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Synthesis of *N*-modified sTn analogs and evaluation of their immunogenicities by microarray-based immunoassay

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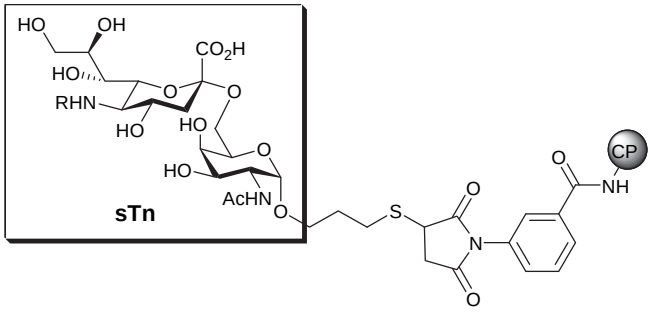
ABSTRACT

A series of sTn derivative-keyhole limpet hemocyanin (KLH) conjugates were synthesized, and their immunogenicities were evaluated by corresponding IgG production. To achieve a high-throughput screening immunoassay, a glycan microarray with sTn and its analogs was used to detect the production of corresponding antibodies in mouse sera. The immunoassay results revealed that the derived sTn antigens are generally more immunogenic than the parent sTn antigen. The *N*-propionyl sTn antigen was the most immunogenic among the sTn derivatives investigated, and its antiserum was cross-reactive with natural sTn. These results indicate that *N*-propionyl sTn may serve as a viable vaccine candidate to produce antibody for detection of sTn antigen.

1. Introduction

Many carbohydrates have been identified as tumor-associated carbohydrate antigens (TACAs),[1] which, as a consequence of their vast overexpression on the surface of tumor cells,[2] may serve as targets for cancer immunotherapy in the form of anti-cancer vaccines.[3] However, TACAs are T-cell independent antigens and exhibit poor immunogenicity, which leads to the formation of low-affinity IgM antibodies and the absence of IgG antibodies.[4] To overcome the T-cell independent properties of TACAs, carbohydrate antigens are conjugated to a strong immunogenic carrier protein, such as ovalbumin (OVA),[5a] tetanus toxoid,[5b] or keyhole limpet hemocyanin (KLH).[5c,d] In these methods, the carrier proteins enhance the presentation of the carbohydrate to the immune system and provide T epitopes that can activate helper T-cells. Additionally, the carrier proteins can elicit an MHC-II T-cell response characterized by IgG antibody production.[5]

Some sialic acid-containing carbohydrates that are overexpressed by certain cancers[1] are considered to be TACAs. In particular, the disaccharide sialyl-Tn (sTn, NeuAcα(2→6)GalNAcα-*O*-Ser/Thr, Figure 1), a mucin-associated carbohydrate antigen O-linked to serine or threonine, is expressed on various types of tumors, including colon, breast, prostate, pancreas, lung, stomach, and ovarian carcinomas.[2]



R	**1** (CP = KLH)	**2** (CP = OVA)
a	CH_3CO-	CH_3CO-
b	CH_3CH_2CO-	CH_3CH_2CO-
c	$CH_3CH_2CH_2CO$-	$CH_3CH_2CH_2CO$-
d	$HOCH_2CO$-	$HOCH_2CO$-

Figure 1. Structures of *N*-modified sTn and conjugate vaccines.

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Expression of sTn is an independent indicator for the prognosis of cancer and has been shown to be effective for cancer vaccine development.[6] Previously, a sTn trimer–KLH conjugate was developed as a therapeutic vaccine (Theratope®) for metastatic breast cancer.[7,8] Unfortunately, this sTn-based vaccine failed in phase III of the clinical trial.[8]

Modification of the C5 *N*-substitutent of sialic acid in sialic-acid-containing antigens has been shown to enhance their immunogenicity.[9–13] For example, Jennings et al.[10] modified the 5-NHAc groups of oligo α(2,8)-sialic acid to form a propionyl amide and achieved a better immune response to the modified antigen. Similar approaches also produced higher antibody titers against modified antigens for GM_3,[11] GD_3,[9] and sTn[12] antigens. In addition, the modification of monomer sialic acid, which is non-antigenic, to an *N*-levulinoylneuraminic acid derivative results in significant antibody production.[13] However, in those approaches, some of the produced antibodies can only recognize the modified antigens, not the parent antigens. Thus, further investigation of antigen modification is needed.

Traditionally, enzyme-linked immunosorbent assays (ELISA) were used to detect and quantify antibodies in the sera of vaccinated animals or humans.[14] However, ELISA is laborious, requires a relatively large amount of serum, and only detects individual antigens. Recently, carbohydrate microarray technology has emerged as a powerful tool for glycobiology since the procedure has enough potency for high-throughput screening.[15] Carbohydrate microarrays provide the capacity to measure several carbohydrate antigens in a single sample, thereby reducing the handling time required for analysis. Additionally, the microarray uses an extremely small volume of material for the analysis, which may be critical for analyzing samples from small animals including mice. As such, microarrays provide a high-throughput tool to obtain a broader and more in-depth profile of immune responses. Therefore, a carbohydrate microarray could be a good alternative for traditional ELISA immunoassays.

To develop a method that enhances IgG production of the low antigenic carbohydrate antigen, sTn was chosen as the target antigen, and its 5-NHAc of sialic acid was modified into unnatural *N*-acyl derivatives followed by conjugation with KLH. The immunogenicities of the modified sTn–KLH conjugates were investigated based the corresponding antibodies detected by a sTn microarray. Our results showed that the propionyl group modified sTn vaccine **1c** exhibited higher antibody production and that the resulting antibodies also bound to the parent sTn antigen. We anticipate that our strategy might circumvent the immunogenic problem of sTn and result in effective antibody production for sTn antigen detection.

2. Results and discussion

2.1. Design of sTn-based vaccines

Despite the significant progress in carbohydrate chemistry in recent years,[16] complex oligosaccharide synthesis still remains difficult, particularly for sialoconjugates. The major problem in the synthesis of sTn and its derivatives is that the sialylation reactions often produce a low yield, low α-stereoselectivity, and an undesirable 2,3-elimination due to the presence of an electron withdrawing group at the anomeric center. Also, there is a lack of participation from the auxiliary substituent adjacent to the anomeric center and a sterically hindered tertiary anomeric center.[17] Previously, our group and other investigators have discovered some synthetic routes with higher yields.[18] In some cases, the syntheses produced better α-selectivity during sialylation by replacing the *N*-acetyl functional group at C-5 with *N*,*N*-diacetyl,[19] 2,2,2-trichloroethoxycarbonyl (*N*-Troc),[20] trifluoroacetyl (*N*-TFA),[21] azido (N_3),[22] *N*-Fmoc,[18e] *N*-phthalimide,[23] and *N*,*N*-Ac,Boc[24] groups in the sialyl donors. Furthermore, an oxazoline derivative at the C4 and C5 positions of sialic acid was also found to be an excellent sialyl donor for forming the α-glycosidic bond.[25]

In this report, we predicted that a Troc protected-sialic acid donor would be feasible for a pivotal approach to variant sialosides due to the Troc group's acid-resistant nature, better sialylation yield, better α-selectivity, and selective cleavability under mild conditions.[20a] In addition, the allyl group was chosen as the appropriate linker for attaching sTn to carrier proteins. Because the Troc group can be selectively removed in the presence of an allyl group, this selectivity facilitates the possibility of introducing a linker at an early stage before the deprotection and acylation of the amino group at C5 of sialic acid. Deprotection and modification of the sialic acid residue at the latest stage can lead to a highly convergent synthetic design. Although several leaving groups, such as phosphite,[26] sulfide,[27] xanthanate[28] or hydroxyl group[29] at the anomeric center have been shown to enhance the α-selectivity of sialyl donors, the sulfide- and phosphite-based donors are most commonly used. The sulfide-based donors have the advantage of being very stable. However, phosphite donors can be activated by a catalytic amount of promoter (usually TMSOTf) and lead to predominant formation of the α-product during glycosylation. Based on precedence, the sialic acid donor with Troc as a protecting group was placed at N-5, phosphite was chosen as the leaving group and an allyl group served as the linker at the sTn reducing end. In addition, we also examined 5-*N*,4-*O*-oxazolidinone-protected sialyl phosphate[30] as a donor for the synthesis of a sTn derivative.

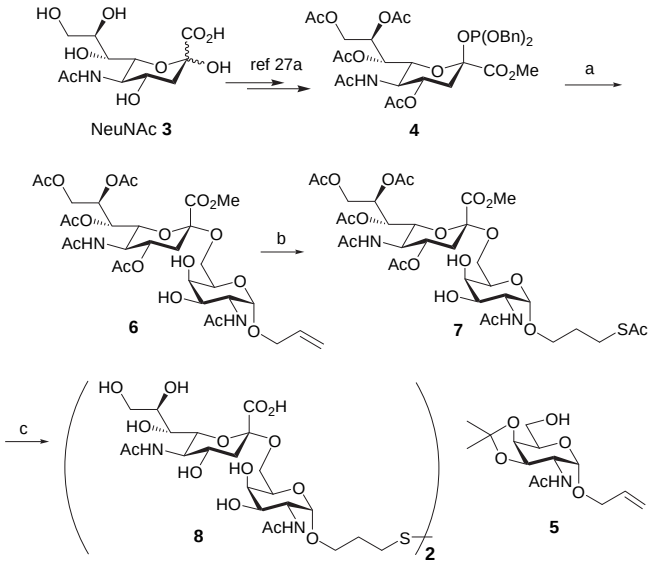
As shown in Figure 1, we synthesized sTn antigen and its analogs, including *N*-acetyl sTn **1a**, *N*-propionyl sTn **1b**, *N*-butanoyl sTn **1c**, and *N*-glycolyl sTn **1d**. Natural sTn and **1b** show few structural differences; the propionyl group is only one carbon longer than an acetyl group. However, a similar structural variation in polysialic acid vaccines showed substantial improvement in immunogenicity. Therefore, it is interesting to evaluate the effect of a propionyl group on the immunological properties of sTn. Butanoyl and glycolyl groups are more structurally different from an acetyl group and likely more immunogenic.

