugation, the supernatant was concentrated and purified by a BioGel P-2 gel filtration column chromatography with or without an additional C18-column chromatography in a high-performance liquid chromatography (HPLC) system to obtain the desired products.

4.3.1. Neu5Acα2–3Le^{a}βProN_3 (6),—203 mg, 85% yield, white powder: ^{1}H NMR (800 MHz, D_2O) δ 5.00 (d, *J* = 4.0 Hz, 1H, H_{Fuc}-1), 4.88–4.84 (m, 1H, H_{Fuc}-5), 4.52 (d, *J* = 8.0 Hz, 2H, H_{Gal}-1 and H_{GlcNAc}-1), 4.06 (t, *J* = 9.6 Hz, 1H), 4.04 (dd, *J* = 3.2 and 9.6 Hz, 1H), 3.98–3.94 (m, 2H), 3.89 (d, *J* = 3.2 Hz, 1H), 3.88–3.51 (m, 18H), 3.49 (t, *J* = 8.0 Hz, 1H), 3.40–3.34 (m, 2H, $OCH_2CH_2\underline{CH_2}N_3$), 2.76 (dd, *J* = 4.8 and 12.8 Hz, 1H, H_{Neu5Ac_eq}-3), 2.04 (s, 3H, NHCO$\underline{CH_3}$), 2.02 (s, 3H, NHCO$\underline{CH_3}$), 1.85–1.81 (m, 2H, $OCH_2\underline{CH_2}CH_2N_3$), 1.76 (t, *J* = 12.0 Hz, 1H, H_{Neu5Ac_ax}-3), 1.16 (d, *J* =6.4 Hz, 3H, H_{Fuc}-6); ^{13}C NMR (200 MHz, D_2O) δ 174.81, 174.11, 173.81, 102.68 (C_{Gal}-1), 100.86 (C_{GlcNAc}-1), 99.24 (C_{Neu5Ac}2), 97.93 (C_{Fuc}-1), 75.93, 75.48, 75.33, 74.63, 72.62, 72.22, 71.82, 71.74, 68.97, 68.66, 68.31, 67.90, 67.66, 67.07, 66.80, 66.72, 62.17, 61.55, 59.58, 51.55, 47.66, 39.89, 28.00, 22.29, 21.92, 15.21, HRMS (ESI) m/z calculated for $C_{34}H_{57}N_5O_{23}$ (M-H) 902.3372, found 902.3366.

4.3.2. Neu5Gcα2–3Le^{a}βProN_3 (7),—37 mg, 82% yield, white powder: ^{1}H NMR (800 MHz, D_2O) δ 5.00 (d, *J* = 4.0 Hz, 1H, H_{Fuc}-1), 4.88–4.85 (m, 1H, H_{Fuc}-2), 4.53 (d, *J* = 8.0 Hz, 2H, H_{Gal}-1 and H_{GlcNAc}-1), 4.11 (s, 2H, NHCO$\underline{CH_2}$OH), 4.08–4.04 (m, 2H), 3.98–3.95 (m, 2H), 3.93 (t, *J* = 10.4 Hz, 1H), 3.91 (d, *J* = 3.2 Hz, 1H), 3.89–3.52 (m, 17H), 3.50 (t, *J* = 8.0 Hz, 1H), 3.40–3.34 (m, 2H, $OCH_2CH_2\underline{CH_2}N_3$), 2.78 (dd, *J* = 4.8 and 12.8 Hz, 1H, H_{Neu5Ac_eq}-3), 2.05 (s, 3H, NHCO$\underline{CH_3}$), 1.86–1.82 (m, 2H, $OCH_2\underline{CH_2}CH_2N_3$), 1.78 (t, *J* = 12.8 Hz, 1H, H_{Neu5Ac_ax}-3), 1.17 (d, *J* = 6.4 Hz, 3H, H_{Fuc}-6); ^{13}C NMR (200 MHz, D_2O) δ 175.58, 174.11, 173.85, 102.70 (C_{Gal}-1), 100.87 (C_{GlcNAc}-1), 99.25 (C_{Neu5Ac}2), 97.94 (C_{Fuc}-1), 75.94, 75.47, 75.34, 74.64, 72.35, 72.22, 71.82, 68.98, 68.67, 68.04, 67.84, 67.67, 67.07, 66.79, 66.73, 62.14, 61.56, 60.86, 59.58, 55.60, 51.26, 47.66, 39.98, 28.01, 22.30, 15.22. HRMS (ESI) m/z calculated for $C_{34}H_{57}N_5O_{24}$ (M-H) 918.3321, found 918.3300.