Conjugation of carbohydrate antigens to an immunogenic carrier protein has enhanced not only the magnitude, but also the quality of the antibody response.[5] KLH, a well-established carrier protein for experimental cancer vaccines, was employed to form glycoconjugate vaccines **1a**–**d** (Fig. 1). Alternatively, OVA conjugates **2a**–**d** were prepared as capture reagents for immunological microarray assays to avoid recognition between produced antibodies and KLH.

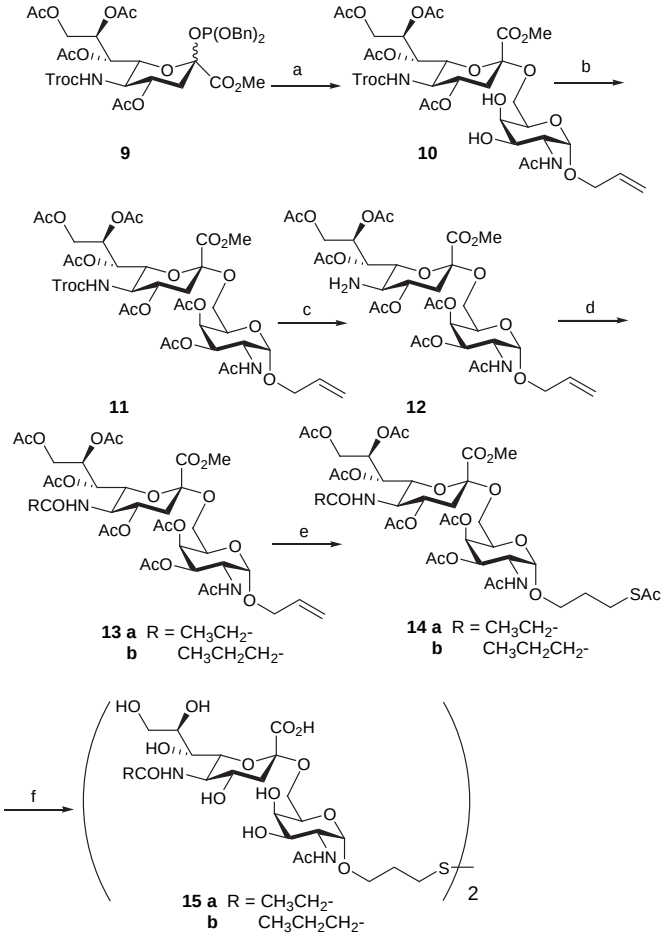
2.2. Synthesis of *N*-acyl sTn derivatives and their KLH and OVA conjugates

The synthesis of native sTn is illustrated in Scheme 1. The phosphite *N*-acetylneuraminic acid donor **4**,[26a] prepared from neuraminic acid **3**, was coupled with acceptor **5**[31] to create a 3:2 (α:β) mixture of the 2,6-linked sialoside with a 65% yield under the conventional conditions.[26b] The α/β isomers were inseparable with column chromatography. Fortunately, after acidic deprotection of acetonide on the isomers, the resulting α product **6** was separated efficiently with a 31% yield over two steps. Then, the thiol functionality was introduced onto the olefin moiety of **6** by a photochemical reaction with thiolacetic acid[32] to provide **7** (91%). Full deprotection of **7** was achieved with NaOMe in MeOH and a 0.1 M NaOH solution at rt. The resultant free thiol group was spontaneously oxidized into a disulfide bond to give dimer **8**, which was purified by a Biogel P2 column and characterized by MS and NMR.

The syntheses of sTn derivatives are shown in Scheme 2. Similar to Scheme 1, the coupling of donor **9**[21a] with acceptor **5** yielded an inseparable α/β mixture of the 2,6-linked sialoside with a 80% yield



Scheme 1. Synthesis *N*-acetyl sTn antigen from *N*-acetylneuraminic acid. (a) (1) TMSOTf, MS 3 Å, MeCN, −40 °C, 30 min, 65% (α/β=3:2); (2) 80% $AcOH_{(aq)}$, 40 °C, 5 h, 31% (two steps); (b) HSAc, AIBN, MeOH, 75 °C, 24 h, 91%; (c) (1) NaOMe, MeOH; (2) 0.1 M $NaOH_{(aq)}$, 90% (two steps).



Scheme 2. Synthesis of *N*-propionyl sTn antigen **15a** and *N*-butanoyl sTn antigen **15b**. (a) (1) **5**, TMSOTf, MS 3 Å, MeCN, −40 °C, 30 min, 80% (α/β=2:1); (2) 80% $AcOH_{(aq)}$, 40 °C, 5 h, 43% (two steps); (b) Ac_2O, pyridine, 24 h, 92%; (c) AcOH, Zn, rt, 4 h, 92%; (d) EDC, HOBt, $NaHCO_3$, MeCN, then acid, rt, 42% for **13a** and 51% for **13b**; (e) HSAc, AIBN, MeOH, 75 °C, 24 h, 66% for **14a** and 91% for **14b**; (f) (1) NaOMe, MeOH; (2) 0.1 M $NaOH_{(aq)}$, 84% for **15a** and 93% for **15b** (yield for two steps).

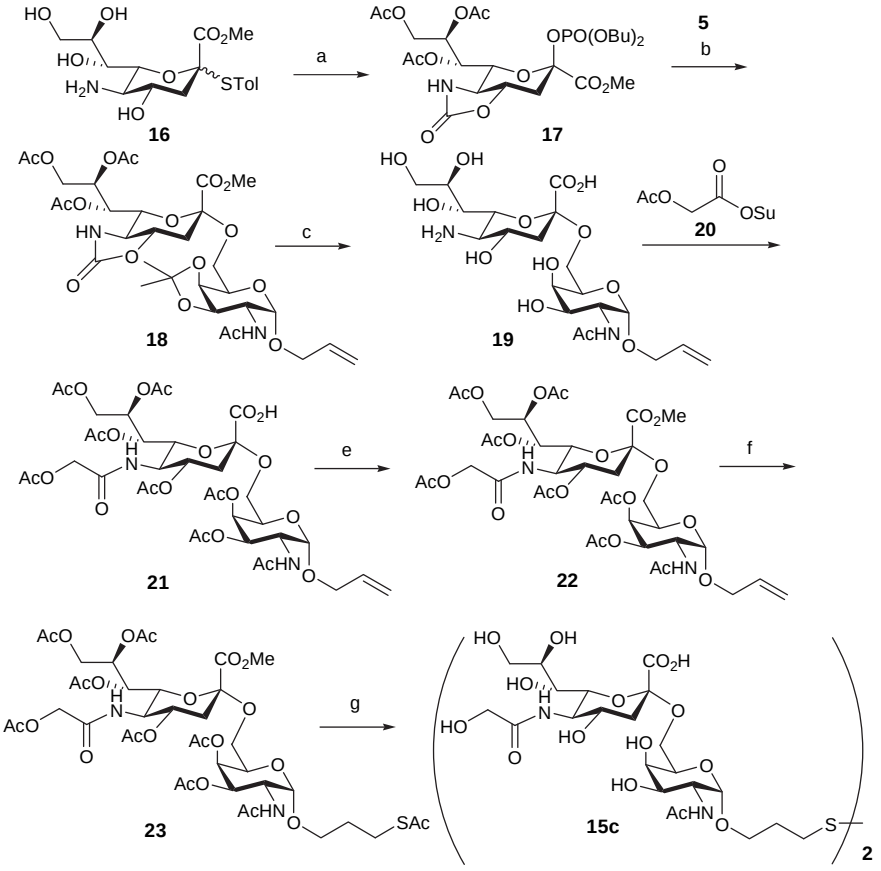
(α:β=2:1). The α/β isomers were easily separated after acidic deprotection of acetonide to yield α isomer **10** with a 43% yield over two steps. Peracetylation of **10** by the acetic anhydride/pyridine protocol generated **11** (92%). Treatment of **11** with zinc dust in glacial acetic acid at rt for 4 h led to the desired compound **12** with a 92% yield.[20] The resulting free amino group of **12** was then coupled with propionic acid and butyric acid, respectively, using EDC/HOBt reagents to produce the *N*-acyl derivatives of sTn: **13a** (42%) and **13b** (51%). The thiol functionality was introduced onto the olefin moiety of **13a**–**b**, followed by full deprotection, using similar procedures as described for the synthesis of **8**, to produce dimer **15a** (84%, for two steps) and **15b** (93%, for two steps).

However, the acyl migration was a serious competition reaction in the preparation of **13**, particularly when glycolic acid was used to couple with **12**. In addition, acyl group migration is also a problem for storing compound **12**. To circumvent this problem, we developed a new approach to synthesize sTnNGc using 5-*N*,4-*O*-carbonyl and 2-phosphate sialic acid donor **17**.[30] As shown in Scheme 3, the phosphate donor **17** was synthesized from **16** by treating it with *p*-nitro phenyl chloroformate, then O-acetylation was performed with Ac_2O/pyridine, followed by sialylation with bisbutyl phosphate in the presence of NIS. Coupling acceptor **5** with glycosyl phosphate donor **17** produced compound **18**, a single α isomer, with a 93% yield. Basic hydrolysis of acyl groups in **18** was followed by acidic (10% hydrochloric acid) workup to afford **19** with a 96% yield (for two steps). In the presence of triethyl amine, compound **19** was then coupled to a *N*-hydroxysuccinamide activated glycolic ester (**20**) followed by peracetylation with Ac_2O/pyridine to produce **21** with an 82% yield (for two steps). Methyl esterification of **21** with methyl iodide, and Cs_2CO_3 produced **22** (93%). Finally, thiolated sTnNGc (**15c**) was obtained by following the procedures as described in Scheme 1.

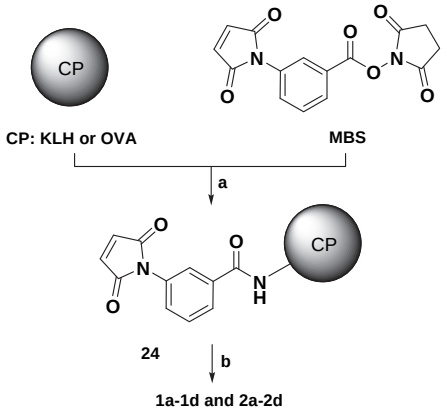
The conjugation of sTn derivatives with carrier proteins was achieved with the bifunctional linker *m*-maleimidobenzoyl-*N*-hydroxysuccinamide ester (MBS). As shown in Scheme 4, the carrier proteins were modified into a maleimide functionality by reacting with MBS under pH 7.0 to produce **24**–KLH and **24**–OVA, respectively. The disulfide bonds of sTn dimers **8** and **15a**–**c** were cleaved with $NaBH_4$ in buffer (pH=7.3) to generate monomers. The thiol functionality of monomers was reacted with the maleimide moiety of carrier proteins through 1,4-Michael addition.[18a] The resulting products were dialyzed against distilled water then freeze-dried to produce the glycoconjugates as white fluffy solids (**1a**–**d** and **2a**–**d**). The sialic acid contents of the glycoconjugates were analyzed by a Warren assay[33] then converted into different levels of carbohydrate loading according to the equation presented in the Experimental Section. In general, the conjugation rates of sTn derivatives with KLH are lower than those of OVA, ranging from 5% to 11% (w/w) of carbohydrate antigens (Table 1).

2.3. Immunological studies of protein conjugates of *N*-acyl sTn derivatives with a carbohydrate microarray

To facilitate a high-throughput assay for immunoscreening, we modified the traditional ELISA immunoassay into a carbohydrate microarray format (Fig. 2). To suppress undesired interaction between antibodies and KLH, the *N*-acyl sTn–OVA conjugates **2a** were used to fabricate a sTn derivative microarray. In addition, the use of *N*-acyl sTn–OVA conjugates **2a** provided specific detection of antibodies against the modified sTns. Although the lysine residues of OVA had been utilized to conjugate sTn derivatives, there are some unreacted lysines that were used to form a Schiff base with the aldehyde groups on a glass slide. To construct a sTn microarray, a high precision contact-printing robot was used to deliver nanoliter volumes of sTn/OVA samples (**2a**–**d**) to the slides, yielding spots with a diameter of ~100 μm. The sTn microarray was irradiated with microwaves (in a domestic microwave oven) for 2 min



Scheme 3. Synthesis of *N*-glycolyl sTn antigen **15c** from amine **16**. (a) (1) NPCC, $NaHCO_3$, MeCN/H_2O, 0 °C, 2 h; (2) Ac_2O, pyridine, rt, 12 h; (3) $HOPO(OBu)_2$, NIS, rt, 2 h, 70% (for three steps); (b) **5**, TMSOTf, MS 3 Å, CH_2Cl_2/MeCN, −78 to −40 °C, 30 min, 93%; (c) (1) LiOH, EtOH/H_2O, 80 °C, 13 h, (2) 10% $HCl_{(aq)}$, 96% (for two steps); (d) (1) DMF, Et_3N, rt, 12 h; (2) Ac_2O, pyridine, rt, 18 h, 82% (for two steps); (e) Cs_2CO_3, MeI, DMF, rt, 4 h, 93%; (f) HSAc, AIBN, MeOH, 75 °C, 24 h, 97%; (g) (1) NaOMe, MeOH; (2) 0.1 M $NaOH_{(aq)}$, 80% (for two steps).



Scheme 4. Conjugation of sTn derivatives to carrier protein. (a) buffer (pH 7.0), rt, 30 min; (b) **8** and **15a**–**c**, buffer (pH 7.3), $NaBH_4$, rt, 20 min.

Table 1
Carbohydrate antigen amounts of glycocojugates

Sample	KLH conjugates				OVA conjugates			
	1a	**1b**	**1c**	**1d**	**2a**	**2b**	**2c**	**2d**
Antigen amount (w/w)%	7	5	5	6	8	8	9	11

to improve the immobilization efficiency of sTn antigens. Furthermore, to quench the unreacted aldehydes on the slide and prevent nonspecific interactions, the slide was blocked with PBS containing 0.2% bovine serum albumin (BSA) for 1 h.

The immunogenicities of the sTn derivative–KLH conjugates were investigated in female BABL/c-J mice at the age of 8 weeks. For immunization, each conjugate containing 2.0 μg of a sTn derivative was mixed with Complete Freund's Adjuvant and injected intraperitoneally. The mice were injected again with the same mixture on day 14 and day 28 following the initial immunization. Sera were collected from the animals' eye sockets on day 1, day 7, day 21, and day 35. Although the raw data allowed for a rough estimate of the relative immunogenicities, an optimal dilution of sera and Cy5-*anti*-IgG and IgM was performed to normalize the data and produce more accurate results. In addition, the optimal concentration diminished the background interference of the glass slide. After the sera were diluted 1:100 with PBS buffer, the samples were incubated with a sTn microarray for 30 min then washed with PBS buffer containing 0.04% Tween 20. Antibodies were detected on the slide by incubating the slide with Cy5-*anti* IgG and Cy5-*anti* IgM (in PBS buffer with 1% BSA) for 30 min and scanning the slide with a chip reader.

As shown in Figure 3, the sera obtained on Day 35 presented the highest IgG antibody titers. Notably, no significant IgM (see Fig. S1) production was observed for all of the sTn–KLH conjugates **1a**–**d**. The cross-reactivity of the antibodies with sTn derivatives was also examined by incubating sera with a sTn microarray (Fig. 4). As expected, all of the sTn derived conjugates exhibited stronger immunogenicity than the native sTn conjugate **1a**. In addition, **1b** presented the highest antibody titer binding with native sTn, indicating that the subtly modified sTn antigen can stimulate antibodies that recognize the parent antigen. Notably, the antibody stimulated by **1b** also recognizes other modified sTn antigens (see Fig. S2). Our results clearly display that subtle structural modification of the sTn antigen results in a stronger immunogenic antigen and that the antibodies stimulated by the modified antigen can bind with the parent antigen.

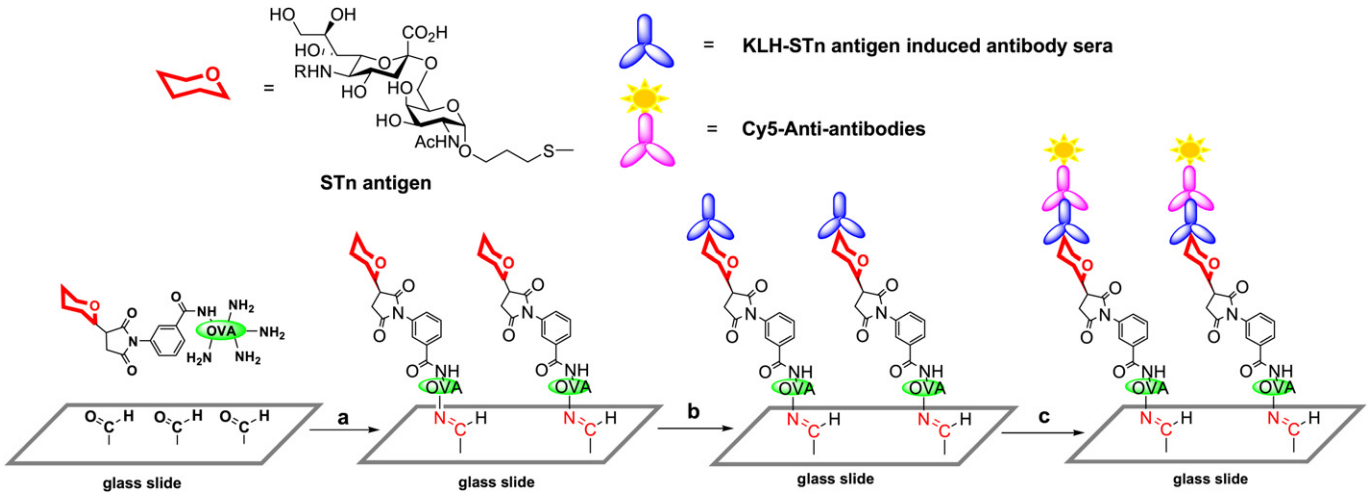


Figure 2. Immunoassay of *N*-acyl sTn derivatives by using carbohydrate microarray. (a) The OVA/conjugated *N*-acyl sTn derivatives were immobilized on the aldehyde-coated glass slides through the microwave-assisted imine formation. (b) The antibody in vaccinated mouse serum binds the corresponding carbohydrate antigen on the sTn microarray. (c) The secondary antibody, tagged a fluorescent tag (Cy5), interacts the mouse antibody. The fluorescent signal can be quantified using a scanning laser microarray reader.