4.3.3. Kdnα2–3Le^{a}βProN_3 (8),—122 mg, 86% yield, white powder: ^{1}H NMR (800 MHz, D_2O) δ 4.99 (d, *J* = 4.0 Hz, 1H, H_{Fuc}-1), 4.87–4.84 (m, 1H, H_{Fuc}-5), 4.51 (d, *J* = 7.2 Hz, 2H, H_{Gal}-1 and H_{GlcNAc}-1), 4.04 (t, *J* = 9.9 Hz, 1H), 4.02 (dd, *J* = 3.2 and 10.4 Hz, 1H), 3.98–3.51 (m, 21H), 3.48 (t, *J* = 8.0 Hz, 1H), 3.40–3.34 (m, 2H, $OCH_2CH_2\underline{CH_2}N_3$), 2.71 (dd, *J* = 4.8 and 12.8 Hz, 1H, H_{Neu5Ac_eq}-3), 2.05 (s, 3H, NHCO$\underline{CH_3}$), 1.85–1.81 (m, 2H, $OCH_2\underline{CH_2}CH_2N_3$), 1.70 (t, *J* = 12.0 Hz, 1H, H_{Neu5Ac_ax}-3), 1.15 (d, *J* = 6.4 Hz, 3H, H_{Fuc}6); ^{13}C NMR (200 MHz, D_2O) δ 174.09, 173.98, 102.74 (C_{Gal}-1), 100.88 (C_{GlcNAc}-1), 99.10 (C_{Neu5Ac}-2), 97.94 (C_{Fuc}-1), 75.97, 75.38, 75.32, 74.64, 73.65, 72.21, 72.04, 71.82, 70.15, 69.61, 68.97, 68.64, 67.66, 67.56, 67.07, 66.72, 66.69, 62.22, 61.56, 59.56, 55.57, 47.66, 39.62, 28.00, 22.29, 15.21. HRMS (ESI) m/z calculated for $C_{32}H_{54}N_4O_{23}$ (M-H) 861.3106, found 861.3115.

4.3.4. Neu5NTFAα2–3Le^{a}βProN$_3$ (9),—64 mg, 80% yield, white powder: ^{1}H NMR (800 MHz, D_2O) δ 5.00 (d, *J* = 4.0 Hz, 1H, H_{Fuc}-1), 4.88–4.85 (m, 1H, H_{Fuc}-5), 4.52 (d, *J* = 8.0 Hz, 1H, H_{GlcNAc}-1), 4.51 (d, *J* = 8.0 Hz, 1H, H_{Gal}-1), 4.06–4.04 (m, 2H), 4.00 (t, *J* = 9.6 Hz, 1H), 3.98–3.94 (m, 2H), 3.90 (d, *J* = 3.2 Hz, 1H), 3.88–3.51 (m, 17H), 3.50 (t, *J* = 8.0 Hz, 1H), 3.39–3.34 (m, 2H, $OCH_2CH_2\underline{CH_2}N_3$), 2.79 (dd, *J* = 4.8 and 12.8 Hz, 1H, H_{Neu5Ac_eq}-3), 2.04 (s, 3H, $NHCO\underline{CH_3}$), 1.85–1.81 (m, 2H, $OCH_2\underline{CH_2}CH_2N_3$), 1.78 (t, *J* = 12.8 Hz, 1H, H_{Neu5Ac_ax}-3), 1.16 (d, *J* = 6.4 Hz, 3H, H_{Fuc}-6); ^{13}C NMR (200 MHz, D_2O) δ 174.11, 173.71, 102.71 (C_{Gal}-1), 100.88 (C_{GlcNAc}-1), 99.22 (C_{Neu5Ac}-2), 97.94 (C_{Fuc}-1), 75.98, 75.43, 75.33, 74.62, 72.21, 71.93, 71.82, 68.97, 68.64, 67.96, 67.84, 67.66, 67.07, 66.72, 62.11, 61.55, 59.57, 52.10, 47.66, 39.97, 28.00, 22.29, 15.21. HRMS (ESI) m/z calculated for $C_{34}H_{53}F_3N_5O_{23}$ (M-H) 956.3089, found 956.3084.

4.3.5. Neu5Ac9NAcα2–3Le^{a}βProN$_3$ (10),—35 mg, 76% yield, white powder: ^{1}H NMR (800 MHz, D_2O) δ 5.00 (d, *J* = 4.0 Hz, 1H, H_{Fuc}-1), 4.88–4.83 (m, 1H, H_{Fuc}-5), 4.54–4.47 (m, 2H, H_{Gal}-1 and H_{GlcNAc}-1), 4.06 (t, *J* = 9.6 Hz, 1H), 4.01 (dd, *J* = 3.2 and 9.6 Hz, 1H), 3.98–3.95 (m, 2H), 3.90 (d, *J* = 3.2 Hz, 1H), 3.88–3.47 (m, 18H), 3.40–3.34 (m, 2H, $OCH_2CH_2\underline{CH_2}N_3$), 3.25 (dd, *J* = 8.0 and 14.4 Hz, 1H), 2.76 (dd, *J* = 4.8 and 12.8 Hz, 1H, H_{Neu5Ac_eq}-3), 2.02 (s, 3H, $NHCO\underline{CH_3}$), 2.01 (s, 6H, 2x$NHCO\underline{CH_3}$), 1.87–1.80 (m, 2H, $OCH_2\underline{CH_2}CH_2N_3$), 1.77 (t, *J* = 12.0 Hz, 1H, H_{Neu5Ac_ax}-3), 1.16 (d, *J* =6.4 Hz, 3H, H_{Fuc}-6); 13C NMR (200 MHz, D_2O) δ 174.75, 174.19, 173.92, 173.80, 102.62 (C_{Gal}-1), 100.86 (C_{GlcNAc}-1), 99.47 (C_{Neu5Ac}-2), 97.93 (C_{Fuc}-1), 75.94, 75.57, 75.33, 74.63, 72.50, 72.22, 71.82, 69.89, 69.45, 68.98, 68.66, 68.27, 67.67, 67.04, 66.88, 66.73, 61.55, 59.59, 55.60, 51.55, 47.66, 41.92, 39.84, 28.01, 22.18, 21.93, 21.66, 15.21. HRMS (ESI) m/z calculated for $C_{36}H_{60}N_6O_{23}$ (M-H) 943.3637, found 943.3641.

4.3.6. Neu5,9Ac$_2$α2–3Le^{a}βProN$_3$ (11),—29 mg, 62% yield, white powder: ^{1}H NMR (800 MHz, D_2O) δ 5.00 (d, *J* = 3.2 Hz, 1H, H_{Fuc}-1), 4.87–4.84 (m, 1H, H_{Fuc}-5), 4.53 (d, *J* = 8.0 Hz, 2H, H_{Gal}1 and H_{GlcNAc}-1), 4.38 (dd, *J* = 2.4 and 11.2 Hz, 1H), 4.18 (dd, *J* = 7.2 and 12.0 Hz, 1H), 4.083.52 (m, 21H), 3.51 (t, *J* = 8.0 and 14.4 Hz, 1H), 3.41–3.34 (m, 2H, $OCH_2CH_2\underline{CH_2}N_3$), 2.76 (dd, *J* = 4.8 and 12.8 Hz, 1H, H_{Neu5Ac_eq}-3), 2.13 (s, 3H, $OCO\underline{CH_3}$), 2.04 (s, 3H, $NHCO\underline{CH_3}$), 2.03 (s, 3H, $NHCO\underline{CH_3}$), 1.87–1.80 (m, 3H, $OCH_2\underline{CH_2}CH_2N_3$ and H_{Neu5Ac_ax}-3), 1.17 (d, *J* = 7.2 Hz, 3H, H_{Fuc}-6); ^{13}C NMR (200 MHz, D_2O) δ 174.76, 174.17, 174.01, 172.66, 102.64 (C_{Gal}-1), 100.85 (C_{GlcNAc}-1), 98.61 (C_{Neu5Ac}-2), 97.92 (C_{Fuc}-1), 75.98, 75.39, 75.34, 74.50, 72.55, 72.20, 71.83, 69.08, 69.00, 68.68, 68.12, 67.85, 67.67, 67.06, 66.74, 66.72, 65.77, 61.44, 59.59, 51.52, 47.67, 39.47, 28.01, 22.27, 21.97, 20.16, 15.21. HRMS (ESI) m/z calculated for $C_{36}H_{58}N_5O_{24}$ (M-H) 944.3477, found 944.3430.