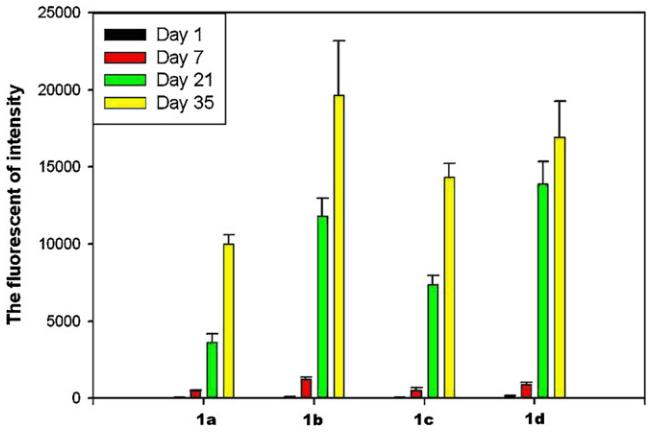


Figure 3. IgG antibodies production by sTn derivative–KLH vaccines. Each bar represents the antibody level in serum pooled from a group of five mice. *anti*-sTn and *anti*-sTn derivative antibody levels were detected by sTn derivative microarray assay as described in Experimental section.

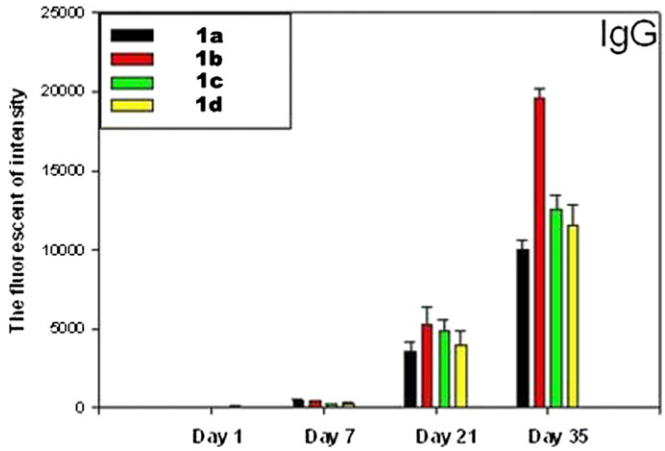


Figure 4. The cross reactivities of the antisera stimulated by sTn derivatives with sTn/OVA. Each bar represents the antibody level stimulated by sTn derivative in recognition with sTn antigen.

In summary, an expedient method was established to synthesize *N*-modified sTn and their protein conjugates. The immunogenicities of these glycoconjugates were studied to explore the impact of structural changes in sTn. To create a high-throughput screening immunoassay, a sTn microarray was made with Schiff base formation and microwave assistance. The immunoassay results revealed that the derived sTn antigens are generally more immunogenic than the parent sTn antigen. The **1b** antigen was the most immunogenic among the sTn derivatives **1b**–**d** investigated, and its antisera were cross-reactive with natural sTn. These results indicate that **1b** may serve as a viable vaccine candidate to produce antibody for sTn antigen detection.

3. Experimental section

3.1. General procedures

^{1}H and ^{13}C NMR spectra were recorded on Bruker AV-400 or DMX-600 MHz. Assignment of ^{1}H NMR spectra was achieved using 2D methods (COSY). Chemical shifts are expressed in parts per million using residual $CDCl_3$ (7.24 ppm), CD_3OD (3.31 ppm) or D_2O (4.67 ppm at 298 k) as an internal standard. Low-resolution and high-resolution mass spectra were recorded under ESI-TOF Mass spectra conditions. Analytical thin-layer chromatography (TLC) was performed on pre-coated plates (Silica Gel 60). Silica gel 60 (E. Merck) was employed for all flash chromatography. All reactions were carried out in oven-dried glassware (120 °C) under an atmosphere of argon unless indicated otherwise. All solvents were dried and distilled by standard techniques.

*3.1.1. O-[Methyl(5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(Allyl2-acetamido-2-deoxy-α-D-galactopyranosyl) (**6**).* TMSOTf (127 μL, 0.70 mmol) was added to a flask containing acceptor **5** (1.7 g, 3.50 mmol), sialic phosphite **4** (4.83 g, 6.57 mmol) and 3 Å MS (5.0 g) in CH_3CN (15 mL) at −40 °C under Ar. The mixture was stirred for 30 min. After the reaction was completed, as determined by TLC analysis, the residue was filtered through Celite. The mixture was concentrated then diluted with EtOAc and washed successively with $NaHCO_{3(aq)}$ and brine. The organic layer was dried over $MgSO_4$ and then concentrated. The residue was purified with flash silica-gel chromatography (EtOAc/hexane=1/1

containing 10% MeOH) to yield a mixture (α/β=3/2, 2.24 g, 65%). R_f 0.2 (EtOAc/hexane=1/1 containing 10% MeOH).

A solution of the above mixture (2.24 g, 2.33 mmol) was dissolved in 80% acetic acid (10 mL) and stirred at rt for 5 h. The mixture was concentrated and then purified with flash silica-gel chromatography (EtOAc/hexane=1/1 containing 20% MeOH) to yield product **6** (1.11 g, 31%, for two steps). R_f 0.1 (EtOAc/hexane=1/1 containing 20% MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ 1.83 (dd, J=12.8, 12.8 Hz, 1H), 1.84 (s, 3H), 1.97 (s, 6H), 1.99 (s, 3H), 2.09 (s, 3H), 2.13 (s, 3H), 2.65 (dd, J=4.8, 12.8 Hz, 1H), 3.60 (dd, J=6.0, 9.0, 1H), 3.76–3.90 (m, 7H), 3.94–4.05 (m, 2H), 4.10 (dd, J=5.2, 12.4 Hz, 1H), 4.14–4.32 (m, 4H), 4.80 (dd, J=4.8, 10.4 Hz, 1H), 4.83 (d, J=3.6 Hz, 1H), 5.18 (m, 1H), 5.30–5.40 (m, 3H), 5.95 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD): 20.83, 21.00, 21.36, 39.04, 50.25, 51.63, 53.53, 63.55, 64.81, 68.79, 69.44, 69.81, 69.91, 70.00, 70.71, 70.88, 73.46, 98.05, 100.19, 117.80, 135.75, 169.69, 171.65, 171.92, 172.58, 173.67, 174.07. HRMS (FAB) calcd for $C_{31}H_{46}N_2O_{18}Na$ $[M+Na]^+$: 757.2632, found: 757.2643.

*3.1.2. O-[Methyl(5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-[(3-thioacetyl) propyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl] (**7**).* HSAc (82 μL, 1.135 mmol) and AIBN (3.1 mg, 0.02 mmol) were added to a solution of compound **6** (63 mg, 0.076 mmol) in dry MeOH (15 mL). The mixture was degassed three times then stirred at 75 °C for 24 h. The mixture was concentrated then purified with flash silica-gel chromatography (EtOAc/hexane=1/1 containing 15% MeOH) to yield **7** (62 mg, 91%). R_f 0.2 (EtOAc/hexane=1/1 containing 15% MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 1.80–1.89 (m, 2H), 1.83 (s, 3H), 1.86 (dd, J=12.8, 12.8 Hz, 1H), 1.97 (s, 3H), 1.99 (s, 3H), 2.00 (s, 3H), 2.10 (s, 3H), 2.13 (s, 3H), 2.32 (s, 3H), 2.64 (dd, J=4.8, 12.8 Hz, 1H), 3.00 (t, J=7.2 Hz, 2H), 3.44 (m, 1H), 3.57 (dd, J=8.8, 12.0 Hz, 1H), 3.74–3.86 (m, 8H), 3.96 (t, J=10.4 Hz, 1H), 4.10 (dd, J=5.6, 12.4 Hz, 1H), 4.14 (dd, J=2.0, 10.8 Hz, 1H), 4.24 (dd, J=3.6, 10.8 Hz, 1H), 4.30 (dd, J=2.8, 12.4 Hz, 1H), 4.78 (d, J=3.6 Hz, 1H), 4.79–4.84 (m, 1H), 5.33 (dd, J=2.0, 8.4 Hz, 1H), 5.37–5.41 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.71, 20.75, 20.78, 21.00, 22.97, 23.05, 25.35, 29.32, 30.60, 37.48, 49.15, 50.79, 50.79, 52.92, 62.44, 63.44, 64.88, 67.47, 68.01, 68.52, 69.05, 70.87, 72.70, 97.14, 98.77, 168.06, 170.06, 170.19, 170.36, 170.80, 170.88, 173.02, 196.24. HRMS (FAB) calcd for $C_{33}H_{51}N_2O_{19}S$ $[M+H]^+$: 811.2807, found: 811.2798.

*3.1.3. O-(5-(Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate)-α-(2,6)-(3-thiopropyl-2-acetamido-2-deoxy-α-D-galactopyranosyl) (**8**).* To a stirred solution of compound **7** (60 mg, 0.07 mmol) in dry MeOH (1.5 mL) was added NaOMe (catalyitic amount). After being stirred at rt for 2 h, the solution was neutralized with Amberlite IR-120 (H^+) resin, filtered, and concentrated to dryness. The residue was then added 0.1 N NaOH (1.5 mL) and allowed to stir at rt for 2 h. The solution was neutralized with Amberlite IR-120 (H^+) resin, filtered, and concentrated. The mixture was purified by P2 biogel to give product **8** (38 mg, 90%). ^{1}H NMR (400 MHz, D_2O): δ 1.53 (dd, J=12.0, 12.0 Hz, 1H), 1.85–1.89 (m, 2H), 1.88 (s, 3H), 1.89 (s, 3H), 2.58 (dd, J=4.4, 12.0 Hz, 1H), 2.71 (t, J=6.7 Hz, 2H), 3.38–3.55 (m, 6H), 3.63–3.78 (m, 6H), 3.83–3.90 (m, 2H), 3.98 (dd, J=3.6, 10.6 Hz, 1H), 4.72 (d, J=3.6 Hz, 1H); ^{13}C NMR (100 MHz, D_2O): δ 22.05, 28.02, 34.91, 40.36, 49.97, 51.88, 62.64, 63.76, 66.43, 67.51, 68.25, 68.52, 69.49, 71.76, 72.56, 97.04, 100.37, 173.39, 174.46, 175.03. HRMS (FAB) calcd for $C_{44}H_{74}N_2O_{28}S_2Na$ $[M+H]^+$: 1193.3829, found: 1193.3810.