4.3.7. Neu5Gc9Acα2–3Le^{a}βProN$_3$ (12),—12 mg, 51% yield, white powder: ^{1}H NMR (800 MHz, D_2O) δ 5.00 (d, *J* = 4.0 Hz, 1H, H_{Fuc}-1), 4.88–4.85 (m, 1H, H_{Fuc}-2), 4.53 (d, *J* = 8.0 Hz, 1H, H_{GlcNAc}-1), 4.52 (d, *J* = 8.0 Hz, 1H, H_{Gal}-1), 4.35 (dd, *J* = 2.4 and 11.2 Hz, 1H), 4.18 (dd, *J* = 6.4 and 12.0 Hz, 1H), 4.11 (s, 2H, $O\underline{CH_2}OCOCH_3$), 4.07–3.51 (m, 21H), 3.50 (t, *J* = 8.0 and 14.4 Hz, 1H), 3.40–3.34 (m, 2H, $OCH_2CH_2\underline{CH_2}N_3$), 2.78 (dd, *J* = 4.8 and 12.8 Hz, 1H, H_{Neu5Ac_eq}-3), 2.13 (s, 3H, $OCO\underline{CH_3}$), 2.04 (s, 3H, $NHCO\underline{CH_3}$), 1.85–1.81 (m, 2H, $OCH_2\underline{CH_2}CH_2N_3$), 1.78 (t, *J* = 12.0 Hz, 1H, H_{Neu5Ac_ax}-3), 1.16 (d, *J* =6.4 Hz, 3H,

H_{Fuc}-6); ^{13}C NMR (200 MHz, D_2O) δ 175.53, 174.25, 174.17, 173.99, 102.77(C_{Gal}-1), 102.69(C_{GlcNAc}-1), 100.87(C_{Neu5Ac}-1), 97.94(C_{Fuc}-1), 76.00, 75.40, 75.35, 75.30, 74.71, 74.54, 72.32, 72.22, 71.83, 70.36, 69.26, 69.00, 68.68, 68.24, 68.05, 67.67, 67.07, 66.73, 65.67, 61.55, 61.49, 60.88, 59.59, 55.59, 51.20, 47.67, 39.70, 28.01, 22.28, 22.16, 20.17, 15.25, 15.21. HRMS (ESI) m/z calculated for $C_{36}H_{58}N_5O_{25}$ (M-H) 960.3426, found 960.3420.

Supplementary Material

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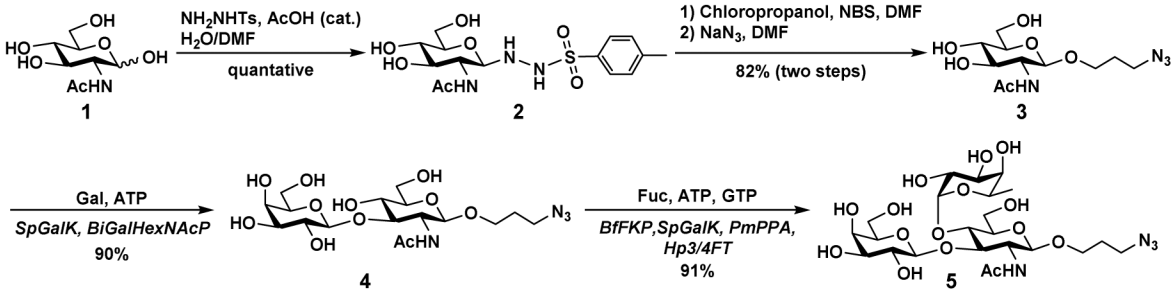
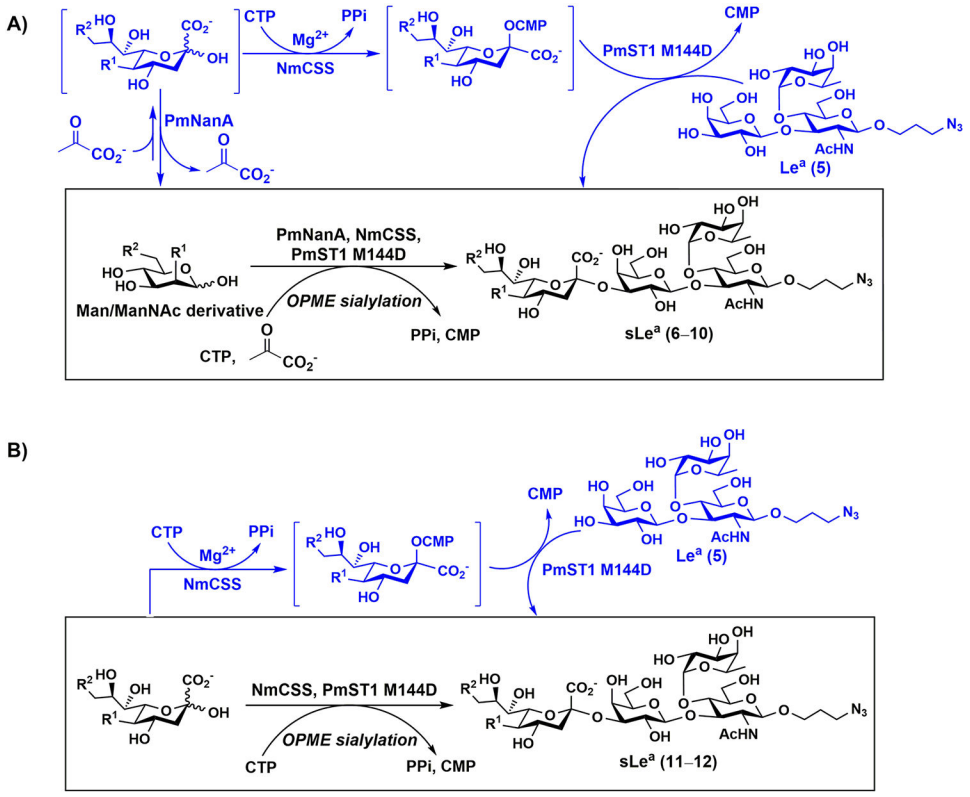
Highlights:

- High-yield protection group-free glycosylation for synthesizing GlcNAcβProN$_3$
- Gram-scale chemoenzymatic synthesis of Lewis a antigen was efficiently achieved
- Le^{a}βProN$_3$ was shown to be a suitable acceptor for PmST1 M144D
- Seven sLe^a antigens containing different sialic acid forms were synthesized
- A high-yield chemoenzymatic method for Le^a and sLe^a synthesis was demonstrated



Scheme 1.

Gram-scale chemoenzymatic synthesis of $Le^a\beta ProN_3$ (**5**). Enzyme abbreviations: SpGalK, *Streptococcus pneumoniae* galactokinase; BiGalHexNAcP, *Bifidobacterium infantis* Dgalactosyl-β1–3-*N*-acetyl-D-hexosamine phosphorylase; BfFKP, *Bacteroides fragilis* strain NCTC9343 bifunctional L-fucokinase/GDP-fucose pyrophosphorylase; PmPpA, *Pasteurella multocida* inorganic pyrophosphatase; Hp3/4FT, *Helicobacter pylori* α1–3/4-fucosyltransferase.

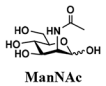
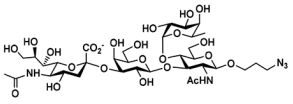
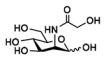
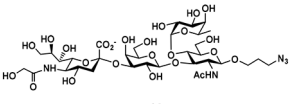
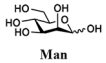
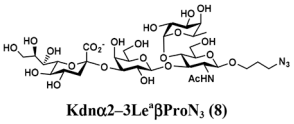
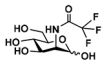
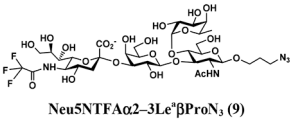
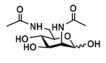
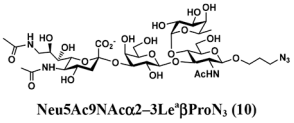
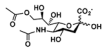
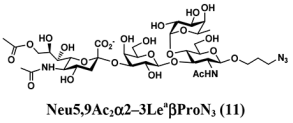
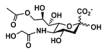
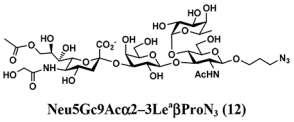


Scheme 2.

Synthesizing sLe^{a}βProN_3 containing different sialic acid forms using one-pot multienzyme (OPME) α2–3-sialylation system containing NmCSS and PmST1 M144D with (**A**) or without (**B**) PmNanA.

Table 1:

One-pot multienzyme (OPME) preparative-scale synthesis of sLe^{a}βProN$_3$ (**6–12**).

Entry	Sialic acid or precursor	Product	Yield (%)
a	 ManNAc	 Neu5Acα2–3Le^{a}βProN$_3$ (6)	**85**
b	 ManNGc	 Neu5Gcα2–3Le^{a}βProN$_3$ (7)	**82**
c	 Man	 Kdnα2–3Le^{a}βProN$_3$ (8)	**86**
d	 ManNTFA	 Neu5NTFAα2–3Le^{a}βProN$_3$ (9)	**80**
e	 ManNAc6NAc	 Neu5Ac9NAcα2–3Le^{a}βProN$_3$ (10)	**76**
f	 Neu5,9Ac$_2$	 Neu5,9Ac$_2$α2–3Le^{a}βProN$_3$ (11)	**62**
g	 Neu5Gc9Ac	 Neu5Gc9Acα2–3Le^{a}βProN$_3$ (12)	**51**