*3.1.4. O-[Methyl(5-(2,2,2-trichloroethoxycarbonylamino)-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl) onate]-α-(2,6)-(allyl2-acetamido-2-deoxy-α-D-galactopyranosyl) (**10**).* Compound **10** (19.0 mg, 43% for two steps) was prepared from **9** following the same procedure as described in the synthesis of **6**. R_f 0.3 (EtOAc/hexane=1/1 containing 15% MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.93 (t, J=12.8 Hz, 1H, H3 of sialic acid), 2.03 (s, 3H), 2.06 (s, 3H), 2.07 (s, 3H), 2.15 (s, 3H), 2.16 (s, 3H), 2.66 (dd, J=4.4, 12.8 Hz, 1H), 3.62–3.72 (m, 2H), 3.70–3.90 (m, 4H), 3.83 (s, 3H), 3.98–4.03 (m, 2H), 4.09–4.16 (m, 1H), 4.21–4.26 (m, 2H), 4.31–4.35 (m, 2H), 4.49 (d, J=12.0 Hz, 1H), 4.88 (dd, J=3.6 Hz, 1H), 4.91 (d, J=12.0 Hz, 1H), 5.00 (dt, J=4.4, 12.8 Hz, 1H), 5.05 (d, J=10.0 Hz, 1H), 5.24 (dd, J=2.0, 10.4 Hz, 1H), 5.31 (dd, J=2.0, 17.2 Hz, 1H), 5.87–5.97 (m, 1H), 6.09 (t, J=7.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.72, 20.76, 20.95, 23.25, 37.58, 50.19, 50.31, 51.38, 52.95, 62.26, 63.54, 67.49, 68.05, 68.28, 68.53, 68.65, 70.39, 72.18, 74.35, 95.39, 96.52, 98.60, 117.65, 133.54, 154.17, 167.96, 170.02, 170.05, 170.34, 170.80, 172.05. HRMS (FAB) calcd for $C_{32}H_{46}Cl_3N_2O_{19}$ $[M+H]^+$: 867.1760, found: 867.1757.

*3.1.5. O-{Methyl[5-(2,2,2-trichloroethoxycarbonylamino)-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl] onate}-α-(2,6)-(allyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl) (**11**).* Acetic anhydrate (2.0 mL) was added to a solution of **10** (200 mg, 0.19 mmol) in pyridine (3.0 mL) at rt under a nitrogen atmosphere. After being stirred overnight, the solvent was removed in vacuo. The residue was purified with flash silica-gel column chromatography (EtOAc/hexane=1/1 containing 10% MeOH) to generate compound **11** as a white syrup (190.0 mg, 90%). R_f 0.45 (EtOAc/hexane=1/1 containing 20% MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.81 (t, J=12.8 Hz, 1H, H3 of sialic acid), 1.96 (s, 3H), 1.97 (s, 3H), 2.01 (s, 3H), 2.02 (s, 3H), 2.08 (s, 3H), 2.13 (s, 3H), 2.14 (s, 3H), 2.57 (dd, J=4.6, 12.8 Hz, 1H), 3.30 (dd, J=6.8, 10.0 Hz, 1H), 3.62 (q, J=10.0 Hz, 1H), 3.76 (s,3H), 3.77–3.82 (m, 1H), 4.00 (dd, J=6.0, 12.6 Hz, 1H), 4.06–4.14(m, 3H), 4.20–4.24 (m, 2H), 4.45 (d, J=12.0 Hz, 1H), 4.49–4.54 (m, 1H), 4.87 (d, J=12.0 Hz, 1H), 4.92–4.98 (m, 2H), 5.14–5.22 (m, 3H), 5.26–5.36 (m, 4H), 5.82–5.92 (m, 1H), 6.35 (d, J=8.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.59, 20.86, 21.29, 22.64, 37.75, 48.13, 51.25, 52.76, 62.08, 62.76, 67.10, 67.19, 67.51, 67.83, 68.29, 68.36, 68.42, 71.88, 74.29, 95.30, 96.25, 98.35, 118.04, 133.26, 153.99, 167.61, 169.50, 169.98, 170.08, 170.20, 170.52, 170.71, 171.30. HRMS (FAB) calcd for $C_{36}H_{50}Cl_3N_2O_{21}$ $[M+H]^+$: 951.1972, found: 951.1953.

*3.1.6. O-[Methyl(5-amino-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(allyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl) (**12**).* Activated Zn (4.3 g) was added to a solution of **11** (285 mg, 0.299 mmol) in acetic acid (30 mL) and then stirred at rt for 4 h. Then the reaction mixture was filtered and concentrated. The residue was diluted with EtOAc then washed with $NaHCO_{3(aq)}$ and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified with flash silica-gel chromatography (EtOAc/hexane=1/1 containing 5% MeOH) to yield product **13** (207 mg, 92%). R_f 0.3 (EtOAc/hexane=1/1 containing 20% MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ 1.62 (t, J=12.2 Hz, 1H), 1.93 (s, 6H), 2.01 (s, 3H), 2.05 (s, 3H), 2.13 (s, 6H), 2.19 (s, 3H), 2.56 (t, J=10.0 Hz, 1H), 2.57 (dd, J=4.8, 12.2 Hz, 1H), 3.36 (dd, J=7.4, 10.0 Hz, 1H), 3.77 (dd, J=7.4, 10.0 Hz, 1H), 3.79 (s, 3H), 3.82 (dd, J=1.2, 10.0 Hz, 1H), 4.05 (dd, J=6.4, 12.8 Hz, 1H), 4.19 (t, J=7.4 Hz, 1H), 4.25–4.33 (m, 3H), 4.42 (dd, J=3.6, 11.6 Hz, 1H), 4.58–4.65 (m, 1H), 4.90 (d, J=3.6 Hz, 1H), 5.16 (dd, J=3.2, 11.6 Hz, 1H), 5.21 (dd, J=1.2, 11.6 Hz, 1H), 5.34 (dt, J=1.2, 17.2 Hz, 1H), 5.38–5.46 (m, 3H), 5.90–6.00 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.46, 20.59, 20.67, 20.71, 20.86, 20.93, 21.17, 22.42, 38.47, 51.83, 53.33, 61.52, 63.13, 63.91, 68.83, 68.88, 69.19, 69.70, 69.82, 72.40, 75.96, 97.89, 99.89, 118.04, 135.23, 169.40, 171.50, 171.94, 172.17, 172.27, 172.69, 173.58. HRMS (FAB) calcd for $C_{33}H_{49}N_2O_{19}$ $[M+H]^+$: 777.2929, found: 777.2915.

*3.1.7. O-[Methyl(5-propionylamino-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(allyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl) (**13a**).* $NaHCO_3$ (16.2 mg, 0.19 mmol), HOBt (2.5 mg, 0.02 mmol), and

EDC (17.3 mg, 0.09 mmol) were added to a solution of **12** (50 mg, 0.06 mmol) and propionic acid (7.3 μL, 0.10 mmol) in CH_3CN (1 mL) at 0 °C. The mixture was stirred at rt for overnight. After the reaction was completed, as determined by TLC analysis, the solvent was removed and purified with flash silica-gel chromatography (EtOAc/hexane=1/1 containing 5% MeOH) to yield product **13a** (27 mg, 50%). R_f 0.3 (EtOAc/hexane=1/1 containing 20% MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.07 (t, *J*=7.6 Hz, 3H), 1.91 (t, *J*=12.8 Hz, 1H), 1.96 (s, 3H), 1.99 (s, 3H), 2.00 (s, 3H), 2.03 (s, 3H), 2.06 (q, *J*=7.6 Hz, 2H), 2.11 (s, 3H), 2.15 (s, 3H), 2.16 (s, 3H), 2.54 (dd, *J*=4.8, 12.8 Hz, 1H), 3.31 (dd, *J*=6.4, 10.0 Hz, 1H), 3.79 (s, 3H), 3.83 (dd, *J*=6.4, 10.0 Hz, 1H), 4.00–4.14 (m, 5H), 4.22–4.29 (m, 2H), 4.57 (ddd, *J*=3.6, 9.6, 11.2 Hz, 1H), 4.84–4.89 (m, 1H), 4.92 (d, *J*=3.6 Hz, 1H), 5.10 (db, 1H), 5.17 (dd, *J*=3.2, 11.2 Hz, 1H), 5.23 (dd, *J*=1.2, 10.4 Hz, 1H), 5,28–5.38 (m, 4H), 5.60 (d, *J*=9.6 Hz, 1H), 5.85–5.94 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.80, 20.70, 20.78, 21.03, 23.34, 29.83, 37.88, 47.81, 49.26, 52.86, 62.37, 62.97, 67.19, 67.51, 67.78, 68.26, 68.51, 68.78, 72.63, 96.70, 98.65, 118.15, 133.44, 167.92, 169.70, 169.92, 170.10, 170.29, 170.55, 170.90, 170.97, 173.89. HRMS (FAB) calcd for $C_{36}H_{53}N_2O_{20}$ $[M+H]^+$: 833.3192, found: 833.3209.

*3.1.8. O-[Methyl(5-propionylamino-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-[(3-thioacetyl) propyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl] (**14a**).* The procedure is similar to the one described for the synthesis of **7**. R_f 0.4 (EtOAc/hexane=1/1 containing 20% MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.06 (t, *J*=7.6 Hz, 3H), 1.81–1.91 (m, 2H), 1.89 (t, *J*=12.8 Hz, 1H), 1.97 (s, 6H), 1.98 (s, 3H), 2.01 (s, 3H), 2.05 (q, *J*=7.6 Hz, 2H), 2.09 (s, 3H), 2.13 (s, 3H), 2.14 (s, 3H), 2.35 (s, 3H), 2.54 (dd, *J*=4.6, 12.8 Hz, 1H), 2.90–3.07 (m, 2H), 3.28 (dd, *J*=6.4, 9.6 Hz, 1H), 3.41–3.47 (m, 1H), 3.77 (s, 3H), 3.79–3.83 (m, 2H), 4.02–4.09 (m, 4H), 4.26 (dd, *J*=2.8, 12.8 Hz, 1H), 4.57 (ddd, *J*=3.6, 9.6, 11.6 Hz, 1H), 4.83 (d, *J*=3.6 Hz, 1H), 4.86–4.92 (m, 1H), 5.13 (dd, *J*=3.2, 11.6 Hz, 1H), 5.14–5.18 (m, 1H), 5.27–5.36 (m, 3H), 5.95 (d, *J*=9.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.55, 20.68, 20.76, 20.99, 23.19, 25.51, 29.26, 29.77, 30.59, 37.84, 47.61, 49.15, 52.84, 62.31, 63.03, 65.89, 67.16, 67.48, 67.75, 68.23, 68.84, 72.61, 97.61, 98.61, 167.84, 169.68, 170.05, 170.26, 170.32, 170.53, 170.86, 170.93, 173.88, 195.71. HRMS (FAB) calcd for $C_{38}H_{57}N_2O_{21}S$ $[M+H]^+$: 909.3175, found: 909.3175.

*3.1.9. O-(5-Propionylamino-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(3-thiopropyl 2-acetamido-2-deoxy-α-D-galactopyranosyl) (**15a**).* The procedures are similar to the ones described for synthesis of **8**. ^{1}H NMR (400 MHz, CD_3OD): δ 1.13 (t, *J*=7.6 Hz, 3H), 1.61 (t, *J*=11.4 Hz, 1H), 1.96–2.04 (m, 5H), 2.27 (q, *J*=7.6 Hz, 2H), 2.81–2.86 (m, 3H), 3.44–3.50 (m, 2H), 3.59–3.94 (m, 12H), 4.21 (dd, *J*=3.6, 11.2 Hz, 1H), 4.84 (d, *J*=3.6 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 10.38, 22.78, 30.04, 30.12, 36.34, 42.73, 51.73, 54.01, 64.13, 64.58, 67.32, 69.28, 69.48, 69.77, 70.35, 70.86, 72.90, 74.51, 98.91, 101.94, 173.84, 174.43, 179.27. HRMS (FAB) calcd for $C_{46}H_{78}N_4O_{28}S_2Na$ $[M+Na]^+$: 1221.4142, found: 1121.4157.

The syntheses of **13b**, **14b**, and **15b** are similar as the syntheses of **13a**, **14a**, and **15a**.

*3.1.10. O-[Methyl(5-butyrylamino-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(allyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl) (**13b**).* R_f 0.3 (EtOAc/hexane=1/1 containing 20% MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, *J*=7.4 Hz, 3H), 1.52–1.63 (m, 2H), 1.89 (t, *J*=12.8 Hz, 1H), 1.95 (s, 3H), 1.98 (s, 3H), 1.99 (s, 3H), 2.01 (s, 3H), 2.06 (q, *J*=7.4 Hz, 2H), 2.11 (s, 3H), 2.14 (s, 3H), 2.15 (s, 3H), 2.54 (dd, *J*=4.8, 12.8 Hz, 1H), 3.30 (dd, *J*=6.8, 10.0 Hz, 1H), 3.78 (s, 3H), 3.83 (dd, *J*=6.8, 10.0 Hz, 1H), 4.00–4.14 (m, 5H), 4.21–4.27 (m, 1H), 4.26 (dd, *J*=2.8, 12.4 Hz, 1H), 4.57 (ddd, *J*=3.6, 9.6, 11.2 Hz, 1H), 4.84–4.89 (m, 1H), 4.91 (d, *J*=3.6 Hz, 1H), 5.13–5.19 (m, 1H), 5.17 (dd, *J*=3.6, 11.2 Hz, 1H), 5.21–5.37 (m, 5H), 5.61 (d, *J*=9.6 Hz, 1H), 5.84–5.91 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.89, 19.02, 20.93, 21.00, 21.06, 21.26, 23.56, 38.11, 38.90, 48.00, 49.38, 53.08, 62.58, 63.15, 67.42, 67.69, 67.95, 68.42, 68.71, 68.98, 72.80, 96.89, 98.82, 118.37, 133.64, 168.12, 169.91, 170.16, 170.35, 170.50, 170.78, 171.06, 171.16, 173.26. HRMS (FAB) calcd for $C_{37}H_{55}N_2O_{20}$ $[M+H]^+$: 847.3343, found: 847.3348.

*3.1.11. O-[Methyl(5-butyrylamino-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-[(3-thioacetyl) propyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl] (**14b**).* R_f 0.4 (EtOAc/hexane=1/1 containing 20% MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.91 (t, *J*=7.4 Hz, 3H), 1.53–1.64 (m, 2H), 1.86–1.93 (m, 2H), 1.90 (t, *J*=12.8 Hz, 1H), 1.99 (s, 6H), 2.00 (s, 3H), 2.03 (s, 3H), 2.08 (q, *J*=7.4 Hz, 2H), 2.11 (s, 3H), 2.15 (s, 3H), 2.16 (s, 3H), 2.35 (s, 3H), 2.55 (dd, *J*=4.8, 12.8 Hz, 1H), 2.92–3.09 (m, 2H), 3.30 (dd, *J*=6.4, 10.0 Hz, 1H), 3.43–3.49 (m, 1H), 3.79 (s, 3H), 3.79–3.85 (m, 1H), 3.83 (dd, *J*=6.4, 10.0 Hz, 1H), 3.99–4.12 (m, 4H), 4.27 (dd, *J*=2.4, 12.4 Hz, 1H), 4.59 (ddd, *J*=3.6, 9.6, 11.2 Hz, 1H), 4.85 (d, *J*=3.6 Hz, 1H), 4.87–4.93 (m, 1H), 5.12–5.20 (m, 1H), 5.16 (dd, *J*=3.2, 11.2 Hz, 1H), 5.29 (dd, *J*=1.6, 8.8 Hz, 1H), 5.33 (dd, *J*=3.2, 5.2 Hz, 1H), 5.34–5.37 (m, 1H), 5.95 (d, *J*=9.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.75, 18.87, 20.77, 20.83, 20.92, 21.09, 23.28, 25.66, 29.41, 30.70, 38.01, 38.77, 47.84, 49.32, 52.93, 62.45, 63.17, 66.06, 67.41, 67.63, 67.94, 68.42, 69.92, 69.98, 72.73, 97.75, 98.75, 167.99, 169.77, 170.20, 170.40, 170.60, 170.88, 171.02, 173.13, 195.95. HRMS (FAB) calcd for $C_{39}H_{59}N_2O_{21}S$ $[M+H]^+$: 923.3328, found: 923.3331.

*3.1.12. O-(5-Butyrylamino-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(3-thiopropyl 2-acetamido-2-deoxy-α-D-galactopyranosyl) (**15b**).* ^{1}H NMR (400 MHz, CD_3OD): δ 0.96 (t, *J*=7.6 Hz, 3H), 1.63–1.66 (m, 1H), 1.59–1.68 (m, 2H), 1.97–2.02 (m, 2H), 1.99 (s, 3H), 2.24 (t, *J*=7.6 Hz, 2H), 2.82–2.86 (m, 3H), 3.48–3.51 (m, 2H), 3.59–3.63, (m, 2H), 3.69–3.72 (m, 3H), 3.76–3.96 (m, 8H), 4.22 (dd, *J*=3.6, 11.2 Hz, 1H), 4.79 (d, *J*=3.6 Hz, 1H); ^{13}C NMR (100 MHz, D_2O): δ 12.77, 18.95, 21.97, 27.96, 34.84, 37.79, 40.40, 49.80, 49.92, 51.72, 62.55, 63.72, 66.37, 67.45, 68.04, 68.33, 68.46, 68.66, 69.44, 71.70, 72.54, 96.98, 100.31, 173.31, 174.40, 178.08. HRMS (FAB) calcd for $C_{48}H_{82}N_4O_{28}S_2Na$ $[M+Na]^+$: 1249.4455, found: 1249.4427.

*3.1.13. Methyl (1-(dibutoxy-phosphoroyloxy)-7,8,9-tri-O-acetyl-5-N,4O-carbonyl-3,5-dideoxy-2-thio-D-glycero-β-D-galacto-non-2-ulopyranoside)onate (**17**).* $NaHCO_3$ (4.2 g, 50.0 mmol) was added to a vigorously stirred solution of **16** (4.3 g, 10.0 mmol) in CH_3CN (35.0 mL) and H_2O (70.0 mL), followed by the slow addition of 4-nitrophenyl chloroformate (5.0 g, 25.0 mmol) in CH_3CN (35.0 mL) through dropping funnel at 0 °C. The mixture was stirred for another 3 h at the same temperature. The aqueous layer was extracted with EtOAc. The combined extract was washed with brine, dried over MgSO4, filtered, and evaporated in vacuo. The residue was purified by flash chromatography, eluting with EtOAc/methanol (from 10:1 to 5:1) to generate a white foam that was acylated with acetic anhydride (10.0 mL) in the presence of pyridine (15 mL). The above acetylated compound was dissolved in dichloromethane (70.0 mL) containing 3 Å MS (5.0 g), and the mixture was stirred for 15 min at rt. Dibutyl phosphate (4.2 g, 20.0 mmol) was added to the solution, and the resulting mixture was stirred for another 15 min. NIS (4.5 g, 20.0 mmol) was added, and the reaction mixture was stirred for 2 h at rt. The reaction was quenched by 5% Na_2SO_3 solution then diluted with dichloromethane followed by extraction with dichloromethane. The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated to a crude product that was purified by flash chromatography, eluting with a EtOAc/hexane (1:1) mixture to produce **17** (4.85 g, 70%) as a colorless liquid. R_f 0.4 (EtOAc/hexane=1:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.92 (t, *J*=7.3 Hz, 6H), 1.37 (m, 4H), 1.62 (m, 4H), 2.00 (s, 3H), 2.03–2.05

(m, 1H), 2.07 (s, 3H), 2.16 (s, 3H), 2.80 (dd, J=3.8, 12.6 Hz, 1H), 3.10 (t, J=9.9 Hz, 1H), 3.83 (s, 3H), 3.95–4.11 (m, 5H), 4.33 (dd, J=4.9, 12.6 Hz, 1H), 4.45 (dd, J=2.0, 12.6 Hz, 1H), 4.52 (td, J=3.8, 12.4 Hz, 1H), 5.15 (dd, J=1.7, 7.0 Hz, 1H), 5.27 (ddd, J=2.0, 4.9, 7.0 Hz, 1H), 5.41 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.50, 13.54, 18.57, 18.62, 20.65, 20.69, 20.94, 29.55, 32.15, 37.17, 53.37, 57.72, 61.70, 67.13, 68.40, 68.43, 69.10, 74.33, 75.64, 99.71, 159.06, 165.71, 170.01, 170.49, 171.32. HRMS (FAB) calcd for $C_{25}H_{41}NO_{15}P$ $[M+H]^+$:626.2214, found: 626.2205.

*3.1.14. O-[Methyl(5-N,4O-carbonyl-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(allyl 2-acetamido-3,4-di-O-isopropyl idene-2-deoxy-α-D-galactopyranosyl) (**18**).* A mixture of glycosyl phosphate donor (619 mg, 0.99 mmol) and acceptor **5** (260 mg, 0.82 mmol) was azeotropically dried three times with toluene in vacuo, and the resulting mixture was dissolved in CH_2Cl_2/CH_3CN (6.0 mL/3.0 mL) containing activated 3 Å MS (900 mg). The reaction mixture was stirred at rt for 10 min under a nitrogen atmosphere then cooled to −78 °C. After 10 min of stirring at −78 °C, TMSOTf (0.18 mL, diluted five times with CH_2Cl_2) was added to the reaction mixture at the same temperature. The temperature was raised to −40 °C, and the mixture was stirred for 2 h. The reaction was quenched with Et_3N and filtered through a pad of Celite followed by evaporation in vacuo. The residue was purified by flash silica-gel column chromatography (hexane/ethylacetate=1/1 containing 2% MeOH) to produce compound **18** as a white foam (550 mg, 93%). R_f 0.4 (hexane/ethylacetate=1/1 containing 1% MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.32 (s, 3H), 1.55 (s, 3H), 2.00 (s, 3H), 2.05 (s, 3H), 2.06–2.09 (m, 1H), 2.16 (s, 3H), 2.17 (s, 3H), 2.90 (dd, J=3.5, 12.0 Hz, 1H), 3.04 (t, J=9.9 Hz, 1H), 3.63 (dd, J=7.0, 9.8 Hz), 3.78 (s, 3H), 3.88 (dd, J=6.4, 9.8 Hz, 1H), 3.92–3.99 (m, 2H), 4.00–4.05 (m, 2H), 4.07–4.13 (m, 2H), 4.15–4.26 (m, 3H), 4.27–4.34 (m 2H), 4.77 (d, J=3.6 Hz, 1H), 5.11 (dd, J=1.7, 9.6 Hz, 1H), 5.21 (dd, J=1.4, 10.4 Hz, 1H), 5.28 (dd, J=1.4, 17.2 Hz, 1H), 5.44 (td, J=2.6, 9.6 Hz, 1H), 5.51 (d, J=9.6 Hz, 1H), 5.81–5.91 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.87, 21.01, 21.19, 23.68, 26.75, 28.18, 37.39, 50.50, 53.21, 58.12, 61.87, 64.46, 66.32, 67.35, 68.55, 69.08, 72.49, 73.86, 74.61, 76.80, 97.09, 100.41, 109.83, 117.93, 133.71, 159.47, 168.32, 169.82, 170.19, 170.73, 171.63. HRMS calcd for $C_{31}H_{44}N_2O_{17}Na$ $[M+Na]^+$: 739.2538, found: 739.2537.

*3.1.15. O-(5-Amino-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(allyl 2-acetamido-2-deoxy-α-D-galactopyranosyl) (**19**).* LiOH (400 mg, 16.74 mmol) was added to a stirred solution of compound **18** (400 mg, 0.56) in ethanol (15.0 mL) and water (15.0 mL) at rt. After being stirred at 80 °C for 13 h, the reaction mixture was cooled to rt. Then 10% HCl was added to neutralize the reaction mixture (checked by pH paper). Solvents were evaporated in vacuo. The residue was purified with flash silica-gel column chromatography (CH_2Cl_2/MeOH=1/1) to yield compound **19** as a white syrup (270 mg, 95%). R_f 0.2 (CH_2Cl_2/MeOH=1/1). ^{1}H NMR (400 MHz, MeOH-d_4): δ 1.56 (t, J=12.0 Hz, 1H), 1.98 (s, 3H), 2.75–2.79 (m, 2H), 3.47 (m, 1H), 3.64–3.72 (m, 4H), 3.78 (dd, J=3.1, 11.0 Hz, 1H), 3.84–3.99 (m, 6H), 4.17 (dd, J=5.0, 13.1 Hz, 1H), 4.25 (dd, J=3.6, 11.0 Hz, 1H), 4.82 (d, J=3.6 Hz, 1H), 5.17 (dd, J=1.5, 10.4 Hz, 1H), 5.30 (dd, J=1.5, 17.2 Hz, 1H), 5.89–5.99 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.94, 42.39, 49.84, 51.66, 54.62, 64.28, 69.21, 69.37, 69.78, 69.96, 70.94, 71.66, 73.56, 75.67, 97.99, 102.07, 117.97, 135.66, 174.06, 174.89. HRMS calcd for $C_{20}H_{34}N_2O_{13}Na$ $[M+Na]^+$: 533.1959, found: 533.1950.

*3.1.16. O-[(5-(2-Acetoxy-acetylamino)-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(allyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl) (**21**).* Activated glycolic ester **20** (50.6 mg, 0.4 mmol) was added to a stirred solution of amino acid **19** (120 mg, 0.24 mmol) in DMF (3.0 mL), in the presence of Et_3N (0.065 mL, 0.48 mmol) under a nitrogen atmosphere at rt. After being stirred for 16 h, the solvent was removed in vacuo. The residue was purified with flash silica-gel column chromatography (CH_2Cl_2/MeOH=2/1) to produce a white syrup, which was treated with pyridine (3.0 mL) and acetic anhydride (2.0 mL) under a nitrogen atmosphere at rt. After being stirred overnight, the solvent was removed in vacuo. The residue was purified by flash silica-gel column chromatography (CH_2Cl_2/MeOH=4/1) to generate compound **21** as a white syrup (166.0 mg, 82%). R_f 0.3 (CH_2Cl_2/MeOH=1/1). ^{1}H NMR (400 MHz, MeOH-d_4): δ 1.63 (t, J=12.0 Hz, 1H), 1.93 (s, 3H), 1.94 (s, 3H), 1.97 (s, 3H), 2.00 (s, 3H), 2.09 (s, 3H), 2.11 (s, 3H), 2.12 (s, 3H), 2.15 (s, 3H), 2.61 (dd, J=12.0, 4.1 Hz, 1H), 3.46 (dd, J=6.1, 10.5 Hz, 1H), 3.87–3.92 (m, 2H), 4.06 (dd, J=13.2, 6.3 Hz, 1H), 4.12–4.16 (m, 1H), 4.24 (m, 1H), 4.29–4.34 (m, 2H), 4.39–4.46 (m, 3H), 4.60 (d, J=10.3 Hz, 1H), 4.92 (d, J=3.5 Hz, 1H), 4.99–5.04 (m, 3H), 5.20 (dd, J=11.1, 2.2 Hz, 2H), 5.29–5.41 (m, 3H), 5.48 (d, J=2.2 Hz, 1H), 5.93–5.99 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.47, 20.74, 20.85, 20.93, 21.32, 22.48, 39.74, 49.65, 50.55, 63.56, 63.63, 64.45, 69.11, 69.38, 69.51, 69.88, 69.94, 71.15, 72.65, 97.71, 101.54, 117.96, 135.30, 170.52, 171.62, 171.80, 171.85, 171.93, 172.23, 172.36, 172.44, 173.51, 174.39. HRMS calcd for $C_{36}H_{50}N_2O_{22}Na$ $[M+Na]^+$: 885.2753, found: 885.2751.

*3.1.17. O-[Methyl(5-(2-acetoxy-acetylamino)-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(allyl-2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl) (**22**).* Cs_2CO_3 (45.3 mg, 0.139 mmol) was added to a mixture of **21** (120.0 mg, 0.139 mmol) and iodo methane (59.3 mg, 0.417 mmol) in dry DMF (2.0 mL). The resulting mixture was stirred for 7 h. Water (10.0 mL) and EA (10.0 mL) were added to the reaction mixture, followed by an extraction with EA (2×10 mL). The combined organic parts were washed with water (10.0 mL), dried over $MgSO_4$, and concentrated in vacuo to produce a crude product that was purified by flash silica-gel column chromatography to furnish **22** (113.4 mg, 93%). R_f 0.3 (EtOAc/hexane=1/1 containing 20% MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.92 (t, J=12.6 Hz, 1H, H3 of sialic acid), 1.97 (s, 3H), 1.99 (s, 3H), 2.01 (s, 3H), 2.04 (s, 3H), 2.14 (s, 3H), 2.15 (s, 3H), 2.17 (s, 3H), 2.19 (s, 3H), 2.57 (dd, J=4.6, 12.6 Hz, 1H), 3.32 (dd, J=6.7, 10.0 Hz, 1H), 3.81 (s, 3H), 3.82–3.87 (m, 1H), 3.99–4.16 (m, 5H), 4.23–4.30 (m, 2H), 4.30 (d, J=15.3 Hz, 1H), 4.55–4.60 (m, 1H), 4.59 (d, J=15.3 Hz, 1H), 4.92 (d, J=3.6 Hz, 1H), 4.92–4.98 (m, 1H), 5.18 (dd, J=3.2, 11.3 Hz, 1H), 5.23–5.30 (m, 2H), 5.33–5.39 (m, 2H), 5.61 (d, J=9.7 Hz, 1H), 5.85–5.96 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.85, 20.94, 21.13, 23.42, 38.05, 47.91, 49.53, 53.02, 62.39, 62.90, 63.16, 67.38, 67.62, 67.89, 68.16, 68.40, 68.63, 68.86, 72.61, 96.82, 98.79, 118.24, 133.55, 167.71, 168.08, 169.71, 169.81, 170.03, 170.40, 170.42, 170.66, 171.06, 171.08. HRMS (FAB) calcd for $C_{37}H_{55}N_2O_{22}$ $[M+H]^+$: 877.3098, found: 877.3090.

The procedures for the syntheses of **23** and **15c** are similar as described in the syntheses of **14a** and **15a**.

*3.1.18. O-[Methyl(5-(2-acetoxy-acetylamino)-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-[(3-thioacetyl) propyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-α-D-galactopyranosyl] (**23**).* R_f 0.4 (EtOAc/hexane=1/1 containing 20% MeOH), ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.81–1.97 (m, 3H, H3 of sialic acid), 2.00 (s, 3H), 2.00 (s, 3H), 2.00 (s, 3H), 2.04 (s, 3H), 2.14 (s, 3H), 2.15 (s, 3H), 2.17 (s, 3H), 2.19 (s, 3H), 2.36 (s, 3H), 2.57 (dd, J=4.8, 12.8 Hz, 1H), 2.93–3.09 (m, 2H), 3.31 (dd, J=6.4, 10.0 Hz, 1H), 3.44–3.50 (m, 1H), 3.81 (s, 3H), 3.81–3.86 (m, 1H), 3.84 (dd, J=6.4, 10.0 Hz, 1H), 3.99–4.11 (m, 3H), 4.13 (dd, J=2.0, 10.0 Hz), 4.28 (dd, J=3.2, 12.4 Hz, 1H), 4.30 (d, J=15.3 Hz, 1H), 4.59 (d, J=15.3 Hz, 1H), 4.59–4.63 (m, 1H), 4.85 (d, J=3.6 Hz, 1H), 4.94 (ddd, J=4.8, 10.0, 12.4 Hz, 1H), 5.16 (dd, J=3.2, 11.2 Hz, 1H), 5.27 (dd, J=2.0, 8.4 Hz, 1H), 5.34–5.38 (m, 2H), 5.83 (d, J=10.0 Hz, 1H), 5.98 (d, J=10.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.93, 21.07, 21.23, 23.41, 25.78,

29.54, 30.83, 38.17, 47.93, 49.62, 53.14, 62.48, 63.01, 63.40, 66.20, 67.52, 67.75, 68.04, 68.30, 68.54, 69.08, 72.74, 97.63, 98.66, 167.68, 167.90, 169.59, 169.67, 170.27, 170.56, 170.93, 195.66. MS (FAB) calcd for $C_{39}H_{57}N_2O_{23}S$ $[M+H]^+$: 953.3072, found: 953.3100.

*3.1.19. O-(5-Glycolylamino-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-α-(2,6)-(3-thiopropyl 2-acetamido-2-deoxy-α-D-galactopyranosyl) (**15c**).* ^{1}H NMR (400 MHz, D_2O): δ 1.65 (t, J=12.1 Hz, 1H), 1.92–1.96 (m, 2H), 1.96 (s, 3H), 2.67 (dd, J=4.5, 12.1 Hz, 1H), 2.79 (t, J=6.9 Hz, 2H), 3.47–3.59 (m, 4H), 3.66–3.76 (m, 3H), 3.77–3.86 (m, 5H) 3.91 (d, J=3.0 Hz, 1H), 3.97 (dd, J=4.2, 7.4 Hz, 1H), 4.04 (br s, 2H), 4.06 (m, 1H), 4.80 (d, J=3.8 Hz, 1H); ^{13}C NMR (100 MHz, D_2O): δ 22.06, 28.07, 34.99, 39.95, 49.97, 51.55, 61.02, 62.78, 63.90, 66.50, 67.58, 67.63, 68.28, 68.59, 69.52, 71.43, 72.47, 97.10, 99.84, 172.48, 174.56, 175.82. HRMS (FAB) calcd for $C_{44}H_{73}N_4O_{30}S_2$ $[M-H]^-$: 1201.3740, found: 1201.3751.

3.2. General procedures for conjugation of sTn derivatives with KLH or OVA

A solution of KLH (or OVA, 5 mg) and MBS/DMF (1.05 mg/70 μL) in 0.01 M PBS buffer (0.5 mL, pH=7.0) was stirred for 30 min at rt. The reaction mixture was purified with a Biogel P-10 column using 0.05 M PBS buffer (pH=6.0) as an eluent to yield KLH/MBS (or OVA/MBS). A solution of **9** or **20a**–**c** (5 mg) and $NaBH_4$ (10 equiv) in 0.05 M PBS buffer (1.0 mL, pH=6.0) was stirred for 20 min at rt and then KLH/MBS (or OVA/MBS) was added. The reaction mixture was adjusted to pH=7.3 and stirred for 3 h at rt. Then the mixture was dialyzed against distilled water for 12 h. The resulting solution was lyophilized to produce a white powder of the desired glycoconjugates (**1a**–**d** and **2a**–**d**).

3.2.1. Analysis of carbohydrate antigen amount on the synthetic glycoconjugate. Reagent A: a solution of periodic acid (0.025 M) in diluted sulfuric acid (0.063 M)(pH 1.2). Reagent B: a 2% sodium arsenite solution in dilute hydrochloric acid (0.5 M). Reagent C: aq thiobarbituric acid (0.1 M) adjusted to pH 9.0 with NaOH. Reagent D: 1-butanol containing 5% hydrochloric acid (12 M). A solution containing exactly 0.5 mg of the glycoconjugate in dilute water (1.0 mL) was mixed with 0.25 mL of reagent A. The mixture was heated in a water bath for 30 min followed by the addition of reagent B (0.2 mL). The solution was heated until the brown color disappeared. Reagent C (2 mL) was added to the above solution. The solution was heated for 15 min in a boiling water bath then cooled in an ice-bath. The solution was extracted with reagent D, and the organic layer was separated from the aqueous layer. The organic layer was transferred to a cuvette, and its absorbance at 549 nm was determined with a UV–vis spectrometer. The sialic acid content of the glycoconjugate was determined against a calibration curve created with the standard NeuNAc solution. The carbohydrate antigen amount of each glycoconjugate was calculated according to the equation shown below.

$$N-\text{Acyl sTn amount}\ (\%) = \frac{\text{amount of sialic acid}\ (\mu g)\ \text{in the sample}}{\text{weight of the glycoconjugate sample}\ (\mu g)} \times \frac{\text{molecular weight of sTn}}{\text{molecular weight of sailic acid}} \times 100\%$$

3.2.2. Construction of the N-acyl sTn microarray. The *N*-acyl sTn–OVA conjugates were dissolved in PBS buffer containing 0.014% Tween 20. The solution was microspotted in predetermined places on an aldehyde-coated glass slide with a distance of 0.6 mm between the centers of adjacent spots. After the printing was finished, the glass slide was placed in a microwave oven and irradiated for 2 min (P=100 watts of microwave power). The slide was immersed in PBS buffer containing 0.2% BSA with gentle shaking for 1 h then washed with PBS buffer containing 0.04% Tween 20 and PBS buffer (each for 2 min). Finally, the slide was dried by a high speed centrifugation.

3.2.3. Immunogenicity studies of the synthetic glycoconjugates. For each sTn–KLH glycoconjugate, five female BABL/c-J mice were immunized when they were 8 weeks old. The immunizations were delivered intraperitoneally with the glycoconjugate containing 2 μg of sTn in 100 μL of saline mixed with 100 μL of Complete Freund's Adjuvant (Sigma). The mice were given identical immunizations on days 0, 14, and 28. Then the mice were bled from the eye socket after immunization on days 1, 7, 21, and 35. Blood was stored at −80 °C.

Sera from five mice per group were diluted 1:100 in PBS buffer with 1% BSA and incubated with sTn microarray glass slides for 30 min. Afterward, the glass slides were washed with PBS buffer containing 0.04% Tween 20 and PBS buffer (each for 2 min). Finally, the glass slides were dried by a high speed centrifuge. The glass slides were incubated with a 1:200 dilution of Cy5-*anti* IgG and Cy5-*anti* IgM, respectively, in PBS buffer with 1% BSA for 30 min. Next, the glass slides were washed with PBS buffer containing 0.04% Tween 20 then PBS buffer (each for 2 min), followed by drying the slides with a high speed centrifuge. Finally, the glass slides were scanned with an ArrayWoRx biochip scanner to visualize the fluorescence. The fluorescence intensity was analyzed using ArrayVision 8.0 software from Imaging Research, Inc.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2010.07.050.

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